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Analysis of the Control of Photosynthesis in C<latex>$_4$</latex> Plants by Changes in Light and Carbon Dioxide [and Discussion]

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Analysis of the control of photosynthesis in C_4 plants by changes in light and carbon dioxide

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Parallel measurements of contents of photosynthetic intermediates, activities of enzymes of photosynthetic carbon assimilation, gas-exchange rates and components of chlorophyll-fluorescence quenching in leaves of C_4 plants are considered in relation to changes in photon flux density (PFD) and CO_2. The influence of varying light and CO_2 concentration upon changes in the amounts of phosphoenolpyruvate (PEP) in leaves of C_4 plants during steady-state photosynthesis are interpreted in terms of the regulatory properties of PEP carboxylase and in terms of feedback interactions between the Calvin cycle and the C_4 cycle. Relations between electron transport and carbon assimilation are discussed in terms of the regulation of the supply of ATP and NADPH and the demands of carbon assimilation. In low light these relations differ in C_3 and C_4 plants. The lag in photosynthetic carbon assimilation in maize that follows a decrease in PFD has been analysed. The changes that occur in enzyme activities, metabolites and components of chlorophyll-fluorescence quenching following the transition from high to low light indicate that diminished production of ATP and NADPH is responsible for the lag in photosynthetic carbon assimilation and may reflect a stimulation of cyclic electron flow to make up a deficit in ATP.

Introduction

Changes in the rate of photosynthetic carbon assimilation that are occasioned by changes in environmental factors such as light, temperature and CO_2 concentration result in changes in the factors that limit photosynthesis and require a redistribution of control between the reactions of electron transport, CO_2 assimilation and product synthesis. At the level of the intact leaf as complete a picture as possible of the behaviour of these components is required if we are to establish how and where regulation occurs during steady-state photosynthesis and during rapid transitions in photon flux density (PFD) and CO_2 concentration. Regulation in intact leaves may be investigated by using intrusive, or destructive, techniques, such as measurement of metabolites, enzyme activities and photosynthetic products, as well as by non-intrusive techniques such as analysis of gas exchange, chlorophyll fluorescence and by the use of spectroscopic methods. We can also extrapolate to the intact leaf from our knowledge of the behaviour of isolated organelles, thylakoids and enzymes.

This paper indicates some of the information that metabolite and enzyme measurements in leaves and in isolated intact chloroplasts can offer to an understanding of function of leaves of C_4 plants *in vivo*, and illustrates how these measurements can not only shed light upon the regulation of carbon assimilation but also upon the regulation of electron transport.

Some principles of metabolite analysis and interpretation

Current studies of the regulation of metabolism in plants are concerned with qualitative and mechanistic approaches, in asking how metabolism is controlled and at which points control is exercised. These form the basis of the considerations presented here. However, those who study photosynthesis have an intrinsic advantage in that they are able to manipulate fluxes readily by changes in light and CO_2 concentration. When coupled with an increasingly sophisticated description of the intracellular and intercellular compartmentation of metabolites and the advent of transgenic plants, the study of photosynthesis can begin to be concerned with asking quantitative questions about control, by asking how much a particular enzyme contributes to the control of flux (Kacser & Burns 1973).

(*a*) *Methods*

Most of the metabolite pools involved in photosynthetic carbon assimilation in leaves of C_3 and C_4 plants turn over in a matter of seconds. Many of the metabolites are also rather labile. Metabolite analysis must therefore be accompanied by a rigorous technique. First, evidence of rapid cessation of metabolism must be provided (ap Rees 1974). In leaves, as in other tissues, this can be achieved by freeze-clamping at the temperature of liquid N_2. A number of devices have been designed for freeze-clamping illuminated leaves (see, for example, Badger *et al.* 1984). All must incorporate gas-exchange chambers with windows of parafilm or kitchen film that can be disrupted by copper rods cooled to the temperature of liquid N_2. The leaf chamber design employed in Sheffield (a modified version of the chamber developed by Harris *et al.* (1983)) includes facilities for temperature measurement and efficient temperature control (down to less than 5 °C) for intact leaves and for systems in which water or other substances such as P_i or glycerol are fed to the leaf via the transpiration stream. The freeze-clamping device is solenoid driven. Illumination is provided by a bifurcated optical fibre, which then allows for movement of the chamber relative to the freeze-clamping device. Chlorophyll fluorescence can be detected with a fibre-optic system from the underside of the chamber by using the Walz pulse-modulated system (H. Walz, Effeltrich, F.R.G.). After the leaf has been freeze-clamped, evidence that killing, extraction and measurement techniques are adequate must be shown by satisfactory recoveries of metabolites added in amounts comparable to those present in the tissue (ap Rees 1974). Evidence must be presented not only for leaves from different plants, but also for radically different developmental stages, as older plants or stressed plants may accumulate large amounts of phenolics, tannins etc. that can interfere with metabolite analysis.

Measurement of amounts of metabolites and estimation of their *in vivo* concentrations permits comparison with *in vitro* data on the properties of enzymes in respect of their sensitivity to substrates and effector molecules. To provide an adequate description we need to know the intercellular and intracellular distribution of metabolites and the volumes of the relevant compartments. Various fractionation procedures have been developed; the fractionation of leaf protoplasts into chloroplastic and extrachloroplastic fractions, for example (Robinson & Walker 1979; Giersch *et al.* 1980; Stitt *et al.* 1980). Intact leaves may be fractionated by non-aqueous procedures into various subcellular fractions (Santarius & Heber 1965; Gerhardt *et al.* 1987), and procedures for rapid separation of bundle-sheath from mesophyll cells have been developed for maize (Leegood 1985; Stitt & Heldt 1985*a*).

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(*b*) *Identification of sites of regulation*

A number of approaches may be used in the identification of regulatory reactions. These are discussed by Denton & Pogson (1972) and Newsholme & Start (1973) and focus on three types of analysis.

(i) Comparison of the mass–action ratio, from *in vivo* contents of metabolites, with the equilibrium constant, K'. This method can be used to identify reactions that are removed from equilibrium *in vivo* and are likely to be regulatory. Examples of the application of this method in plants are the identification of regulatory reactions in gluconeogenesis (Leegood & ap Rees 1978) and in the pathway of sucrose synthesis in leaves (Heldt & Stitt 1987).

