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Ribulose Bisphosphate Carboxylase-Oxygenase: Its Role in Photosynthesis [and Discussion]

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Ribulose bisphosphate carboxylase–oxygenase: its role in photosynthesis

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Synthesis of triose phosphate by the chloroplast requires three substrates: light, CO_2 and orthophosphate (P_i). In the response of the rate of carbon assimilation to the concentration of CO_2, the kinetic properties of RuBP carboxylase–oxygenase (Rubisco) constitute the main limitation at low CO_2 concentrations, while at higher concentrations of CO_2 the limitation is shifted towards the reactions leading to the regeneration of the substrate, RuBP, driven by electron transport. In these circumstances, light or P_i, or both, can become limiting.

The characteristics of Rubisco that can affect photosynthesis fall under three main headings: (1) amount and kinetic constants; (2) activation state; and (3) regulation of catalysis (including the role of effectors, such as P_i and glycerate 3-phosphate (PGA)). These characteristics are analysed, and the role of changes in activity of the enzyme is discussed in the context of limitation and regulation of the photosynthetic process. Other factors considered are the regeneration of RuBP and its relation to electron transport, P_i supply, and photorespiration. The influence that expected increases in atmospheric CO_2 concentration, and/or genetic improvements in the characteristics of the enzyme, may have on the present balance between the partial processes of photosynthesis, is discussed.

Introduction

Carboxylation is an energy-requiring reaction. There is also very little CO_2 in the atmosphere. If the contemporary biochemist could create overnight what evolution has fashioned over millenia, he would therefore undoubtedly incoporate two prime features. He would devise an 'energy-rich' substrate which, when offered CO_2 in the presence of a suitable catalyst, would conveniently form a new carboxylated compound in a reaction accompanied by a large decrease in free energy. This would ensure an extremely favourable equilibrium position. Secondly he would design a catalyst with an extremely high affinity for CO_2, such that the reaction would continue at an unabated rate in extremely low concentrations of carbon dioxide.

When the carboxylation of ribulose bisphosphate (RuBP) was first described it was soon obvious that the first criterion had been met. The decrease in free energy was some 8 kcal per mol, and the reaction was virtually irreversible. On the other hand, the affinity of the enzyme involved for CO_2 was ridiculously low, with some 6% CO_2 required for half-saturation. This enigma was resolved by Lorimer *et al.* (1976) who found that the purified enzyme requires activation by CO_2, Mg^{2+} and high pH. At about the same time it became clear that chloroplast stroma, released into suitable media, also displayed carboxylase activity with appropriate characteristics. The whole picture was rounded out once it was understood (Bowes & Ogren 1972) that the carboxylase is also an extremely active oxygenase (figure 1). This aspect will be considered more fully in the following paper by Alfred Keys. The biochemical 'engineer' might conceivably improve on nature but it seems to us perfectly reasonable that the original carboxylase evolved in atmospheres containing large quantities of CO_2 and virtually no oxygen.

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It has proved so successful that we are here to pay tribute to its achievements in an atmosphere in which the relative proportion of these gases has been reversed. Seen in this light, the massive concentration of RuBP carboxylase–oxygenase in the chloroplast stroma could be regarded as an evolutionary response to decreasing CO_2 (and increasing O_2) in the atmosphere. Similarly, photorespiration can be regarded as a classic example of making a virtue out of a necessity. Some at least of the 'lost' carbon finds its way back into the cycle, and some useful by-products are formed.

In the early 1970s carboxydismutase (or ribulose diphosphate carboxylase) as it was then called, was still an unresolved enigma. What problems does it continue to pose as ribulose bisphosphate carboxylase–oxygenase (Rubisco) in 1985? Since these problems will be addressed in detail in subsequent papers it falls to us to take a broadly physiological view.

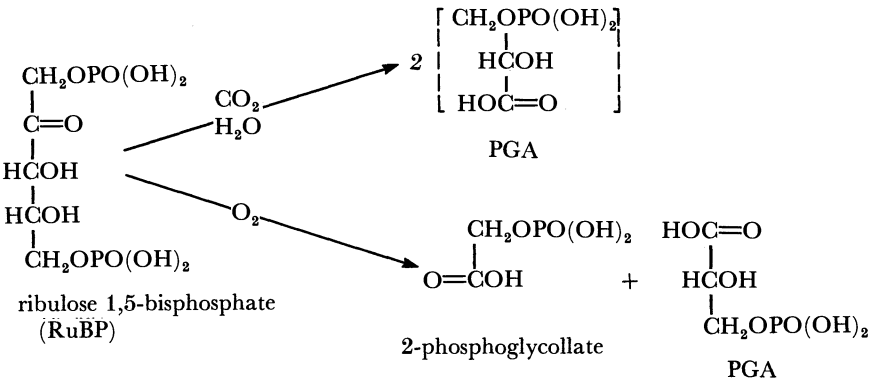

FIGURE 1. Carboxylation and oxygenation: the two reactions catalysed by Rubisco.

REGULATION VERSUS LIMITATION

During the last 15 years there have been great advances in our understanding of the characteristics of Rubisco. However, if we are to understand its regulation *in vivo* we must think in terms of factors which influence both its activation and its catalytic activity. Here also it continues to be as enigmatic as ever. Before 1970, it appeared to be inadequate for its task; however, once the requirement for activation had been established, there was no doubt that, at least in some species, there was more than enough Rubisco to account for *maximal* photosynthesis (Lilley & Walker 1975). Inevitably there will always be doubts about the adequacy of extraction from many species and the degree of activation. Viewed teleologically, there is also the fact that since RuBP carboxylase is a major resource investment (which may constitute 50% or more of soluble leaf protein in C_3 species) it is surprising that a plant can afford more than it appears to need. In other words, it does not make sense that, having made this investment, the same plant may not utilize all of its carboxylating capacity, particularly since the photosynthetic carbon cycle appears to be richly endowed with other regulatory mechanisms. Within certain limits, a plant may be capable of adjusting the composition of its photosynthetic machinery to the growth conditions which it encounters during its development (for review see Björkman 1981). However, environmental conditions also change markedly during the day, sometimes in a matter of seconds. Short term under-utilization of parts of this photosynthetic machinery may therefore be inevitable, the price the plant pays for its immobility and total dependence on a changing environment.

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When light is plentiful but conditions do not otherwise favour carbon assimilation, mechanisms exist that can dissipate light energy (e.g. pseudocyclic electron transport), although the phenomenon of photoinhibition demonstrates that their capacity may be insufficient in extreme conditions. Conversely, the activity of enzymes can be adjusted to the prevailing light intensity. The multiplicity of regulatory mechanisms known to affect the activity of all the enzymes involved in photosynthesis (with many new factors found every year), is probably part of this seemingly wasteful adjustment of every part of the machinery to the prevailing rates of the others. A single factor (light intensity, metabolites, pH) or a combination of these can decrease the activity of an enzyme substantially. However, a low activity of a particular enzyme is not necessarily proof that the photosynthetic process is limited by it. It can equally well be interpreted as a regulatory and useful adjustment to the activity of the rest of the photosynthetic apparatus. Thus, regulation is not synonymous with limitation and, although it is easy to accept that a certain waste of enzymic capacity occurs in the short term, it seems unlikely that photosynthetic rates could be limited during long periods by the activation state of a single enzyme, especially when the enzymic protein is already there and the nitrogen and energy needed to form it have already been invested. In spinach the isolation of highly intact chloroplasts has long since been a routine procedure and possibly more is known about Rubisco and its activation from this source than from any other. We must therefore be on good ground when we assert that, *in saturating* CO_2, the rate of carboxylation is approximately 2.5 times that of coupled electron transport from H_2O to NADP or the rate of photosynthesis displayed by the parent tissue (Lilley & Walker 1975). To the extent that it has been studied (and with less certainty) we can suggest that the same is probably true of other species. Conversely, in air, the kinetics of CO_2 assimilation *in vivo* approximate to the RuBP-saturation kinetics of Rubisco and the reported activity and properties of this enzyme appear to be the limiting factors in a number of species. This conclusion is central to the model of von Caemmerer & Farquhar (1981), which interprets the relation between [CO_2] and rate in terms of the RuBP-saturated kinetics of the carboxylase at low CO_2 concentration and the regeneration of RuBP at high CO_2 concentration (see also Laisk & Oja 1976; Laisk & Walker 1986). Seen in this context, the reported low activation status of the carboxylase (Perchorowicz & Jensen 1983) could well be an example of short term under-utilization, with the carboxylase only partly activated when the regeneration of the substrate is limiting.

