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ENZYMATIC REGULATION OF PHOTOSYNTHETIC CO_2 FIXATION IN C_3 PLANTS*

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"*Reality is only an approximation to our models.*"
–Henry Miziorko, at a workshop on Rubisco, the University of Arizona, April 1987.

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*Abbreviations: CA-1-P—2-carboxyarabinitol 1-phosphate; CABP—2-carboxyarabinitol 1,5-bisphosphate; DHAP—dihydroxacetone phosphate; G3P—glyceraldehyde 3-phosphate; G6P—glucose 6-phosphate; F26P—fructose 2,6-bisphosphate; F6P—fructose 6-phosphate; FBP—fructose 1,6-bisphosphate; FBPase—FBP phosphatase; PGA—3-phosphoglyceric acid; PCR-cycle—photosynthetic carbon reduction cycle; Pi—orthophosphate; PSI—photosystem 1; PSII—photosystem 2; Q_A—the primary acceptor of PSII; Ru5P—ribulose 5-phosphate; Rubisco—RuBP-carboxylase/oxygenase; RuBP—ribulose 1,5-bisphosphate; SBPase—sedoheptulose 1,7-bisphosphate phosphatase; SPS—sucrose phosphate synthase; UDPG—UDP-glucose

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INTRODUCTION

The physiological process of photosynthetic CO_2 uptake by plant leaves is a manifestation of reactions catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). In this review we consider the metabolic and biochemical factors that exert "local control" over these reactions in vivo. Mechanisms of regulation (particularly of Rubisco) play a prominent role here, but the phenomenon of central interest is the overall regulation of the biochemical process of photosynthesis. Our goal is to show how some of these mechanisms, which effect "local" control of this key enzyme, are linked to other steps in the photosynthetic process, thus forming "regulatory sequences" that connect the velocity of the carboxylation and oxygenation reactions to independent factors of the environment (e.g. the intensity of light or the concentration of CO_2). These sequences bring us, inescapably, to consideration of limiting factors in photosynthesis—a topic that has been considered mostly in qualitative terms. To facilitate a natural progression toward application of quantitative methods we pay particular attention in this part of the review to quantitative procedures that have been used to examine control of complex metabolic systems by co-limiting factors. Finally, we note that our approach to this problem is conditioned in part by the view that, through evolutionary processes, the photosynthetic system has been selected to achieve efficient use of limited resources for primary production. We conclude our review with consideration of some general ways in which the regulation of CO_2 fixation conforms to this view.

BIOCHEMICAL INTERPRETATION OF NET CO_2 UPTAKE

Studies of the biochemical regulation of net CO_2 uptake in photosynthesis must focus first upon the operation of Rubisco in vivo. This enzyme is the gatekeeper standing between the internal metabolic systems of the mesophyll cells and the observer, who may monitor the rate of net gas exchange with the environment. Obviously, the more known about the rules governing passage

through the gate, the more we may infer concerning the internal operation of the system. In recent years a number of reviews of Rubisco have appeared (5, 121, 137, 158, 166, 174, 261). Here, we consider some aspects of the enzyme from C_3 plants that are relevant to understanding its function in vivo.

The CO_2 Compensation Point, a Special Case

To presume that physiological responses of intact leaves can be interpreted from biochemical studies of Rubisco involves a leap of faith. Our confidence for making this leap may be bolstered by reviewing studies that have established the biochemical basis of a single, well-defined physiological property—the CO_2 compensation point (Γ). When a leaf of a C_3 plant is illuminated in a closed space it will either take up or evolve CO_2 (depending upon the initial concentration of CO_2) until the CO_2 concentration reaches a stable steady-state value ($\Gamma \approx 45$ μbar CO_2 at 25°C and 210 mbar O_2) at which the rate of net CO_2 assimilation (A) is zero. The value of Γ is independent of the intensity of illumination (above a threshold intensity), strongly dependent upon $[O_2]$ and temperature, and under any given condition, similar for leaves of all C_3 plants. Tregunna and coworkers (252) postulated that the responses of Γ to O_2 and temperature indicate that the rates of photorespiration and gross CO_2 uptake are somehow linked. The basis for this linkage became clear with the discovery that Rubisco catalyzes the first step in both metabolic sequences (19, 20).

Laing et al (147) derived an equation relating the ratio of the rates of carboxylation (v_c) and oxygenation (v_o) of RuBP to the concentration of the substrates, CO_2 and O_2,

$$\frac{v_c}{v_o} = \frac{V_c/K_c}{V_o/K_o} \cdot \frac{[CO_2]}{[O_2]} = \tau \cdot \frac{[CO_2]}{[O_2]}, \qquad 1.$$

where v_c and v_o are the rates of carboxylation and oxygenation, K_c, K_o, V_c, and V_o are the corresponding K_m and V_{max} terms for the carboxylase and oxygenase functions of Rubisco, respectively, and τ is a constant used to abbreviate the ratio of kinetic constants. From the equation for net CO_2 exchange (64)

$$A = v_c\left(1 - 0.5\,\frac{v_o}{v_c}\right) - R_d \qquad 2.$$

it may be deduced that $v_o/v_c = 2$ at a $[CO_2]$ and $[O_2]$ where $A = -R_d$, the rate of normal respiration continuing in the light. Ignoring the respiration term for the moment we may write that $\Gamma \approx 0.5[O_2]/\tau$. Laing et al (147) evaluated τ from measurements of the kinetic constants conducted over a range of tem-

peratures and showed that the observed dependence of Γ values on [
temperature could be approximated using this model and kinetic constants derived from in vitro studies with purified Rubisco. Jordan & Ogren (126) developed a more accurate procedure for determining τ based upon a simultaneous assay of the v_c/v_o ratio. These workers demonstrated much better agreement between Γ values predicted from their measurements of τ and Γ values for C_3 plants (18, 127, 128, 134). Furthermore, they showed that τ is not significantly affected by the concentration of RuBP, the pH of the medium, or the activation state of Rubisco, all of which may vary in vivo (128).

The next step in the development of this quantitative test fell to the physiologists. As first indicated by Laisk (149), the "true" compensation point of photosynthesis is not at Γ where $A = 0$ but at a slightly lower CO_2 concentration where $A = -R_d$. He defined this point as Γ* and showed that $\tau = 0.5$[
significant given the precision of the measurements of τ by Jordan & Ogren (128). Brooks & Farquhar (22) developed gas exchange procedures to measure Γ* for leaves of spinach as a function of temperature, and the corresponding values of τ obtained by this procedure are compared with those obtained in vitro by Jordan & Ogren (128) in Figure 1. The close agreement

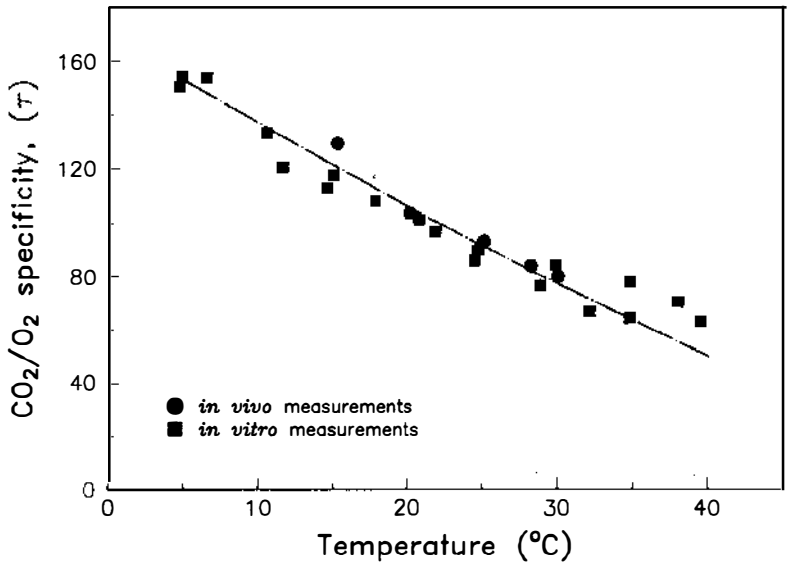

Figure 1 A comparison of the temperature dependence of the CO_2/O_2 specificity of spinach Rubisco. Values were obtained from measurements of the CO_2-compensation point (Γ*) of intact leaves by Brooks & Farquhar (22) and from simultaneous determination of RuBP-carboxylase and RuBP-oxygenase activities with purified spinach Rubisco in vitro by Jordan & Ogren (128). The dashed line is drawn using the Q_{10}=0.74 (Table 1).

between the two sets of data provides some confidence that kinetic measurements with isolated Rubisco conducted in vitro are relevant to interpreting physiological responses of intact leaves.

Experimental Approaches

The experimental approaches that provide the basis for mechanistic analyses of the rates of CO_2 uptake during steady-state photosynthesis include: (*a*) biochemical studies of the components of the system—particularly Rubisco; (*b*) physiological measurements of the systemic properties of the intact tissue at defined steady states; and (*c*) predictive or interpretive models of the relevant processes. It is not practical to provide a complete review of these approaches here. Nevertheless, a brief introduction to these topics may be useful.

KINETIC PROPERTIES OF RUBISCO There are many reports of measurements of the kinetic constants of Rubisco from C_3 species [for a compilation see Keys (137)]. Most of these measurements have been made with preparations having somewhat lower activity than the native enzyme as a result of damage during preparation or aging. Of course what one wishes to know are the values of the kinetic constants that apply in vivo, and there is no completely sound basis for selecting such constants. The values listed in Table 1 are calculated from measurements of the enzyme from spinach. There may, however, be small significant differences in the kinetic properties of Rubisco from different species of C_3 plants (59, 217). The set of constants provided here—the $K_m(CO_2)(K_c)$, the inhibitory constant for oxygen with

Table 1 Suggested values of kinetic constants to approximate the response of net CO_2 uptake by Rubisco of C_3 plants in vivo.

Constant	Symbol	Y_{25}, value at 25°C	Q_{10}	Reference
CO_2/O_2 specificity	τ	88 M M^{-1}	0.74[b]	(22, 128)
		2360 bar bar^{-1}	0.67[b]	
$K_m(CO_2)$	K_c	9 μM	1.8[c]	(128, 212)
		270 μbar	2.1[c]	
$K_i(O_2)$ on K_c	K_o^i	535 μM	1.0	(128)
		400 mbar	1.2	
K_m(RuBP)	K_R^1	28μM	1.9	(9)
Activity	V_c	3.6 μmol $mg^{-1}min^{-1}$	2.4[c]	(9, 212)
	k_{cat}	3.3 $s^{-1}site^{-1}$		

[a]These values are based upon measurements conducted in vitro with Rubisco and have been selected assuming that the activity of the native enzyme is greater than or equal to that observed in vitro. The Q_{10} values approximating the temperature dependence of these constants may be used to calculate the value (Y_T) at any temperature (T) between 5 and 35°C according to $Y_T = Y_{25} \cdot Q_{10}^{(T-25)/10}$, where Y_{25} is the value at 25°C.

[b]See also a polynomial given in Ref. 22.

[c]To extrapolate below 15°C use: $Y_T = Y_{25} \cdot Q_{10}^{-1} (1.8Q_{10})^{(T-15)/10}$.

respect to CO_2 on the carboxylase (K_o^i), the K_m(RuBP) (K_R), and the catalytic constant for the carboxylase function (k_{cat})—are generally all that is required to calculate v_c and v_o. Direct measurements of the kinetic constants for the oxygenase function are not as accurate as those for the carboxylase function, and v_o can be defined from v_c by an expression—$v_o = v_c[O_2]/\tau[CO_2]$—which is based upon the more precise measurements of the specificity factor.

The constants for the gaseous substrates are given (Table 1) both in terms of the dissolved concentration and on an equivalent partial pressure basis. The latter are convenient for interpretation of gas exchange measurements. Some controversy has arisen concerning the use of partial pressure as a basis for expressing these constants (90). Either basis is valid, and Henry's law can be used to convert between them. Note that the apparent temperature coefficients (Q_{10}) for the constants are slightly different depending on the convention used. Difficulties in controlling the concentration of the gaseous substrates dissolved in the aqueous phase during in vitro assays (and in the case of CO_2, errors in equating experimental measurements obtained in terms of total dissolved inorganic carbon with an equivalent level of CO_2) severely limit the reliability of the K_c and K_o^i values. Jordan & Ogren (128) used precision gas mixing equipment to obtain the $[CO_2]/[O_2]$ ratios in their assays of τ. The K_R value in Table 1 is the apparent K_m (RuBP) and does not take into account chelation of the active form $RuBP^{4-}$ with Mg^{2+} present in the assay system (30). Inhibitory constants for various phosphorylated compounds with respect to RuBP summarized by Ashton (6) and Jordan et al (125) are also apparent constants valid only at 5–10 mM Mg^{2+}. Provided $[Mg^{2+}]$ does not vary significantly during steady-state photosynthesis, these apparent constants are adequate approximations of the operative values. Von Caemmerer and coworkers (254, 256) present kinetic expressions that take into account chelation by Mg^{2+}, and the true constants can be calculated knowing the dissociation constants for the respective Mg^{2+} complexes. The k_{cat} of Rubisco reported here (Table 1) was obtained from assays of whole-leaf extracts and an assay for Rubisco protein (see below). Since the apparent k_{cat} values decline rapidly after extraction of Rubisco from the leaf, the values of purified Rubisco seldom approach this activity, and the adequacy of these constants must be judged, among other things, on how well they account for in vivo responses.

GAS EXCHANGE STUDIES Obtaining accurate measurements of the net flux of CO_2 uptake is now routine. For the present purposes, it is important to obtain estimates of the velocity of specific reactions in the photosynthetic process. For example, the rate of carboxylation (v_c) can be obtained from measurements of A and R_d, if the $[CO_2]/[O_2]$ ratio under that condition is known. By combining Equations 1 and 2, we may write that

$$v_c = \frac{A + R_d}{1 - 0.5[O_2]/\tau[CO_2]}. \quad 3.$$

The rate of other biochemical steps of photosynthetic carbon metabolism that are linked to CO_2 uptake or photorespiration can be calculated from v_c and the v_o/v_c ratio using stoichiometric relationships summarized by Farquhar & von Caemmerer (255). This requires that one know the activity of CO_2 and O_2 in the chloroplast stroma during steady-state photosynthesis. (Unless otherwise noted, terms in square brackets denote the chemical activity of that species in the chloroplast.)

The partial pressures of CO_2 and O_2 can be measured only for the bulk air surrounding the leaf. Whenever there is a net flux, the mole fraction CO_2 must be lower (and that of O_2 must be higher) in the stroma than in the bulk air. Note that in the special case where A is zero (at Γ) the diffusion gradients are negligible. The CO_2-diffusion gradient can be calculated from the net flux, knowing the conductance of the diffusion pathway. Simultaneous measurements of the rates of assimilation (A) and transpiration (E) permit measurement of the conductance of the stomata and boundary layer (12, 255) and may be used to obtain reliable estimates of the concentration of CO_2 in the intercellular air spaces (224).

