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Limitation to Photosynthesis in Water-stressed Leaves: Stomata *vs.* Metabolism and the Role of ATP

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Decreasing relative water content (RWC) of leaves progressively decreases stomatal conductance (g_s), slowing CO_2 assimilation (A) which eventually stops, after which CO_2 is evolved. In some studies, photosynthetic potential (A_{pot}), measured under saturating CO_2, is unaffected by a small loss of RWC but becomes progressively more inhibited, and less stimulated by elevated CO_2, below a threshold RWC (Type 1 response). In other studies, A_{pot} and the stimulation of A by elevated CO_2 decreases progressively as RWC falls (Type 2 response). Decreased A_{pot} is caused by impaired metabolism. Consequently, as RWC declines, the relative limitation of A by g_s decreases, and metabolic limitation increases. Causes of decreased A_{pot} are considered. Limitation of ribulose bisphosphate (RuBP) synthesis is the likely cause of decreased A_{pot} at low RWC, not inhibition or loss of photosynthetic carbon reduction cycle enzymes, including RuBP carboxylase/oxygenase (Rubisco). Limitation of RuBP synthesis is probably caused by inhibition of ATP synthesis, due to progressive inactivation or loss of Coupling Factor resulting from increasing ionic (Mg^{2+}) concentration, not to reduced capacity for electron or proton transport, or inadequate trans-thylakoid proton gradient (ΔpH). Inhibition of A_{pot} by accumulation of assimilates or inadequate inorganic phosphate is not considered significant. Decreased ATP content and imbalance with reductant status affect cell metabolism substantially: possible consequences are discussed with reference to accumulation of amino acids and alterations in protein complement under water stress.

Key words: Photosynthesis, relative water content (RWC), ATP synthesis, stomata, amino acid metabolism, ribulose bisphosphate synthesis, protein synthesis, chaperones.

INTRODUCTION

The photosynthetic rate (A) of leaves of both C_3 and C_4 plants decreases as their relative water content (RWC) and water potential (ψ) decrease (Chaves, 1991; Cornic, 1994; Kramer and Boyer, 1995; Lawlor, 1995; Cornic and Massacci, 1996) (Fig. 1). However, the relative importance of stomatal conductance (g_s) in restricting the supply of CO_2 to metabolism (stomatal limitation), and of metabolic impairment which decreases the potential rate of A (A_{pot}, non-stomatal limitation), is unclear and discussion has become polarized. Stomatal limitation is considered to decrease both A and CO_2 concentration in the intercellular spaces of the leaf (C_i), which inhibits metabolism (Kaiser, 1987; Downton *et al*., 1988; Cornic, 2000) (Fig. 2). Thus, Kaiser (1987) and Kaiser and Foster (1989) concluded that nitrate reductase (NR) activity, and Vassey *et al*. (1991) that sucrose phosphate synthase (SPS) activity, were inhibited by low C_i or the associated low rate of A. Limited A decreases consumption of electrons released from water as a consequence of the light reactions: the ensuing excess of excitation energy is dissipated by non-photochemical quenching by the xanthophyll cycle in the photosystem antennae (see Lawlor, 2001). A key feature in this analysis is that the CO_2 concentration in the atmosphere (C_a) can be increased so that the CO_2 concentrations in the intercellular spaces and in the chloroplast stroma (C_c) rise, and restore A to A_{pot}. This occurs over a range of RWC from 100 to 80 % (note that RWC values are generalizations for mesophytes), but at more severe stress A_{pot} is decreased (eventually ceasing at approx. 40 % RWC) and cannot be restored by elevated C_a. The decrease in A_{pot} at low RWC (Chaves, 1991) has been considered to be a consequence of low C_i.

Another point of view (Keck and Boyer, 1974; Lawlor, 1995; Escalona *et al*., 1999; Flexas *et al*., 1999; Pankovič *et al*., 1999) considers that A_{pot} and metabolism decrease progressively, i.e. elevated C_a is less and less able to restore A_{pot} to the unstressed rate as RWC falls. Thus, metabolism is inhibited. This is not regarded as a consequence of the effect of low C_i or C_c on NR or SPS. Rather, decreased ATP synthesis by the enzyme ATP synthase (Coupling Factor, CF) is considered to be the primary effect of decreasing RWC (Keck and Boyer, 1974; Tezara *et al*., 1999), due largely, but not exclusively, to the effects of increasing ion (specifically Mg^{2+}) concentrations in the chloroplast as RWC falls (Younis *et al*., 1979); CO_2 depletion is not the primary effect (Tang *et al*., 2002). Metabolic limitation is often observed and correlates with loss of ATP content, which starts to decrease with mild water stress (Tezara *et al*., 1999; Flexas and Medrano, 2002). Thus, the limitation to A_{pot} is caused by inadequate ATP not CO_2, with major consequences for metabolism; excess excitation energy is dissipated via non-photochemical quenching.

Whilst emphasizing the overall loss of photosynthetic potential, this is not to deny that the initial decrease in A in

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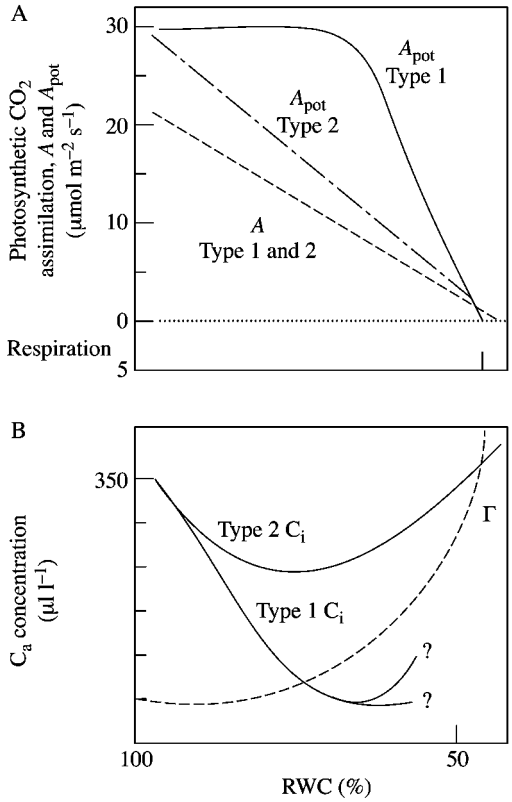

FIG. 1. A, Schematic of the basic responses of actual photosynthetic rate (A) in air (360 μmol CO_2 m^{-2} s^{-1}) and potential photosynthetic rate (A_{pot}) measured at elevated CO_2 concentration, to relative water content (RWC). Type 1 and 2 responses of A_{pot} are shown. In the Type 1 response, A_{pot} is unaffected until a 20–30 % decrease in RWC occurs, when it becomes metabolically limited. In Type 2, the change is linear, showing progressive metabolic limitation. In both types in well-watered leaves, photosynthetic rate (A) is stimulated by elevated CO_2. Elevated CO_2 maintains A at the potential rate (A_{pot}) in the Type 1 response as RWC decreases; but at RWC below approx. 80 % A_{pot} decreases in Type 1. Elevated CO_2 simulates A progressively less as RWC decreases in Type 2, showing that A_{pot} is inhibited. B, Scheme of the changes in CO_2 inside the leaf (C_i) during steady-state A, as stomatal conductance (g_s) decreases with falling RWC, associated with Type 1 or Type 2 photosynthetic response (1 with C_i decreasing to compensation point; 2 with C_i decreasing but not to compensation point). The equilibrium compensation point, Γ, associated with Type 1 response is indicated. There are differences between experiments, with C_i not decreasing, or decreasing somewhat, or substantially. This may reflect different methods of assessing C_i.

air can be due to lower g_s, although metabolic effects become increasingly dominant (e.g. Tezara *et al*., 1999). It is essential to understand how A_{pot} is affected because the nature and sensitivity of metabolic processes determine the responses of plants to water deficits, and the processes required to prevent damage. These protective mechanisms allow plants to function in terms of productivity, reproduction and ecological fitness in different environments and under varying water balance. Development of a generally acceptable model (Fig. 2) of what may be called the 'water deficiency syndrome', to emphasize the complex nature of the problem and the wide range of responses to changing conditions, is an important but rather distant goal. Despite many years of effort to understand the causes of the myriad changes in cellular biochemistry, e.g. accumulation of 'stress metabolites' such as amino acids including proline (Samaras *et al*., 1995) and citrulline (Yokota *et al*., 2002), and changes in gene expression and protein synthesis (see Bray, 2002), resulting from decreased RWC, conditions within the cell responsible for altered metabolism are poorly known. Understanding of the interactions between photosynthesis and other metabolic processes, illustrated in Fig. 2, is advanced; ultimately, a more quantitative approach will be required. Changes in gene expression and protein synthesis are being studied intensively, as mechanisms of cellular adaptation to drought, and major attempts made to genetically modify plants to be more drought tolerant or resistant. The long-term goals of stabilizing and increasing yields under drought may be more successful given clearer understanding of conditions within cells at decreased RWC.