(ii) Studies of the properties of enzymes and reactions to identify reactions that are non-equilibrium (although this does not mean that enzymes that catalyse equilibrium reactions are not regulatory), those enzymes which have regulatory properties and those that have maximum catalytic activities of the same order of magnitude as the fluxes they catalyse.

(iii) Measurement of changes in flux and their relation to changes in metabolites. The change in the substrate concentration alone is sufficient for identification of a regulatory reaction, i.e. it is necessary to observe that the substrate changes in a direction opposite to that of the flux. The fact that the substrate changes in this way does not necessarily lead to the conclusion that the enzyme is regulatory if other substrates or cofactors are involved. Thus glycerate 3-phosphate (PGA) may rise as the photosynthetic flux decreases, but this may merely mean that ATP and NADPH are in short supply (Dietz & Heber 1984). The crossover theorem of Chance & Williams (1956) is not strictly applicable to metabolic systems. The change that occurs in the product concentration is superfluous because it may change quite independently of the reaction under study (Denton & Pogson 1972; Newsholme & Start 1973). Changes in metabolite ratios have been employed in some recent work to detect regulatory reactions. The use of fructose 1,6-bisphosphate/fructose 6-phosphate (FBP/F6P) ratios (Dietz & Heber 1984; Kobza & Edwards 1987*a*, *b*) and ribulose 1,5-bisphosphate/3-phosphoglycerate (RuBP/PGA) ratios (Usuda 1987*b*; Kobza & Edwards 1987*a*, *b*) requires clarification. For the reaction catalysed by fructose 1,6-bisphosphatase (FBPase), all that need be considered is the concentration of FBP, as discussed above. The same applies to the ratio RuBP/PGA in that the concentration of PGA can, in theory, vary quite independently of the concentration of RuBP (e.g. it can be converted to phosphoenolpyruvate (PEP) and/or transported to the mesophyll in C_4 plants). However, there may be an added justification for using the ratio RuBP/PGA as an indicator (indeed as an amplifier) of regulation by the reactions that regenerate RuBP, because factors that decrease the RuBP pool also often lead to an increase in PGA. In low CO_2, for example, RuBP increases whereas PGA declines, and during P_i limitation *in vivo* RuBP declines and PGA increases (Leegood & Furbank 1986; Sharkey *et al.* 1986). However, these relations are not necessarily of universal application.

Metabolite ratios can serve two further purposes. First, they can be used to indicate the compartmentation of metabolites such as hexose phosphate. If the ratio of glucose 6-phosphate/fructose 6-phosphate (G6P/F6P) is around 4–5, this indicates a cytoplasmic location, whereas ratios around 1–2 indicate a chloroplastic location and a predominance of starch synthesis (Stitt *et al.* 1987). Secondly, metabolite ratios can be employed to estimate other intracellular metabolite concentrations or ratios. Thus if it is assumed that lactate dehydrogenase catalyses a reaction that is close to equilibrium ($K' = 1.11 \times 10^{-4}$), then the

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cytoplasmic NADH/NAD^+ ratio in animal tissues can be calculated indirectly from measurements of the amounts of pyruvate and lactate (Williamson *et al.* 1967; Newsholme & Start 1973). Thus $[NAD^+]/[NADH] = [\text{pyruvate}]/[\text{lactate}] \times K'_{LDH}$, where K'_{LDH} is the equilibrium constant for lactate dehydrogenase. In a similar manner the mitochondrial NADH/NAD^+ ratio may be determined by the ratio of β-hydroxybutyrate to acetoacetate.

Analysis of this type is limited in leaves because lactate dehydrogenase is absent, and no other suitable enzyme reaction has yet been found with which to estimate the cytoplasmic NADH/NAD^+ ratio (e.g. glycerol phosphate dehydrogenase is present both in chloroplasts and cytoplasm and appears to be regulated (Leegood *et al.* 1988)). On the other hand, the phosphorylation potential is as difficult to estimate as are free NADH and NAD^+, partly because of the presence of a large vacuole in leaf cells that precludes accurate estimation of the cytoplasmic P_i concentration. Heber *et al.* (1986, 1987) have therefore developed the concept of assimilatory force. Electron transport provides ATP and NADPH for the reduction of PGA to triose phosphate. The ratio between the amounts of triose phosphate and PGA can be taken as an indication of the strength of this driving force provided by electron transport, the assimilatory force (F_A). If the reactions catalysed by PGA kinase, glyceraldehyde phosphate dehydrogenase and aldolase approach equilibrium under all flux conditions, as they have been shown to do in chloroplasts isolated by non-aqueous means from leaves (Dietz & Heber 1984; Prinsley *et al.* 1986*b*), then

$$([\text{PGA}]\,[\text{ATP}]\,[H^+][\text{NADH}])/([\text{triose phosphate}]\,[P_i]\,[\text{ADP}]\,[NAD^+]) = K' = 9.8 \times 10^{-6}.$$

The assimilatory force is given by

$$[\text{ATP}]\,[\text{NAD(P)H}]/([\text{ADP}]\,[P_i]\,[\text{NA(D)P}^+]) =$$

$$9.8[\text{triose phosphate}] \times 10^{-6}/([\text{PGA}]\,[H^+]) = F_A.$$

This approach has a number of potential objections, perhaps the most important being the possibility that stromal H^+ concentration may change substantially in photosynthetic systems. However, Heber *et al.* (1986) have argued that the stromal pH saturates at rather low PFD and that under most circumstances it will be relatively constant.

(*c*) *Total amounts of metabolites*

A knowledge of the total amounts of different classes of metabolites can provide important information. In C_4 plants, such as *Amaranthus edulis*, the pool of aspartate is photosynthetically active, in contrast to the malate pool in maize, and the C_4 cycle as a whole constitutes a pool that is metabolically connected to other metabolic processes, including the Calvin cycle, via the interconversion of PGA and PEP (figure 1). The sum of the intermediates can be used to infer what factors regulate the activity of the C_4 cycle and its interaction with the Calvin cycle (Leegood & von Caemmerer 1988).