What limits photosynthesis?

'God' is still a good answer to the question 'what limits photosynthesis?' but we can make the question more scientifically useful if we define the CO_2 concentration. In air, the characteristics of the carboxylase would seem to be paramount and herein lies the current importance attached to the possibility of genetic manipulation of these characteristics. In higher concentrations of CO_2, or at low temperatures, or in otherwise stressed leaves, there is almost certainly co-limitation and therefore a real prospect that any engineered improvement in the characteristics of the carboxylase might simply replace one limitation by another.

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The characteristics of Rubisco

Broadly speaking, the limitations imposed by the characteristics of Rubisco fall under three headings:

(*a*) the amount and kinetic constants of the enzyme;

(*b*) the activation state of the enzyme (including factors which may maintain or change activation);

(*c*) the regulation of catalysis (including the role of effectors).

(*a*) *The amount*

Interpretation of metabolite and enzyme measurements rests largely on the calculations in which they are then used but neither the measurements nor the calculations are easy. Apart from the difficulties involved in extracting from the parent tissue all of the metabolite or the enzyme, without destroying the former or inactivating the latter, calculations of molarities rely on further assumptions. RuBP carboxylase is a prime example. Many stromal metabolite concentrations are often related to ubiquitous '4 mM RuBP binding sites'. This figure is frequently quoted with the air of authority usually reserved for statements such as 'the atmosphere contains about 350 µl CO_2 per litre', but it was never intended to be more than a good guess and ought to be subjected to periodic reappraisal. It was based by Jensen & Bahr (1977) on the values of 6 mg of carboxylase per milligram chlorophyll (from Lyttleton & Ts'o 1958), and 25 µl of stromal volume per milligram chlorophyll (from Heldt & Sauer 1971). This calculation gives a concentration of 0.4–0.5 mM carboxylase and, with eight binding sites for RuBP per molecule, '3–4 mM binding sites'. Data for stromal volumes have not changed much over the years (they range between 25 and 45 µl per milligram chlorophyll: Hampp *et al.* 1982; Wirtz *et al.* 1980). Alternative estimates of the Rubisco content have been based on extraction of whole leaves or algae, subsequent reaction with [^{14}C]carboxypentitol bisphosphate (CABP) and separation of the resultant Rubisco–CABP complex by immunoprecipitation (Collatz *et al.* 1979) or by treatment with polyethylene glycol and $MgCl_2$ (Yokota & Canvin 1985). Collatz *et al.* (1979) reported binding site concentrations of about 50 nmol mg chlorophyll^{-1} (*ca.* 2 mM), while Yokota & Canvin reported a value of 97 nmol mg chlorophyll^{-1} (*ca.* 4 mM). Naturally, a good deal of variation is to be expected but these and other data (Ku *et al.* 1979) are largely consistent with the 4 mM figure. However, none of these authors presented adequate evidence that all of the Rubisco protein had been recovered in the soluble fraction of the leaf homogenate. There are good grounds for doubt here, because extraction in buffers containing Mg^{2+} results in association of Rubisco with membranes, a situation which may lead to sedimentation during centrifugation (McNeil & Walker 1981). It is salutary to note that the soluble protein content of spinach chloroplasts has been put at 18 mg per milligram chlorophyll (Lilley *et al.* 1975) and if this value (based on 80 measurements) is correct it could increase the concentration of catalytic sites by a factor of 3–4 (most of the stromal protein is Rubisco). In all the above estimates the recovery of protein is crucial. While loss by binding to, and co-precipitation with, insoluble residues seems inescapable, it seems hardly sufficient to account for two- to threefold differences in amount. If the different values are taken at face value it would follow that very large differences in carboxylase content might exist, as might be expected (see above). Moreover, chloroplast volumes (and no doubt protein content) vary widely according to species and environment if chloroplast areas, as seen in micrographs, can

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be taken as a guide. It should also be noted that some workers have used stromal volumes of 35 µl per milligram chlorophyll to calculate metabolite concentrations and the value of 25 µl (implicit in the 4 mM figure) for carboxylase.

An important consequence of the presence of these very high concentrations of Rubisco in the stroma is that when RuBP is low (e.g. in high CO_2 or during induction), sequestration of stromal metabolites, particularly fructose bisphosphate (FBP), sedoheptulose bisphosphate (SBP) and NADPH, may reduce their free concentration by more than tenfold (Ashton 1982), although Gutteridge & Keys (1985) argue that Ashton's values are considerable underestimates of the actual extent of binding.

Several enzymes of the reductive pentose phosphate pathway (RPPP) show changes in kinetic constants following light activation (such as the decrease in K_m of the two bisphosphatases for their substrates: Leegood *et al.* 1985). In view of the fact that inactive Rubisco has a high $K_m(CO_2)$ and the activated enzyme has a low $K_m(CO_2)$, surprisingly little attention has been paid to the possibility of variations in $K_m(CO_2)$ due to changes in activation state under physiological conditions, although the available evidence indicates that the $K_m(CO_2)$ is constant between darkened and illuminated spinach chloroplasts (Bahr & Jensen 1974) or between darkened and illuminated leaves of wheat (Mächler & Nösberger 1980).

There is considerable interspecific variation in the $K_m(CO_2)$ of Rubisco (Yeoh *et al.* 1980, 1981). The enzyme from C_3 and CAM species exhibits lower $K_m(CO_2)$ values (12–25 µM) than that extracted from C_4 species (28–34 µM) or aquatic species (30–70 µM); a high $K_m(CO_2)$ therefore appears to be associated with the presence of a CO_2-concentrating mechanism.

(*b*) *The activation state of Rubisco*

Estimation of the *in vivo* activation state is a difficult problem. No test can adequately demonstrate that the activation state of Rubisco is not altered by exposure to a change in pH, vacuolar contents etc., during extraction, although there is reasonable evidence that, once extracted, the activation state can be maintained for short periods in crude extracts (see, for example, Perchorowicz *et al.* 1982; Servaites & Torisky 1984). Although comparisons of activation state between treatments are likely to be valid providing the extraction procedure is kept uniform, clearly too much emphasis should not be placed on *precise* values for the 'percentage activation' of Rubisco.