This review considers the evidence for the stomatal and non-stomatal regulation of A, and inhibition of A_{pot} in leaves of C_3 plants in relation to RWC. Changes in photosynthetic metabolism and the role of ATP synthesis are discussed. Conditions in stressed cells and consequences of changed ATP and A are related to metabolism, particularly amino acid and protein synthesis.

PHOTOSYNTHESIS UNDER STRESS

The 'classical' response of A to decreasing RWC and water potential, whether induced by decreasing the water supply to the roots and thence leaves by soil drying or application of osmotica, is shown in Fig. 1 for leaves of mesophytes. It is assumed, for discussion, that incident photosynthetically useful photon flux (PPF, wavelength 400–700 nm) saturates A, and that excess energy captured is dissipated non-photochemically. Between 100 and 90 % RWC (the control, unstressed state), g_s is maximal but with C_a of 350 μl l^{-1} CO_2 and 210 ml l^{-1} O_2, A is not saturated with CO_2. Raising C_a to >1000 μl l^{-1} saturates A, giving A_{max}, the maximum metabolic capacity, or potential (A_{pot}) of the system. If metabolic processes are inhibited then A_{pot} is decreased. When the tissue is unstressed at large RWC, A_{pot} = s A_{max}, but at small RWC, $A_{pot} < A_{max}$ of unstressed leaves; A and A_{pot} approach or become zero at approx. 40 % RWC.

Decreasing RWC causes g_s and A to decrease, approximately in parallel, although at small values of RWC, g_s reaches a minimum but A may continue to decrease. In some experiments (see Cornic, 2000), when A decreases as RWC falls initially, elevated C_a (50 up to 150 ml l^{-1}, equivalent to 5 to 15 %) restores A to A_{max}, i.e. A_{pot} is unaffected. Thereafter A drops, and stimulation by elevated CO_2 is smaller, showing that A_{pot} is limited by metabolic factors at low RWC. This has been called a Type 1 response (Lawlor and Cornic, 2002). In other studies, A falls progressively as RWC falls, but is also less stimulated by elevated CO_2. This shows that A_{pot} is progressively inhibited, and the effect of g_s diminished, with increasing stress. This is called a Type 2 response (Lawlor and Cornic, 2002).

What causes the differences in response of A_{pot} to RWC between experiments? Are differences in techniques responsible? Are there fundamental differences between species?

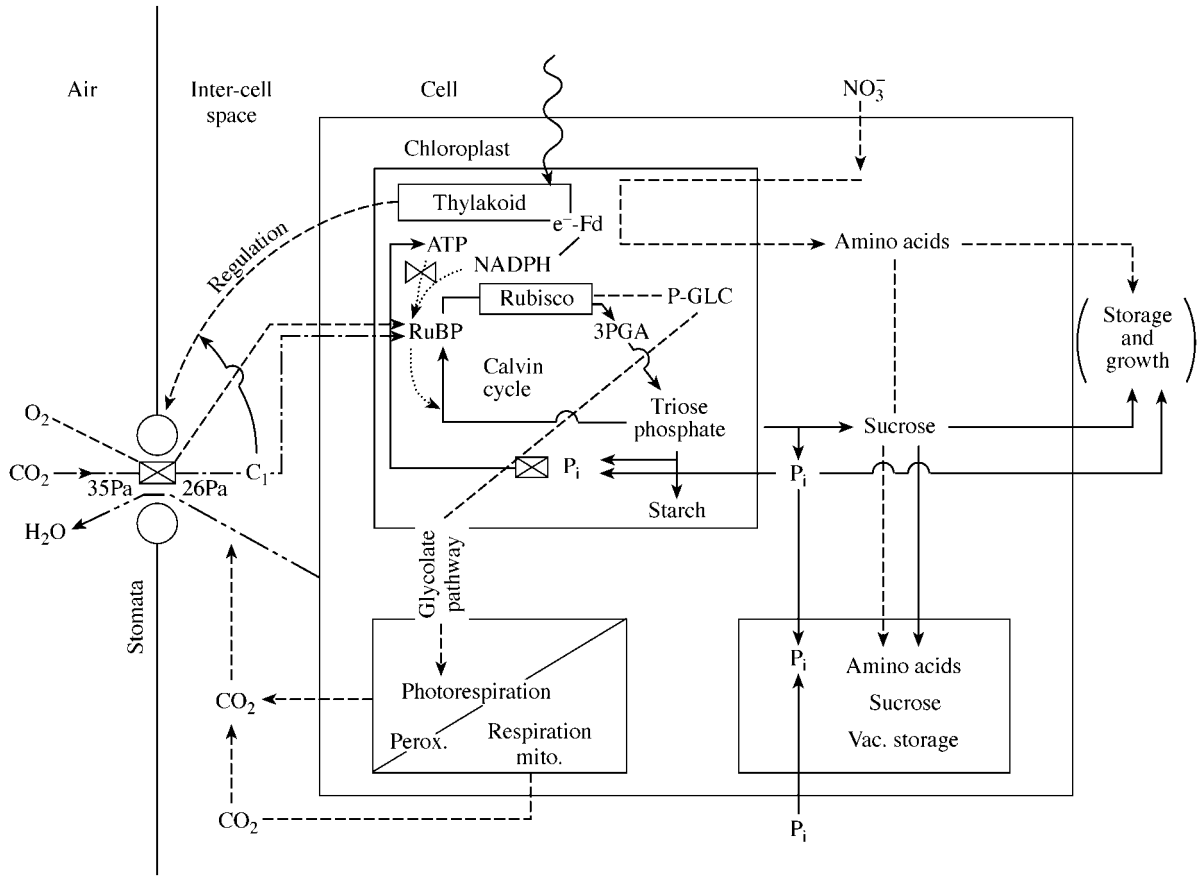

FIG. 2. Schematic of the reactions of photosynthesis, showing sites of possible limitation to photosynthesis offered by the stomatal pore, in ATP synthesis and supply, and inorganic phosphate recycling. These are discussed more fully in the text. Other metabolic processes that are linked to photosynthesis are indicated.

Are there conditions in the plant's environment, either during growth or experimentation, which can explain the conflicting information and interpretation? Lawlor and Cornic (2002) consider these questions at length; no clear reasons for the difference in response to elevated C_a are apparent. However, the decreased A_{pot} in both types of response leads to the important question: what are the metabolic causes of inhibited A_{pot}? There is a substantial body of information related to this question; analysis of it may help to clarify the problem and mechanisms responsible.

Interpretation of A/C_i curves

The cuticle and stomata limit CO_2 diffusion into the well-watered leaf, so C_i in air is approx. 0·7-times smaller than C_a when A is 30 μmol m^{-2} s^{-1}. Also, C_c, which is taken as equal to the CO_2 concentration at the active site of the carboxylating enzyme, ribulose bisphosphate carboxylase-oxygenase (Rubisco), is approx. 0·7-times smaller than C_i. This is caused by limitations to CO_2 transport from the intercellular spaces across the cell walls, membrane, cytosol, and chloroplast envelope and stroma (von Caemmerer and Evans, 1991). Capacity for transport through this combined pathway is referred to as mesophyll conductance. Thus, C_c/C_a is approx. 0·5 (see von Caemmerer, 2000 for details and references). The calculated C_i is close to that measured directly by attaching a chamber to one side of a photosynthesizing leaf with large g_s (Sharkey *et al.*, 1982; Lauer and Boyer, 1992). From response curves of A/C_i in saturating light (1200 μmol m^{-2} s^{-1} PPF), A (equal to A_{max}) saturates at C_i >600 μl l^{-1} and is related to the limitation imposed by ribulose bisphosphate (RuBP) regeneration by the photosynthetic carbon reduction (PCR) cycle. The initial slope is the carboxylation efficiency (c.e.), related to CO_2 limitation. Generally, as RWC decreases, the decrease in c.e. and A_{max} is similar, e.g. in wheat and sunflower, illustrated in Fig. 3 (Lawlor and Khanna-Chopra, 1984; Giménez *et al.*, 1992). Eventually, at very small RWC (approx. 40 % but varying with species, growth conditions, etc.), net CO_2 exchange is zero; if the deficit is more severe, CO_2 is emitted. This is *prima facie* evidence of inhibition of A by altered metabolism, as the effect of g_s is eliminated. The progressive decrease in A_{max} with stress, at C_i that normally saturates A, suggests that RuBP regeneration rather than CO_2 supply or Rubisco activity limits A. This is confirmed by increasing C_i to 2500 μl l^{-1} and greater in some experiments (e.g. Tezara

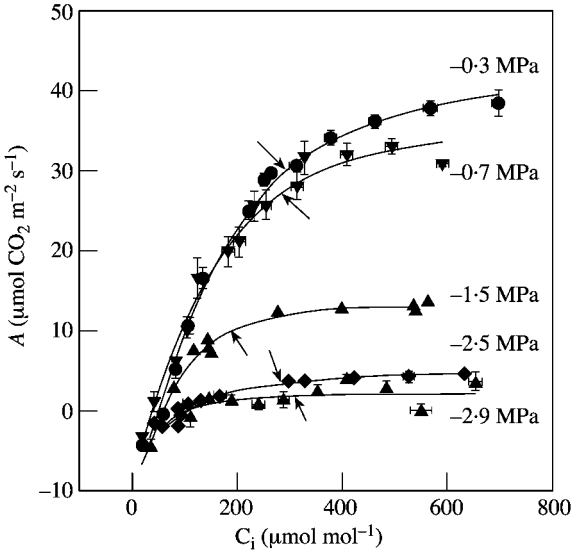

FIG. 3. Typical relationship between photosynthetic rate (A) and the CO_2 concentration inside the leaf (C_i), the A/C_i curves, for leaves of different water potential given on the curves (from Tezara *et al*., 1999). Arrows denote the operating point achieved in air of 360 μl l^{-1} CO_2.