A knowledge of total amounts of phosphorylated intermediates (i.e. all glycolytic intermediates, plus intermediates of the Calvin cycle and adenylates) can be employed to study the phosphate status of the extravacuolar compartments, and in the short term to estimate changes that occur in the amount of P_i in these compartments, as transport of P_i out of the vacuole is very slow (Bligny *et al.* 1989). Thus Sharkey *et al.* (1986) have shown that the cytoplasmic P_i concentration might be decreased by as much as 4 mM following the transition from 20% to 2% O_2. Work in Sheffield shows that acclimation to low temperature involves

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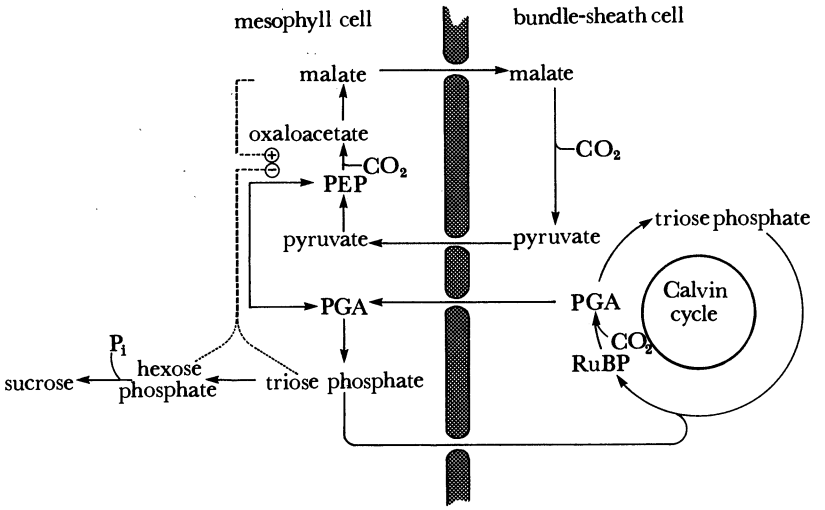

FIGURE 1. Scheme for photosynthetic carbon assimilation in an NADP-malic enzyme type C_4 plant such as maize, showing the C_4 cycle and the nature of some of its interactions with the Calvin cycle. Note that PGA can be converted to PEP via 2-phosphoglycerate (not shown) and that hexose phosphate and triose phosphate act as activators of PEP carboxylase, whereas C4 acids act as inhibitors.

changes in phosphate status that are accompanied by a large increase in total phosphorylated intermediates, suggesting efflux of P_i from the vacuole in response to lowered temperature (data not shown). Similarly, feeding a phosphate-sequestering agent such as glycerol or choline induces a rapid loss of P_i from the cytoplasm and a corresponding accumulation of, for example, glycerol 3-phosphate (Bligny *et al.* 1989; Leegood *et al.* 1988).

REGULATION OF C_4 PHOTOSYNTHESIS IN RELATION TO CHANGES IN IRRADIANCE AND CO_2 CONCENTRATION

Coordination of the rate at which the Calvin and C_4 cycles fix CO_2 is necessary if photosynthesis is to proceed efficiently under different environmental conditions. This could occur in a variety of ways. First, in $NADP^+$-malic enzyme plants such as maize the C_4 cycle is obligatorily coupled to the Calvin cycle, because NADPH generated in the reaction catalysed by $NADP^+$-malic enzyme is reoxidized in the bundle-sheath chloroplast by the reduction of PGA. This does not occur in aspartate formers such as the NAD^+-malic enzyme plant *Amaranthus edulis*, in which no reducing power is exported from the mesophyll to the bundle sheath. Secondly, electron transport in the mesophyll chloroplasts not only powers conversion of pyruvate to malate in the C_4 cycle but also drives PGA reduction. Thirdly, interconversion of PGA and PEP, catalysed by phosphoglycerate mutase and enolase, occurs in the mesophyll and results in metabolic communication between the C_4 cycle and the Calvin cycle (Furbank & Leegood 1984) (figure 1). This is exemplified by a strong positive correlation between the pools of PGA and PEP in leaves of maize and *Amaranthus edulis* (Leegood & von Caemmerer 1988). Fourthly, products of PGA exported from the Calvin cycle, such as triose phosphate and ultimately hexose phosphate, act as positive effectors of PEP carboxylase (figure 1). Both can relieve inhibition of the activity of the enzyme by C4 acids (Doncaster & Leegood (1987), and references therein). Modulation of the activity of PEP carboxylase by triose phosphate and hexose phosphate will undoubtedly be important in determining PEP carboxylase activity in

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response to the supply of PGA (which can be considered to be the metabolite 'message' from the Calvin cycle) and to the rate of triose phosphate utilization by sucrose synthesis in the mesophyll cytosol and to triose phosphase consumption in the Calvin cycle. In leaves of maize and *Amaranthus edulis*, the amount of triose phosphate is always closely related to the assimilation rate whether the flux is changed by alterations in PFD, CO_2 concentration or temperature (Usuda 1987*a*, *b*; Usuda *et al.* 1987; Leegood & von Caemmerer 1988).

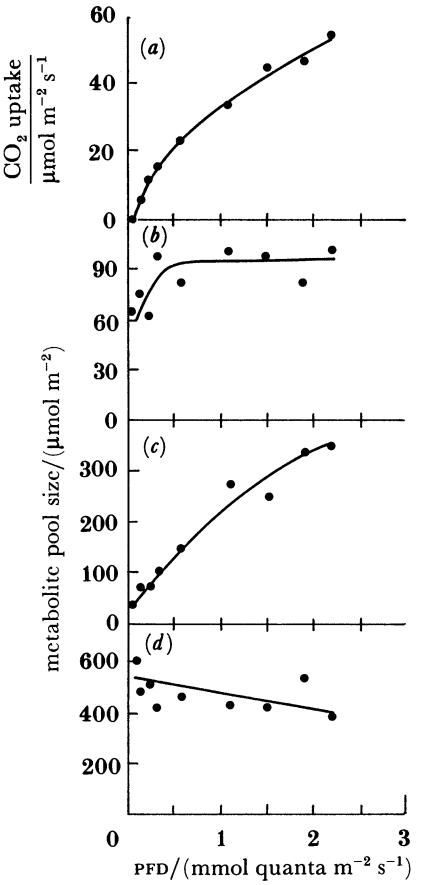

FIGURE 2. Influence of PFD on (*a*) the rate of CO_2 assimilation and the amounts of (*b*) PEP, (*c*) triose phosphate and (*d*) aspartate in leaves of *Amaranthus edulis* during steady-state photosynthesis. The temperature was 28 °C and the attached leaves were illuminated in ambient CO_2. From Leegood & von Caemmerer (1988).