There must, however, be an additional gradient across the cell wall and aqueous phase pathway that separates the intercellular air spaces and the stroma. Theoretical arguments have led to conflicting opinions as to the magnitude of this gradient (172, 193). Direct estimates of this gradient have been obtained recently by Evans et al (60), who conducted simultaneous measurements of A and E (which permit calculation of the gradient of CO_2 between the bulk air and the intercellular spaces), and carbon isotope discrimination (which permits calculation of the gradient of CO_2 between the bulk air and the stroma) by gas exchange techniques (60). They found that the concentration of CO_2 in the stroma could be as much as 60 μbar less than that in the intercellular air spaces during active CO_2 uptake (e.g. $A \approx 30$ μmol $m^{-2}s^{-1}$) and calculated that the aqueous-phase conductance of wheat leaves (g_{aq}) is 0.5–1 mol $m^{-2}s^{-1}$. This conductance should be a constant for a given leaf. There have not been sufficient measurements of this parameter yet to permit any general conclusions concerning the value of g_{aq} for leaves of different species or as a function of developmental factors such as light intensity or nutrient status during growth. Uncertainty concerning the value to use for g_{aq} is a significant problem, but provided this conductance is similar to that of wheat, the chloroplast [C
parameter. Corrections of the O_2 partial pressure are only significant at very low [O
levels.

FREEZE-CLAMP STUDIES Development of the freeze-clamp apparatus (11) opened the way for studies of the metabolic basis of steady-state photosynthesis. This device is designed to obtain metabolite flux measurements from gas exchange studies, and while the leaf is under steady-state conditions in the gas exchange cuvette, to take a sample of the leaf by clamping it between two cold metal pistons that can be brought into contact with both surfaces of the leaf. Metabolic processes of the tissue are arrested in <1 s, and the leaf sample can be divided and extracted to assay for enzyme activities and metabolite pool sizes (11, 168, 213, 226, 254). The measured pools include material from all compartments of the leaf. Studies of the subcellular compartmentation of metabolites in protoplasts (83) and non-aqueous fractionation of the frozen leaf samples (46, 47, 83) have been important in determining the distribution of metabolites among the stroma, cytosol, and vacuole.

THE RUBISCO CONTENT The concentration of active sites is widely assumed to be ~4 mM (121), but as pointed out by Walker et al (261), this may be too low. Crystals have been observed in electron microscopy of chloroplasts (235), and the concentration of crystalline Rubisco has been estimated to be ~8–10 mM (184). The Rubisco content of leaf extracts may be quantified in terms of activity or protein. Activity measurements are adequate to interpret measurements of CO_2 response kinetics, but an estimate of the concentration of Rubisco catalytic sites is needed to interpret the significance of RuBP pool size measurements and to calculate the apparent k_{cat} of Rubisco. The molar concentration of Rubisco in an extract can be obtained by incubating with ^{14}C-carboxyarabinitol-1,5-bisphosphate (CABP), which binds very tightly and specifically to Rubisco sites. Determination of protein-bound ^{14}C by immunoprecipitation, gel filtration, or precipitation with polyethylene glycol (35, 91, 285) permits a direct estimate of the molar concentration of Rubisco. Immunoelectrophoresis (129) has also been used to quantify Rubisco in crude extracts. Procedures based upon in vivo assays (267) or separation and quantification of denatured protein may eventually supplant present methods.

RUBISCO ACTIVATION STATE The activation level of Rubisco may vary over a wide range according the prevailing environmental conditions. Very rapid extraction of quick-frozen leaf tissue and processing the extract at ice temperature in buffer containing a low [C
level of activation for at least 1 min (160, 218). Assays for Rubisco activity conducted immediately after extraction and again after incubation of an aliquot of the extract with saturating concentrations of Mg^{2+} and CO_2 at room temperature give an estimate of the steady-state activation level. An alterna-

tive approach takes advantage of the observation that CABP forms a much tighter complex with the active than with the inactive form of Rubisco (91). Thus, ^{14}C-CABP bound to an inactive Rubisco catalytic site can be displaced by exchange with unlabeled CABP, while that bound to an activated Rubisco catalytic site cannot be displaced (91). The latter method, since it is not based on catalytic activity, can be used when the inhibitor CA-1-P is present (214).

CA-1-P CONTENT Extracts of some species may contain 2-carboxyarabinitol-1-phosphate, a strong inhibitor of Rubisco activity (16, 89, 214). Generally, when this compound is present, one observes diurnal variations in the extractable activity of Rubisco (164, 257). The concentration of the compound can be measured by titrating the activity of a known quantity of Rubisco with the solution of CA-1-P (16). Provided the CA-1-P concentration is less than that of Rubisco, analysis of the quantity of Rubisco and its catalytic activity in a leaf extract can be used to estimate the CA-1-P concentration (140, 214).

MODELS OF CO_2 FIXATION Knowledge of physiological and biochemical mechanisms of photosynthetic metabolism have been drawn together in the form of conceptual or mathematical models of the photosynthetic process. The usefulness of models stems primarily from the complexity of the photosynthetic system and from the strong emphasis on quantitative analysis of photosynthetic responses. The two essential components of any such model are: (*a*) the structure and stoichiometric relationships of the component reactions of the pathway, and (*b*) kinetic expressions for the controlling steps. The review of Farquhar & von Caemmerer (63) provides a comprehensive introduction to these essentials. Models differ primarily in their complexity and in the details of the kinetic expressions used. The model of Farquhar et al (64), for example, assumes that kinetic control of the rate of CO_2 uptake resides either with Rubisco or with the light-dependent reactions that regenerate RuBP, and the intervening reactions are not considered. The models of Woodrow (273) and Laisk & Walker (151) include reactions of the PCR cycle and the synthesis of sucrose and starch. However, while these models are useful for analyzing regulatory responses within the photosynthetic system there is not yet enough information regarding the activities and kinetic constants of the constituent enzymes to predict accurately the responses of the pathway to changes in external variables. In this regard a simpler model (93) that focuses upon the main points where environmental factors interact with the system may be more useful. In the following sections we develop a general kinetic model for Rubisco, then consider regulatory sequences that link this enzyme to the remainder of the photosynthetic process.

Kinetic Analysis of Rubisco in vivo

A GENERAL KINETIC MODEL FOR RUBISCO In order to analyze the multiple levels of kinetic interactions known for this enzyme, we may write a general expression of the form,

$$v_c \simeq (E_t \cdot k_{cat}) \times f_1([CO_2],[O_2]) \times f_2(R_t,E_t) \times f_3(C_t) \times f_4(X), \qquad 4.$$

where the functions $f_1 \ldots f_4$ are factors ($0 \leq f \leq 1$) giving the fractional activity as influenced by: 1) the concentration of gaseous substrates; 2) the total concentration of RuBP (R_t) and competitive inhibitors (with respect to RuBP) in relation to the Rubisco site concentration (E_t); 3) the stromal concentration of the tight binding inhibitor, 2-carboxyarabinitol-1-phosphate (C_t); and 4) the effectors (X) that influence the activation of Rubisco. The rate (v_c) can be approximated by substituting the measured "local" concentration values into Equation 4 and is related to the rate of net CO_2 uptake according to Equation 3. We emphasize, however, that Equation 4 serves only as a simple and clear way to approximate the Rubisco rate equation and that the multiplicative properties of these terms has not been rigorously established.

RESPONSES TO CO_2 AND O_2 The influence of the gaseous substrates has generally been assumed to follow the classical Michaelis-Menten expression for an enzyme with a single substrate (CO_2) in the presence of a competitive inhibitor (O_2):

$$f_1 = \frac{[CO_2]}{[CO_2] + K_c(1 + [O_2]/K_o^i)}. \qquad 5.$$

This equation is based upon in vitro studies in the presence of a saturating concentration of RuBP, but there has been some debate as to whether these kinetics are appropriate when RuBP is not saturating or the enzyme is not fully activated. More complex kinetics could apply, depending upon the order of substrate binding. Farquhar (61) developed equations to describe these kinetics assuming that the reaction mechanism is ordered with: (*a*) CO_2 binding first, (*b*) RuBP binding first, or (*c*) either binding first (i.e. random). From analysis of kinetic experiments in vitro, Badger & Collatz (9) were able to eliminate the first possibility but were not able to choose between the second and third alternatives. In his general kinetic model, Farquhar (61) assumes the second possibility, in part because the random mechanism does not lend itself to an analytical solution.

Recent experiments have established that the reaction mechanism is strictly ordered, with RuBP binding first, followed by a slow step—enolization of

RuBP on the enzyme (185). This apparently creates the binding site for CO_2 or O_2 (186), making it improbable that the apparent $K_m(CO_2)$ is dependent on the level of RuBP or the activation level of the enzyme. The latter should affect the number of functional sites available for reaction with the gaseous substrates, but the apparent K_m for CO_2 or O_2 of these sites should be independent of the steady-state population of functional sites. Therefore, the model of Farquhar (61) should provide a good approximation for the kinetics of Rubisco.

In order to test whether the Rubisco kinetic constants with respect to CO_2 and O_2 calculated from in vitro studies are valid for the in vivo enzyme, the modulation of enzyme activity by factors 2–4 must be eliminated (i.e. $f_2, f_3, f_4 = 1$). If this is not done, the physiological response may become saturated more abruptly and at a lower $[CO_2]$ than would be expected from in vitro measurements. Laisk & Oya (150) used a creative experimental design to avoid this problem. CO_2 was provided to a leaf in short (1 s) pulses interspersed with CO_2-free periods of variable duration selected to keep the time-average rate of CO_2 uptake constant. In this way the $[CO_2]$ during the pulse could be increased to saturating levels while the time-average rate of net CO_2 uptake remained essentially constant and well below the maximum that could be attained under steady-state conditions. When the rate of net CO_2 uptake was plotted against $[CO_2]$ in the pulses, Laisk & Oya (150) obtained a classical rectangular hyperbola with an apparent $K_c = 28$ μmol and $V_{max} = 2.8$ μmol mg^{-1}protein.

These kinetic constants for Rubisco in the complete photosynthetic system agree fairly well with those presently measured in vitro. This is remarkable, in part because these measurements were made in the early 1970s, before the activation process was understood. These experiments also provided a clear demonstration that the abrupt (nonrectangular hyperbola) saturation curve for the steady-state CO_2 uptake vs $[CO_2]$ in vivo is a reflection of regulation of Rubisco by factors 2–4. Laisk (149) suggested that the rate of RuBP synthesis becomes limiting at high $[CO_2]$. Walker and coworkers (157) independently came to the same conclusion by comparing the $[CO_2]$ response of CO_2 uptake by illuminated intact chloroplasts to that obtained upon lysing the chloroplasts into a medium containing a rate-saturating concentration of RuBP.

Most comparisons of the dependence of CO_2 uptake on CO_2 and O_2 have been restricted to low $[CO_2]$ and high light, conditions where $f_2, f_3, f_4 \approx 1$. The use of such low substrate concentrations precludes calculation of the apparent K_c, K_o^i, and k_{cat} values. It is possible, however, to approximate the rate of CO_2 uptake using the Rubisco rate equation, measurements of the concentration of substrates, and kinetic constants estimated from in vitro studies. Seemann et al (213, 217) confirmed the validity of this approximation in experiments with spinach leaves at a low $[O_2]$ and a $[CO_2]$ of 100

μbar. In this study no allowance was made for the gradient in [C
finite aqueous phase conductance, but the uncertainty introduced by this omission is not larger than that introduced by uncertainty in the magnitude of the kinetic constants. In a conceptually similar a series of studies, Evans and coworkers (54–56, 58) analyzed the slope of the [CO_2] response curve at the CO_2-compensation point ($(dA/dc)_\Gamma$) of *Triticium aestivum* (wheat) using the derivative of the rate expression for CO_2 uptake (see 255). The observed values of $(dA/dc)_\Gamma$ are highly correlated with the Rubisco content of the corresponding leaves, and these values are quantitatively consistent with the response predicted from in vitro kinetic measurements. Evan's studies, however, did not resolve differences between genotypes in g_{aq} (estimated by ^{13}C discrimination) and in the k_{cat} of Rubisco by analyzing $(dA/dc)_\Gamma$ in vivo. A significant concern in experiments of this type is the assumption that Rubisco is fully activated. Von Caemerer & Edmondson (254) show that the activation state of well-nourished leaves of *Raphanus sativa* decline at [CO_2]$<100\mu$bar. Furthermore, phosphate-deficient leaves do not fully activate their Rubisco—even at normal ambient CO_2 and saturating light (21, 161). Variation in the activation level may underlie the rather large variation in $(dA/dc)_\Gamma$.

DEPENDENCE ON RuBP In assessing the degree to which regulatory mechanisms influence the rate of CO_2 assimilation it is necessary, among other things, to have a clear picture of the range over which changes in the concentration of RuBP significantly affect the activity of Rubisco. The kinetics of the Rubisco-catalyzed reaction do not obey a simple rate equation with respect to RuBP, partly because the concentrations of the enzyme and substrate are similar (34, 61, 121). Peisker (181) derived a rate equation for conditions where the concentration of RuBP (R_t) is less than that of the enzyme (E_t). Farquhar (61) used similar conditions in a general kinetic model for Rubisco according to which the response of the catalytic velocity to R_t at a constant CO_2 concentration has the form of a nonrectangular hyberbola. This relationship defines the second term in Equation 4 and is given by:

$$f_2 = \frac{(E_t + K'_R + R_t - [(}{2E_t}, \qquad 6.$$

where R_t is the total concentration (free and bound) of RuBP, and K'_R the apparent Michaelis constant for RuBP. Farquhar et al (64) also noted that, since K'_R is small in comparison to E_t in vivo, the solution to this equation can be approximated by

$$f_2 = \begin{cases} R_t/E_t, & \text{for } R_t < E_t \\ 1, & \text{for } R_t \geq E_t \end{cases}. \qquad 7.$$

Initial results supporting this interpretation (34, 85, 104, 272) have been contradicted by studies with intact leaves which demonstrate that R_t does not apparently control the rate of CO_2 uptake when that rate (at constant $[CO_2]$) declines from the "light-saturated" level in response to a decrease in light intensity. It is generally accepted that regulation of Rubisco activity as described by f_3 and f_4 is the primary reason for this observation. Nevertheless, it will be important to establish the role of other factors, such as competitive inhibitors with respect to RuBP.

Since the activation state of Rubisco changes relatively slowly ($t_{1/2} \approx 5$ min) in response to an alteration in external conditions, it is possible to conduct short-timeframe experiments on Rubisco in vivo during which the rate of CO_2 assimilation may change but the activation state remains approximately constant. Mott et al (169) conducted a series of such experiments and assumed that the rapid (≈30 s) adjustments to the rate of photosynthesis in response to a change in light intensity results entirely from modulation of the [RuBP]. They used freeze-clamp experiments at a constant $[CO_2]$ to demonstrate that the relationship between the rate of carboxylation and [RuBP] approximates a nonrectangular hyperbola (Figure 2). Freeze-clamp samples taken following a transition to a low light intensity, however, showed that after the rapid drop in R_t there was a rise in the level of this substrate until, in the steady state, R_t is approximately equal to that occurring prior to the decrease in light. Prinsley et al (192) reported similar results, but these workers suggested that increases in [PGA] and [Pi] (competitive inhibitors with respect to RuBP) also play a significant role in determining the rate of photosynthesis during the transient.

Effects of phosphorylated compounds on K'_R can be described by an equation of the general form

$$K'_R = K_R\left(1 + \frac{P_1}{K_{P1}} + \frac{P_2}{K_{P1}}\ldots\right), \qquad 8.$$

where P_n is the activity of any phosphorylated compound present in the stroma and K_{P_n} is its corresponding K_i with respect to RuBP. Note that the concentrations used here should be the activity of the free compound, which may differ significantly from the total concentration if the compound binds tightly to Rubisco or another ligand (6, 254). The expected relationship between v_c and R_t according to Equation 6 are plotted (Figure 2) for the case where only RuBP is present, and where the total phosphate moieties in RuBP and PGA are assumed constant (i.e. [PGA] + 2 × [RuBP] = 60 mM). The latter simulation, which should approximate the situation in vivo, indicates that an RuBP concentration approximately twice that of Rubisco active-sites

is sufficient to sustain a v_c of 80% of the RuBP-saturated velocity. It should be noted that the presence of inactive Rubisco or of the inhibitor CA-1-P must be considered when using Equation 6 to interpret in vivo measurements because (*a*) inactive Rubisco binds RuBP (125, 146), thus rendering a portion of the measured RuBP unavailable for catalysis; and (*b*) the operative value for E_t should reflect the activated sites that are free of inhibitor.