et al., 1999), and strongly indicated in others (Graan and Boyer, 1990). Of course, C_c may still be very low, but A would be expected to increase gradually with increasing C_i. This is further evidence that A_{pot} is inhibited. If CO_2 transport were impaired at low RWC, then A_{pot} would not have remained constant but would have risen as C_i increased. Calculation suggests that the C_a required to overcome the minimum g_s measured in mesophytes is relatively modest, a few thousand μl l^{-1} CO_2. Application of very high C_a, 5 to 10 % (50 to 100 ml l^{-1}), increased A (and A_{pot}) to A_{max} in many studies, but not in others. Indeed, in some, 10 % CO_2 substantially or totally inhibited A and A_{pot} even at high RWC (Graan and Boyer, 1990; Tezara *et al*., 1999), showing that CO_2 does indeed reach the site of metabolism, and that small g_s and inadequate CO_2 supply do not limit A; rather A_{pot} is inhibited.

Relative importance of stomatal and non-stomatal effects on A

Limitations caused by g_s and mesophyll regulation may be calculated from A/C_i curves. Several similar methods have been suggested (Cornic *et al*., 1983; Jones, 1985; Assmann, 1988). The stomatal (L_s) and mesophyll (L_m) limitations for sunflower are given for three RWCs (based on Giménez *et al*., 1992), calculated as described in Fig. 4. For the unstressed control leaf L_s is 18 %, with moderate stress 25 % and at very severe stress 20 %. The metabolic limitation, L_m, is zero in the control by definition, 46 % at moderate stress and 87 % at severe stress. g_s limits A in well-watered leaves but is only responsible for 29 and 3 %, respectively, of the reduction in A_{pot} at moderate and very severe stress. Martin and Ruiz-Torres (1992) demonstrated that the non-stomatal limitations to A_{pot} (calculated similarly to the above example) and c.e. increased as RWC fell for wheat. Of course, where A_{pot} is unaffected over a wide range of RWC before decreasing, the relative limitations will show only g_s limitation followed by a progressive increase in L_m.

Validity of A/C_i *curves.* Analyses of A/Ci curves are only valid if they represent the relationship between A and C_i correctly. There are uncertainties in calculating C_i in stressed leaves (Cornic, 2000) arising, for example, from technical difficulties in measuring small A at large CO_2 concentrations, but agreement between different methods (e.g. infrared analysis, uptake of $^{14}CO_2$ of known specific radioactivity: Tezara *et al*., 1999) suggests that the errors do not invalidate assessments of A_{pot} (Lawlor and Cornic, 2002). Further analysis is required: for example, of the effect of different water vapour and CO_2 transport in the cuticle, which invalidates the assumption that the ratio of diffusivities in air can be applied (Boyer *et al*., 1997) and may affect estimates of C_i. However, this error is most serious at very small g_s when A is very small. At intermediate stress, where A is decreased but g_s is not minimal, the effect is probably small (Tezara *et al*., 1999), and has little effect on A_{max}, but may affect c.e.

Heterogeneous ('patchy') distribution of g_s, particularly if some areas have closed and others open stomata, invalidates calculation of C_i and so has been the basis of the rejection of differences in the A/C_i curves with stress. Experimentally, patchiness has been assessed in different ways (Pospíšilová and Šantrúcek, 1997; Weyers and Lawson, 1997). Direct observation of stomatal aperture, and use of solutions of different viscosities that enter pores of different apertures, show that heterogeneity occurs. Indirect measurements, e.g. chlorophyll *a* fluorescence and $^{14}CO_2$ assimilation, demonstrate heterogeneity of CO_2 assimilation in the leaf, although the causes may not necessarily be directly related to heterogeneity of g_s. Patchiness occurs when abscisic acid (ABA) is applied in the transpiration stream (Mott and Buckley, 2000), but evidence for water-stressed leaves is less compelling. It is surprising, given the frequent, consistent observation of decreased A_{pot} as a consequence of stress, that patchiness has not been more clearly demonstrated. Patchiness probably does not have a major influence on A/C_i curves for leaves with low RWC, as concluded by Gunasekera and Berkowitz (1992) and Giménez *et al*. (1992), and does not adequately explain inhibition of A_{pot}. In any case, elevated C_a should overcome the effects of patchiness; that it does not shows metabolic limitation of A_{pot}.

I conclude that decreased A_{pot}, in experiments where elevated CO_2 does not restore it to the control value, is caused by a mesophyll limitation that increases in magnitude as RWC drops. Where elevated C_a restores A to A_{max} (the control A_{pot}), overcoming reduced g_s, then clearly mesophyll processes are not inhibited, so control is entirely via g_s. However, even with this type of response, a RWC is reached below which metabolism is impaired.

Compensation point at low RWC

As RWC falls initially and g_s decreases, C_i generally falls when measured in air (350 μl l^{-1} CO_2, 210 ml l^{-1} O_2) under

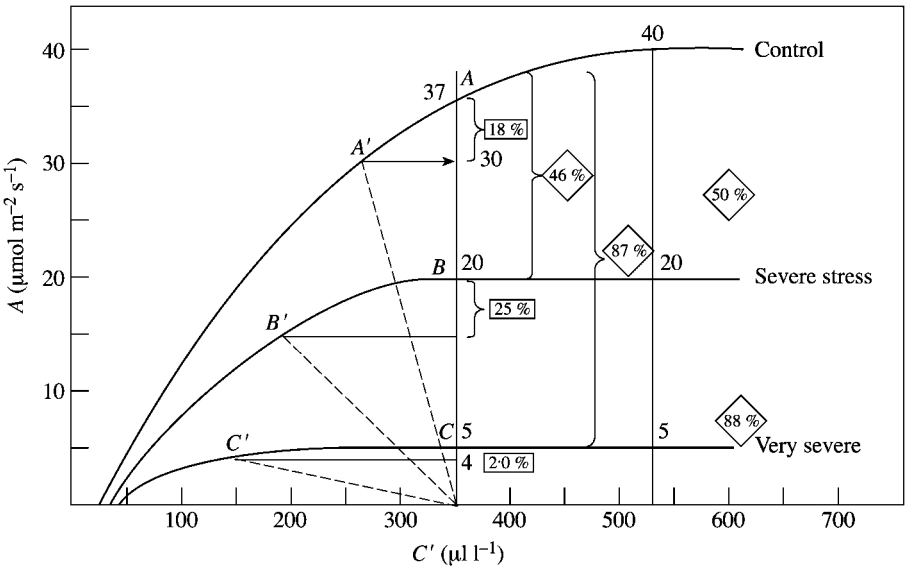

FIG. 4. Simplified analysis of relative limitation by stomatal and mesophyll factors (L_s and L_m) applied to well-watered, or severely, or very severely stressed leaves. L_s is calculated from measurements at C_a of 350 μl l^{-1} CO_2. For the unstressed control leaf, $L_s = [(A - A')/A] \times 100 = 18$ %, where A is the rate at 350 μl l^{-1} CO_2 , i.e. with infinite g_s, and A' is the rate corresponding to the C_i with finite stomatal conductance. At severe stress, $L_s = [(B - B')/B] \times 100 = 25$ %, and at very severe stress $L_s = [(C - C')/C) \times 100 = 20$ %. By definition, L_m is zero in the control; at severe stress $L_m = [(A - B)/A) \times 100 = 46$ %, and at severe stress $L_m = [(A - C)/A) = 87$ %. When the limitation by g_s is related to the metabolic limitation, it is at severe stress $[(B - B')/(A - B)] \times 100 = 29$ %, and at very severe stress $[(C - C')/(A - C)] \times 100 = 3$ %.

steady-state fluxes (Lawlor, 1995). The decrease in C_i agrees with carbon isotope discrimination in water-stressed leaves (Donovan and Ehleringer, 1994); however, the magnitude varies. Analysis from chlorophyll *a* fluorescence of stressed leaves indicates that C_i decreases substantially to the compensation point (Cornic and Briantais, 1991), as the effects can be simulated by decreasing C_a. Gas exchange data suggest a much smaller decrease in C_i. However, with further fall in RWC, C_i generally increases again (Lawlor, 1995), e.g. in sunflower (Lauer and Boyer, 1992; Tezara *et al*., 1999) and other plants (Brodribb, 1996). Also, the equilibrium compensation point, Γ (measured in a closed system when A and CO_2 production are in balance), increases as RWC falls. The increase is relatively small with initial loss of RWC in some studies, but is greater in others, and is very substantial at RWC of 80 % and below (Lawlor, 1976; Lauer and Boyer, 1992; Tezara *et al*., 1999). This decrease in C_i, followed by an increase, is probably due to A drawing down C_i as g_s decreases, followed by increased CO_2 production from respiration relative to A, so C_i and Γ rise. As respiration remains relatively constant (or may decrease) as RWC falls (Lawlor and Fock, 1975; Lawlor, 1976; Tezara *et al*., 1999), increased Γ and C_i show unequivocally that A_{pot} is impaired.