Figures 2 and 3 illustrate some of the metabolic changes that reflect coordinated metabolism during changes in light and concentration of CO_2. Coordination of the C_4 cycle with the Calvin cycle during photosynthesis is achieved by modulation of PEP carboxylase activity by light and by metabolites. PEP carboxylase from maize shows a light-dependent decrease in $S_{0.5}$ (PEP) (the substrate concentration that gives half V_{max}), from 2.25 to 1.6 mM, and a decreased sensitivity to inhibition by malate ($I_{0.5}$ increases from 5 to 12 mM) (Doncaster & Leegood 1987). The data in figure 2 demonstrate the stability of the pool of PEP observed in leaves of *Amaranthus edulis* in relation to PFD. It is clear that the PEP pool is maintained despite a large decrease in the flux. This can only be achieved by modulation of the activity of the enzyme. It is possible that this change is entirely brought about by the changes that occur in the $S_{0.5}$ (PEP). However, figure 2 illustrates that rates of PEP carboxylation may also be regulated

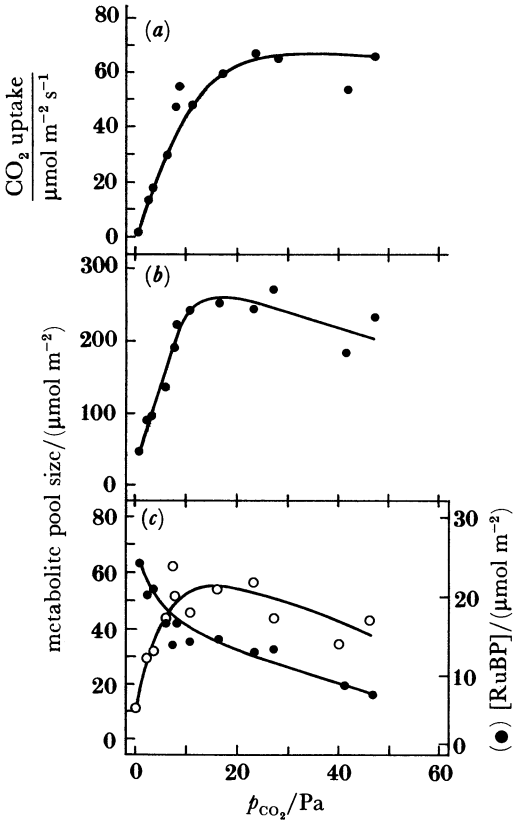

FIGURE 3. Influence of the intercellular p_{CO_2} upon (*a*) the rate of CO_2 assimilation, and the amounts of (*b*) PGA and (*c*) PEP and RuBP in leaves of *Zea mays*. The temperature was 28 °C and the PFD 1600 μmol quanta m^{-2} s^{-1}. From R. C. Leegood & S. von Caemmerer (unpublished data).

both by stability of the aspartate pool, which inhibits activity of the enzyme, and by decreases in the levels of the activators, triose phosphate and hexose phosphate (figure 2).

Figure 3 shows the relation between the assimilation rate and photosynthetic intermediates in maize as a function of the intercellular concentration of CO_2. It is apparent that, together with the amount of HCO_3^-, the concentration of PEP *co-limits* the rate of photosynthesis at low intercellular concentrations of CO_2. It is known that PEP is largely confined to the mesophyll cells (Leegood 1985; Stitt & Heldt 1985*a*) and that about 50% of the PEP pool is cytoplasmic (Usuda 1988). At low CO_2 concentrations the pool of PEP was 20 μmol m^{-2}, corresponding to a cytoplasmic concentration of 1 mM, which can be compared with an $S_{0.5}$ (PEP) of PEP carboxylase from illuminated leaves of 1.6 mM in the absence of metabolite effectors and of between 1 and 10 mM in a simulated cytoplasmic environment (Doncaster & Leegood 1987). As the assimilation rate increases with increasing CO_2 concentration the pool of RuBP falls, but the amount of PEP (and metabolites of the C_4 cycle) actually rises (see also Leegood & von Caemmerer 1988). This behaviour is unexpected in view of the limitation of PEP carboxylase by both its substrates and suggests that a feedback loop from the Calvin cycle is operative *in vivo*. The simplest explanation is that the amount of PEP rises when the assimilation rate rises because the amount of PGA exported to the mesophyll increases (figure 1).

Some relations between electron transport and carbon assimilation in C_4 plants

Electron transport provides ATP and NADPH for the assimilation of CO_2. Conversely CO_2 assimilation can be regarded as consuming that assimilatory force (Heber *et al.* 1987). To understand the interactions between electron transport (supply) and CO_2 assimilation (demand), we need to be able to measure aspects of electron transport *in vivo*, including the redox state of the various components and the degree of energization of the thylakoid membrane, either by direct measurements of ΔpH (which is possible only in isolated chloroplasts and thylakoids) or by estimating energization from light-scattering measurements, from the electrochromic shift (A_{518}) (Crowther *et al.* 1983), or from the non-photochemical component of chlorophyll fluorescence quenching (Krause *et al.* 1982, 1983). There are, however, additional metabolic probes. The first is the measurement of $NADP^+$-dependent malate dehydrogenase activity as a probe of the redox state of thioredoxin, and hence of ferredoxin, in photosystem I. In many instances there is an excellent correlation during steady-state photosynthesis between the redox state of Q_A and the activation state of malate dehydrogenase (Leegood & Walker 1983; Scheibe & Stitt 1988). The second is the use of PGA/triose phosphate ratios as a probe of the supply of assimilatory force, as discussed above. In maize the pyruvate pool is a third metabolic probe of demand. Isolated maize mesophyll chloroplasts are capable of reducing oxaloacetic acid (OAA) to malate (at the expense of NADPH), PGA to triose phosphate (at the expense of ATP and NADPH) and pyruvate to PEP (at the sole expense of ATP).