Light, rather than CO_2, appears to be the major factor responsible for changes in the activation state of Rubisco in leaves. The activity of the carboxylase increased more or less linearly with irradiance in wheat leaves up to 1000 µmol photons m^{-2} s^{-1} (i.e. 50% of full sunlight) (Perchorowicz & Jensen 1983). Rather similar observations have been made in work with soybean leaves (Servaites *et al.* 1984). These results have been interpreted as an effect of higher stromal pH (Heldt *et al.* 1973) and Mg^{2+} (Portis 1981) activating Rubisco at the higher irradiances (Perchorowicz & Jensen 1983). However, our knowledge of the dependence of stromal pH and [Mg^{2+}] on light intensity is very scant. The only available indication of possible changes in stromal pH and [Mg^{2+}] comes from light scattering data in leaves. Light scattering is considered to indicate the magnitude of ΔpH in leaves (Köster & Heber 1982; Kobayashi *et al.* 1982). The light-intensity dependence of light scattering varies considerably between different leaves, different species and between sun and shade leaves, but half-maximum light scattering was observed to occur in air in spinach and bean leaves at about 100 W m^{-2} (Dietz & Heber 1985*b*). What is not clear, however, is the relation between ΔpH and stromal pH.

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In chloroplasts the stromal pH is more sensitive to a lowering of the irradiance than is ΔpH, due to the efficient buffering capacity of the stroma (Heldt *et al.* 1973). If the same relationship holds in leaves, then changes in stromal pH and $[Mg^{2+}]$ could well be important over a wide range of irradiance. However, it is also clear from the work of Perchorowicz & Jensen (1983) that changes in the concentration of CO_2 up to about 1500 μl l^{-1} had little influence on the activation state of Rubisco, even at low irradiance. Since an increase in $[CO_2]$ decreases light scattering drastically at lower light intensities (although less so at higher irradiance) (Krause 1973; Dietz & Heber 1985*b*; Sivak *et al.* 1985*b*), Perchorowicz & Jensen (1983) were led to ask if changes in pH and $[Mg^{2+}]$ alone were sufficient to explain the observed changes in Rubisco activation state in the leaf.

There are several other observations that are not readily accommodated by the notion that the activation state is wholly determined by pH and $[Mg^{2+}]$ within the stroma. For example, Perchorowicz & Jensen (1983) noted that the activity of the carboxylase in the dark was higher than in low light. Bahr & Jensen (1978) observed that light-activation of Rubisco was inhibited by D,L-glyceraldehyde (an inhibitor of carbon assimilation rather than electron transport). Chastain & Ogren (1985) have shown that mutants of *Arabidopsis thaliana* that have lesions in the photorespiratory pathway display a photorespiration-induced decrease in the activation state of Rubisco. This inhibition was attributed to changes in the amount of a photorespiratory metabolite. On the other hand, Vu *et al.* (1983), working with soybean leaves, have observed an effect of illumination on the activity of Rubisco which was observed even when it was assayed *in vitro* with saturating $[Mg^{2+}]$ and $[CO_2]$, implying that light exerts a regulatory effect independent of the activation by CO_2 and Mg^{2+}.

Servaites *et al.* (1984) explain the inability to bring about light activation of Rubisco *in vitro* by the presence of a phosphorylated inhibitor. The inhibitor is presumably synthesized in the dark and appears to be degraded in the light, and may be analogous to binding-site inhibitors such as substrate RuBP and xylulose 1,5-bisphosphate. Studies by Somerville *et al.* (1982), of a mutant of *Arabidopsis thaliana* that lacks activation of Rubisco *in vivo*, have shown that the mutation does not affect Rubisco directly, since the properties of the enzyme purified from the wild-type and from the mutant were indistinguishable. Rather, the results indicated the participation of an enzyme-specific regulatory agent. Whether this agent is the same as the one recently proposed by Servaites *et al.* (1984), remains to be elucidated (but see also Ogren *et al.* this symposium).

The dark form of Rubisco, which is not susceptible to activation *in vitro* by CO_2 and Mg^{2+}, has been found in a number of species, and there are also considerable differences in the degree of light-activation between C_3, C_4 and CAM species (Vu *et al.* 1984). C_4 species, except *Panicum maximum*, showed little light activation of Rubisco, while CAM species, such as *Ananas comosus*, showed very extensive (30-fold) light activation (see also Littlejohn & Ku 1984). Among C_3 species there was considerable variation, with species such as wheat showing no light activation (but see Mächler & Nösberger 1980), in contrast to the enzyme from leaves of soybean, tobacco and tomato (Vu *et al.* 1984).

(*c*) *The regulation of catalysis*

In terms of regulation, the activation state is not the sole determinant of enzymic activity, and other aspects of the enzyme's environment must be taken into account. Considerable emphasis has been placed on the behaviour of the activation state of the carboxylase in response

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to fluctuations in light intensity (Perchorowicz *et al.* 1981) or after the transition from 20% O_2 to 2% O_2 (Schnyder *et al.* 1984). Little attention has been paid to an assessment of changes in the activity of the carboxylase under such circumstances when every aspect of the stromal environment is considered. In a light–dark transition, for example, there will not only be a fairly rapid change in stromal pH and $[Mg^{2+}]$, but there will also be rapid, and very large, increases in the content of glycerate 3-phosphate and 6-phosphogluconate and a corresponding fall in the content of RuBP. It is well known that the enzyme is strongly competitively inhibited by sugar phosphates, glycerate 3-phosphate (PGA), NADPH, or 6-phosphogluconate (Badger & Lorimer 1981). These effects may be particularly important at limiting RuBP concentrations. For example, it has been shown in this laboratory that, under these conditions, increasing the concentration of PGA from 5 to 20 mM decreased the rate of *in vitro* $^{14}CO_2$ fixation by Rubisco by 60% (C. Foyer & R. Furbank, personal communication). These changes could therefore lead to a very rapid reduction in the activity of the carboxylase quite apart from relatively slow changes in the enzyme's activation state.

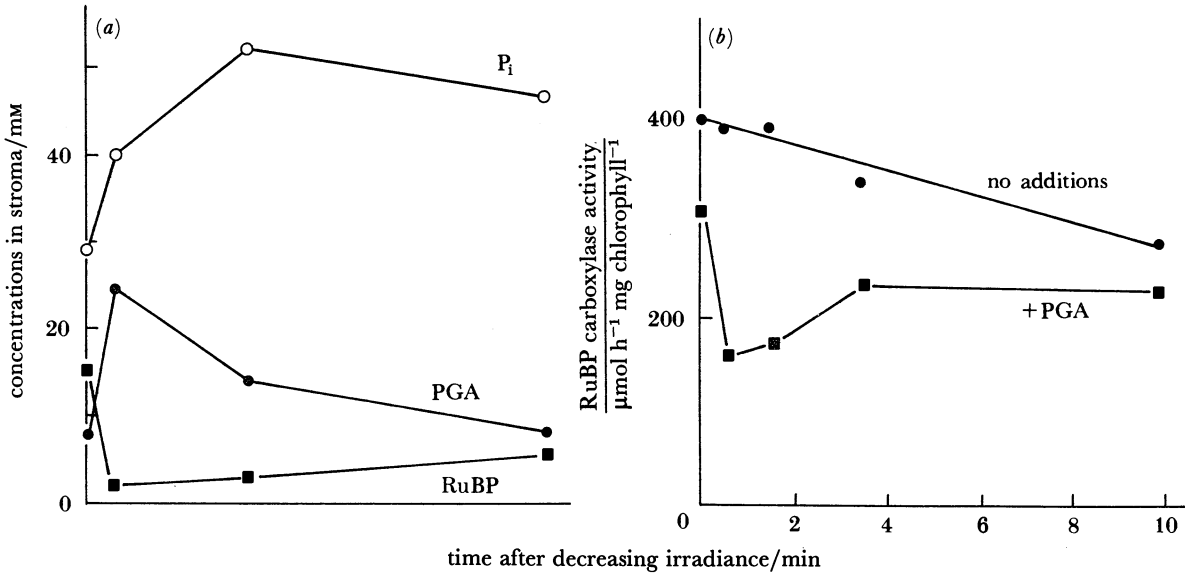

FIGURE 2. (*a*) Changes in the concentrations of RuBP, PGA and P_i (calculated) in spinach chloroplasts (isolated by non-aqueous means from leaves photosynthesizing in air) after a decrease in irradiance. (*b*) Changes in the activation state of Rubisco (rapidly extracted from leaves following a decrease in irradiance) assayed in the presence and absence of the PGA concentrations measured in (*a*). The irradiance was decreased from 250 to 25 W m^{-2} at time zero. The stromal volume is assumed to be 25 μl mg $chlorophyll^{-1}$. Redrawn from Prinsley *et al.* (1986) and from unpublished data.