Photorespiration and day respiration. Photorespiration (PR) plays an important role in stressed C_3 leaves as a route for energy consumption, and is examined here briefly. Rubisco oxygenase activity catalyses the reaction of RuBP with O_2, resulting in 3PGA and phosphoglycolate (PG) synthesis (Fig. 2). Metabolism of PG via the glycolate pathway is a complex but well understood process: PG is dephosphorylated to glycolate, which is metabolized and transaminated to glycine. In the mitochondria, glycine is decarboxylated by glycine decarboxylase, producing the CO_2 released as PR. The remaining carbon from glycine forms serine, which is metabolized to glycerate and re-enters photosynthetic metabolism. The proportion of PR to A increases as the ratio of Rubisco oxygenase to carboxylase activity rises, and thus with increased O_2/CO_2 ratio at the active site (Farquhar, 1979; von Caemmerer, 2000). This relative increase in PR is important at low C_i as electrons from decarboxylation of glycine are transferred to oxygen, forming water, and the associated electron transport is coupled with ATP synthesis in the mitochondria (Siedow and Umbach, 1995). Photorespiration plays a key role in protection of leaves against excessive reductant and uses energy when other methods of dissipation (e.g. CO_2 assimilation) are restricted (Kozaki and Takeba, 1996; Osmond *et al*., 1997). However, note that the absolute rate of A decreases with low C_i, but the ratio PR/A increases: at the compensation point the rates are equal. Hence, the relative increase in PR to A seen in early studies of CO_2 assimilation at low RWC (Lawlor and Fock, 1975, 1977; Lawlor, 1976) was explained by the increased oxygenase to carboxylase ratio from low C_i. It is important to emphasize that inadequate RuBP supply limits the rates of A and PR, but will not affect the proportion in the way that the ratio of CO_2 to O_2 does.

PR does not maintain normal electron fluxes because the absolute magnitude of the processes is much smaller in stressed than in unstressed leaves. Clearly this is the case if C_c decreases to the compensation point at low RWC, as suggested by chlorophyll *a* fluorescence (Cornic and Briantais, 1991). This assumed that RuBP supply to Rubisco was unimpaired, so increased fluorescence was a

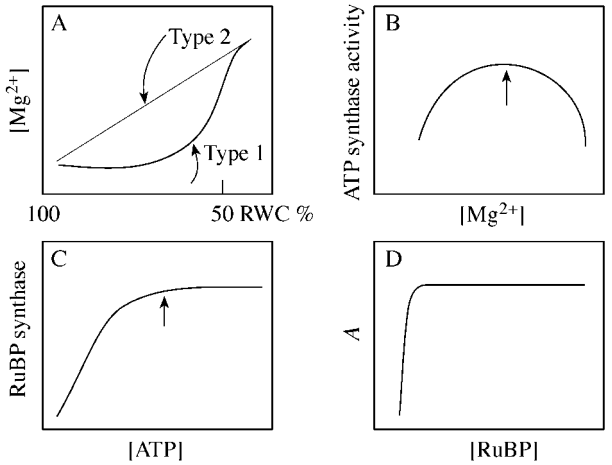

FIG. 5. Speculation, in diagrammatic form, about the possible causes of decreased metabolic limitation of A_{pot} by decreased RWC to explain the differences in Type 1 and 2 response shown in Fig. 1. The chain of events causing decreased A is suggested to be: Fig. 1A. Decreasing RWC results in increased Mg^{2+} concentration in plants that cannot maintain the concentration (homeostasis). Other plants are able to maintain the concentration over a wide range of RWC. With a response of ATP synthesis by Coupling Factor to Mg^{2+} concentration as shown in Fig. 5B, and a relation of RuBP synthesis to ATP concentration as in Fig. 5C, and of CO_2 assimilation to RuBP as in Fig. 5D, then Type 1 and 2 responses of A_{pot} to RWC as shown in Fig. 1 will result.

result of low C_c. However, if RuBP synthesis is limited, and A thereby decreased, then fluorescence increases as energy is not used. An analogy is provided by the effect of deficient phosphate supply (Jacob and Lawlor, 1992) and low PRK (phosphoribulokinase) activity (Paul *et al*., 1995) which would appear as a CO_2 limitation but impair metabolism. Thus, the decrease in A_{pot} as a consequence of reduced RuBP would appear to be due to low C_i based on fluorescence alone. Further analysis of the effects of RWC on A_{pot} in relation to RuBP concentration (changed, for example, by using PRK and CF transformants, altered phosphate supply, inhibitors of CF) and different C_i is desirable.

Photorespiration is the main, but not the only, source of respiratory CO_2. 'Day' respiration from mitochondria [and probably equivalent to dark respiration with CO_2 released by the tricarboxylic acid (TCA) cycle] also contributes. This is shown by analysis of the CO_2 released, in the light, from leaves that had assimilated radiocarbon-labelled CO_2 (Lawlor and Fock, 1975). Photorespiratory CO_2 is derived from very recently formed assimilates, so its specific activity (^{14}C/total C ratio) rapidly becomes that of the CO_2 supplied [relative specific activity (RSA) = 1]. This should be the case at all PR/A ratios unless alternative sources of unlabelled CO_2 are assimilated. However, RSA was much less than 1 at low RWC, even after a long period. This shows either that carbon entering the glycolate pathway was derived from non-radioactive sources, or that the CO_2 emitted was a mixture of PR and 'day' respiration, which was maintained at low RWC (Lawlor and Fock, 1975). Mathematical simulation of the C fluxes in this system (Lawlor and Pearlman, 1981) showed that with a small increase in stress, Rubisco characteristics could simulate the changes in specific activity, but not at low RWC where an additional source of CO_2 was required. Continuation of non-photorespiratory CO_2 release accords with the increased C_i and equilibrium compensation points observed at low RWC (Lawlor, 1976; Brodribb, 1996; Tezara *et al*., 1999).

METABOLIC BASIS OF THE RESPONSE OF A_{POT} TO RWC

The Type 1 and Type 2 responses of A_{pot} to RWC may be extremes of a much broader range, or continuum, of response; the causes of the difference are not understood. Speculation about the causes may help to guide thinking about the mechanisms to be addressed. (1) Photosynthetic metabolism is more sensitive to changing RWC and cellular conditions in some types of plants than in others. This is not likely as sunflower responds in both ways depending on experiments. (2) Conditions such as increased osmotic or ionic concentrations within the metabolic compartments (Fig. 2), particularly chloroplasts, may result from dehydration and may affect the metabolic reactions. In the Type 1 response, conditions may not change with the initial loss of RWC, perhaps because of regulatory mechanisms which control ion concentrations, etc. If within the regulatory tolerances of metabolism, then no change occurs in A_{pot}. The Type 2 response may be caused by the inability of the regulatory mechanisms to maintain ionic and osmotic homeostasis as RWC decreases, so metabolism is impaired with loss of A_{pot}. This aspect is illustrated in Fig. 5 and discussed later.

Metabolic causes of decreased A

What metabolic reactions, from the capture of light energy to CO_2 fixation, are impaired by changing conditions at low RWC and reduce A_{pot}? Sites at which photosynthetic metabolism may be impaired include (Fig. 2): (1) Rubisco enzyme activity; (2) regeneration of RuBP by the PCR cycle; (3) supply of ATP and NADPH to the PCR cycle; (4) electron transport and generation of the proton gradient across the thylakoid membrane; (5) light capture and transduction in the photosystems; and (6) use of assimilation products outside the chloroplast.

Limitation by Rubisco

From the A/C_i curves, the decreased c.e. and A_{max} suggest loss of Rubisco activity with decreasing RWC. However, the amount of Rubisco protein is generally little affected by moderate or severe stress (Flexas and Medrano, 2002), even if experienced over a period of many days (e.g. Medrano *et al*., 1997; Tezara *et al*., 1999). Restoration of A_{pot} to A_{max} by rehydration also suggests that Rubisco (and, of course, other potential limitations) is not impaired irreversibly. A reduction in the amount of Rubisco under stress (Majumdar *et al*., 1991) may be related to stimulation of senescence, which is difficult to distinguish from a direct effect of low RWC. However, more prolonged severe stress often

decreases Rubisco activity, e.g. in subterranean clover both initial and total activity fell, associated with substantially lower activation state and catalytic constant (Medrano *et al*., 1997). Loss of Rubisco activity is probably more related to inhibition or to non-activation of enzyme active sites (Medrano *et al*., 1997; Parry *et al*., 2002). Studies with transgenic plants provide an interesting analogy to the water-stress effects: decreasing Rubisco protein by antisense genetic modification, thereby lowering total enzyme activity (although with increased activation state of the enzyme), decreased A_{pot} (Hudson *et al*., 1992). However, over 75 % of wild-type activity was removed whilst A_{pot} fell by 50 % (although this depends on environment), suggesting that the large changes in A_{pot} under stress would require substantial reduction of Rubisco protein and activity. Future analyses of the effects of water stress on Rubisco require careful measurements of Rubisco protein amounts per unit leaf area and per unit cell and chloroplast volume, together with activity and activation state of the enzyme (Parry *et al*., 2002).