Figure 4 illustrates the response of the electrochromic shift (A_{518}) in maize mesophyll chloroplasts to the addition of pyruvate (Crowther *et al.* 1983). The enhanced electrogenic slow phase ($A_{518,s}$) indicates rapid turnover of the cyclic pathway of electron flow. The uncoupler, nigericin, then increases the rise rate and amplitude of $A_{518,s}$, as would be expected by relief of a restriction of ΔpH upon cyclic electron flow in the presence of pyruvate alone. During pyruvate conversion by intact chloroplasts, both plastoquinone and Q_A are maintained in a highly reduced state, as indicated by chlorophyll fluorescence induction studies and by the

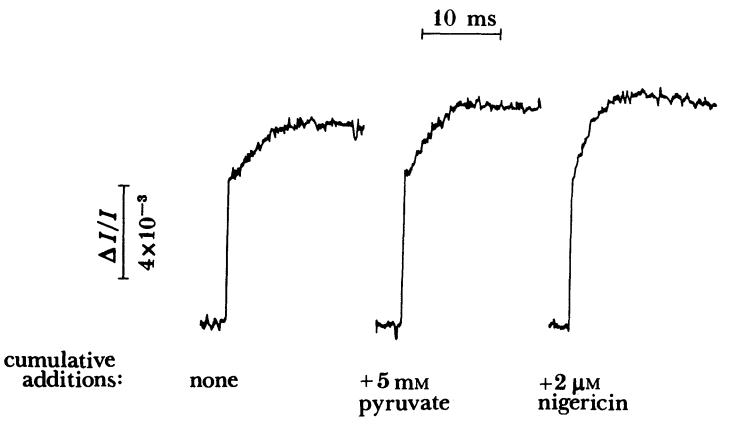

Figure 4. Flash-induced absorption changes (I = amount of light absorbed) at 518 nm in maize mesophyll chloroplasts under aerobic conditions. Additions to the sample were made as shown. Flash-induced absorption changes were made as described by Leegood *et al.* (1983). The actinic flash had a duration of 4 μs at half amplitude. The flash frequency was 2 Hz. Traces represent an average of 128 records. From Crowther *et al.* (1983).

effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) addition upon steady-state chlorophyll fluorescence (Leegood & Walker 1983; Leegood *et al.* 1983) and ΔpH, as indicated by the extent of non-photochemical quenching and by 9-aminoacridine fluorescence, is low (Leegood & Walker 1983; Leegood *et al.* 1983). The ATP/ADP ratio is also drastically reduced in chloroplasts metabolizing pyruvate (Fernyhough *et al.* 1983).

It is important to note that in C_4 plants the part of the C_4 cycle that operates within the mesophyll is catalysed by reactions that are regulated directly by changes in energy supply, i.e. pyruvate, P_i dikinase by phosphorylation in response to changes in adenylates (Burnell & Hatch 1985), PEP carboxylase by triose phosphate and PEP (Doncaster & Leegood 1987) and possibly phosphorylation (Nimmo *et al.* 1987) and $NADP^+$-malate dehydrogenase by $NADP^+$ (in addition to thioredoxin) (Ashton & Hatch 1983). The levels of all these cofactors and metabolites are directly dependent upon the supply of ATP and NADPH. This raises the possibility that electron transport-mediated changes in such effectors may be as influential in C_4 plants as the thioredoxin system is in C_3 plants in modulation of the activity of CO_2-fixing enzymes.

(*a*) *Regulation in response to changes in* CO_2 *concentration*

Figure 5 illustrates the influence of the intercellular partial pressure of CO_2 (p_{CO_2}) upon the amount of FBP, the ratio of triose phosphate to PGA, $1-q_Q$ (photochemical quenching), non-photochemical quenching and the activity of $NADP^+$-dependent malate dehydrogenase in

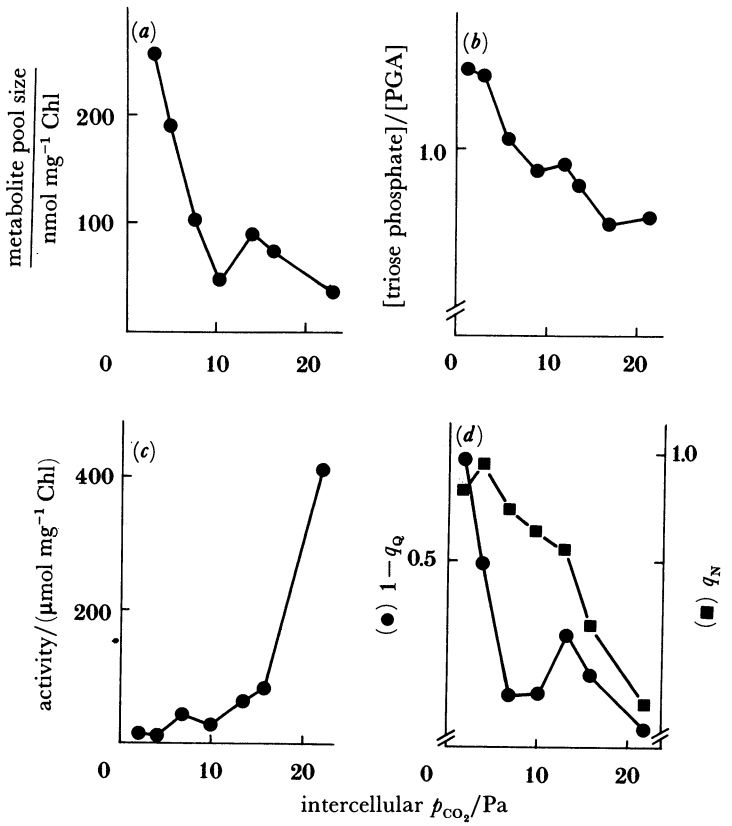

FIGURE 5. Influence of the intercellular p_{CO_2} upon (*a*) the amount of FBP, (*b*) the ratio of triose phosphate to PGA, (*c*) the activity of $NADP^+$-dependent malate dehydrogenase and (*d*) $1-q_Q$ and non-photochemical quenching (q_N) in leaves of *Zea mays*. The temperature was 25 °C and the PFD 1000 μmol quanta $m^{-2}\ s^{-1}$.