When the irradiance is lowered suddenly, the activation state of Rubisco declines extremely slowly, taking about 20 min to reach a new steady state (Perchorowicz *et al.* 1981), while the rate of RuBP regeneration declines immediately. It has been proposed (see, for example, Mott *et al.* 1984) that, in these circumstances, deactivation of the carboxylase would help to keep a steady concentration of RuBP and other metabolites of the reductive pentose phosphate cycle, thus avoiding a situation of 'continuous induction'. This situation may also arise when P_i supply or the maximum capacity of electron transport are limiting. However, it should be noted that in circumstances in which RuBP regeneration limits photosynthesis, [PGA] rises and [RuBP]

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falls (Badger *et al.* 1984; Dietz & Heber 1985*a*; Prinsley *et al.* 1986; Leegood & Furbank 1986), and this will have an effect on the activity of Rubisco. For example, there is a large decrease in the RuBP pool (Perchorowicz *et al.* 1981; Mott *et al.* 1984) and large increases in the pools of PGA and P_i when the irradiance is lowered (figure 2; see also Prinsley *et al.* 1986). Prinsley *et al.* (1986) have investigated the effects of these changes in metabolites on the activity of carboxylase rapidly extracted from spinach leaves after a reduction in irradiance. The effect of an increase in PGA concentration in the assay mixture is to change completely the response of Rubisco activity to a decrease in irradiance. Rather than a slow change in activation state (i.e. a mismatch between RuBP regeneration and its consumption) there is a rapid decrease in the activity of Rubisco (figure 2). We would therefore expect the consumption of RuBP to be reduced immediately, not only because of the decrease in the amount of RuBP but also because of inhibition of Rubisco activity by metabolites such as PGA and P_i.

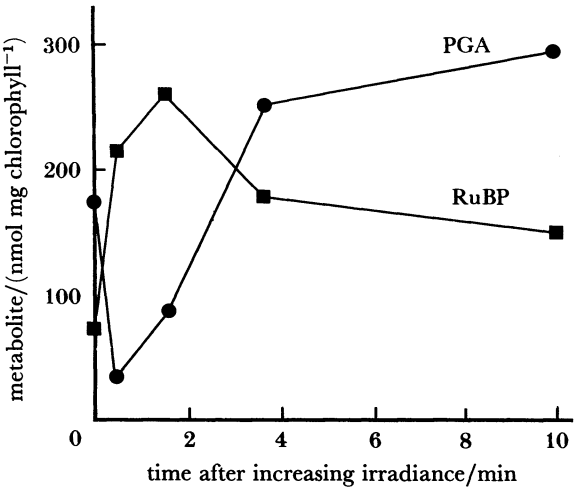

FIGURE 3. Changes in the amounts of RuBP and PGA measured in spinach leaves photosynthesizing in air, after an increase in irradiance from 15 to 150 W m^{-2}. From Prinsley (1985).

On the other hand, the opposite effect may be envisaged in circumstances where the irradiance is suddenly increased and the constraint on RuBP regeneration is lifted. Then the activity of Rubisco is initially inadequate, and the RuBP pool rises to a peak and then falls (figure 3, and Perchorowicz *et al.* 1981), reflecting a temporary imbalance between the rate of assimilation and the rate of RuBP production. However, we could again argue that the sudden decline in [PGA] caused by the increase in assimilatory power allows a more or less immediate increase in Rubisco activity, and this again minimizes the imbalance, while the change in the activation state (Perchorowicz *et al.* 1981) occurs somewhat more slowly.

In certain circumstances a transition from air to 2% O_2 may result in inhibition of the rate of carbon assimilation. This behaviour (the converse of the usual increase in photosynthesis occasioned by inhibition of photorespiration in 2% O_2) has been attributed to a restriction in the supply of P_i released from phosphoglycollate (Harris *et al.* 1983, and see below). In leaves taken from plants grown at higher temperatures but measured in high light at lower temperatures, the transition to 2% O_2, when associated with inhibition of CO_2 uptake, is accompanied by a sudden decrease in the rate of RuBP regeneration, and is characterized by a dramatic rise in [PGA] and a fall in [RuBP] (Leegood & Furbank 1986). The transition

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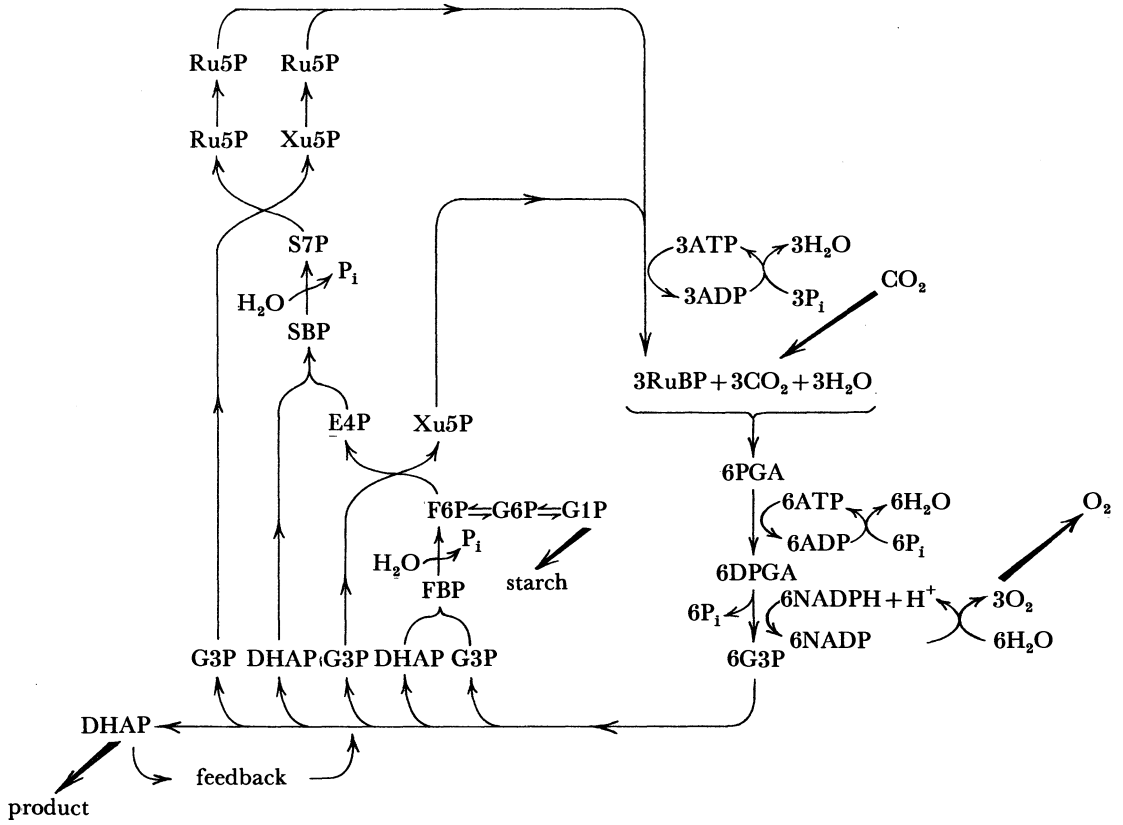
is also marked by a decrease in the activation state of the carboxylase (Schnyder *et al.* 1984), although the kinetics of this change have not been reported. However, the activation state of the enzyme was not sensitive to 2% O_2 when leaves of white clover were grown at lower temperatures (Schnyder *et al.* 1984). This lack of response could, of course, represent genuine adaptation in the structure and properties of Rubisco in plants grown in a cooler regime (see, for example, Huner & Macdowall 1978, 1979), but it might also have more to do with the phosphate status of the system, since phosphate deficiency is known to result in a decrease in the activation state of Rubisco (Heldt *et al.* 1978; Parry *et al.* 1985; Brooks 1986).