Most measurements of the relationship between RuBP and the rate of CO_2 uptake under normal steady-state conditions indicate that the total concentra-

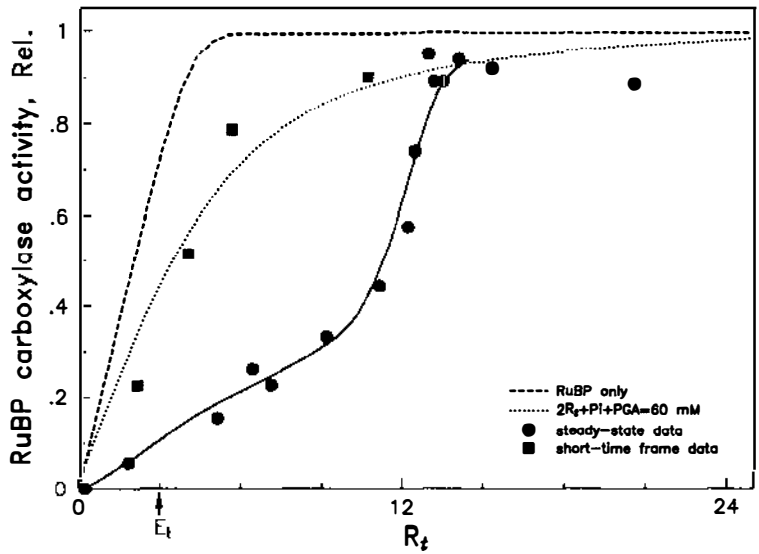

Figure 2 The relationship between the relative activity of RuBP-carboxylase and the total concentration of RuBP (R_t) at constant [CO_2]. The dotted and dashed lines are simulations showing the expected influence of physiological levels of PGA and Pi, which are competitive inhibitors with respect to RuBP. The data points are from freeze-clamp studies with intact leaves that show the response in short-time frame experiments where the activation state of Rubisco remains essentially constant (■) or in experiments where time is sufficient for the activation state of Rubisco to adjust to a new steady-level at each rate (●). The solid line (not a simulation) is drawn to illustrate the sigmoid relationship observed under steady-state conditions. The simulations are drawn according to Equation 6, where: (dashed line) there are no inhibitors; or (dotted line) the concentration of inhibitors (Pi+PGA) varies with R_t such that the total of phosphate moieties in the two pools is constant at 60 mM (estimated from Ref. 254). In the latter case the value of K_k' in Equation 6 was adjusted for each value of R_t using K_i(PGA+Pi) = 0.9 mM and [PGA+Pi] = 60 − $2R_t$ in Equation 8. The steady-state data points are taken from von Caemmerer & Edmondson (254), and the short-time frame experiments are from Mott et al (169). The rates of CO_2 uptake were scaled assuming that v_c was 95% saturated with respect to RuBP at "rate saturating" light, and the concentration values were scaled to give E_t = 3 mM assuming 21.5 μmol Rubisco m^{-2} and 60 nmole Rubisco mg^{-1} chl for the two sets of data, respectively.

tion of RuBP is only less than the concentration of Rubisco active sites when light intensities are very low (i.e. well below the light intensity required for half maximum assimilation rates) or when CO_2 partial pressures are very high. Furthermore, when light intensity changes are used to perturb the steady-state assimilation rate, there is a significant range over which assimilation changes and RuBP either remains constant (11, 183, 254) or declines (46, 47, 182). Low levels of RuBP have been measured in isolated cells and chloroplasts (34, 85, 104, 272), and these probably reflect a transient rather than the true steady-state condition. Von Caemmerer & Edmondson (254) estimated that, at constant $[CO_2]$, the relationship between v_c and [RuBP] has a sigmoid form (Figure 2; see also Figure 4 in Ref. 11). Such sigmoid relationships are normally associated with cooperative binding of a substrate to the enzyme, but in vitro studies of Rubisco indicate no evidence for such kinetics. We discuss this phenomenon in greater detail in a later section.

REGULATION BY CA-1-P An inhibitor of Rubisco, carboxyarabinitol-1-phosphate (CA-1-P), has been detected in extracts of *Solanum tuberosum* and *Phaseolus vulgaris*, and the same (or a similar) compound has been detected in many (but not all) species (214, 220, 257). The substance binds tightly to the activated form of Rubisco [K_D = 32 nM (16)] inhibiting the activity of Rubisco, and the third term of Equation 4 is given by, $f_3 = 1 - EI/E_t$ where EI is the concentration of enzyme-inhibitor complex. Substituting the equation for the concentration of EI (16), we may write that

$$f_3 = 1 - \frac{(C_t + E_t + K_D) - [(C_t + E_t + K_D)^2 - 4C_tE_t]^{1/2}}{2E_t}, \qquad 9.$$

where C_t is the level of CA-1-P, E_t the concentration of Rubisco sites, and K'_D the apparent dissociation constant. For values of $C_t < 0.8E_t$ the concentration of EI can probably be approximated by assuming $EI = C_t$. Effects of compounds that may compete with CA-1-P for binding to Rubisco have not been investigated.

Evidence obtained with *Phaseolus vulgaris* implicates CA-1-P as an important regulator of Rubisco activity (214). In leaves held overnight in darkness, the concentration of this compound may approach or exceed that of Rubisco sites, and the activity of Rubisco extracted from these leaves is only 10% of the normal value. When a leaf is illuminated, however the level of CA-1-P declines to a steady-state value that depends on the intensity of illumination (213, 214). After prolonged exposure to saturating light, the compound is not detectable (213, 214). It is proposed that the concentration of CA-1-P is regulated by a futile cycle (16) involving simultaneous synthesis and degradation. Little is known about the synthetic reaction, but it appears that the rate of

the degradative reaction is dependent upon the turnover of PSII (214). This would be sufficient to regulate the steady-state concentration of CA-1-P.

Kobza & Seemann (140) examined the combined roles of CA-1-P, the activation state, and the concentration of RuBP in controlling the rate of CO_2 uptake in species that, under a given set of conditions, have different levels of CA-1-P. In *Phaseolus vulgaris,* most of the regulation of Rubisco seems to be via CA-1-P. The uninhibited Rubisco sites generally remain in the activated form, although at high [CO_2] the CO_2-Mg^{2+}-dependent level of activation may fall (225). *Spinacea oleracea* apparently does not produce CA-1-P under any circumstance, but this compound is found in the related species, *Beta vulgaris*. In contrast to *P. vulgaris,* regulation of Rubisco in *B. vulgaris* also involves the CO_2-Mg^{2+}-dependent control of the activation state. In all three species, however, the net result was similar: The activity of Rubisco (as controlled by CA-1-P and/or the activation state) changes with light intensity, and the concentration of RuBP is apparently saturating, except at strongly limiting light. The level of RuBP in *P. vulgaris* responds more with light than that in *S. oleracea* which may reflect some interaction between the RuBP and CA-1-P pools.

CONTROL OF ACTIVATION STATE A specific lysine residue of Rubisco (ly 201 of the spinach enzyme) participates in a reversible reaction in which CO_2 and Mg^{2+} add to the inactive Rubisco (E) to form a catalytically active ternary complex (ECM):

$$E + CO_2 + Mg^{2+} \rightleftharpoons ECM + H^+ \qquad 10.$$

The mass action equilibrium expression for this reaction solved for the fraction of Rubisco in the activated form provides a good approximation to experimental data for activation as a function of pH at air levels of CO_2 in vitro (Figure 3). When RuBP is present, the activation level is more sensitive to pH (Figure 3). Mott & Berry (167) attribute this additional pH dependence to the involvement of a protonated group on the enzyme in forming the binary enzyme–RuBP(ER) complex ($E + H^+ + R \rightleftharpoons ER$). An equilibrium expression assuming the overall release of two H^+ in the activation of the ER complex approximates the pH dependence of the activation level in the presence of RuBP (Figure 3).

Several other phosphorylated compounds (particularly 6-phosphogluconate and NADPH) affect the activation process, apparently by binding preferentially to the E or the ECM forms of Rubisco (10, 31, 95, 146, 163). Kinetic studies indicate that these compounds interact with the catalytic site (10, 125) and are competitive with respect to RuBP. High concentrations of RuBP typically present in vivo would therefore tend to suppress any effect of

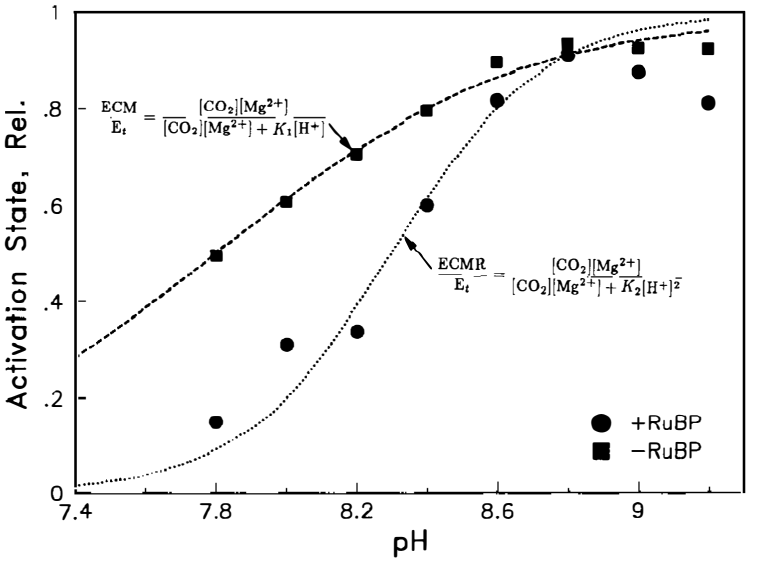

Figure 3 The fraction of Rubisco in the activated state as a function of pH at air level CO_2 in the presence or absence of RuBP. These data are taken as the initial and steady-state rates observed in time-course experiments when the 0.5 mM RuBP was added to Rubisco preincubated at air level CO_2 in the assay mixture at the indicated pH [from Mott et al (167, 168)]. Theoretical expressions for the equilibrium activation level assuming that the activation reaction involves a stoichiometry of one H^+ (in the absence of RuBP) or 2 H^+ (in the presence of RuBP) approximate the observed responses where K_1 = 170 M and K_2 = 1.31 × 10^{11}. The mechanism leading to a lower steady-state Rubisco activity in the presence of RuBP has not been established and may involve another inactive form (i.e. not the ER complex as assumed here). Nevertheless, the activity of Rubisco at physiological pH appears to be lower in the presence than in the absence of RuBP.

these substances. At very low light intensities, when [RuBP]<E_r, these compounds could affect activation, but this effect must be offset against their effect as competitive inhibitors of catalysis (166, 249). Parry et al (179) propose that the effect of Pi is allosteric (as distinct from the above mechanism), but there is no evidence that there is a separate effector binding site for Pi on the enzyme (see 125). A particularly interesting and unresolved matter concerns the possible role of light-dependent changes in [Ca^{2+}] in the stroma in regulating of Rubisco activity. There are light-dependent changes in the stromal [Ca^{2+}] (145), but studies on the effects of this ion on the activation state or activity of Rubisco are contradictory (cf. 14, 30, 180).

The equilibrium expressions provide a basis for calculating the activation level as a function of [CO_2], [Mg^{2+}], [RuBP], and the pH of the medium (e.g. 256). Studies attempting to relate the responses of activation state to the levels of Rubisco effectors in situ, however, have highlighted several phe-

nomena that are difficult to reconcile with our current knowledge of the equilibrium process. First, it is well established that $[Mg^{2+}]$ should increase and $[H^+]$ decrease in the stroma upon illumination (101). These changes taken in combination should increase the activation level of Rubisco. Nevertheless, the strong light-dependent activation of Rubisco occurs over a range of light intensities (>100 μmol $m^{-2}s^{-1}$) where changes in these factors are assumed to be "saturated" (53, 175, 187, 189). Second, it is difficult to explain how the enzyme can be fully activated at normal atmospheric levels of CO_2, because calculations based on the equilibrium (Equation 10) indicate that it should be strongly $[CO_2]$-dependent and less than 50% activated [unless, as pointed out by Mott et al (169), the stromal pH $\geq$ 8.6]. Moreover, recent evidence (254) indicates that the activation level is not significantly affected by CO_2 until $[CO_2] < 100$ μbar. Third, it is well established than an **increase** in $[CO_2]$ can result in a **decrease** in the in vivo activation level—directly contradicting the results of in vitro studies. Such a decrease was observed, for example, by Sharkey et al (225) when $[CO_2]$ was increased from near 300 to 500 μbar under optimal conditions for photosynthesis. Fourth, Heldt et al (102) showed that light-dependent activation of Rubisco in intact chloroplasts requires the presence of an optimal concentration of Pi in the suspending medium. This result is not explicable by any known direct effect of Pi on the activation equilibrium. Finally, the rate of catalysis in vitro decays with time (146). An unpublished experiment (G. Lorimer and J. Pierce) shows that this decline is not explained by a loss of the ECM form. The possibility that Rubisco is regulated by yet another mechanism cannot be ignored.

Despite uncertainty about the mechanisms, it is possible to consider the level of Rubisco activation on an ad hoc basis. The recent study of von Caemmerer & Edmondson (254) gives complete response curves for CO_2 uptake by leaves of *Raphanus sativa* to light at constant $[CO_2]$ and to $[CO_2]$ at saturating light. They also present information on the activation state of Rubisco, concentrations of RuBP and other PCR cycle intermediates, and total enzyme levels. We have analyzed these measurements according to Equation 4 and find (data not shown) that these measurements are quantitatively sufficient to account for >95% of the variation of net CO_2 uptake with light or $[CO_2]$. These studies demonstrate that gas exchange analysis of steady-state metabolic flux together with freeze-clamp studies of metabolite concentrations and Rubisco activation levels provide an adequate basis upon which to interpret the local control of CO_2 uptake by Rubisco in vivo.

RUBISCO-ACTIVASE Ogren and coworkers propose that a chloroplast protein—"Rubisco-activase"—functions to maintain Rubisco in the activated form in vivo (174). According to their hypothesis, this protein catalyzes an

energy-dependent activation of the Rubisco-RuBP complex (190, 202) driven by ATP (248):

$$ER + CO_2 + Mg^{2+} + ATP \xrightarrow{\text{activase}} ECMR + ADP + Pi. \qquad 11.$$

While both the mechanism of the activase reaction and its kinetics remain to be established, there is substantial evidence for this activity. A mutant of *Arabidopsis thaliana* that lacks peptides identified as Rubisco activase has a low level of Rubisco activation in the light and requires high [CO_2] for growth (191, 202, 233). Consistent with the proposed model, the level of activation of the mutant is normal in the dark (in the absence of RuBP) and is much lower than that of the wild type in the light (when RuBP is present) (203). Antibodies to Rubisco activase from spinach have been used to demonstrate the existence of a cross-reacting protein in other photosynthetic organisms, including C_4 plants and green algae (204).