RuBP concentrations and Rubisco activity. The rate of photosynthesis depends on synthesis of RuBP and activity of Rubisco. Therefore the decrease in RuBP content of leaves at low RWC (Giménez *et al*., 1992; Gunasekera and Berkowitz, 1993; Tezara *et al*., 1999) is significant. There was a strong sigmoidal dependence of *A* on RuBP in water-stressed sunflower (Giménez *et al*., 1992), with little change in Rubisco activity, which suggests that A_{pot} was determined by RuBP content. Rubisco exhibits a Michaelis–Menten rectangular hyperbolic relationship between the carboxylation rate and free RuBP, with a K_m of 20 μM and saturation at 200 μM free RuBP. Stromal concentration of Rubisco protein is considerable (100–250 g l^{-1} or kg m^{-3}, 0·25–0·5 mM), with active site concentrations (Et) from 2 to 5 mM, so the RuBP concentration is also large. Indeed, total RuBP concentration (Rt) may be two- to three-fold greater than Et in unstressed chloroplasts. The carboxylation rate increases linearly and steeply with increasing Rt/Et ratio until it reaches 1–1·5, when saturation occurs. The transition between RuBP-dependence and RuBP-saturation is very sharp (von Caemmerer, 2000). This was shown in transgenic tobacco (at large RWC) with reduced activity of the PCR cycle enzyme glyceraldehyde-3-phosphate dehydrogenase when Rt/Et dropped substantially (GAPDH; Price *et al*., 1995). This also occurred in leaves exposed to low or elevated CO_2, which, respectively, increased and decreased RuBP content (von Caemmerer and Edmondson, 1986), although Rt/Et did not fall below 1. The response of *A* to RuBP content was linear below 40 μmol m^{-2}, in both subsaturating and saturating CO_2, indicating that the supply of RuBP determines *A* in different CO_2 concentrations. Interaction between Rubisco activity and RuBP supply is well illustrated by studies on unstressed tobacco leaves with normal amounts of Rubisco (Laisk and Oja, 1974; von Caemmerer, 2000). The RuBP pool increased in leaves in bright light when the CO_2 concentration was decreased transiently, so that on return to CO_2 greater than approx. 300 μl l^{-1}, *A* was much greater than the steady-state rate for a short time, until the RuBP was consumed. Thus, under steady-state conditions, RuBP supply limited the rate of CO_2 assimilation in CO_2 >300 μl l^{-1}. However, equivalent measurements have not been made on water-stressed leaves. Given the large decrease in Rubisco activity required to decrease A_{pot} (see earlier discussion), the rather small changes in Rubisco amount and activity in stressed leaves, and the sigmoidal relationship between *A* and RuBP observed as a consequence of water stress, I conclude that inadequate RuBP supply limits A_{pot}. Limited RuBP could result from inadequate supply of ATP and/or NADPH to the PCR cycle, or from a decreased rate of cycle turnover caused by low enzyme activity.

Role of Rubisco activase

Rubisco activase is an abundant protein (Salvucci and Ogren, 1996) that regulates the active site conformation of Rubisco and removes inhibitors allowing rapid carboxylation. Inhibitors are generally analogues of RuBP, generated as 'miss-fire' products of the Rubisco carboxylation and oxygenation reactions or produced in other metabolic processes (Edmondson *et al*., 1990). They bind to enzyme sites not occupied by RuBP (Portis *et al*., 1995), so to minimize inhibition and obtain optimal Rubisco activity, Rt/Et must exceed 1·5 under a range of conditions. Hence, the large concentration of RuBP required in the stroma to achieve maximum rates of CO_2 assimilation, mentioned earlier. If RuBP synthesis decreases and Rt/Et falls, then the enzyme may become inhibited. Activase releases tight-binding inhibitors from the Rubisco active sites, so increasing activity. The reaction requires ATP (Robinson and Portis, 1988), so decreased activity and activation state of Rubisco at low RWC may be related to inadequate ATP concentrations (see below). There is evidence that Rubisco activase activity decreases as RWC falls, consistent with decreased ATP concentration (see below; Parry *et al*., 2002).

Limitation of A_{pot} by the photosynthetic carbon reduction cycle

Decreased RuBP content of stressed leaves is not expected if CO_2 supply is the limiting factor, suggesting inhibition of RuBP synthesis. However, low RuBP content is not definitive proof of inhibition of RuBP synthesis, either by limited ATP and/or NADPH synthesis, or by impaired PCR cycle enzyme reactions, because *A* has decreased so the autocatalytic cycle cannot regenerate RuBP. However, RuBP concentration increased in unstressed leaves in low CO_2, despite lack of cycle turnover (von Caemmerer and Edmondson, 1986; Hudson *et al*., 1992). Also, the ratio of RuBP to 3PGA increased as the low CO_2 concentration indicating that lack of CO_2 prevented consumption of RuBP. In bean, Sharkey and Seeman (1989) showed that RuBP decreased with severe, but not mild, water stress, and 3PGA decreased strongly at both stresses, suggesting that synthesis was limited. However, the RuBP/3PGA ratio increased as a consequence of stress. The authors concluded that CO_2, and thus g_s, was limiting *A*, not RuBP synthesis. In

contrast, RuBP decreased but 3PGA remained essentially constant in the study by Tezara *et al*. (1999), so RuBP/3PGA fell. This is expected if factors other than g_s, such as RuBP synthesis, decreased *A*. As the decrease in RuBP relative to 3PGA was measured on whole tissue and 3PGA is not unique to the PCR cycle, 3PGA synthesis outside the PCR cycle could affect the conclusion. However, the low RuBP/3PGA ratio with decreasing RWC suggested that regeneration of RuBP was inhibited. In stressed, isolated chloroplasts, RuBP content but not concentration decreased, and ATP behaved similarly, showing that the osmotic stress did not decrease these aspects of metabolism (Sharkey and Badger, 1982). Comparing RuBP in stressed leaves with the effects of different CO_2 concentrations on unstressed leaves and antisense modifications to the PCR cycle discussed earlier, which increased RuBP, strongly suggests that impaired RuBP synthesis is responsible for the reduction in A_{pot}. It is perhaps of significance that decreased Rubisco activity (Tezara *et al*., 1999) is consistent with generation of inhibitors and inability of the activation mechanisms to maintain active enzyme with progressively lower RWC. Another interpretation of the decreased RuBP under stress (Gunasekera and Berkowitz, 1993), namely that PCR cycle activity was impaired, was based on the assumption that ATP (and NADPH) supply was unaffected, so the conclusion is questionable. The observation of Gunasekera and Berkowitz (1993) that RuBP increased in transformed plants with low Rubisco and *A* but decreased under water stress is, I contend, very important evidence of impaired RuBP synthesis. Combined evidence from stressed leaves is consistent with decreased RuBP concentration, but the differences between experiments suggest the need for caution and further analysis.

There are two main sites for inhibition of RuBP synthesis: the enzymes of the PCR cycle, or supply of ATP and/or NADPH to the cycle.

PCR cycle enzymes. Reduction of RuBP content at low RWC could result from a limitation in one or more enzymes of the PCR cycle. There is little direct evidence regarding the response of the individual enzymes of the regenerative part of the PCR cycle to decreasing RWC. Measurement of the enzymes potentially responsible for regulating the flux of carbon through the PCR cycle *in vivo* is a considerable technical challenge, and analyses *in vitro* may not reflect the *in vivo* behaviour, especially under conditions relevant to low RWC. Rather, the approach has been to interpret changes in metabolites (see previous discussion) to establish if limitation occurs in the PCR cycle or elsewhere. Thus, Sharkey and Seeman (1989) concluded that low CO_2, not enzyme(s), decreased cycle activity. In contrast, Gunasekera and Berkowitz (1993) concluded that PCR cycle activity limited A_{pot}. Analogy with unstressed transgenic leaves shows that reduced PCR cycle activity (low Rubisco activity) decreases *A* but increases RuBP (Hudson *et al*., 1992). This provides additional evidence that limitation of PCR cycle capacity is not the cause of low A_{pot} at low RWC. Similarly, decreased ATP with low RWC observed in some studies (Lawlor and Khanna-Chopra, 1984; Tezara *et al*., 1999) suggests that the PCR cycle is not substantially impaired: if low C_i were the cause then ATP content should rise. Again, an analogy is provided by tobacco with antisense to PRK (Paul *et al*., 1995): a 95 % decrease in PRK substantially decreased *A* and RuBP content but increased ATP content (see below). Thus, there is strong evidence that the PCR cycle *per se* is not the cause of decreased *A* with low RWC.