It is evident from the above that the activity of Rubisco will be modulated by fluctuations in the rate of RuBP regeneration and that such effects may be more complex than simple adjustment to changes in the supply of RuBP *per se*. Before addressing again the question 'what limits photosynthesis?', and having discussed the characteristics of Rubisco, we will summarise the reactions which lead to the regeneration of the substrate, RuBP.

The regeneration of RuBP

Figure 4 (Edwards & Walker 1983) outlines the reactions that lead to the regeneration of RuBP. On the right, three molecules of RuBP combine with three molecules of CO_2 and three molecules of water to give six molecules of PGA. These are phosphorylated at the expense of



$$3CO_2+2H_2O+P_i \rightarrow CH_2OH.CO.CH_2OPO(OH)_2+3O_2$$

Figure 4. The regeneration of RuBP in the reductive pentose phosphate pathway (RPPP). For details see text (from Edwards & Walker 1983).

ATP and the resulting diphosphoglycerate (DPGA) is reduced by NADPH to glyceraldehyde 3-phosphate (G3P). Part of this is converted to its isomer, dihydroxyacetone phosphate (DHAP). Aldol condensation of these two triose phosphates gives a molecule of fructose bisphosphate (FBP) which undergoes hydrolysis to fructose 6-phosphate (F6P). This hexose phosphate is also the precursor of glucose 6-phosphate (G6P) and glucose 1-phosphate (G1P), which after further transformation, gives rise to starch. The F6P also enters the first transketolase reaction, donating a 2-carbon unit to G3P to form xylulose 5-phosphate (Xu5P) and erythrose 4-phosphate (E4P). The process of condensation, phosphorylation and 2-carbon transfer is repeated yielding sedoheptulose bisphosphate (SBP), sedoheptulose 7-phosphate (S7P) and two more molecules of pentose phosphate respectively. All three molecules of pentose monophosphate are finally converted to ribulose 5-phosphate (Ru5P), which is phosphorylated to RuBP. The entire cycle can be divided into three phases. The initial carboxylation is followed by reduction to triose phosphate; five of these C_3 molecules are then rearranged to regenerate three C_5 molecules of CO_2-acceptor. It should be noted that the cycle consumes nine molecules of ATP and six molecules of NADPH in the formation of one triose phosphate product (which can also feed back into the cycle to promote autocatalytic acceleration). In total, five molecules of H_2O are consumed in the cycle proper and three are released in the generation of assimilatory power. If the triose phosphate product were hydrolysed to give free triose in a reaction consuming one molecule of H_2O, there would be no net P_i consumption and the entire sequence would simplify to the classic overall equation for photosynthesis.

In an absolute sense, photosynthesis is ultimately limited by what Arnon called assimilatory power (i.e. light-generated ATP and NADPH). 'Non-cyclic photophosphorylation' and associated NADP reduction, if freed of all external constraints, will proceed at the same rates as whole-leaf photosynthesis whereas (as noted above) *maximal* carboxylation capacity exceeds this rate by a factor of 2 to 3 (Lilley & Walker 1975). This implies that the ultimate ceiling is imposed by the intrinsic rate of electron transport. Photosynthetic phosphorylation is, however, constrained by the availability of NADP ('cyclic photophosphorylation', in the absence of artificial cofactors such as pyocyanine, appears to be slower than 'non-cyclic photophosphorylation') and by the availability of ADP and P_i. Whether *in vivo* photosynthesis is ever limited by the concentration of P_i at the site of photophosphorylation, or whether declining $[P_i]$ first affects the [ATP]:[ADP] ratio and thereby the availability of NADP for reduction, is still a matter for argument (Sivak & Walker 1985*a*, 1986; Walker & Sivak 1985*b*). What is clear is that, when other constraints are removed, P_i feeding can bring about substantial increases in the rate of photosynthesis (see below and Walker & Sivak 1985*b*; Sivak & Walker 1986).

In certain circumstances, e.g. in saturating light and saturating CO_2, major oscillations in rate can be observed if photosynthesis is interrupted or perturbed by a short period of darkness or a change in the gas phase surrounding the leaf (Walker *et al.* 1983; Sivak & Walker 1985*a*, and references therein). The peaks in CO_2 uptake or O_2 evolution then observed can exceed quasi-steady-state photosynthesis by 50% or more (figures 5 and 6, and see Walker *et al.* 1983; Sivak & Walker 1985*a*, *b*). These peaks are of interest because they (and not the quasi-steady-state values) must reflect the total electron transport and carboxylation capacity of the system. In the short-term, these oscillations dampen to lower values implying that photosynthesis is regulated *down* to lower values. The precise form of this regulation is still uncertain but there is no doubt that oscillatory behaviour can be stimulated by inadequate P_i supply or decreased by P_i feeding (Walker & Sivak 1985*a*, *b*; Sivak & Walker, 1985*a*, 1986). Similarly, light-scattering

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(Heber 1969) has indicated (Sivak *et al.* 1985*a*), and direct measurement confirmed (Furbank & Foyer 1986), that [ATP] : [ADP] ratios also oscillate in these circumstances, giving credence to the proposal (Walker *et al.* 1983; Sivak & Walker 1985*a*) that these ratios play a key role in oscillations by their effect on the reduction of [PGA] and hence the availability of NADP for reduction.