If one accepts the activation reaction given in Equation 11, and that both activation and inactivation occur by the equilibrium shown in Equation 10, then the steady-state proportion of active Rubisco will be a function of the rate constants for the three partial reactions. Assume, for example, that the rates of these reactions are first order with respect to the active [E_a] and inactive [E_i] forms of the enzyme present in the system (i.e. ER and ECMR when RuBP is saturating). The rates of the partial reactions are: $v_{act} = k_{act}[E_i]$; $v_{on} = k_{on}[E_i]$; $v_{off} = k_{off}[E_a]$, where the subscripts *act*, *on*, and *off* designate the activase reaction and the forward and reverse partial reactions of Equation 10, respectively. The steady-state proportion of active enzyme is thus given by

$$f_4 = \frac{E_a}{E_a + E_i} = \frac{k_{act} + k_{on}}{k_{act} + k_{on} + k_{off}}. \qquad 12.$$

Note that when k_{act} is small compared to $k_{on} + k_{off}$ the activation level should be determined by effects of Mg^{2+}, CO_2, and pH on the values of k_{on} and k_{off} (146, 159), but when $k_{act} >> k_{on} + k_{off}$ the activation level can approach 1.0. Recent experiments by Streusand & Portis (248) indicate that k_{act} (all else being equal) may increase as a sensitive function of the ATP/ADP ratio. It may also be significant that RuBP has been shown to be a strong inhibitor of both k_{on} and k_{off} in the equilibrium activation reaction (125, 146). Thus, for a given value of k_{act}, the steady-state level of activation should be higher in the presence of a high concentration of RuBP, which is consistent with the apparent RuBP requirement for the "activase effect" (190).

Activase may function to link the activation of Rubisco to the light intensity (174, 190, 248). In this regard, it should be noted that the ATP/ADP ratio in

vivo is, among other things, affected by the activity of Rubisco, since ATP use is linked to the production of PGA. For example, an immediate effect of a rise in light intensity would be an increase in the ATP/ADP ratio and the rate of PGA reduction. Activation of Rubisco and the other "light-activated" enzymes would follow, and this concomitant rise in activation and flux would tend to stabilize the concentration of PCR cycle intermediates (see below). Since the reaction catalyzed by PGA kinase is close to equilibrium, the steady-state change in the ATP/ADP ratio would be relatively small. The effectiveness of this homeostatic regulatory mechanism will depend upon the degree of change of other effectors such as CA-1-P, Mg^{2+}, and H^+ and their effects on Rubisco and the "light-activated" enzymes. An increase in the $[CO_2]$ may, however, be expected to have the opposite effect on the activation state of Rubisco because the increased flux will tend to reduce the ATP/ADP ratio. This concept seems to explain the observation noted above that increasing CO_2 in vivo can result in a decline of Rubisco activation state. Sharkey et al (226) measured a decrease in the ATP/ADP ratio of intact leaves during the transient following an increase in $[CO_2]$, and this change in ATP/ADP ratio correlates with deactivation of Rubisco during the transient (199). These results support the notion that Rubisco-activase participates in regulation of the activation state of Rubisco in vivo.

SUMMARY Measurements of the rate of CO_2 fixation by intact leaves can now be accounted for fairly well by measurements of several elements known to exert "local" control over Rubisco. Experimental approaches to quantifing the extent of "local" control by these different elements have been developed and demonstrated, but many details concerning the mechanisms and kinetics of reactions that affect the V_{max} of Rubisco (by controlling the concentration of CA-1-P or by controlling the activation state) remain to be elaborated. The studies discussed in this section provide some indication of how the "local" control of Rubisco may be linked to other elements of the photosynthetic system. These links are developed below.

REGULATORY SEQUENCES

We have given Rubisco a central position in the foregoing discussion because it is strategically placed to regulate the rate of photosynthesis. As the catalyst of the first largely irreversible reaction in the CO_2-fixation sequence, Rubisco alone (if we ignore diffusion of CO_2 to the site of reaction) will determine the flux unless subject to feedback regulation. There are now clear indications that under some conditions (e.g. high light and limiting CO_2) the levels of Rubisco effectors are such that feedback inhibition of enzyme activity is negligible and the rate of photosynthesis is determined solely by the amount of Rubisco. Under other conditions (e.g. low light and high CO_2) Rubisco

activity is held well below its maximum. What remains now is to describe our current understanding of the sequences of regulatory events that lead to changes in the levels of Rubisco effectors and to define the conditions under which these "regulatory sequences" are most operative.

The most fundamental regulatory sequences influencing Rubisco activity can be defined by considering the structure of the photosynthetic system (Figure 4). This system can be described broadly as a convergent metabolic pathway (266). One branch mediates the input of CO_2 from the atmosphere and the other the input of quantum energy for the ultimate reduction of CO_2 to sucrose, the principal product of photosynthesis. A fundamental requirement for a steady state in such a system is that the two input fluxes conform to a specific stoichiometry and that the input flux does not exceed the capacity of the distal reactions to sustain that flux. If the converging sequences contain largely irreversible reactions, there must be regulatory mechanisms that can coordinate the rate of these reactions such that the soichiometric requirements are fulfilled. In the photosynthetic system, the input of CO_2 is mediated by a series of reversible diffusion processes (62) and the largely irreversible reactions catalyzed by Rubisco. The absorption of quanta involves processes in which quantum energy is either dissipated as heat and fluorescence or used for photochemistry within a few picoseconds (50). From that point on, the input flux is essentially irreversible, although there are other distal mechanisms by which energy can be dissipated (8). There are, therefore, three fundamental regulatory sequences that must be in place to ensure that steady-state photosynthesis can be approached. First, at low light intensities, Rubisco activity must be modulated such that it balances the input of quantum energy. Second, at high light intensity when energy input exceeds the capacity of Rubisco to fix CO_2, a regulatory sequence must ensure that excess energy is dissipated. Finally, if under certain circumstances the maximum activity of reactions involved in product synthesis is approached, then a sequence that can reduce both the rate of energy input and Rubisco activity must be activated. In the previous section, we have discussed several mechanisms whereby the reactions of CO_2 fixation are regulated in response to a change in the rate of energy input. We elaborate upon the details of this sequence in the following discussion and describe some of the recent advances in our understanding of regulatory sequences linking the activity of Rubisco to the input of quantum energy and the reactions of the sucrose synthetic pathway to both input processes. Since the PCR cycle plays a crucial role in mediating all three regulatory sequences, we first examine some of the salient features of this cycle and then link these to events occurring in the cytosol and the thylakoids.

Regulatory Properties of the PCR Cycle

Many of the important regulatory properties of the PCR cycle have been discussed in detail in reviews by Walker (258), Edwards & Walker (52), and

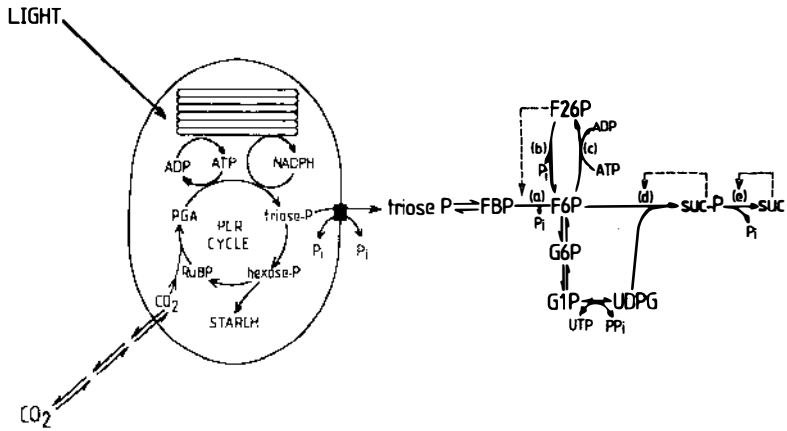

Figure 4 A diagram showing the structure of the photosynthetic system. Inputs of fixed carbon, mediated by Rubisco, and reducing equivalents, mediated by PSII, converge in the photosynthetic carbon reduction (PCR) cycle. Two major branch points of the cycle lead to the production of starch in the chloroplast and the export of triose-P to the cytosol via the phosphate translocator. Synthesis of sucrose (suc) in the cytosol is linked to the release of Pi which is returned to the stroma via the phosphate translocator in exchange for triose-P. Enzymes of the cytosolic pathway are: (a) fructose 1,6-bisphosphatase; (b) fructose 2,6-bisphosphatase; (c) fructose 6-phosphate, 2-kinase; (d) sucrose phosphate synthase, and (e) sucrose phosphate phosphatase—the dashed lines indicate possible feedback mechanisms.

Leegood et al (155), and in a paper by Woodrow (273). We do not elaborate upon these discussions, but draw attention to two basic properties of the PCR cycle that are critical for understanding how the cycle mediates the regulatory interactions among the cytosol, the electron transport system, and Rubisco.

The first property involves the constancy of total stromal phosphate (esterified plus inorganic), which is a consequence of the compartmentation of the PCR cycle and the fact that the main protein catalyzing metabolite export from the chloroplast—the phosphate translocator—does not sustain a net phosphate flux (68, 69). This conservation of phosphate is of great importance because it requires that a change in the level of any phosphorylated intermediate be compensated by an equal and opposite change (in terms of phosphate) elsewhere in the cycle (66, 273). Therefore, a change in the activity of any enzyme in the PCR cycle can affect both the substrate concentrations and the activities of other enzymes in the chloroplast regardless of whether they are adjacent on a metabolic scheme or whether they are connected by a classical allosteric (or other) feedback mechanism. For example, a change in the concentration of RuBP would most likely be associated with balancing changes in PGA and Pi, since these compounds account for about 80% of the stromal phosphate (83). This association was discussed

previously with respect to the relationship between the apparent K_m(RuBP) of Rubisco and the RuBP concentration (see Figure 2). But it may also be of great significance in connecting the activity of Rubisco (which influences the steady-state [RuBP]) to the level of stromal Pi and thus to the rate of electron transport. We discuss this interaction and the importance of phosphate conservation in cytosol-stroma interactions in the following sections. It is important to note that the compartmentation of the cytosol by membranes that are only slowly permeable to Pi may also result in the conservation of total cytosolic phosphate in the minutes-to-hours range. The same rules of balancing esterified and inorganic phosphate concentrations would therefore be relevant to consideration of the regulation of sucrose synthesis.

The second property of the PCR cycle involves regulatory responses at the two major branch points. At the first of these, triose-P is withdrawn from the chloroplast for the ultimate synthesis of sucrose; and at the second, hexose-phosphate is used for the synthesis of starch (Figure 4). In contrast to a linear divergent pathway, the competing reactions must carry a specific range of fluxes for the inputs and outputs to balance and the system to approach a steady state [see reviews by Walker (258), Edwards & Walker (52)]. Laisk (149) and Woodrow et al (278) proposed that a corollary to this need for restricting the steady-state fluxes at the branch points is that enzymes catalyzing the reactions adjacent to these branch points must adopt a restricted range of kinetic parameters in order for the system to adopt a stable steady state. With the exception of the phosphate translocator, the kinetic properties of these competing reactions (namely, stromal aldolase, transketolase, and hexose phosphate isomerase) are subject to regulation only through modulation of their product pool sizes. It is therefore more relevant to consider the required balance at the branch points in terms of the kinetic parameters of enzymes catalyzing the adjacent largely irreversible reactions. This means that in the stroma the FBPase, SBPase, Ru5P kinase, and ADP-glucose pyrophosphorylase will be important in determining metabolite partition at both branch points; and in the cytosol the FBPase and the phosphate translocator (which is also subject to regulation by cytosolic Pi) can influence the competition for triose phosphate (see 273).

Should a regulatory mechanism perturb the balance between the kinetic constants of these latter enzymes, a transient state would occur during which either metabolite export or recycling is favored. The effect of this perturbation would be to change the relative levels of esterified and inorganic phosphate in the stroma until a new steady state is attained. For example, a rise in the cytosolic Pi concentration (cytosolic triose-phosphate and PGA levels remaining constant) would affect both the apparent K_m and V_{max} of the phosphate translocator with respect to stromal triose-phosphate (84, 188) such that this enzyme, at least temporarily, could out-compete the enzymes of the stroma

for triose-phosphate, and proportionally more Pi would be imported from the cytosol than is incorporated into the esterified phosphate pool via the Ru5P kinase reaction (Figure 4). As the concentration of stromal Pi increases and that of esterified phosphate decreases, a new steady state would be approached in which the ratio of Pi to esterified phosphate in both compartments is altered and the rate of starch synthesis reduced. Changes in the levels of cytosolic triose-phosphate and PGA can similarly affect the kinetic characteristics of the phosphate translocator, the partition to starch and sucrose, and the ratio of Pi to esterified phosphate in the stroma.

This ability of regulation of the branch-point reactions to modulate the ratio of esterified to inorganic phosphate (within the confines of phosphate conservation), and the steady-state rates of starch synthesis and photosynthetic CO_2 assimilation has been clearly demonstrated in a series of experiments with isolated chloroplasts where the properties of the phosphate translocator were altered by adding Pi, PGA, and triose-phosphate to the external medium [see reviews by Walker & Crofts (259), Walker (258), Edwards & Walker (52)]. These fundamental stoichiometric relationships and regulatory responses of the PCR cycle will be important in describing regulatory sequences that permit reactions in the sucrose synthetic pathway to influence both the partition of carbon and the initial events of photosynthesis. Details of these mechanisms are elaborated in the following discussions.

Feedback Regulation of Photosynthesis

Many biosynthetic pathways contain product-sensitive feedback sequences that can inhibit early irreversible reactions in the pathway and thus stabilize the supply of product (253, 284). A natural consequence of such feedback loops is that, over a wide range of conditions, the flux is sensitive to the activities of some or all the enzymes distal to the first irreversible reaction (209). This sensitivity, as mentioned in the introduction to this section, becomes significant when the flux approaches the maximum activity of these distal enzymes. In the following discussion, we first examine the biochemical evidence for a sequence linking sucrose—the primary end product exported from leaf mesophyll cells—to the photoacts and to Rubisco. We then look at evidence of the operation of this sequence, and of whether, under normal conditions, the activities of cytosolic enzymes contribute significantly to the determination of the rate of photosynthesis.

THE STRUCTURE OF THE FEEDBACK SEQUENCE The enzymological evidence for a metabolic feedback loop originating from sucrose (see Figure 4) is somewhat variable. Both sucrose phosphatase (96) and sucrose phosphate synthase (SPS) (115, 119, 133, 201) from a variety of species are inhibited by sucrose, but there are some exceptions to this rule (3, 72, 92, 115, 116, 118,

271). The effect of sucrose-phosphate on SPS is also variable. There is evidence that in spinach sucrose-phosphate is an inhibitor of SPS activity (3), but in wheat germ this compound exerts little influence over SPS (200). The enzymes involved in the synthesis and degradation of fructose 2,6-bisphosphate in spinach also appear not to be affected by sucrose (243).

The structure of the sequence linking the activity of SPS to chloroplast metabolism is, however, relatively well defined and involves the recently identified inhibitor of cytosolic FBPase, fructose-2,6-bisphosphate (F26P) (43, 44, 111, 241, 246). The concentration of F26P is regulated by a futile cycle involving F6P,2-kinase and a specific F26P phosphatase. The former enzyme synthesizes F26P from F6P and ATP (41) and the latter catalyzes the production of F6P and Pi from F26P (43). A fall in the activity of SPS (relative to that of the cytosolic FBPase) should lead to accumulation of F6P, UDPG, and metabolites close to equilibrium with these compounds (e.g. G6P). This rise in F6P concentration would probably effect an amplified (i.e. greater than proportional) rise in F26P because the former compound stimulates F6P,2-kinase and inhibits the phosphatase (43). This amplified response was verified by Stitt et al (244), who showed that, under conditions that reduce the activity of SPS, a 50% rise in F6P was accompanied by a doubling of F26P. These authors also measured a decrease in the metabolic flux through the cytosolic FBPase and a build-up of cytosolic DHAP [which is presumably in equilibrium with FBP (83)]. An increase in substrate concentration together with a reduction in metabolic flux is unequivocal evidence that FBPase is regulated under these conditions (144).