Limitation of RuBP regeneration by NADPH and ATP availability. Synthesis of RuBP depends on the supply of substrates for the PRK reaction, ribose 5-phosphate from the PCR cycle and ATP (Lawlor, 2001). NADPH is consumed only by the triosephosphate dehydrogenase reaction in the PCR cycle, for the reduction of glycerate 1,3-bisphosphate derived from 3PGA. If NADPH were limiting at low RWC then it would decrease glyceraldehyde-3-phosphate and thus ribose 5-phosphate, the same effect as decreasing the activity of an enzyme in that portion of the cycle. Inadequate ATP would decrease the PCR cycle's ability to regenerate RuBP by PRK, so glyceraldehyde-3-phosphate would increase and RuBP decrease. This was the effect of decreased PRK activity (Paul *et al*., 1995), but ATP increased in the transgenics, in sharp contrast to the case of water stress in some studies, as discussed earlier.

Measurements of NADPH and other pyridine nucleotides in photosynthesizing leaves over a range of RWC are few. NADPH content remained relatively constant (Lawlor and Khanna-Chopra, 1984; Tezara *et al*., 1999) and NADH increased (Lawlor and Khanna-Chopra, 1984) as RWC decreased, indicating that the electron transport capacity is sufficient to maintain and increase the reduction state of these pyridine nucleotides. The redox balance of photosynthetic cells at low RWC thus appears to be in a substantially reduced state. The concentration of NADPH in the chloroplast stroma under stress is not known, nor is how it relates to the requirements of triosephosphate dehydrogenase reaction *in vivo*. However, it is unlikely that availability of NADPH to the PCR cycle limits its capacity to form RuBP.

The behaviour of ATP in water-stressed cells and tissues has been assessed in several studies. Boyer and co-workers (Keck and Boyer, 1974; Boyer *et al*., 1987) concluded over two decades ago that ATP synthesis was the major limitation for *A* at low RWC because photophosphorylation by CF was inhibited in chloroplasts from water-stressed leaves of sunflower. In addition, Younis *et al*. (1979) provided strong evidence that photophosphorylation was inhibited by increased concentration of Mg^{2+} ions. Mg^{2+} is likely to increase in the chloroplast stroma as RWC falls. Coupling Factor has one portion, CF_0, within the thylakoid membrane and the other, CF_1, projecting into the chloroplast stroma; the latter is probably sensitive to Mg^{2+} concentration. Other evidence of the sensitivity of CF_1 to stress conditions and loss of photophosphorylation capacity with decreased RWC is provided by Meyer and de Kouchkovsky (1992) and Meyer *et al*. (1992). However, loss of CF protein was not considered the cause, although the amount of CF_1 decreased in stressed leaves (Tezara

et al., 1999). Photophosphorylation requires ΔpH and passage of H^+ through CF_0, and also activation of CF_1 by the thioredoxin system (which occurs rapidly at very low light; Haraux and de Kouchkovsky, 1998). Most studies suggest that ΔpH is large and adequate for ATP synthesis, even at low RWC, e.g. measurements based on 9-aminoacridine fluorescence calibrated against ΔpH (de Kouchkovsky and Meyer, 1992; Meyer and de Kouchkovsky, 1992; Meyer *et al*., 1992). The decrease in ATP content of leaves occurs with relatively small loss of RWC (see Flexas and Medrano, 2002), although ATP content is not reduced to zero even at very low RWC when *A* has virtually stopped (Tezara *et al*., 1999).

Measurements of ATP in stressed tissues vary considerably between studies. ATP decreased in osmotically stressed mesophyll cells of *Xanthium* in some, but not all, experiments, and it was not considered to limit CO_2 assimilation (Sharkey and Badger, 1982). Sharkey and Seeman (1989) observed no differences in mildly stressed leaves of bean, and concluded that ATP was not the limiting factor for photosynthesis. In contrast, Lawlor and Khanna-Chopra (1984), Meyer *et al*. (1992) and Tezara *et al*. (1999) observed a progressive decrease in ATP as RWC fell. The ATP content should increase if the PCR cycle were inhibited, as ATP is not consumed. This is the case, as mentioned above, when the PCR cycle is inhibited by antisense to PRK (Paul *et al*., 1995), and also when leaves are exposed to low C_a. Thus, the decrease in ATP observed (Lawlor and Khanna-Chopra, 1984; Tezara *et al*., 1999) agrees with inhibition of ATP synthesis. The possibility that increased ATP consumption is the cause is unlikely given the low *A* and general loss of metabolic activity.

The rate of ATP synthesis depends on the light reactions, generation of the trans-thylakoid pH gradient (ΔpH), availability of ADP and P_i, and activity of CF. Synthesis of RuBP depends on ATP and NADPH concentration, and on PCR cycle activity, or more specifically on PRK activity, and concentration of the substrates ATP and ribose 5-phosphate. The ATP concentration in unstressed chloroplasts in bright light, which saturates *A*, probably exceeds that required to saturate PRK, but as ATP decreases (e.g. as light intensity falls) the ATP concentration drops and with it RuBP synthesis. Decreased ATP content in leaves at low RWC, and the correlation between ATP content and *A* (Tezara *et al*., 1999), show that ATP was less than that required to achieve the maximum rate of RuBP regeneration. If dependence of RuBP synthesis on ATP follows a Michaelis–Menten response, then RuBP synthesis would only respond at sub-saturating ATP, so the consequences of lower ATP content will depend on the characteristics of PRK.

Further studies (Ortiz-Lopez *et al*., 1991; Wise *et al*., 1990; Ort *et al*., 1994) however concluded that limitation of A_{pot} was not caused by loss of ATP. The effects of water deficits on photophosphorylation were measured *in vivo* with a spectrophotometer, which detects the rapid relaxation of the 518 nm electrochromic signal from carotenoids, located in thylakoids. The signal changes as the pH of the thylakoid lumen alters. In laboratory conditions, the dissipation of ΔpH in stressed leaves in darkness, when CF_1 is oxidized, following the generation of the gradient by light flashes, indicated that CF_1 activity was impaired with an increased energy requirement for activation, and thus ATP synthesis would have been decreased (Ortiz-Lopez *et al*., 1987; Boyer *et al*., 1987). These conditions were similar to those used by Keck and Boyer (1974), and thus confirmed inhibition of photophosphorylation noted in earlier work using different methods under comparable conditions. However, in the field, the effect of drought was not observed as the signal was unaffected when CF_1 was reduced and activated, and so would have permitted ATP synthesis in droughted leaves (Ortiz-Lopez *et al*., 1991). Wise *et al*. (1990) detected no change in the 515 nm signal, and so no inhibition of ATP synthesis in watered and droughted field-grown sunflower. However, *A* decreased substantially during the mid-afternoon when C_i increased, i.e. there was non-stomatal inhibition. Therefore, Wise *et al*. (1990) concluded that *A* was down-regulated (its activity decreased by regulatory processes) as an adaptive response to drought. Such loss of capacity did not cause short- or long-term effects on A_{pot}.

As a result of these studies, the role of ATP has been rejected as a factor in decreasing A_{pot} in stressed plants (Ort *et al*., 1994), yet decreased ATP content, loss of Coupling Factor, etc. suggest otherwise. Thus, there is conflicting evidence regarding the behaviour of ATP synthesis under stress. Resolution of this conflicting evidence and interpretation is essential. Although technical problems (Cornic, 2000) have been suggested to be responsible for the observed changes in ATP content and CF_1 as RWC diminishes (e.g. in Tezara *et al*., 1999), they are unlikely to affect the results substantially (Lawlor and Cornic, 2002). The correlation between decreasing ATP content and decreasing RWC is strong and appears linear (Tezara *et al*., 1999), suggesting loss of ATP synthesis at large RWC. However, it is argued (G. Cornic, pers. comm.) that linearity is spurious, caused by scatter of the data, and that ATP content is unaffected at large RWC. Similarly, the limited number of points for CF_1 cannot provide information about events during the initial loss of RWC. It is thus argued that any decrease in ATP occurs only at small RWC, and as a consequence of metabolic changes induced by low C_i. The spectroscopic measurement of the rapidly decaying 518 nm signal (Wise *et al*., 1990) is accepted as closely related to ATP synthesis, but may not be related closely to the ATP pool. Ideally this method should be compared with biochemical measurements of photophosphorylation, and with ADP, P_i and ATP concentrations in chloroplasts. Also, the amounts, activities and oxidation states of CF_1 under a range of RWCs under different conditions—controlled and field—are required.

Electron transport, O_2 emission and formation of ΔpH. Light harvesting and electron transport are maintained at low RWC. Although there is evidence of damage to the light reactions and photosystems in some studies (e.g. Giardi *et al*., 1996), generally these primary events are largely unaffected by water deficit (Cornic *et al*., 1992), although they may be damaged at unphysiologically low

RWC where photoinhibition occurs (Flexas and Medrano, 2002). The potential rate of electron transport in thylakoids is maintained even at low RWC, based on chlorophyll *a* fluorescence (Cornic and Briantais, 1991), O_2 emission in the O_2 electrode with large CO_2 concentrations and mass spectrometric measurements of O_2 isotope exchange (see Lawlor and Cornic, 2002). Continued O_2 emission at high CO_2 is taken as evidence that 'photosynthesis' (and by analogy, CO_2 assimilation) is not affected by water stress. However, there may be some disparity between CO_2 assimilation and O_2 emission at low RWCs where *A* has virtually ceased (Tezara *et al*., 1999).