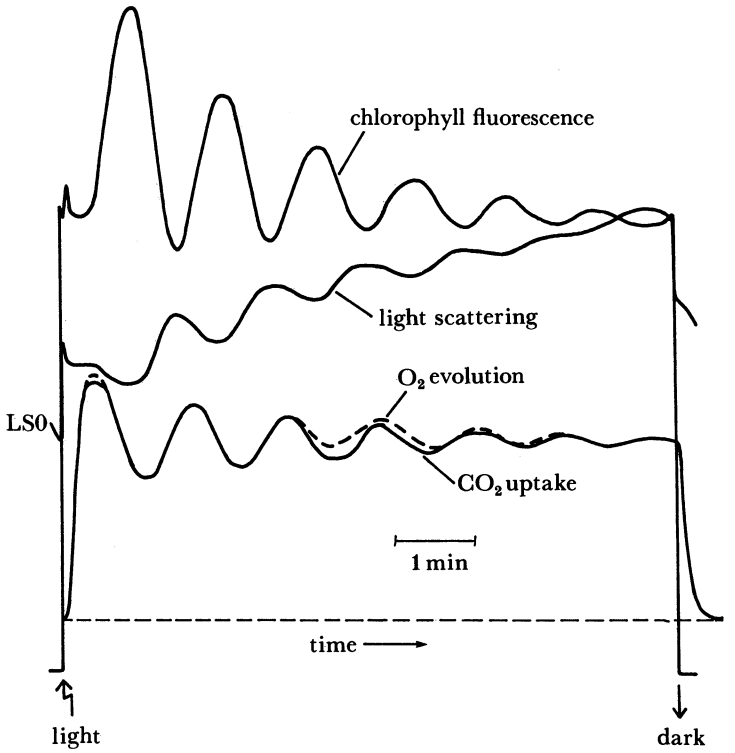

FIGURE 5. The relation between chlorophyll fluorescence, light scattering (Heber 1969), and rates of CO_2 fixation and O_2 evolution during oscillatory behaviour. Borage (*Borago officinalis*) leaf, photosynthesizing at 20 °C in 2% O_2 and 0.55% CO_2 (balance N_2, obtained using a Signal Blender series 850, Signal, U.K.); light (red) was near saturation (approx. 150 W m^{-2}) and gas was supplied at a flow of 75 ml min^{-1}. Carbon dioxide was measured by infrared gas analysis in a specially modified apparatus (ADC, Hoddesdon, U.K.). Oxygen was measured with a Draeger electrode (Draeger Transoxode, Draegerwerk, AG Luebeck, Germany) but similar results were obtained with a Clark-type electrode plus extensive, stable pre-amplification; stable suppression of a large fraction of the signal is required with either electrode. (See also Sivak *et al.* 1985*a*; Sivak & Walker 1985*a*,*b*; Walker & Sivak 1985*b*.) LS0 marks the light scattering before re-illumination and the broken horizontal line marks the dark values of the O_2 and CO_2 rates. The fluorescence signal rises, on illumination, from the point indicated by the 'light' arrow.

Figure 5 offers a major challenge to anyone wishing to arrive at all-embracing scheme that would account for the regulation of photosynthesis (see also Walker *et al.* 1983; Sivak & Walker 1985*a*,*b*; Sivak *et al.* 1985*a*). The kinetics illustrated in this figure were derived in saturating CO_2, 2% oxygen, and high light (Walker *et al.* 1983), i.e. in circumstances in which CO_2 fixation might be expected to be limited by the rate of regeneration by RuBP. This indeed is our interpretation (Sivak & Walker 1985*a*,*b*) but there are alternative explanations and there is no real reason for supposing that only one mechanism is involved. The salient features are these:

(*a*) as noted above, the maximum rate of CO_2 fixation greatly exceeds the quasi-steady-state rate that is approached as the oscillations dampen out;

(*b*) the changes in rate are considerable (from +35% to −20% in figure 5, and from +70% to −70% in figure 6) and even larger changes are not uncommon;

(*c*) there is a remarkable congruence of the O_2 and CO_2 signals throughout most of the period of measurement;

(*d*) during most of the period of measurement, chlorophyll fluorescence displays a broadly reciprocal relationship with carbon assimilation but changes in fluorescence anticipate the changes in oxygen. Light scattering measurements (Heber 1969), an indicator of the transthylakoid ΔpH (Kobayashi *et al.* 1982; Köster & Heber 1982), suggest (Sivak *et al.* 1985*a*) that it is the energy-dependent quenching component (q_e) that accounts for this anticipation, thereby implying that one of the earliest events in this sequence is a change in the proton gradient across the thylakoid membrane.

What can be inferred from these relations? First, it is clear that both the carboxylase and the other enzymes of the pentose phosphate cycle can reach a very high level of activity within a minute (or less) of re-illumination. Thereafter, either their activation status or their catalytic activity can change considerably in a matter of seconds or (alternatively or additionally) the flux of metabolites through the cycle may fluctuate for other reasons. It is possible that the activation status of Rubisco (and other enzymes of the reductive pentose phosphate pathway) may parallel the availability of RuBP (see above). Similarly, it is possible that changes in metabolites that act as inhibitors or modulators of the carboxylase may be involved. Our own inclination is to apply Occam's razor and conclude that what we see reflects changes in the RuBP supply and that the accommodation of enzymic activity is secondary. The argument, which has been frequently stated (Walker *et al.* 1983; Sivak & Walker 1985*a*; Sivak *et al.* 1985; Walker & Sivak 1985*a*) is as follows. On illumination after a brief dark interval, a swift rise in PGA reduction would be accompanied by an equally swift increase in [Ru5P] and [RuBP]. Associated oxygen evolution and CO_2 fixation would rise to a peak. As this peak was approached, ATP consumption would outstrip ATP regeneration (Robinson & Walker 1979; Carver *et al.* 1983; Edwards & Walker 1983). The fall in the [ATP] : [ADP] ratio would slow PGA reduction and RuBP regeneration. Oxygen evolution and CO_2 fixation would decline until overtaken by an improving [ATP] : [ADP] ratio. The entire process would then be repeated with less and less overshoot until the oscillations had dampened out. Consistent with this interpretation is the fact that P_i feeding dampens oscillations (presumably by allowing maximal discharge of PGA and triose phosphate from stroma to cytosol and/or directly limiting ATP synthesis) and increases the CO_2 and light intensity thresholds below which they are not observed (Harris *et al.* 1983; Sivak & Walker 1985*b*, 1986; Walker & Sivak 1985*a*,*b*; Walker & Osmond 1986; Laisk & Walker 1986; Leegood & Furbank 1986). Similarly, sequestration of cytosolic P_i by mannose and 2-deoxyglucose has converse effects (Walker & Sivak 1985*a*,*b*; Sivak & Walker 1986).

The role of phosphate in the regeneration of RuBP

Whether or not the above interpretation is correct, there is little doubt that P_i availability plays a major role in RuBP regeneration. This suggests that the interpretation of rate against [CO_2] curves, in terms of the kinetic characteristics of the carboxylase and regeneration of RuBP, is an over-simplification. Any discussion of cause and effect must take into account that, at any point during oscillations, photosynthesis can be increased dramatically until it

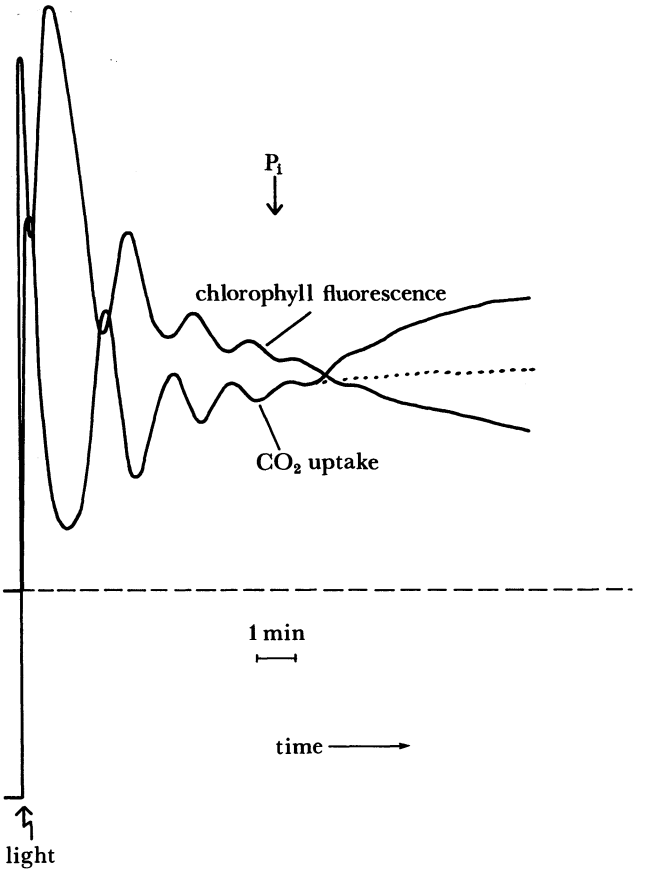

FIGURE 6. Stimulation of quasi-steady-state photosynthesis by P_i feeding (via the petiole) to a young barley leaf in saturating light and saturating CO_2 (0.55% CO_2, 20% O_2, balance N_2, at a temperature of 20 °C). Under these conditions re-illumination induces marked oscillations in CO_2 uptake (note that the rate at the first trough is about 15% of the rate at the peak) and broadly reciprocal but phase-shifted oscillations in fluorescence (figure 5; see also Walker *et al.* 1983; Sivak & Walker 1985). After P_i feeding the amplitude and frequency of the oscillations is decreased (not shown, but see Walker & Sivak 1985*b*; Sivak & Walker 1986). CO_2 uptake measured by infrared gas analysis, as in figure 5, at 20 °C. Gas flow 100 ml min^{-1}, illumination with red light, 180 W m^{-2}. The dotted line indicates the course of CO_2 uptake in the absence of P_i feeding; the horizontal broken line marks the dark value of CO_2 uptake.