When assessing the sensitivity of this mechanism to either a build-up of products or a reduction in SPS activity, one must consider two other regulatory properties. First, it is probable that the accumulation of esterified phosphates in the cytosol is compensated by a reduction in the level of Pi. This opposite movement of the G6P-F6P and Pi pools together with the influence of all three metabolites on SPS activity (49) probably results in a relatively sensitive relationship between the F6P concentration and the catalytic velocity of SPS. In other words, a large change in SPS activity may only result in a relatively small increase in substrate concentration and thus a relatively weak feedback effect. Second, the rise in triose phosphate and fall in Pi that accompany a build-up of F6P would tend to affect F26P phosphatase and F6P,2-kinase in a manner opposite to that of F6P (41, 242). The sensitivity of the F26P level to F6P may thus be somewhat reduced.

The notion that changes in cytosolic Pi and esterified phosphates balance each other such that there is no net change in the level of cytosolic phosphate (i.e. that cytosolic phosphate is a conserved moiety) depends upon there being slow exchange between the cytosol and adjacent compartments. Significant exchange with the chloroplast is unlikely because the phosphate translocator

does not sustain a net phosphate flux (69), and other forms of exchange are thought to be slow (97). Although it is thought that the large vacuolar Pi pool serves to buffer the cytosolic Pi pool from changes in phosphate supply from the environment (73, 74, 194) and perhaps to maintain cytosolic Pi at a level optimal for photosynthesis, most evidence indicates that exchange between these compartments requires several hours to equilibrate (194, 277). It appears, therefore, that cytosolic Pi can fluctuate over the short term and act as a regulatory intermediate. In this regard the short-term variation in Pi considered here differs in a fundamental way from a nutritional deficiency of phosphorus (21, 161).

The decrease in the cytosolic Pi/triose phosphate ratio and the inactivation of FBPase exert their influence over chloroplast metabolism by altering the kinetic characteristics of the phosphate translocator, as discussed earlier. Under these conditions the reactions of the PCR cycle are more able to compete for triose phosphate, and recycling of carbon is favored over export to the cytosol. The ultimate effect of this change appears to be an increase in the stromal PGA/Pi and ADP/ATP ratios, activation of several stromal enzymes, and an increase in the rate of starch synthesis (265, 273, 278). In addition, if the feedback is strong enough, there are mechanisms in place by which both the rate of photosynthetic electron transport and the activity of Rubisco can be attenuated. In the next sections, we focus especially on the role of Pi in regulatory mechanisms that can affect the activities of both the electron transport system and Rubisco.

REGULATION OF ELECTRON TRANSPORT The initial step in the electron transport chain is the abstraction of an electron from water by a reaction linked to the photoact of PSII. As noted in the introduction to this section, these electrons must ultimately be used to reduce products of carbon metabolism, and to a lesser extent, alternative electron acceptors (8). If, under conditions where the PCR cycle or output pathway activity is limiting, the input of quantum energy increases, then the initial steps in the electron transport system must be regulated such that the excess energy is dissipated and the required rate of electron transport is adhered to. Duysens & Sweers (51) suggested that the primary acceptor of PSII (Q_A) could accumulate in the reduced form under conditions of strong illumination or in the absence of suitable electron acceptors and lead to an increase in fluorescence and a corresponding decrease in the yield of electrons. These workers were careful to state, however, that another mechanism not involving redox feedback or increased fluorescence might also be important in vivo. Evidence for this view comes from studies showing that, at high light when the ΔpH and the phosphorylation potential (i.e. [ATP]/[ADP][Pi]) are relatively large, the NADPH/NADP ratio is actually less than that occurring in low light (99,

250). A feedback mechanism involving the redox state of the adenine nucleotide pool is difficult to reconcile with these measurements, and studies in which the ΔpH was modified during steady-state photosynthesis in isolated chloroplasts demonstrated that an excessive proton gradient could reduce the rate of electron transport (231, 232). It was also shown that ΔpH could be modified by altering the rate of ATP turnover (230–232).

In analyzing the regulatory sequence involving ATP turnover and the ΔpH, it is important to recognize that comparisons of phosphorylation potentials in isolated thylakoid systems (e.g. 142) and intact chloroplasts undergoing even limited photosynthesis (85) suggest that the reaction catalyzed by the ATP synthase is significantly displaced from equilibrium during steady-state photosynthesis [for a more detailed discussion, see Horton (114)]. If this is true, then ΔpH will be strongly influenced by modification of the substrate levels of this reaction. Attention has focused on the influence of Pi because the ADP concentration apparently remains quite constant over a broad range of conditions (46, 98, 99).

The theory that a reduction in the stromal Pi concentration restricts the rate of photophosphorylation and ultimately the rate of CO_2 fixation has been questioned on the basis that the ATP synthase is apparently, under most conditions, saturated with Pi (229). The apparent K_m(Pi) measured in several studies (cf 77, 130, 219, 251) is less than 1mM (60–600 μM), and metabolite measurements indicate a stroma Pi concentration of 4–35 mM (cf 46, 77, 156, 205, 272). Changes in the Pi level are, on this basis, unlikely to affect ATP synthase activity, but two points must be considered when evaluating this proposal.

First, there is evidence that the concentration of free Pi influencing the activity of the ATP synthase is significantly less than that estimated from the Pi content of non-aqueously fractionated leaf tissue (46, 205). Part of this discrepancy is thought to result from contamination of the chloroplast extracts by Pi from the vacuolar pool (46, 247). Other evidence indicates that a considerable proportion of stromal Pi could be bound to both the protein (6, 27, 76, 179) and membrane (195) components of the chloroplast. In addition to this presumably freely exchangeable component, Furbank et al (78) have shown that there may be a pool of up to 2 mM Pi that is unavailable—at least for several minutes—to the ATP synthase. These authors suggest that "shielding" of the latter enzyme by Rubisco may be the mechanism underlying the exclusion of Pi (see also 195).

Second, in assessing whether a change in the stromal Pi concentration could significantly affect the level of the other substrates of the reaction (ADP and H^+) we must have a clear understanding of whether the calculated K_m(Pi) values for the ATP synthase (1, 130, 219) accurately represent the operative in vivo K_m(Pi). At present this value is difficult to assess because it is a

function of both the intra-thylakoid and stromal pH (1, 251) and of the concentration of other stromal elements. Experiments with sollen "leaky" chloroplasts showed that both the apparent K_m(ADP) and K_m(Pi) values are significantly higher than those calculated using isolated thylakoids (77). The effect may be caused by binding of certain substrates to Rubisco and by a process related to Rubisco binding to the thylakoid membrane (76). If the free stromal Pi concentration is in the apparent K_m(Pi) range and there is a decline in its concentration, the immediate effect would be a rise in the ADP concentration and ΔpH in order to maintain the flux through the reaction. Similarly, a decrease in ADP concentration in the range of the K_m(ADP) may also cause acidification of the thylakoid space. In either case, the acidification itself would not reduce the steady-state rate of photosynthesis unless it is linked to a process that can alter the input of quantum energy. Clearly, more work is required to clarify whether changes in the Pi and ADP levels can elicit a feedback response through effects on ΔpH.

Recent experiments on functional photosynthetic systems have yielded new information regarding the mode by which PSII is regulated by feedback sequences. Weis et al (268) used chlorophyll fluorescence to show that the primary acceptor of PSII, Q_A, can remain mostly oxidized—even at light intensities sufficient to "saturate" the rate of photosynthesis (see also 48, 143, 269). To explain how the steady state is maintained under these conditions, Weis et al (268) postulate an alternative feedback mechanism that would involve reversible conversion of PSII centers to a form with reduced photochemical and fluorescence yields, and they suggest that acidification of the thylakoid space is the "local" condition that controls this mechanism. Studies using fluorescence procedures to quantify both the redox feedback and this "pH-dependent" feedback on PSII were able to account quantitatively for the regulation of PSII during steady-state photosynthesis by intact leaves when light, [CO_2] or [O_2] were varied (269). More work is required to assess this proposal and the significance of possibly contradictory observations (78, 79, 178). Other regulatory mechanisms that balance the ratio of production of NADPH and ATP to their consumption, the distribution of quanta to PSI and PSII, and regulation of other quenching mechanisms need to be considered. Nevertheless, the sequence of events linking changes in the cytosolic and stromal [Pi] to the thylakoid pH and in turn to the photochemical efficiency of PSII seems to provide a plausible explanation for regulation of the irreversible input of quantum energy (Figure 4).

REGULATION OF RUBISCO We do not have a clear mechanistic view of the regulation of Rubisco activity in response to the stromal changes effected by feedback from the sucrose synthetic pathway. In experiments with isolated

chloroplasts, both Heldt et al (102) and Furbank et al (78) showed that a drop in stromal Pi is accompanied by a rise in RuBP and a decline in flux. This result indicates that Rubisco is subject to regulation under these conditions, but only Heldt's group detected a decrease in the apparent activation state of the enzyme. It is conceivable that the lower ATP/ADP ratio under feedback conditions brings about a change in Rubisco activity through the "activase" system (174), or that there is an allosteric effect of the change in Pi on enzyme activity (179). As outlined in the earlier section on Rubisco, the clarification of this part of the feedback regulatory sequence awaits further information on the basic regulatory properties of Rubisco and associated chloroplast proteins.

Operation of the Feedback Sequence

MANIPULATION OF CYTOSOLIC Pi The potential importance of cytosolic Pi in regulating photosynthesis was first demonstrated in experiments with isolated chloroplasts in which the external Pi concentration was directly manipulated (33). Since that time, similar studies have been made of photosynthesis in intact leaves by modulating the concentration of Pi in the cytosol using a variety of techniques. Infiltration of photosynthetic tissues with mannose, 2-deoxyglucose, or glucosamine—all of which sequester cytosolic Pi as phosphorylated compounds that are not readily metabolized—causes an increase in the rate of starch synthesis and a depression of the rate of photosynthesis (29, 94, 108, 109). This inhibition can be overcome by feeding Pi to mannose-treated leaf discs (229, 263, 264); and at CO_2 partial pressure well above ambient, Pi feeding can actually stimulate the rate of photosynthesis (262).

The importance of cytosolic Pi in regulating photosynthesis has also been reinforced by studies of oscillations in the rate of CO_2 fixation. These complex transients generally appear after a perturbation to the photosynthetic system under conditions of high light and CO_2 (e.g. 173, 228, 263). Oscillation can be restricted by feeding Pi to leaves and enhanced by feeding mannose (229, 263), a result suggesting that modification of the cytosolic Pi concentration can influence the distribution of flux control among elements of the photosynthetic system (see also 175, 279) and that, under these conditions, feedback from the cytosol is significant in determining the rate of photosynthesis. Stitt (237) conducted an experiment along similar lines where photosynthesis was interrupted by a brief period of low light. During this period it is thought that a depletion of cytosolic metabolites results in a temporary increase in the cytosolic Pi concentration. Upon re-illumination, there was a transient enhancement of the rate of photosynthesis which was interpreted to indicate that stromal Pi can restrict photosynthesis under steady-state conditions of high light and CO_2.

The cytosolic Pi status may also be involved in the lack of stimulation of photosynthesis observed, under some conditions, when the O_2 concentration is lowered and photorespiration restricted. This effect was first noticed in experiments with leaves from plants grown at "normal" temperatures that were illuminated at a lower temperature (26, 36, 122, 123, 165). More recent studies have involved a combination of high CO_2 partial pressure and depressed temperature. This "O_2 insensitivity" is thought to occur when the activities of enzymes in the sucrose synthetic pathway partially limit the rate of photosynthesis—i.e. when there is a significant feedback from the cytosolic reactions. Upon transfer from normal to low O_2 the feedback mechanism affects Rubisco activity such that both v_c and v_o decline and the net rate of CO_2 assimilation remains unchanged. Feedback inhibition of the rate of electron transport must also occur in order to balance the changes in v_c and v_o. The hypothesis that the cytosolic Pi pool mediates the feedback sequence under O_2-insensitive conditions is supported by experiments showing that feeding of Pi can restore some O_2 sensitivity to previously O_2-insensitive tissues, whereas the reverse can be effected by feeding the tissues with mannose (94). Studies of metabolite pool sizes under O_2-insensitive conditions show that, compared to control plants, there is a rise in the PGA/triose phosphate and ADP/ATP ratios (153, 226). Assuming that the reactions catalyzed by PGA kinase and G3P dehydrogenase are close to equilibrium and that the stromal pH is constant (46), Leegood & Furbank (153) calculated that there is a kinetic restriction placed upon the rate of electron transport and photophosphorylation.

Sharkey and coworkers (286) employed changes in CO_2 under "O_2- insensitive" conditions to examine the engagement of this feedback mechanism. Upon increasing the [CO_2], the electron transport rate decreased by 25–30%. An accompanying and immediate decline in the level of photochemical quenching of fluorescence indicates that the degree of redox-linked feedback regulation of PSII increases under these conditions. Over the next few minutes, however, they observed compensating changes in the levels of fluorescence quenching (photochemical and nonphotochemical) indicating an acidification of the thylakoid space and a relaxation of the redox feedback on Q_A. As there was little change in the rate of electron transport, it appears that the feedback regulation of PSII was maintained, and the feedback sequence became increasingly mediated by ΔpH. Dietz et al (48) also observed a rise in nonphotochemical quenching on increasing CO_2 from near ambient levels to $\sim$3000 μbar. These results are consistent with the general interpretation that stromal Pi may reach a suboptimal steady-state level in low [O_2] or high [CO_2] or under a combination of both conditions, but there is apparently an initial period when the primary feedback may occur by another mechanism. Furbank et al (79) suggested that an alternate feedback sequence may involve

the redox state of the pyridine nucleotide pool. In experiments with isolated chloroplasts, these authors showed that an increase in ΔpH is not necessarily associated with a reduction in stromal Pi.

Sharkey et al (225) and Leegood & Furbank (153) monitored the effect of "O_2-insensitive conditions" on Rubisco activity and the RuBP concentration in leaves. The former group demonstrated that, upon transfer to low [O_2], Rubisco is inactivated to a similar extent to the drop in electron transport. They speculated that this may be the result of the lower ATP/ADP ratio reducing "Rubisco-activase" activity (216). They also noted that the change in activation state occurs relatively slowly and that the initial decline in v_o and v_c probably results from a transient drop in the RuBP concentration (199). Leegood & Furbank (153), however, measured a relatively small decline in RuBP, which is probably insufficient to account for the change in flux through the Rubisco reactions.

These studies of whole tissues verify the hypothesis that feedback inhibition of both the rate of electron transport and Rubisco activity can occur and that cytosolic Pi plays a central role in this regulatory sequence (107, 109, 223, 260). Nevertheless, a clearer view of whether this sequence affects the rate of CO_2 fixation (and not just assimilate partition) under more natural conditions comes from studies in which sucrose is allowed to accumulate or is externally supplemented.