Continuation of electron transport is suggested by maintenance of large NAD(P)H content even at low RWC, although, of course, reductant status may be high even when the fluxes are small, providing demand is less than supply. Also, a large ΔpH is maintained at low RWC (Meyer and de Kouchkovsky, 1992) and by the large non-photochemical quenching that depends on ΔpH, so energy is available for ATP synthesis (Haraux and de Kouchkovsky, 1998) at low RWC. Coupling between electron transport and H^+ release in, or transport into, thylakoids is probably not impaired. Thus, decreased photophosphorylation (Keck and Boyer, 1974) and CF activity (Meyer and de Kouchkovsky, 1992) suggest that it is the amount of enzyme or inhibition of activity that limits ATP synthesis. This conclusion should be tempered by the evidence that ΔpH dissipation is unaltered at low RWC (Ortiz-Lopez *et al*., 1991).

Electron transport is determined by the capacity of the sinks, *A*, PR and possibly the Mehler ascorbate peroxidase reaction. At high RWC, most electrons are used in CO_2 assimilation, with only a small proportion being used by PR, and probably fewer or none by the Mehler ascorbate peroxidase reaction (Ruuska *et al*., 1998, 2000). As RWC decreases and *A* and C_i fall, so a larger proportion of the electrons is used in PR and possibly by the Mehler–ascorbate–peroxidase reaction (Biehler and Fock, 1996; Haupt-Herting and Fock, 2002). If PR decreases in absolute magnitude as A_{pot} is inhibited, then it is possible that the Mehler reaction will become more significant. One consequence of reduction of O_2 by the Mehler reaction is generation of oxygen radicals (superoxide, peroxide, etc.), which are very reactive and potentially very damaging to components of the thylakoids, etc. Generation of potentially damaging amounts of these radicals does not necessarily require large electron flux but could be a major cause of a reduction of A_{pot} in stressed leaves over the long term. However, short-term photoinhibition is not important in water-stressed sunflower (Sharp and Boyer, 1986; Tezara *et al*., 1999). Generalizing, electron transport continues under low RWC within the thylakoid, generating ΔpH, reducing acceptors such as NAD(P)H and probably generating oxygen radicals and other reactive compounds within the photosynthesizing cell which are potentially damaging to metabolism.

Triose export from the chloroplast and P_i import. In unstressed leaves, at low O_2 concentrations, *A* reaches a maximum at C_i of about 250–300 μl l^{-1} CO_2, and RuBP is limiting. Increasing C_i further may decrease *A*. This has been attributed to P_i deficiency (see Harley and Sharkey, 1991). Transport of triosephosphate out of the chloroplast, via the triosephosphate translocator in counter-exchange with P_i, is the principal route for P_i transport into the stroma, and so is essential for photophosphorylation, otherwise ATP synthesis and thus RuBP synthesis are impaired (Jacob and Lawlor, 1992). If phosphorylated intermediates are not exported they may be converted to starch, so enabling P_i to be recycled, but this system does not have sufficient capacity to fully alleviate lack of triose export. Thus, accumulation of phosphorylated intermediates leads to P_i deficiency and decreases *A* at very high CO_2. *A* is insensitive to O_2 concentration under these conditions because the regeneration of RuBP is limiting. Similar effects have been suggested to account for the progressive inhibition of A_{pot} as RWC falls. However, the situation may differ. With low *A*, the flux of triosephosphate from chloroplast to cytosol must be small, and there is much less (often no) accumulation of intermediates and starch. Therefore, a shortfall in P_i for ATP and RuBP synthesis is unlikely. Harley and Sharkey (1991) suggested that release of P_i in the chloroplast because of dephosphorylation of PG from the glycolate pathway (see earlier discussion) would allow ATP synthesis if the glycerate from the pathway was not recycled into the chloroplast but consumed outside, e.g. in amino acid synthesis. Under elevated CO_2, this would be a way of overcoming RuBP limitation, and it could operate at low RWC if P_i is deficient, but the likely decrease in absolute rates of PR (see earlier) would limit the capacity of this mechanism. Cells of leaves well supplied with adequate P_i at high RWC might suffer P_i deficiency at low RWC because of altered fluxes of intermediates and P_i. However, there is no evidence for this: too little is known about P_i fluxes and pools in unstressed and stressed cells. Experimental analysis of P_i pools and fluxes in stroma and cytosol is required. Currently, P_i deficiency seems an unlikely cause of decreased A_{pot}, particularly if ATP synthesis is inhibited.

CELLULAR HOMEOSTASIS AND INHIBITION OF ATP SYNTHESIS

If, as I have argued, the failure to synthesize ATP is the fundamental problem for RuBP regeneration, arising from large Mg^{2+} concentration, then many questions inevitably arise. How is Mg^{2+} concentration in the chloroplast stroma regulated, and under what conditions does regulation fail so that the function of CF_1 is impaired? Once availability of ATP limits the ion ATPases which provide the energy for the regulation of cellular ion balance (Sze *et al*., 2001), is metabolic disruption inevitable? Speculation is warranted that the Type 1 and 2 responses relate to the ability of cells to regulate ion (particularly Mg^{2+}) concentrations. Perhaps, as RWC falls, plants with Type 1 response maintain ionic homeostasis so that CF_1 is unaffected, ATP content is unchanged, RuBP is synthesized and A_{pot} continues. In contrast, in the Type 2 response, regulation of ionic homeostasis may fail as RWC decreases, inhibiting CF_1,

then ATP concentration and RuBP synthesis fall, together with A_{pot}. The response to elevated CO_2 would then depend on regulation of ions in the photosynthetic cell. The differences between plant species, or between growth conditions (field *vs*. greenhouse, or nutritional status, for example) or tissue age, might be traced back to such alterations in the cell. Prehistory of the plants is clearly important, for 'hardening' occurs if stress has been experienced. The adaptations in mechanisms responsible are largely unknown, but may protect ATP and RuBP synthesis. In Fig. 5, theoretical relationships between different components of the system determining RuBP concentration and *A* are depicted. A difference in the regulation of Mg^{2+} concentration in the chloroplast (Fig. 5A), when related to the dependence of CF_1-CF_0 activity on Mg^{2+} concentration (Fig. 5B), and the relationship between RuBP synthesis and ATP concentration (Fig. 5C), and between *A* and RuBP (Fig. 5D), will produce the Type 1 and 2 responses shown in Fig. 1A. Experimental examination of such concepts, in the framework of a model of the system, may help progress understanding of regulation of photosynthesis under drought.

IMPACTS OF DECREASED ATP SYNTHESIS ON METABOLISM

It is a truism that ATP plays a vital role in metabolism. Imbalance between ATP and NAD(P)H synthesis may be a fundamental cause of changes in metabolism generally regarded as 'stress responses'. In the case of ATP, the multiple mechanisms of synthesis, pools and uses will obscure causal relationships, and correlations may be relatively weak. Many changes in metabolite concentrations occurring in tissues with low RWC are not well understood in terms of conditions in cells or mechanisms responsible. Here, links between accumulation of 'stress metabolites', a fundamental feature of leaves at low RWC, and photosynthesis are explored.

Amino acids

Accumulation of amino acids, particularly glycine, serine, glutamate and proline (Samaras *et al*., 1995; Lawlor and Cornic, 2002) provides a case study of how metabolism is integrated, regulated and modified in stressed photosynthetic tissues. The case of citrulline is discussed by Yokota (2002). Amino acid synthesis in photosynthesizing cells involves both the chloroplasts and mitochondria, and carbon and nitrogen metabolism. Reduction of nitrate ions by nitrate reductase (NR) forms nitrite, which is further reduced to ammonia by nitrite reductase; the reductant is from photosynthetic electron transport. The ammonia formed is assimilated by the glutamine synthase–glutamate synthase (GS-GOGAT) reactions in which glutamine is formed from glutamate and ammonia, and the glutamine then reacts with 2-oxoglutarate to form glutamate. The reaction requires ATP so, when ATP is limited, assimilation of ammonia by GS-GOGAT is likely to be slowed or inhibited; this has not been examined. However, as mentioned in the Introduction, NR activity is inhibited at low RWC (Kaiser, 1987; Kaiser and Foster, 1989) so ammonia production would be much decreased or stopped. Where does the ammonia required for the increased pools of amino acids come from? Protein degradation and catabolic reactions are possible. What route is used for glutamate synthesis if ammonia is available but GS-GOGAT is inactivated by the lack of ATP? An alternative route of glutamate synthesis would be via glutamate dehydrogenase (GDH), an abundant enzyme linked with catabolic activities (e.g. protein degradation), and associated with mitochondria. GDH forms glutamate by reacting ammonia with 2-oxoglutarate produced by the tricarboxylic acid (TCA) cycle and consuming NAD(P)H. TCA cycle activity in the mitochondria produces organic acids, deriving the carbon from sucrose or storage carbohydrates, which are consumed in severely stressed leaves. The TCA cycle is coupled to respiratory electron transport and ATP synthesis. It is probably more important relative to *A* in stressed cells, as 'day' or dark respiration continue at relatively constant rates when *A* is small. Possibly the GDH reaction becomes more important as RWC drops because glutamate accumulates (Lawlor and Fock, 1977), particularly when A_{pot} is very small.