leaves of maize. C_4 plants such as maize are a particularly suitable system for such studies because of the abrupt transition which occurs between CO_2-limited and CO_2-saturated photosynthesis and because of the lack of light saturation of photosynthesis below full sunlight. In limiting CO_2, light effectively saturates photosynthesis, whereas in saturating CO_2, light limits photosynthesis. In this respect, CO_2 transitions may therefore be expected to show parallel with light transitions. Increased energization at low p_{CO_2} is evidenced by higher non-photochemical quenching and higher triose phosphate/PGA ratios. When the changes in redox state are examined, it can be seen that Q_A becomes more reduced and that $NADP^+$ malate dehydrogenase becomes more oxidized as the concentration of CO_2 decreases, i.e. there is no longer a strict correspondence between the redox state of the acceptors of the two photosystems. As malate dehydrogenase in C_4 plants is directly involved in the fixation of CO_2, its activity must decrease at lower concentrations of CO_2, but the observations are entirely consistent with the occurrence of photosynthetic control and the notion that high ΔpH in low CO_2 concentrations restricts intersystem electron transfer. It should also be noted that the amount of FBP rises dramatically at low CO_2 concentration, reflecting regulation of Calvin cycle turnover (Leegood & von Caemmerer 1988).

(*b*) *Regulation in relation to* PFD

The supply of assimilatory force $(ATP/ADP \times P_i)\,(NADPH/NADP^+)$ declines drastically following a transition from high to low light (Prinsley *et al.* 1986*b*) and would be expected to fall at low PFD in the steady state. However, in spinach leaves chloroplastic triose phosphate/PGA ratios increase with a decrease in PFD, either in high CO_2 (Dietz & Heber 1984) or in ambient CO_2 concentrations (Heber *et al.* 1986, 1987). Figure 6 illustrates data from barley in which a similar phenomenon is observed; the triose phosphate/PGA ratio falls as the PFD is decreased, but then rises again at very low PFD. A similar phenomenon is observed in the response of bean leaves, calculated from the data of Badger *et al.* (1984). Thus in leaves of C_3 plants at low PFD increases in photosynthetic flux are apparently accompanied by a decrease in the assimilatory force, and hence the driving force on photosynthesis (Heber *et al.* 1986, 1987). An explanation for this behaviour in C_3 plants is that the enzymes of the Calvin cycle become less active in low light (Heber *et al.* 1986, 1987), and the consumption of assimilatory force becomes the dominant regulatory component. This is evidenced by the dramatic rise in the amount of FBP that occurs at low PFD (figure 6) (Dietz & Heber 1984).

By contrast, in leaves of maize (figure 6) triose phosphate/PGA ratios continued to fall in low light, and there was no rise in the amount of FBP corresponding to that occurring in the leaves of C_3 plants and at low p_{CO_2}. Similar observations have been made in leaves of *A. edulis* (Leegood & von Caemmerer 1988). If the above explanation for the behaviour of triose phosphate/PGA ratios in C_3 plants is accepted, then these observations indicate that regulation of photosynthetic flux in relation to light in C_4 plants is less reliant upon direct modulation of enzyme activity by light (e.g. by thiols) than it is in C_3 plants. That is, regulation of demand appears to be more important in C_3 plants. Triose phosphate/PGA ratios in the leaves of C_4 plants are also very much higher than those observed in the leaves of C_3 plants. This reflects the high ATP/ADP ratios generated in the mesophyll cells of maize leaves in the light (Leegood 1985; Stitt & Heldt 1985*b*).

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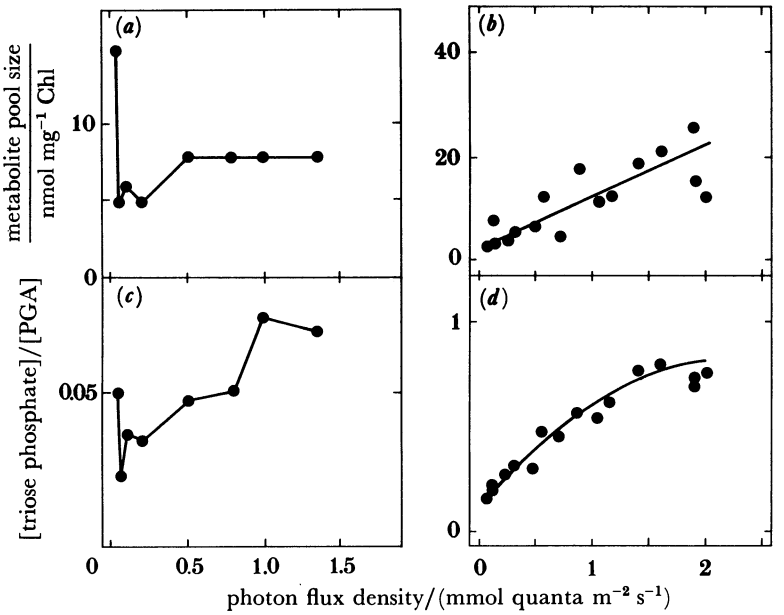

FIGURE 6. Influence of changes in PFD upon (*a*, *b*) ratios of triose phosphate to PGA and (*c*, *d*) amounts of FBP in leaves of (*a*, *c*) barley and (*b*, *d*) maize. Data for maize from R. C. Leegood & S. von Caemmerer (unpublished data). Data for barley are the means of three replicates.