THE SENSITIVITY OF CO_2 FIXATION TO CYTOSOLIC Pi When considering the effectiveness of the cytosolic feedback sequence in regulating the rate of CO_2 fixation, one must recognize that it would be theoretically possible for cytosolic feedback to alter the fluxes of the photosynthetic system such that the PCR cycle pool sizes and rate of CO_2 fixation remain unchanged. This would involve parallel and compensating changes in the activities of the cytosolic and stromal FBPases and the ADP-glucose pyrophosphorylase, assuming that the reaction catalyzed by aldolase, the phosphate translocator, hexose phosphate isomerase, and phosphoglucomutase are at equilibrium (273). Nevertheless, there are two factors that complicate this simple means of altering the relative rates of assimilate export. First, there is no evidence for an effector that can initiate such a sequence without affecting several other important reactions in the chloroplast (e.g. Pi also affects the phosphate translocator, G3P dehydrogenase, SBPase, Ru5P kinase, Rubisco, and the ATP synthase). Second, the starch and sucrose synthetic pathways do not carry true export fluxes because they are coupled to the release of Pi which, in turn, can affect chloroplast metabolism.

If the regulation of assimilate partition and CO_2 fixation are inextricably associated through the effect of Pi on both processes, it is important to distinguish between conditions that affect metabolite partition and those that

affect electron transport and Rubisco activity and thus the rate of CO_2 fixation. Experiments with isolated chloroplasts by Steup et al (236) and Heldt et al (103) addressed this problem. In both studies, the properties of the phosphate translocator were modulated by changing the external Pi concentration. Figure 5 shows the sensitivity of both CO_2 assimilation and starch synthesis to the concentration of external Pi [data from Steup et al (236)]. It is important to note that there is a significant range of Pi levels over which the sensitivity of CO_2 assimilation is close to zero, whereas that of starch synthesis is at its maximum. Although the presence of relatively high levels of PGA and triose phosphate in the cytosol may complicate this relationship in intact tissues, there is probably also a range of Pi/triose phosphate ratios over

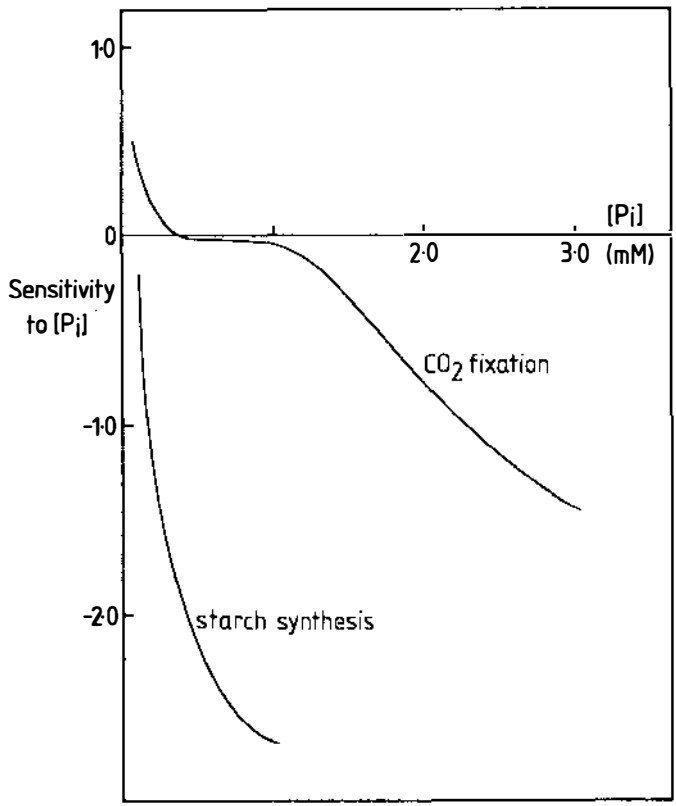

Figure 5 The sensitivity of CO_2 fixation and starch synthesis to the concentration of Pi in a suspension of isolated chloroplasts. The data of Steup et al (236) were used in the calculations. The sensitivity is defined as: $\partial v/\partial[\text{Pi}] \times [\text{Pi}]/v$ where v is the rate of either CO_2 fixation or starch synthesis. There is a range of Pi levels over which the rate of CO_2 fixation is insensitive to Pi but the rate of starch sythesis is extremely sensitive.

which assimilate partition is altered but CO_2 fixation remains relatively unaffected.

FEEDBACK FROM SUCROSE Plants vary considerably in the degree to which they favor starch and sucrose as their major storage compound. In barley, for example, sucrose is the major storage product (88, 227) and appears to be located in the vacuole (65, 81, 132, 177). Sugarbeet, on the other hand, only accumulates sucrose for a short period after the onset of illumination (70, 71) and stores most assimilate as starch (80, 82). The relative sizes of the starch and sucrose pools may also vary during the course of a day and night (28, 71, 83, 106) which, to some degree, may reflect the changes in the level of extractable SPS (106, 115, 136, 197) or F6P,2-kinase activity (136). In some species, these diurnal changes appear to be the result of a light-related regulatory mechanism (227, 238), while in others there is evidence for an endogenous rhythm mechanism that is independent of light-intensity changes (117, 118, 136, 197, 238). Nevertheless, it is by no means clear whether, superimposed upon these "coarse" control mechanisms, there is a classical metabolic feedback sequence that can moderate the rate of CO_2 assimilation in response to a change in the level of sucrose [see reviews by King et al (138), Neales & Incoll (170), Herold (107), Gifford & Evans (86)].

THE EFFECT OF SUCROSE Experiments with leaves, leaf discs, and protoplasts have shown a correlation between high levels of sucrose and a change in the partition of assimilate between starch and sucrose, but the evidence for a depression of the rate of assimilation is not conclusive. Herold et al (110) showed that the rate of CO_2 assimilation is not altered but starch synthesis is stimulated when sugarbeet leaf discs are floated on a sucrose solution. Similar changes in partition were demonstrated by Stitt et al (244, 245) in experiments with spinach leaf discs where sucrose was allowed to build up in the tissue. There was, however, some depression of the rate of photosynthesis, but it should be noted that the experiments were done at levels of CO_2 well in excess of ambient. Because they detected a decline in the rate of sucrose synthesis and an accompanying build-up of UDP-glucose and fructose 6-phosphate, these authors concluded that the mechanism altering metabolite partitioning involved, in part, the inhibition of SPS.

Further evidence for inhibition of SPS activity during a build-up of sucrose was found by Clausen & Lenz (32) and Rufty & Huber (197) using leaves of eggplant and soybean, respectively. But similar experiments with spinach leaves showed no change in the extractable activities of either SPS or cytosolic FBPase (197). The rates of photosynthesis did not change during the sucrose build-up in the spinach leaf experiments, nor was there a marked temporal correlation between the decline in SPS activity and photosynthetic

rate in soybean leaves (197). The effect of sucrose on photosynthesis in protoplasts also varies between species. Foyer et al (72) found that sucrose pretreatment of spinach protoplasts depressed the rate of CO_2 fixation but did not affect the extractable activities of SPS or cytosolic FBPase. Wheat and barley protoplasts, on the other hand, showed little metabolic response to the sucrose treatment. Hills (113) examained the metabolism of freshly isolated asparagus cells and concluded that the inhibition of sucrose synthesis immediately upon cell isolation was unlikely to result from feedback inhibition by sucrose.

There is clearly a need for more work to resolve whether a build-up of sucrose can initiate a feedback sequence by inhibiting the latter reactions of the sucrose synthetic pathway. But even if this can be shown, the evidence from most of the whole-tissue studies indicates that the resulting feedback may exert its greatest effect on the partition of assimilates and have little effect on the rate of CO_2 fixation. The important matter in this context, therefore, concerns the nature of the regulatory mechanisms that affect partition and CO_2 assimilation and the degree to which they can operate independently.

SUMMARY There is a considerable amount of evidence from both the biochemical and whole-tissue studies that classical product feedback, or feedback originating from diurnal changes in the activities of enzymes such as SPS (198), exerts its greatest effect on metabolite partition and has little effect on the rate of CO_2 fixation. Nevertheless, there is evidence that under some relatively extreme conditions, such as shortly after a transition to a low temperature (153) or under conditions of water stress (cf 223), there may be a period during which the feedback regulatory sequence affects the rate of CO_2 fixation. It will be important to establish whether feedback under such conditions can be sustained in the long term, or whether processes such as adjustments of the total cytosolic phosphate pool by exchange with the vacuole (e.g. 73, 194) can minimize the cytosolic limitation and reinstate energetically efficient photosynthesis (see 226, 229).

Feedback on Energy Input from Rubisco

The quantity and kinetic properties of Rubisco largely determine the maximum capacity to provide electron acceptors at any given $[CO_2]$, $[O_2]$, and temperature. It is important that, when the absorbed flux of quantum energy is sufficient to "saturate" this capacity, a feedback mechanism comes into play to allow the dissipation of excess energy. We have noted that the "local" conditions involved in feedback regulation of the photochemical reactions are associated with acidification of the thylakoid space and redox regulation of

the photochemical traps. Engagement of these regulatory mechanisms has been examined in intact leaves by varying the intensity of illumination over the range encompassing both "rate-limiting" and "rate-saturating" levels or by varying the concentration of CO_2 over a corresponding range. Weis et al (268) showed that large changes in the scattering of light at 540 nm by intact leaves begin at a light intensity or [CO_2] that marks the transition between these light-limited and -saturated states and continue to increase as the intensity of illumination is increased or the [CO_2] is decreased. This indicates that, as the absorbed flux of quantum energy approaches the maximum capacity of Rubisco to sustain that flux, there is a marked rise in ΔpH which appears to be associated with regulation of the ATP synthase. They also used light-induced absorbance changes to show that the reaction centers of PSI, which are mostly in the active, reduced form in limiting light, accumulate in the oxidized form as the intensity of illumination exceeds saturation. This accumulation correlates with the apparent acidification of the thylakoid space, and may be the result of an effect of the pH gradient on the transport of electrons from PSII by the cytochrome-*b*/*f*-complex (cf 40, 270). Nonphotochemical quenching of fluorescence also increases with the light scattering changes, indicating an effect of the ΔpH (or some associated change) on the reaction centers of PSII. Significantly, the primary acceptor of PSII remained mostly in the active, oxidized configuration until the intensity of illumination was 3–4-fold saturating [see also Dietz et al (48)]. This indicates that regulation of PSII follows a hierarchical pattern with redox mechanisms becoming important after the pH-dependent mechanism becomes fully engaged.

Dietz & Heber (46, 47) examined the reactions catalyzed by PGA kinase and G3P dehydrogenase during changes in light intensity and CO_2 concentration. They showed that the overall mass action ratio for these reactions does not increase substantially as light becomes "rate-saturating," and that the reactions are not greatly displaced from equilibrium. Changes in the levels of PGA, DHAP, and Pi can therefore affect the steady-state ATP/ADP and NADPH/NADP ratios, which along with Pi could affect the electron transport system by a feedback mechanism. As discussed above, the conservation of total chloroplast phosphate provides a mechanism by which any PCR-cycle enzyme can affect the Pi, PGA, and DHAP concentrations. Von Caemmerer & Edmondson (254), for example, note that the total level of phosphate in PGA and RuBP in intact leaves increases with light intensity and reaches the apparent upper limit at a light intensity slightly lower than that required to saturate CO_2 assimilation. It is conceivable that a concomitant decline in Pi could initiate feedback inhibition of the photoacts by the mechanisms alluded to above. This hypothesis is consistent with the optical studies of the state of the thylakoid described in the preceding paragraph.

Feedforward Regulation of Enzyme Activity

It is necessary, under certain circumstances, to regulate the input of energy into the photosynthetic system to keep pace with limiting steps in the PCR cycle and carbon export pathways. A more common occurrence, however, is that the input of quantum energy limits the rate of photosynthesis and that Rubisco activity (v_c and v_o) must be regulated such that the stoichiometric relationship between CO_2 fixation and energy transduction is fulfilled. This requirement could in theory be met by simply reducing the size of intermediate pools in parallel with the flux and letting the RuBP concentration regulate Rubisco activity. However, in a previous section we indicated that the RuBP concentration does not vary over a wide range of irradiances and that a mechanism controlling the degree of activation of Rubisco appears to be an important mode by which v_c and v_o are modulated to match the rate of energy input. Feedforward activation of other PCR-cycle enzymes (namely, SBPase, FBPase, Ru5P kinase, and G3P dehydrogenase) to match the light-dependent flux may also play a role in the sequence regulating Rubisco. In this section, we do not expand upon the mechanistic details of the regulatory sequence connecting the input of quantum energy to Rubisco outlined in previous sections and other reviews (e.g. 4, 23, 42, 114, 152) but briefly examine the implications and possible advantages of "light regulation" of enzyme activity as a means of feedforward control of the PCR cycle and CO_2 fixation.

The current view of the importance of light regulation of the activity of PCR cycle enzymes (other than Rubisco) has been summarized by Leegood et al (155). (*a*) It may serve as a mechanism preventing enzymes from becoming oxidized (and thereby inactivated) by certain products of chloroplast metabolism. (*b*) Inactivation of enzymes in darkness prevents the operation of several energy-wasteful futile cycles. (*c*) Modulation of enzyme activities may serve to maintain a balance between carbon export and the regeneration of RuBP, and to regulate PCR-cycle metabolite levels and the flux. There is little doubt regarding the value of inactivating enzymes in darkness to prevent the waste of energy stored during periods of illumination, but what remains puzzling is that changes in enzyme activity occur over a wide spectrum of light intensities (e.g. 135, 148, 152, 154, 278) and that steady-state activation states are approached relatively slowly (e.g. 135, 148, 199, 281, 283).

Regulation of the activation state of the PCR-cycle enzymes in parallel with changes in the rate of photosynthesis has a significant effect on the steady-state relationship between substrate concentration and reaction velocity. Woodrow (274) used a model of the regulatory mechanism of SBPase (280, 282) to predict that concomitant changes in the enzyme activation state and the CO_2 fixation flux would result in a sigmoidal relationship between SBP concentration and the flux through the SBPase reaction, even though the in

vitro substrate kinetics are hyperbolic. He also predicted that a more pronounced sigmoidal relationship would result if parallel changes in pH and the Mg^{2+} concentration were considered. Similar predictions can also be made regarding the relationship between the flux and the substrate concentration of all the light-regulated enzymes. The degree of sigmoidicity of the substrate-velocity curves will depend upon the steady-state kinetic properties of the enzymes and the range of photosynthetic fluxes over which variations in activation state and effector concentrations occur. In Figure 2, we show how parallel changes in the Rubisco activation state and the rate of photosynthesis can result in a highly sigmoid substrate response. This curve is quite different from that expected from the steady-state rate equation of active Rubisco (also shown in Figure 2).

One of the most remarkable characteristics of the PCR cycle is the constancy of the intermediate pool sizes, despite large changes in the rate of photosynthesis [see review by Leegood et al (155)]. This observation supports the notion that multivariate interactions in vivo lead to apparent sigmoidal substrate kinetics where there is a large range of fluxes over which changes in substrate concentrations are small. The discrepancy between the pool sizes anticipated by the rate equations for active enzyme species and those occurring in vivo has been confirmed in experiments that take advantage of slow changes in enzyme activation state. For example, the activation state of Rubisco requires several minutes to assume a new level after a change in light intensity. Mott et al (169) showed that when the light intensity is reduced the RuBP concentration drops quickly in response to the lower flux, but soon rises again to the "normal" level as the enzyme activation state declines.