approaches the maximum rate (reached during the first or second peak) if P_i supply is increased by petiole feeding (figure 6 and see Walker & Sivak 1985*b*; Sivak & Walker 1986). P_i sequestration or feeding does not change the quantum efficiency of the photosynthetic process, but only the maximum photosynthetic rate and the light intensity required to reach it (figure 7, and see Walker & Osmond 1986; Sivak & Walker 1986). It is tempting to speculate that P_i could modulate the activation status of Rubisco directly in these circumstances (see above) providing the link between RuBP regeneration, limited in this case by P_i, and carboxylation.

It has been known for many years that the chloroplast exports triose phosphate in exchange for imported P_i, and it has been repeatedly suggested that sucrose synthesis from triose phosphate in the cytosol is the principal means of recycling P_i (Walker & Robinson 1978). The vacuole, containing much of the cellular P_i, has to be involved in the regulation of P_i availability (see Walker & Sivak 1985*b*; Sivak & Walker 1986 and references therein). Photorespiration may play a similar role.

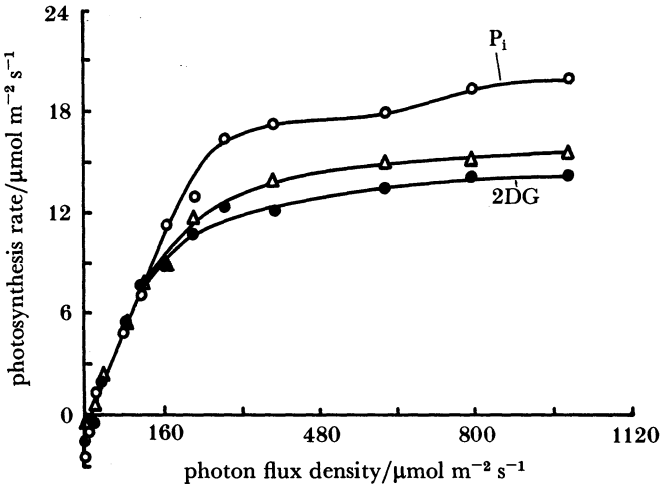

FIGURE 7. Stimulation of photosynthesis by P_i feeding and inhibition by 2-deoxyglucose (2DG) feeding, shown as a function of light intensity (PFD: photon flux density). No changes in rate were observed at the lower intensities (i.e. the quantum yield, which can be determined from the initial slope, was unchanged). This experiment was carried out at 20 °C on a 10 cm^2 disc cut from a spinach leaf. The leaf disc was fed (between measurements) for 20 min; first with 20 mM 2-deoxyglucose and then with 20 mM P_i at pH 5.8, through its midrib. No increase in rate was seen in otherwise similar experiments in which discs were provided with water rather than P_i or 2DG. (Oxygen measured with a Hansatech leaf disc electrode (Delieu & Walker 1983) at 20 °C in 5% CO_2; illumination with red light (Sivak *et al.* 1985*a*, *b*; Sivak & Walker 1985*a*, *b*).) See also Walker & Osmond 1986; Sivak & Walker 1986.

PHOTORESPIRATION AND THE REGENERATION OF RUBP

Photorespiration interacts with RuBP regeneration in three ways. Firstly oxygenation of RuBP decreases the formation of PGA from which RuBP is regenerated. Secondly phosphoglycollate formed by oxygenation is hydrolysed in the chloroplast thereby recycling P_i (cf. internal recycling of P_i in starch synthesis from triose phosphate). Finally, glycerate which is formed (as shown in figure 8) from exported glycollate, is re-imported into the chloroplast and phosphorylated to PGA, thereby consuming ATP and contributing, once again, to the regeneration of RuBP. These latter processes can be regarded as scavenging mechanisms which mitigate the losses in PGA and P_i which would otherwise result from photorespiration.

Figure 8 shows the reactions of the RPPP and photorespiration, the integrated pathway in detail at the CO_2 compensation point. It should be noted that there is no net product under these conditions. The reactions to the left of the dotted line are exactly the same as those depicted in outline in the RuBP regenerative phase (figure 4). Similarly, the carboxylation and reduction of PGA to triose phosphate involves precisely the same reactions as in figure 4 (i.e. as in the RPPP proper) but the flow of carbon through these reactions is changed. Thus only one RuBP is carboxylated instead of three, so only four molecules of PGA are formed at this stage. The two molecules of phosphoglycollate that are formed instead, by the oxygenase reaction (figure 1), enter the sequence glycollate → glyoxylate → glycine → serine → hydroxypyruvate → glycerate. Finally, the glycerate is phosphorylated, restoring a fifth molecule of PGA so that five molecules of triose phosphate can be formed. These can regenerate the CO_2-acceptor.

To implicate photorespiration in P_i recycling is not to question the view of Lorimer & Andrews (1973) that oxygenation is an inevitable consequence of carboxylation or that the rest