Glycine and serine synthesis is directly, but not exclusively, related to photorespiration, as shown by the rapid incorporation of $^{14}CO_2$ in photosynthesis. Accumulation of glycine and serine at low RWC occurs at low A_{pot} and the PR/*A* ratio is large (Lawlor, 1976; Lawlor and Fock, 1977). However, accumulation of glycine and serine occurs when PR is not increased in absolute terms, and probably results from changes in regulation within the glycolate pathway, and decreased use, e.g. in synthetic processes.

Proline concentrations increase many-fold at low RWC, where A_{pot} approaches zero but day respiration continues. Proline is derived from glutamate (which, as discussed, accumulates and is not derived from recently formed carbohydrates or, probably, NH_4^+); the reactions require ATP and NADPH (Morot-Gaudry *et al*., 2001). Synthesis is also from ornithine, although it may be a minor pathway: it does not require ATP or NADPH. If redox components are very reduced at low RWC, as shown earlier, this may enhance or even trigger proline formation from glutamate as enzymes of the pathway are induced although the mechanisms are not known (Kishor *et al*., 1995; Morot-Gaudry, 2001). The requirement for ATP makes synthesis from glutamate less likely. However, ATP content is not zero, even at low RWC when A_{pot} approaches zero. ATP synthesis in mitochondria is coupled to electron transport to O_2 (Siedow and Umbach, 1995; Møller, 2001) so ATP may be synthesized close to the site of proline synthesis. Proline synthesis and accumulation illustrate the potential links between photosynthetic processes and other aspects of metabolism, and how the changes in cellular conditions may alter the composition of tissues. Examination of these links, to test the validity, is required.

Accumulation of amino acids, including proline, is a damage response from the perspective of altered photosynthetic metabolism. The protective function of glutamate and proline at low RWC is then negligible, and does not constitute an evolved process with clear benefits. Such a

view is supported by accumulation occurring predominantly at very low RWC in mesophytes: if it were protective then it should appear very early in stress development. However, a more common rationale for accumulation of amino acids, especially proline, is that it confers advantages, protecting membranes, proteins, etc. when RWC decreases, particularly against increasing ionic concentrations. Amino acids may also be a source of C and N for metabolism following rehydration, although the amounts are relatively small. Advantages are documented, e.g. in bacteria and for plant membranes *in vitro*, and increased proline accumulation in genetically altered plants confers osmotolerance (Kishor *et al*., 1995) although the mechanisms have been disputed (see Blum *et al*., 1996). These views are not mutually exclusive: rapid stress may cause a damage response, whereas slower stress development may alter metabolism sufficiently to be advantageous and so delay, decrease or prevent damage to metabolism. A model based on changes in photosynthetic metabolism at low RWC provides testable hypotheses about the factors regulating glutamate and proline synthesis.

Protein synthesis. At low RWC the amount of many proteins is decreased, probably due to repressed synthesis or increased turnover, caused by an incorrect environment. Increased ionic concentration may be important. Polysomes are lost or not formed, and incomplete and improperly folded proteins are made. Loss of sucrose phosphate synthase activity at low RWC was mentioned in the Introduction. Native SPS is a relatively unstable dimeric protein; perhaps the changed cellular environment, e.g. increased ionic concentration, causes instability or increased amino acids inhibit activity (Huber and Huber, 1996). Also, altered protein turnover might be responsible for loss of activity in the long term (Vassey *et al*., 1991). Loss of activity has been ascribed to slow A caused by low C_i, as it is reversed by elevated CO_2 and is simulated in well-watered plants by low C_i (Vassey *et al*., 1991). SPS is active when a regulatory site at serine-158 is dephosphorylated (Huber and Huber, 1996), so reduced ATP concentration at low RWC would not explain the low activity and reversal by elevated CO_2. Other analyses show that osmotic stress increases activity of SPS from spinach, although it is probably not caused by dephosphorylation but by phosphorylation of another site which regulates serine-158. The structure and regulation of SPS are so complex that many factors could affect its activity *in vivo* (Huber and Huber, 1996). Similar considerations apply to loss of nitrate reductase activity in stressed leaves (Kaiser and Foster, 1989). Therefore, no satisfactory, unified model of the factors controlling SPS and NR activity is possible.

Many polypeptides and proteins accumulate, and some may be induced. Such *de novo* synthesis under stress is regarded as being of great significance for adaptation of cellular functions to dehydration (although induction under stress may mean that the proteins are not normally detectable, e.g. by two-dimensional gel analysis and silver-staining, but increase so substantially that they are easily detected). Accumulation of dehydrins, for example, is considered to protect metabolic functions at low RWC (Close *et al*., 1993), as judged by enhanced plant performance under stress conditions. Aquaporins are proteins integral to cell membranes which facilitate diffusion of water (and also other small molecules) into the cell. Their functions are regulated by metabolic processes, such as reversible phosphorylation, which depend on apoplastic water potential (Maurel and Chrispeels, 2001): this raises the possibility that ATP concentration is involved, and would link factors regulating water balance with metabolism. Some proteins induced by low RWC are also induced by ABA (Bray, 2002), e.g. RAB17 which may be part of nuclear protein transport, and ASR(s714) may function in maintenance of nucleic acid structure. Some enzymes involved in important metabolic pathways are induced: enolase and NAD-malate dehydrogenase are involved in glycolysis, TCA cycle and the oxidative pentose phosphate pathway. Importantly, from the aspect of this review examining the role of ATP under stress conditions, Umeda *et al*. (1994) observed induction of some enzymes of ATP-generating reactions, including triose phosphate isomerase. Riccardi *et al*. (1998) point out the importance of maintaining cellular homeostasis, emphasizing the role of such proteins in energy production.

Production of correctly structured proteins and their maintenance is clearly of great importance for efficient cellular function. The concept that low RWC impairs protein structure explains the importance of molecular chaperones, which accumulate under a range of stresses, including drought. Chaperones have an important role in the folding and assembly of proteins during synthesis, and in the removal and disposal of non-functional and damaged proteins. Production of chaperones, best analysed in the case of heat stress (Schöffl *et al*., 1998), involves a cascade of regulated events, with heat shock transcription factor conveying changes in cellular metabolism to the transcriptional machinery, possibly through decreased phosphorylation. ATP is essential in several aspects of chaperone function, e.g. low ATP stimulates production of heat shock proteins. Unsurprisingly, therefore, interruption of ATP synthesis by chemicals, heat, etc. (Wiengarden *et al*., 1996) prevents protein folding and triggers the synthesis and accumulation of chaperones. Water stress, operating via ATP supply, may trigger chaperone synthesis.

I suggest that decreased ATP under low RWC impairs protein synthesis, through inadequate energy supply, but may increase some types of proteins, e.g. molecular chaperones, because their production is regulated in different ways. Changes in proteins under stress conditions may relate to the effects of altered cellular conditions on gene transcription, translation and post-translational modification of proteins. However, specific conditions in cells and their impact on such basic processes are poorly understood. The central role of ATP in the regulation of metabolism has been explored in other areas of biology. Adaptation of bacterial and animal cells to dormancy, adjustment to adverse conditions, etc. involves reduction of the metabolic rate, and often almost complete cessation of metabolism. This is achieved by regulation of the rate of ATP synthesis and the corresponding adjustments in all parts of metabolism (see

Storey, 2001). Such studies have not been pursued with higher plants under drought stress. More attention has been paid to gene expression and describing changes in proteins as a consequence of decreased RWC and ABA applications (Bray, 2002). The role of ATP in adaptation is likely to be important and should not be neglected.

CONCLUSION ON FACTORS DETERMINING A_{POT} WITH DECREASING RWC

Limitation of CO_2 supply by reduced g_s is responsible for the lower rate of A resulting from a small loss of RWC in probably all plants under the current CO_2 concentration. However, the response of A_{pot} to RWC differs between studies. A Type 1 response is identified in which low g_s causes C_i to fall, possibly to the compensation point, and elevated C_i restores A to A_{pot}. Only after substantial loss of RWC is metabolism and thus A_{pot} impaired. In a Type 2 response, C_i also decreases with initial loss of RWC but A_{pot} and metabolism are progressively impaired as RWC falls, and elevated C_i cannot fully restore A_{pot}. In both responses, C_i rises again at low RWC and the equilibrium CO_2 compensation point increases greatly due to non-photorespiratory CO_2 evolution. Metabolic limitation of A_{pot} is caused by decreased RuBP synthesis, not primarily by loss of Rubisco or other PCR cycle enzymes. RuBP synthesis is limited by impaired ATP synthesis due to alterations in catalytic ability or possibly the amount of CF caused by altered ionic concentrations, specifically Mg^{2+}. Electron transport potential, generation of NADPH and the ΔpH gradient are little affected by stress, and are certainly less affected than A_{pot}. Loss of A_{pot} decreases PR as well as A, so that sinks for electrons are limited, and non-photochemical quenching of excitation energy increases. Due to low A_{pot}, non-photorespiratory CO_2 release becomes relatively large with decreasing RWC, leading to increased C_i and equilibrium compensation point. Limited synthesis of ATP and a large reductant state alters cell metabolism, including ionic balance, protein synthesis and accumulation of 'stress metabolites', such as amino acids.

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