We may speculate that this "normal" state offers advantages in terms of the steady-state efficiency of photosynthesis or the ability of the system to respond to a change in environmental conditions. For example, we have developed arguments that Pi mediates at least part of the feedback sequence influencing the input of quantum energy. Given an unregulated PCR cycle, changes in esterified phosphates would occur in parallel with changes in the flux, and since total stromal phosphate is conserved, the concentration of Pi would change over the full range of fluxes. These changes in Pi could well effect proportional changes in the strength of feedback inhibition of energy input. Mechanisms that stabilize the levels of esterified and inorganic phosphate may therefore "poise" the PCR cycle such that feedback effects on the electron transport system are minimized and energetic efficiency is maximized over the widest range of fluxes. Regulation of Rubisco is especially important in this regard because (*a*) the RuBP pool contains a relatively large proportion of stromal phosphate, and (*b*) this pool is effectively isolated from the other intermediates of the PCR cycle by the largely irreversible reactions that regulate its synthesis and consumption (273). Therefore, phosphate can

be "stored" in RuBP without greatly affecting the partition of assimilate between starch and sucrose.

Modulation of the activation state of PCR-cycle enzymes may also serve to prevent the sequestrations of important chloroplast metabolites. Rubisco, for example, can bind several metabolites (such as NADPH) (e.g. 6), and should the RuBP or CA-1-P concentration drop significantly, a proportion of the compounds would become unavailable for normal function.

The slow imposition of changes to the activation state of the "light-regulated" PCR-cycle enzymes is also puzzling because, if this mechanism serves to aid energetically efficient photosynthesis, it is logical that it should operate as quickly as possible. Certain suggestions regarding the significance of slow "hysteretic" responses have been made (e.g. 75), but these have not stood up to critical examination (e.g. 209). One possibility is that the slow steps in the activation/inactivation mechanisms enhance the sigmoidal substrate responses by enabling a degree of "kinetic cooperativity" (2, 282). Some insight may also be gained by examining the "cost" versus the "benefit" of these enzyme activation mechanisms. If modulation of enzyme activity in response to irradiance serves to increase the overall efficiency of photosynthesis, then this "benefit" must be offset against the energetic and nutritional "cost" of the regulatory mechanisms. Consider, for example, the basic reaction describing the activation/inactivation of the enzymes regulated by thioredoxin (Th) (234):

$$E_i + Th_{red} \underset{k_i}{\overset{k_a}{\rightleftharpoons}} E_a + Th_{ox}, \qquad 13.$$

where E_i and E_a are inactive and active enzyme forms, respectively. The relaxation time (r) of the system is approximated by

$$r = (k_a[Th_{red}] + k_i[Th_{ox}])^{-1}. \qquad 14.$$

A rapid response thus requires a relatively high concentration of thioredoxin. But as the size of the reduced thioredoxin pool grows, the energetic cost of maintining it also grows, because oxidation of thioredoxin can occur either directly or indirectly through, for example, interaction with O_2 (154). Rapid activation/inactivation of Rubisco may similarly require high levels of "activase" and rates of ATP turnover. The relatively slow changes in activity of the light-regulated enzymes may therefore simply reflect an optimal balance between energetic and nutritional "costs" and "benefits" and may not have an important regulatory significance in the non-steady state. The energy cost of such regulatory mechanisms has been analyzed in animal systems, and it has

been shown that up to 20% of the basal respiratory rate of the cell may be used by regulatory, energy-requiring futile cycles (87, 141).

CONCLUSIONS One of the most intriguing aspects of the regulatory sequences linking the sucrose synthetic pathway, the PCR cycle, and the electron transport chain is the existence of several feedforward mechanisms. Regulation of the "light-activated" enzymes, cytosolic FBPase (see also 239, 240), ADP-glucose pyrophosphorylase, and SPS by feedforward processes ensures that these enzymes can transmit a large range of metabolic fluxes with relatively small changes in their substrate pool sizes. We have suggested that one of the main functions of these mechanisms is to reduce the overall "strength" of the feedback sequences and thus to maintain the energetic efficiency of photosynthesis over a wide range of irradiances. If this proposition is true, it means that, over this range of irradiances, the rate of photosynthesis will not be sensitive to the activities of enzymes of the PCR cycle and starch and sucrose synthetic pathways since changes in these activities would produce minimal changes in intermediate pool sizes. In other words, the flux will be determined by the rate of quantum energy input and the efficiency of energy transduction rather than the activities of any distal enzymes. Assessments of the degree to which enzymes of the photosynthetic system determine the flux therefore not only provide fundamental information about flux control (and how the flux can be altered) but also provide a measure of the strength of the feedback regulatory sequences. In the following section we examine studies of the determination of the photosynthetic flux by enzymes of the photosynthetic system.

CONTROL OF THE RATE OF PHOTOSYNTHESIS

A great many studies have sought to define the factors that limit the rate of photosynthesis. If one ignores for the moment any limitation imposed by light, temperature, the availability of water, and stress factors, the answer seems to be protein. Nitrogen, a major constituent of proteins, is the mineral nutrient that plants require in greatest quantity, and nitrogen is the nutrient that most often limits plant growth. Field & Mooney (67) reported a strong correlation between the light-saturated rate of net CO_2 assimilation (A_{max}) measured in the natural environment and the total protein nitrogen content of the leaves of C_3 plants with a wide variety of growth forms. This correlation is not surprising because key reactions of the photosynthetic process are associated with specific assemblages of polypeptides, and it is estimated that 75% of the total leaf protein is associated with the chloroplast (54). This sort of correlative evidence does not, however, provide an insight into the degree to which individual proteins might limit the rate of photosynthesis, because A_{max}

is also highly correlated with changes in the levels of Rubisco, Ru5P-kinase, NADP-G3P-dehydrogenase, cytochrome *f*, coupling factor, and carbonic anhydrase (7, 17, 54, 57, 162). As we have discussed here, such an insight can only be gained from experiments involving direct manipulation of the levels or activities of individual enzymes in the context of the whole system or from studies of the regulation of the photosynthetic system and the kinetic properties of the constituent enzymes.

Taking an evolutionary view, one may reasonably propose that selective mechanisms have tended to allow the expression of genes for proteins such that optimal rates of photosynthesis can occur and no single step is limiting—at least under an assimilation-weighted average condition for the natural environment. A theoretical treatment by Cowan (37) illustrates this point. He considered the optimal partitioning of nitrogen between two enzymes—carbonic anhydrase (CA) and Rubisco—that are directly involved in CO_2 fixation. The presumed role of CA in higher-plant leaves is to facilitate transport of CO_2 to the sites of its fixation by Rubisco, and previous studies had estimated that the high activity of CA present in chloroplasts increases the rate of CO_2 fixation of a given amount of Rubisco in the chloroplast by about 10% (120). Cowan derived a kinetic expression for CO_2 uptake of a leaf mesophyll cell as a function of nitrogen partitioning, assuming that a given total amount of nitrogen could be partitioned to the two functions (i.e. $N_{CA} + N_R$ = a constant). The theoretical optimum $N_{CA}:N_R = 1:22$) is very close to the observed content of CA in several species. According to this logic the most "expensive" components (i.e. those with the lowest specific activities and highest molecular weights) should be the most limiting with respect to the rate of CO_2 fixation. But it must be emphasized that the optimal distribution of protein, and therefore the degree to which various enzymes limit the flux, is also strongly dependent upon the characteristics of the various regulatory sequences.

We have discussed evidence indicating that feedback inhibition of Rubisco and the electron transport system may be quite weak under most "natural" conditions. In view of this finding, the relatively low specific activity of Rubisco, and the large amount of protein devoted to Rubisco and the light-harvesting function [Evans (54) estimated that leaves devote similar amounts of protein to these components], it is not surprising that both protein complexes have emerged as important points of flux control in this and other analyses of the regulation of photosynthesis. Nevertheless, the argument of Cowan illustrates that control is likely to be shared among all proteins that participate in the process, albeit to different extents. Furthermore, given the large variation in the intensity of environmental factors that affect the rate of photosynthesis in a typical natural habitat, the distribution of flux control is likely to change dramatically over the course of a day. It is therefore neces-

sary to develop a quantitative approach to assess how this control is shared, and how this may change with environmental conditions.

Definition of Limitation

It was recognized some time ago that certain enzymes are particularly important in determining the metabolic flux. Krebs (144) called these enzymes "pacemakers" and specified that they must catalyze reactions the velocities of which are restricted by the activities or concentrations of enzymes and not by substrate concentrations. A similar definition was elaborated by Bücher & Rüssmann (24), who used the term "rate limiting" and recognized that several such enzymes could coexist in one pathway. It therefore became necessary to develop a quantitative means of expressing limitation in order to be able to compare the relative importance of the enzymes whose activities are important in determining the flux. Higgins (112) proposed that the "control strength" of an enzyme could be defined as the increase in flux brought about by an infinitesimal increase in enzyme activity. As a differential, this expression is relevant to a particular steady state and does not define the effect of large changes in enzyme activity on the flux.

Differential coefficients of this kind, which express the sensitivity of the steady-state flux to "independent" variables such as enzyme concentration and kinetic constants, have been applied widely in analyzing and characterizing metabolic systems (e.g. 38, 100, 131, 171, 206–208, 210). Using the nomenclature of Burns et al (25), the "flux control coefficient" for an enzyme (E) is given by

$$C_E^J = \frac{\partial J/J}{\partial P/P}, \qquad 15.$$

where J is the metabolic flux of interest and P the independent variable whose change brings about a change in the flux. The latter variable is typically enzyme concentration or V_{mzx}. The control coefficient is a dimensionless quantity because the increments are expressed as fractions, and can be positive, negative, or zero. A coefficient of unity, for example, indicates that small changes in the parameter P effect proportional changes in the flux. Kacser & Burns (131) have demonstrated that the control coefficients for each enzyme in a system with respect to a single flux sum to unity as long as P is chosen such that it is proportional to the velocity of the respective reactions.

There has recently been debate about the use of the control analysis of Kacser & Burns (131) for determining control coefficients from the kinetic properties of individual enzymes (e.g. 39, 211). These criticisms, however, have not questioned the validity or usefulness of the control coefficient for characterizing flux control in the steady state, but rather have expressed the

need for caution when making the assumptions required to predict systemic properties from those of individual components. Crabtree & Newsholme (38) have expressed some reservations about the use of the control coefficient for describing metabolic systems because of the possibility that a high control coefficient may be incorrectly interpreted as indicating that the relevant enzyme must be important during a change in flux resulting from a change in the level of an external effector. They also stress that, as a differential and a property of a given steady state, the control coefficient does not yield information about the effect on the flux of relatively large changes in enzyme activity or, conversely, the degree to which an enzyme is limiting during a large change in flux (see 124).

There are no strict rules that dictate how and with respect to which parameters the determination or limitation of a metabolic flux is defined, but the most useful definitions are made with respect to the independent variables of the delimited system because system variables (e.g. intermediate pool sizes) can only be altered by changing the independent variables.

Control of the Rate of CO_2 Uptake

Before examining some of the studies of enzymes of the PCR cycle and sucrose synthesis, it is worth noting that, unlike "downstream" reactions, the processes mediating CO_2 diffusion to the stroma can limit the rate of photosynthesis simply by restricting the supply of this substrate. Many of the quantitative studies of the limitation to the rate of photosynthesis have in fact been directed at these elements, and reviews by Farquhar & Sharkey (62) and Jones (124) provide an assessment of many of them. Several more recent studies have successfully developed expressions, involving both empirical and imposed equations, for the control coefficients of the stomata and aerodynamic boundary layer (13, 275). An alternative approach advocated by Sharkey (223) defines the limitation due to CO_2 diffusion to the carboxylation site as the difference between the steady-state rate of photosynthesis and that which would occur if the diffusive conductance to CO_2 were infinite. This expression defines the maximum value of the limitation imposed by stomatal conductance and has the disadvantage that it is a complex function of several independent variables and is therefore not useful in evaluating the impact on an existing system of changes to these variables.

FLUX CONTROL BY RUBISCO The most widely accepted appraisal of the role of Rubisco in determining the rate of CO_2 assimilation was put forward by Farquhar et al (64). These authors proposed that the rate of steady-state CO_2 fixation is determined either by the maximum capacity of Rubisco at the prevailing $[CO_2]$ and $[O_2]$ or the light-dependent capacity to regenerate RuBP. This model has been of great value because not only does it define the

criteria for high-gain photosynthesis with respect to CO_2, but it also provides a basis for linking separate kinetic models of Rubisco and carbon metabolism with the input of photochemical energy from the light reactions. It was originally proposed that it would be possible simply to determine the state of the system by measuring the RuBP pool size (64). However, it is now clear that this measurement, taken by itself, is meaningless because v_c and v_o are also controlled in vivo by additional mechanisms that regulate both the amount of active Rubisco and the apparent kinetic properties of the active enzyme species.

In the light of these new findings regarding the regulation of Rubisco activity, it is necessary to redefine the criteria used to assess the degree to which Rubisco determines the rate of photosynthesis. Assuming that we now have an accurate picture of the mechanisms by which Rubisco can be regulated, unequivocal evidence that the quantity of Rubisco is limiting can be obtained by demonstrating that the enzyme is fully active, and the RuBP closely approaches a rate-saturating level (from our earlier discussion, this would appear to be close to twice the active-site concentration). Such a demonstration is technically possible, and several published experiments (213, 215, 254) can be analyzed to show that Rubisco is at least the primary limitation to the photosynthetic rate when the irradiance is saturating and CO_2 is at or below normal ambient levels. However, it becomes more difficult to determine the degree to which Rubisco limits the rate of photosynthesis at subsaturating light intensities or high levels of CO_2 because attenuation of Rubisco activity by regulatory mechanisms becomes more significant.

We have stated a case suggesting that feedforward regulation of Rubisco and other light-activated enzymes is in part a means of "poising" the PCR cycle such that feedback inhibition of the electron transport system is minimized and the efficiency of photosynthetic electron transport maximized. If one were to propose that the progressive inactivation of Rubisco observed with decreasing irradiance actually detracts from the rate of photosynthesis, then it follows that (*a*) the feedforward mechanism is not "tuned" for maximum high-gain photosynthesis, and (*b*) a quantitative assessment of the degree to which Rubisco activity limits the rate of photosynthesis will show a significant degree of limitation at low irradiances.

The complexity of the Rubisco regulatory system makes it very difficult to use kinetic models to draw quantitative conclusions concerning the limitation to the rate of photosynthesis imposed by Rubisco. Woodrow et al (276; see also 279) have taken advantage of the fact that both the internal CO_2 and O_2 levels can be manipulated as fixed (independent) variables. By changing the levels of these gases, it is possible to simulate the effect on the photosynthetic system of changing the maximum activity of Rubisco. The advantage of using this approach is that, from what we understand of the kinetics of Rubisco, the

$K_m(CO_2)$ and $K_m(O_2)$ values do not vary greatly with changes in the level of RuBP or the activation state, and therefore it is possible to predict relatively accurately the fractional effects of CO_2 and O_2 changes on both the carboxylase and oxygenase activities of Rubisco over a range of conditions. The authors derived expressions for control coefficients which reflect the effects of small changes in both the Rubisco activities on the net rate of CO_2 fixation and the RuBP flux (J_R).