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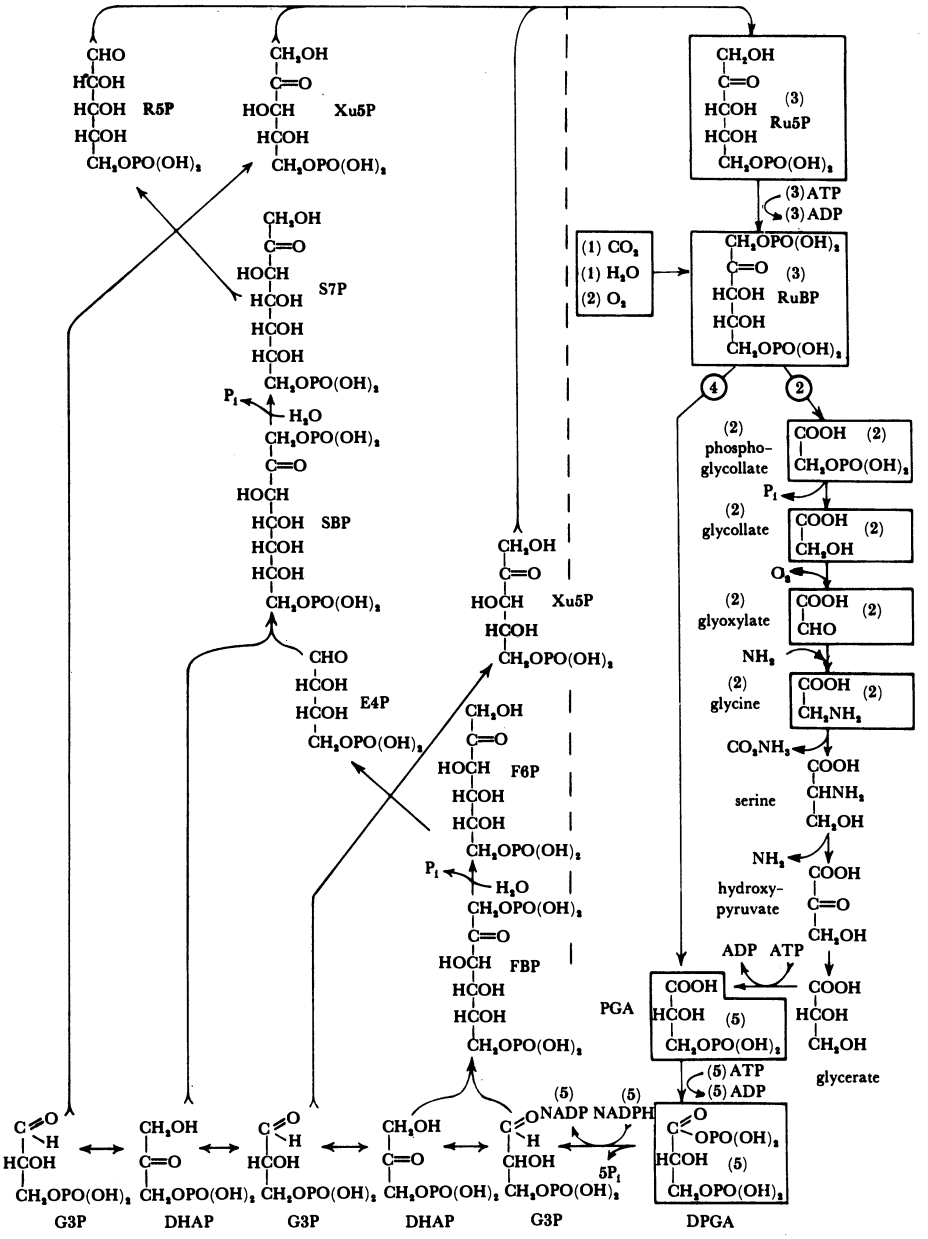

FIGURE 8. Integration of carbon assimilation and photorespiration. For details see text (from Edwards & Walker 1983).

of the photorespiratory sequence is, in effect, an attempt to 'make the best of a bad job' by returning carbon skeletons to the cycle (as glycerate) which would otherwise be lost (as glycollate). If photosynthesis in air is limited by the characteristics of Rubisco, it is tempting to view improved rates of photosynthesis as a consequence of genetically engineered improvements in these characteristics. Tampering with reactions subsequent to the oxygenation of RuBP is now generally thought to be counterproductive because it ensures that once carbon is lost as glycollate it will stay lost rather than be scavenged or recycled. Improvements in the properties of the carboxylase, however, are more often questioned on the grounds of feasibility

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than desirability, and the ominous fact that the leaves may not use their existing carboxylase to capacity is often neglected (see above). It is true, of course, that CO_2 enrichment improves productivity in crops such as cucumber, and that much of this improvement is due to suppression of photorespiration. There is, however, a real possibility that photorespiration may also affect P_i supply not only *in vitro* (Usuda & Edwards 1982) but also *in vivo* (Harris *et al.* 1983; Badger *et al.* 1984; Leegood & Furbank 1986). Phosphoglycollate is hydrolysed prior to the release of glycollate to the cytosol, so recycling P_i within the chloroplast. In some circumstances, for example when CO_2 is in relative excess and when P_i supply is relatively low in relation to demand, 2% O_2 is *inhibitory*; this suggests that photorespiration might then play a useful role in P_i recycling (Harris *et al.* 1983). In this case, restriction of photorespiration, instead of increasing the rate of photosynthesis as expected, is only decreasing the threshold at which P_i limits photosynthesis, replacing one limitation by another. The situation is complicated, however, by the fact that O_2 concentration seems to have very similar effects at CO_2 concentrations which do not favour photorespiration (Walker *et al.* 1983; Walker & Sivak 1985*b*; Sivak *et al.* 1985*b*; Sivak & Walker 1986).

In any event, it is becoming increasingly evident that RuBP regeneration cannot be separated from P_i recycling or supply. Decreased photorespiration (if achieved through decreased Rubisco oxygenase activity) should decrease the light and CO_2-concentration thresholds at which P_i becomes limiting, firstly because less P_i will be made available through photorespiration, and secondly because increased carboxylation will make higher demands on the supply of P_i, itself a substrate of photosynthesis. This conclusion has obvious implications for fundamental research that has improved carboxylation (and decreased oxygenation) as its ultimate practical goal. There is every reason, therefore, to suppose that the catalytically functional Rubisco content of the leaf, defined in turn by existing affinities for CO_2 and O_2, will determine the rate of photosynthesis in air (in high light) and that a decrease in the enzyme affinity for O_2, combined with an increase in its affinity for CO_2, would be a laudable objective in plant breeding or less conventional genetic manipulation (Ogren & Hunt 1978). At the same time, if practical considerations are paramount, it is worth remembering that, according to recent estimates, atmospheric CO_2 is destined to double within the next 25–50 years and that any man-made improvements in the carboxylase will also tend to push crops away from 'carboxylation kinetics limitation' and towards 'RuBP regeneration limitation'. Experiments performed at high CO_2 concentrations, of which those shown in figures 5 and 6 are examples (see also Walker *et al.* 1983; Sivak & Walker 1985; Walker & Sivak 1985*b*; Sivak & Walker 1986), are, in a way, mimicking the situation of increased atmospheric CO_2 concentrations and improved carboxylase. For all of these reasons, therefore, it would be sensible to encompass RuBP regeneration in any strategy designed to improve the effectiveness of RuBP carboxylation.

Conclusions

It is evident that, although we have learned a great deal about the role of Rubisco in photosynthesis from *in vitro* studies, and although its central role in carbon assimilation is unquestionable, it catalyses only one reaction in a complex cycle. Accordingly, while there are entirely valid reasons for seeking to increase its affinity for CO_2 and decrease its affinity for O_2,

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there are equally good reasons for clarifying its activation, its regulation and the processes involved in the regeneration of its substrate. Among these last processes the participation of P_i appears to be paramount.

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Discussion

G. Voordouw (*Department of Biochemistry, Agricultural University, Wageningen, The Netherlands*). You mentioned the uncertainty in the concentration of Rubisco active sites in the stroma of the chloroplast as ranging possibly from 2 to 12 mM. What is the theoretical maximum value, that is the concentration of active sites, in a crystal of Rubisco?

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D. Eisenberg (*Molecular Biology Institute, U.C.L.A., Los Angeles, California* 90024, *U.S.A.*). In response to Dr Voordouw's question, one can compute the concentration of active sites in crystalline Rubisco. In the form III tobacco crystals, it is 9 mM.

D. A. Walker, R. C. Leegood and M. N. Sivak. A similar value (10.4 mM ± 0.1 mM) for the 'upper limit of the active site concentration likely to be found in the chloroplast' has been calculated by Pickersgill (1986). It should be noted, however, that although such calculations undoubtedly set the upper limit they are not inconsistent with the values of 18 mg protein (mg chlorophyll)$^{-1}$ determined by Lilley *et al.* (1975) and are in agreement with electron micrographs of spinach chloroplasts showing crystalline bodies. As indicated in the text, the concentration of active sites has many implications in regard to metabolite binding and a value of 4–10 mM would, in our view, be much more realistic and acceptable than the value of 4 mM that is so often quoted.

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