(*c*) *Regulation following the transition from high to low* PFD

When illuminated leaves are subjected to a sudden decrease in PFD, a pronounced lag is apparent in the rate of photosynthetic carbon assimilation before it proceeds at a new steady-state rate. Two phenomena are concealed by this lag. The first is a post-lower-PFD CO_2 burst, which results from the continued turnover of photorespiratory intermediates that persist from the period spent at high PFD (Vines *et al.* 1982, 1983). The second is a true lag in the rate of photosynthetic carbon assimilation (Prinsley *et al.* 1986*a*, *b*). During a light transition, regulation must act to minimize imbalances between the reactions of electron transport (supply) and those of photosynthetic carbon assimilation (demand). The presence of a lag reflects regulatory imbalances within the system. A number of factors have been proposed to contribute to such a lag, for example, an overshoot in sucrose synthesis (Prinsley *et al.* 1986*a*), but this is unlikely to be a principal factor. Prinsley *et al.* (1986*b*) noted that the supply of assimilatory force (indicated by the triose phosphate/PGA ratio) was reduced below that subsequently reached in the steady state in low light following the transition. The mechanism(s) responsible for this undershoot in assimilatory force have not been elucidated, but various possible explanations have been advanced. For example, on the basis of a relatively slow decrease in non-photochemical quenching and light scattering following a decrease in PFD in leaves of *Hedera helix*, Sivak & Walker (1987) proposed that a high ΔpH present under high PFD relaxed only slowly following the transient, resulting in a temporary inhibition of electron transport by back pressure from the excessive proton gradient. On the other hand, Heber *et al.* 1986) have suggested, on the basis of metabolite measurements made in isolated spinach chloroplasts, that triose phosphate oxidation may account for the lag in carbon assimilation because triose phosphate builds up to high concentrations in the medium.

Recent work on light transitions in maize has resulted in the emergence of a clearer

description of the events occurring during a high to low light transition. Figure 7 shows some of the changes in maize leaves that accompany a 12-fold decrease in PFD. First, there was a typical transient inhibition of the assimilation rate, which lasted several minutes (Prinsley *et al.* 1986*a*). Secondly, the activity of $NADP^+$-malate dehydrogenase declined rapidly in response to lowered PFD, but the activity of pyruvate, P_i dikinase only slowly declined to its new steady-state activity in low light. Thirdly, the amount of pyruvate rose dramatically following a lowering of the PFD, as it does when maize leaves are darkened (Leegood & Furbank 1984). Fourthly, the triose phosphate/PGA ratio declined rapidly and for a considerable period remained well below the value subsequently reached in the steady state in low light, as in spinach leaves (Prinsley *et al.* 1986*b*). However, the information that the triose phosphate/PGA ratio provides about the regulation of the system is ambiguous. It does not reveal whether the problem is one of *supply* (i.e. electron transport is unable to meet the requirement for ATP and NADPH, a situation which might obtain if, for example, ΔpH persisted following the transition) or whether the problem is one of excessive *demand* for ATP and NADPH.

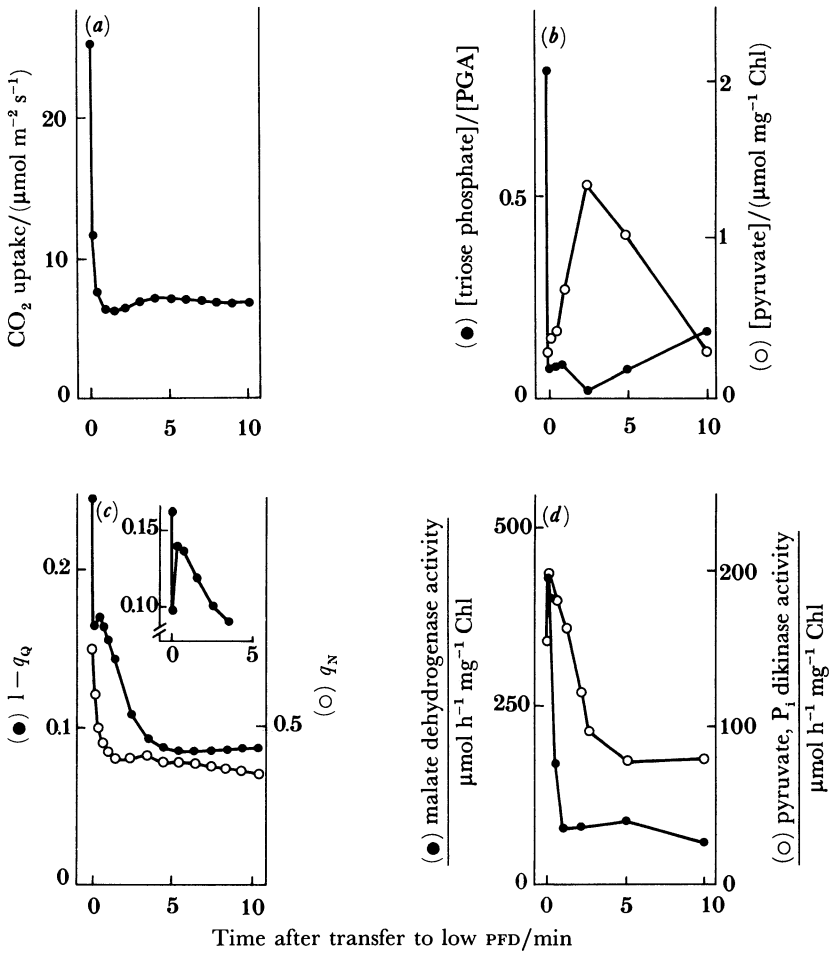

FIGURE 7. Influence of a decrease in PFD from 1700 to 140 μmol quanta m^{-2} s^{-1} upon (*a*) the CO_2 assimilation rate, (*b*) the amounts of pyruvate and the ratio of triose phosphate to PGA, (*c*) q_Q and non-photochemical quenching and (*d*) the activities of $NADP^+$-dependent malate dehydrogenase and pyruvate, P_i dikinase in leaves of *Zea mays*. The inset in (*c*) shows the change in $(1-q_Q)$ in more detail over the first 5 min. The temperature was 25 °C. From H. D. Doncaster & R. C. Leegood (unpublished data).

The rise in pyruvate concentration that occurs following the transition to low light could result either from a lack of the ability of pyruvate, P_i dikinase to metabolize pyruvate or from a lack of ATP (or P_i). The former is unlikely as pyruvate, P_i dikinase remained active following the transition and during the transient inhibition of photosynthesis; indeed its activity 2 min after the transition was the same as under high PFD. Modulation of pyruvate, P_i dikinase occurs by a complex mechanism that involves phosphorylation of the enzyme and is critically dependent upon the adenylate status, with ADP both a substrate for inactivation and a potent inhibitor of activation. Any accumulation of pyruvate, resulting in an increase in the ratio of pyruvate to PEP, tends to maintain the enzyme in an active state (Burnell & Hatch 1985; Burnell *et al.* 1986). (It should be noted that the PEP pool remains constant following a high to low light transition, after a brief transient decline (data not shown).) It can therefore be inferred from these observations that the build-up of pyruvate results from a lack of ATP to metabolize it.

We now need to examine how electron transport behaves following the light transition. In maize, interpretation is simplified because the vast majority of variable chlorophyll fluorescence emanates from the mesophyll (see Leegood *et al.* 1983; Crowther *et al.* 1983). Following a transition from high to low light, Q_A (shown as $1-q_Q$) was rapidly oxidized in the first few seconds (see also inset panel in figure 7*c*). Q_A was then re-reduced (within 10–15 s) and this was followed by a slow oxidation over a period of up to 5 min, with Q_A achieving a new steady-state value coincidentally with the recovery of the assimilation rate. The immediate oxidation of Q_A upon lowering the PFD is to be expected, but the re-reduction requires explanation and could reflect at least two possibilities. The first would be a rapid re-reduction of intersystem electron acceptors such as plastoquinone, which would in turn block the oxidation of Q_A. The second is a reversal of the q_E-dependent quenching mechanism that keeps Q_A in an oxidized state, as proposed by Weis & Berry (1987).

What evidence is there from non-photochemical quenching of chlorophyll fluorescence? Non-photochemical quenching is dependent upon a number of different processes, including membrane energization (Krause *et al.* 1982), state transitions, photoinhibition and plastoquinone-dependent quenching (for this last see Vernotte *et al.* (1979)). Horton & Hague (1988) and Demmig & Winter (1988) have identified three components in the dark relaxation of non-photochemical quenching, of which only the fastest component is likely to reflect ΔpH (see also Leegood *et al.* 1988). Following the transition from high to low PFD, the relaxation of non-photochemical quenching was also triphasic in maize when plotted on a semilogarithmic scale (data not shown). This plot reveals a very rapid phase of the relaxation, complete within about 30 s, which can also be seen in figure 7. If the rapid decay of non-photochemical quenching does reflect decay of ΔpH, than it provides a maximum estimate for the time taken for such a decay. This suggestion that ΔpH does decay rapidly could explain why Q_A undergoes rapid re-reduction according to the hypothesis advanced by Weis & Berry (1987), but it cannot readily explain why $Q_A^{\bullet}$ should be only slowly reoxidized thereafter if such slow reoxidation is the result of a restriction of intersystem electron flow by a high ΔpH.

A situation therefore obtains following a decrease in PFD in which (*a*) ΔpH may be low (indicated by the low triose phosphate/PGA ratio, the large pool of pyruvate and the rapid decay of non-photochemical quenching); (*b*) the $NADP^+$ pool remains relatively oxidized (indicated by the change in the activity of $NADP^+$-malate dehydrogenase); (*c*) Q_A is transiently oxidized and re-reduced, but nevertheless remains oxidized relative to its redox state in high

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light. The conclusion to be drawn by analogy with the behaviour of isolated chloroplasts is that the build-up of pyruvate results in a stimulation of cyclic electron flow to meet the shortfall in ATP, and that it lowers ΔpH and leads to reduction of plastoquinone and Q_A. The accumulation of pyruvate exacerbates a situation in which the assimilatory force is already in short supply owing to the build up of PGA. A stimulation of cyclic electron flow under these conditions could explain the transient inhibition of the rate of carbon assimilation.

Cyclic electron flow is very sensitive to redox poise and is delicately balanced with linear electron flow (Crowther *et al.* 1983). Inhibition of cyclic electron flow readily results from over-reduction by photosystem II. Conversely, over-oxidation ensues when the input of electrons from photosystem II is weak, or is blocked by DCMU, a situation that may be exacerbated by the presence of electron acceptors. Presumably the system remains favourable to cyclic electron flow in this case (*a*) because despite the fact that $NADP^+$ is oxidized, and might generate a potentially unfavourable redox poise, input from photosystem II is also cut drastically, and (*b*) because demand for ATP is so high, and ΔpH is so low, the driving force on H^+ translocation outweighs fine regulation by redox state. In this regard it is worth noting a further feature of the A_{518} data in figure 4. In the presence of pyruvate and nigericin, cyclic electron transport remains poised between flashes (lasting for a total period in excess of 1 min at a frequency of 2 Hz) despite the fact that the metabolic demand for ATP has been abolished by the inclusion of an uncoupler (if the order of additions is reversed by adding nigericin before pyruvate, then $A_{518,s}$ is not induced). This means that, once poised under these conditions, cyclic electron flow is surprisingly resilient to oxidation by molecular O_2, even during the half second spent in the dark between flashes (compare with observations in Heber *et al.* (1985)).

This view of the transition between high and low light suggests that transient inhibition of photosynthetic carbon assimilation in maize leaves is caused by an excessive demand for ATP, which is met by cyclic electron transport. This is a hypothesis which is amenable to testing *in vivo* by flash spectroscopy, particularly as the transient behaviour extends over several minutes. It is clear that regulation of the response is shared between electron transport and carbon metabolism. It is also worth asking to what extent the lag observed in C_3 plants following a lowering of the PFD results from a transient increase in the amount of glycerate (an ATP sink) that may accompany the photorespiratory burst (Vines *et al.* 1982, 1983).

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Discussion

R. Hill (*Department of Biochemistry, University of Cambridge, U.K.*). Free radicals damage the photosynthetic apparatus. They are formed in material containing a variety of light absorbing substances when the light intensity is very strong. High light intensity would therefore produce an inhibition of photosynthesis.

The negatively charged oxygen molecule ($O_2^{-\cdot}$) gives cyclic ATP production through the Mehler reaction, which could account for the observed accumulation of ATP at high light intensity.

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R. C. Leegood. Both these questions highlight the multiplicity of factors that contribute to the control of photosynthesis *in vivo*. No one factor can 'explain' the lags in carbon assimilation any more than one enzyme can regulate a metabolic sequence. With respect the first question, it should be noted that the high light intensity employed in this study does not saturate the photosynthetic rate, although production of free radicals may be increased at such light intensities. The second question is also important, because it may very well be the case that the inability to meet the demand for ATP in low light is partly caused by a diminished capacity to form ATP via the Mehler reaction.

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