Variation in the rate of CO_2 uptake (proportional to J_R and the control coefficient with respect to the V_{max} for Rubisco ($C^{JR}_{Rubisco}$) to light intensity is shown in Figure 6. At a rate-saturating light intensity the control coefficient is over 0.75, indicating that Rubisco is the major controller of the flux. However, as the light intensity is decreased, control by Rubisco decreases until, at less than 400 μmol quanta $m^{-2}s^{-1}$, this step exerts no flux control. One may conclude that small changes in Rubisco activation or the level of CA-1-P (both of which modulate V_{max}) would not affect the flux at low light intensities. These changes would have a much larger effect at

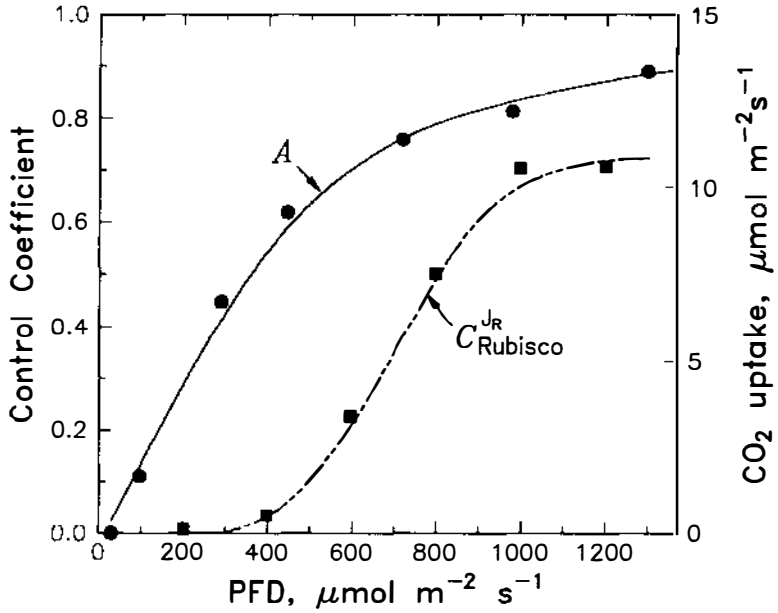

Figure 6 The rate of CO_2 uptake for an intact soybean leaf as a function of the incident photon flux density (*PFD*) and the corresponding control coefficient of the RuBP-flux ($v_c + v_o$) with respect to Rubisco. Values of the control coefficient were calculated from gas-exchange measurements of the response coefficients of CO_2 uptake with respect to $[CO_2]$ (e.g. $\partial A/\partial[CO_2]\times[CO_2]/A$) and $[O_2]$ at the indicated *PFD* levels and ambient levels of CO_2 and O_2. For details of the measurements and calculations see Woodrow et al (276).

saturating light, but as most studies indicate that Rubisco is already fully active at high light (160, 182, 254), regulatory mechanisms that control the activation state of Rubisco may also not be significantly limiting under these conditions. Systematic studies of control coefficients and the level of Rubisco activation over a wide variety of conditions are required to develop a picture of whether, at intermediate light intensities, for example, regulatory mechanisms can actually detract from the rate of photosynthesis. This example, which is typical of healthy C_3 plants, would argue that feedback inhibition of energy input and transduction due to suboptimal poising of enzyme activation states is minimal. Nevertheless, from published responses, one could be fairly confident that mechanisms regulating Rubisco activity will come much more into play with leaves at higher temperatures (139) and under conditions of phosphorus deficiency (21).

FLUX CONTROL BY OTHER ENZYMES From the foregoing discussion of regulatory sequences, it is evident that all of the enzymes of the PCR cycle, the starch and sucrose synthetic pathways, and probably the electron transport system have the potential to affect Rubisco activity and therefore the rate of CO_2 fixation. At low light intensities, for example, Rubisco activity is clearly highly regulated by feedback processes and, in view of the almost linear relationship between irradiance and CO_2 uptake (Figure 6) under these conditions, the rate of photosynthesis is most probably highly limited by the quantum efficiencies of PSI and PSII. Under conditions of intermediate light intensities, however, both Rubisco and the photosystems may be subject to feedback inhibition, some of which may originate from the PCR cycle and export pathways. It remains a challenge to quantify the degree to which these enzymes limit the rate of photosynthesis, because the analysis of Woodrow et al (276) suggests that, in a healthy C_3 plant, the influence of this component is relatively small.

Numerous conclusions concerning the importance of enzyme activities in limiting the rate of photosynthesis have been drawn from relationships between substrate pool sizes and the flux. A strategy of comparing equilibrium constants with mass action ratios was employed by Bassham & Krause (15) in a study of steady-state photosynthesis in *Chlorella*. From the calculated free-energy changes, it was concluded that Rubisco, FBPase, SBPase, and Ru5P kinase are the potentially regulatory enzymes of the PCR cycle, and, except for Rubisco, these enzymes participate in the regulatory sequence affecting the branch points of the PCR cycle. Heldt & Stitt (105) and Gerhardt et al (83) have made similar calculations for enzymes of the sucrose synthetic pathway and have estimated that the cytosolic FBPase, SPS, and sucrose phosphatase have relatively high, negative free-energy changes and therefore are potentially regulatory enzymes. Comparison of phosphorylation potentials and ΔpH values in thylakoid suspensions where ATP is not consumed and

in isolated chloroplasts undergoing photosynthesis revealed that the reaction catalyzed by ATP synthase is also generally displaced from equilibrium (85, 142). Dietz & Heber (46) showed that, over a range of fluxes, there was little change in the mass action ratio for the combined PGA kinase-G3P dehydrogenase reaction and the stromal aldolase reaction, and concluded that these enzymes probably do not limit the rate of photosynthesis. However, the reactions catalyzed by stromal hexose phosphate isomerase and phosphoglucomutase both show some deviation from their calculated equilibrium constants and therefore have the potential to regulate the rate of starch synthesis and presumbly, to some degree, the rate of photosynthesis (45). There have been numerous other studies of the relationships among PCR pool sizes [see review by Leegood et al (155)], the ATP/ADP and NADPH/NADP ratios, ΔpH [see review by Horton (114)], and the flux. While these have greatly expanded our understanding of the regulation of photosynthesis, their interpretation in terms of flux limitation has been complicated by two fundamental problems. 1. Regulation of the activation state of the PCR cycle enzymes and the ATP synthase results in a very complex relationship between metabolite pool sizes and the flux that is not easily interpreted. 2. There is still no clear definition of the nature and strength of feedback processes influencing the initial irreversible steps in photosynthesis (i.e. the quantum yield of the photosystems and the activity of Rubisco). This information is essential if we are to make estimates of the degree to which enzymes limit the flux from information regarding the kinetic and regulatory properties of the constituent enzymes.

The implicit logic of most of these studies of pool sizes is that "nonregulatory" and "potentially regulatory" enzymes can be distinguished simply by comparing the equilibrium constant and mass action ratio for a given reaction under a defined set of conditions (196). It has not always been appreciated, however, that regulatory enzymes identified by this and other means are not necessarily important in determining the flux (196). In fact, Kacser & Burns (131) showed that there is **no** general correlation between the mass action ratio/equilibrium constant quotient and the flux control coefficient (see below). This conclusion is not only relevant to control coefficients but will hold for any quantitative or qualitative expression relating the activity of an enzyme or process to the determination of a flux. These authors also showed that if a reaction is at equilibrium, the relevant enzyme does not limit the flux. Nevertheless, the problem with any arbitrary division between equilibrium and nonequilibrium reactions is that the relationship between the displacement from equilibrium and the control coefficient (or any similarly based quantitative expression) is dependent upon the nature and strength of the feedback sequence linking the substrate pool with the initial, largely irreversible steps in the system. Thus, if the substrate of a reaction causes a severe and sensitive

inhibition of the first largely irreversible reaction in a sequence, even the smallest movement of the mass action ratio (which could occur when the reaction is close to equilibrium) would have a profound impact on the flux. Quantitative and qualitative measurements of the sensitivity both of pool sizes to changes in the activity of an enzyme and of the feedback sequence to alterations in pool sizes are required to draw valid conclusions regarding flux limitation.

Computer models of photosynthesis have also been used to examine the sensitivity of the rate of CO_2 assimilation to various parameters (151, 273). There are of course many assumptions underlying the construction of such models and there are not enough data concerning the regulation of the system for such models to predict accurately the impact on the flux of changes in the activities of the enzymes. Nevertheless, both models do yield valuable insights into the way the rate of photosynthesis is limited under a variety of conditions. The Laisk & Walker (151) model was designed to simulate photosynthesis under high light and CO_2 conditions. Their analysis and that of Woodrow (273) show that under these conditions the limitation of the photosynthetic rate may be shared among several elements of the system, and Laisk & Walker (151) conclude that the activity of the sucrose synthetic enzymes and therefore the rate of cytosolic Pi production may be of great importance in this regard. Although the model of Woodrow (273) does not include all of the sucrose synthetic pathway and cytosolic Pi is not a variable, the accompanying analysis does show an interesting relationship between the flux control coefficients of the cytosolic (C_1) the stromal FBPases (C_2) and the ADP-glucose pyrophosphorylase (C_3):

$$\frac{C_2}{v_2} + \frac{C_3}{v_3} = \frac{C_1}{v_1} \qquad 16.$$

where v_1, v_2, and v_3 are the fluxes through the cytosolic and stromal FBPase and the ADP-glucose pyrophosphorylase catalyzed reactions, respectively. The control coefficients of at least these three enzymes therefore cannot change independently of each other, and limitation of the rate of photosynthesis by the enzymes of the sucrose synthetic pathway may by necessity be accompanied by a significant degree of limitation by the other enzymes at the triose phosphate and hexose phosphate branch points of the PCR cycle.

Part of the difficulty in interpreting experiments aimed at examining limitation by elements other than Rubisco and the photosystems is that the answer is going to be quantitative (i.e. relatively small) over the full range of light intensities (276). In order to overcome this difficulty, many studies have been undertaken using elevated CO_2 concentrations or depressed temperatures. The Walker group had considerable success, using a step change in conditions and

the ensuing transient rate, in showing that the limitation to the rate of photosynthesis under these conditions is both widely distributed and in part involves the reactions of the sucrose synthetic pathway. The most direct evidence comes from their experiments involving the feeding of mannose and Pi to intact tissues (229, 262, 263). The stimulation of steady-state photosynthesis and alteration of the condition under which oscillations in the rate of photosynthesis occur, which can be elicited by feeding Pi, demonstrates that, at high CO_2, the cytosolic reactions that liberate Pi (this will include all of the reactions of the cytosol to a greater or lesser degree because of the conservation of cytosolic Pi and presumably any processes involved in the movement of Pi from the vacuole to the cytosol) partially limit the rate of photosynthesis. Similar conclusions have been drawn by workers examining the sensitivity of the rate of photosynthesis to O_2 either at high CO_2 levels and slightly depressed temperatures (221, 222) or at ambient CO_2 and a low temperature (153).

CONCLUDING REMARKS

The major function of the mature leaf is to supply carbon to the rest of the plant for a range of metabolic and storage functions. In the long term, the requirements of these "sink" tissues for carbon may vary considerably, but over the shorter term a more or less uniform supply of carbon to the developing tissues may contribute to greater metabolic efficiency and therefore may be of adaptive advantage. It may also be of adaptive advantage for the photosynthetic system to be able to make best use of the available light and CO_2 and therefore to produce the greatest amount of end product for growth. Since the conditions in most natural habitats tend to fluctuate frequently over the course of a full day, there is ostensibly a fundamental contradiction between the need of the photosynthetic system to have a high gain (i.e. to be sensitive to variations in external input parameters) and the need to produce a relatively continuous supply of sucrose for export to developing tissues. The mechanisms by which linear metabolic systems deal with this conflict are discussed by Savageau (209) and broadly involve the operation of feedback loops that can partially desensitize the rate of product supply from changes in the substrate levels (253, 284). In other words, there is a trade-off between efficient use of substrates and stable substrate supply.

The photosynthetic system appears, however, to have adapted efficiently to the conflicting demands placed upon it. It is responsive to fluctuations in the range of light intensities and CO_2 partial pressures likely to be encountered in the natural habitat, and the supply of sucrose over the course of a full day generally remains uniform (e.g. 70). The main reason for these properties is that rather than being a simple linear sequence, the photosynthetic system has

two major branch points which terminate in the relatively large transient storage pools of starch and sucrose. By channeling assimilated carbon into these pools, the plant can insulate the rate of sucrose supply to developing tissues from the changes in the rate of photosynthesis that occur during the course of a day and night.

Nevertheless, the system cannot operate with maximum gain by simply eliminating feedback sequences because, when the rate of energy input exceeds the maximum capacity of the PCR-cycle enzymes to sustain the corresponding flux, a feedback mechanism must operate to facilitate the dissipation of excess energy and thus to maintain the condition for a steady state and to protect against photoinhibition (cf 17, 143). The most energetically efficient feedback mechanisms would therefore be largely inoperative over a large spectrum of light intensities and only switch on when the maximum capacity of the enzymes of the PCR cycle and adjoining pathways has been closely approached.

Biological responses are rarely discontinuous, but a "metabolic switch" can be approximated by strongly sigmoidal kinetics. Such enzyme responses have been termed "ultra-sensitive" because there is an appreciable range of substrate or effector concentrations over which there is little or no change in the catalytic velocity, but once the "threshold" has been reached, there is a rapid increase to almost the maximum velocity (141). There is no evidence of cooperative kinetics in the PCR cycle extreme enough to constitute a metabolic switch. However, we have discussed how parallel variation in the levels of several effectors of a single enzyme in response to irradiance, for example, can result in highly sigmoid substrate response characteristics. Such responses appear to have the capacity to reduce significantly the feedback strength of regulatory sequences originating from the PCR cycle and the sucrose synthetic pathway.

The subcellular compartmentation of reactions and the resulting conservation of stromal and cytosolic phosphate play an important role in these and other regulatory responses. For example, we have developed the argument that Pi mediates at least part of the feedback sequence influencing the input of quantum energy, and that these initial steps of photosynthesis may be attenuated when the stromal concentration of Pi declines from its optimal range. While the kinetics of this mechanism are not yet clear, it is apparent that the "ultra-sensitive" responses of certain stromal enzymes must play a major role in this mechanism through their influence on the concentration of esterified intermediates and, consequently, Pi. For example, the sigmoid response of Rubisco to RuBP levels in vivo is such that the photosynthetic flux may change over a wide range without an appreciable change in RuBP (see Figure 2). Among other things, this would tend to maintain a stable—and perhaps near optimal—level of stromal Pi. But as the flux approaches the maximum

capacity of Rubisco, the level of RuBP must increase by a large amount to attain an additional increment of flux. The level of stromal Pi would thus become correspondingly sensitive to changes in flux in this range and initiate feedback inhibition of the photoacts. The regulatory mechanisms underlying the sigmoid responses of Rubisco and other enzymes of the photosynthetic system are therefore important in affecting the extent to which feedback mechanisms may detract from the efficient use of absorbed light energy when light is a factor limiting the rate of photosynthesis.

Amplified responses of enzyme activity may also result from the subcellular compartmentation of reactions and the resulting conservation of stromal and cytosolic phosphate since several photosynthetic enzymes are both activated by phosphate esters and inhibited by inorganic phosphate. The best example of this is the highly sensitive regulatory sequence governing assimilate partition. The three critical enzymes involved in this process (namely, ADP-glucose pyrophosphorylase and cytosolic and stromal FBPase) are all, either directly or indirectly, activated by esterified phosphates and inhibited by Pi. Opposite changes in these effectors permit assimilate partition to be altered—for example, by feedback from the sucrose pathway—almost independently of CO_2 assimilation, despite the capacity of this sequence to affect the electron transport system and possibly the activation state of Rubisco.

The participation of futile cycles in these amplified control mechanisms is also noteworthy. Regulation of cytosolic FBPase through the futile F6P-F26P cycle is the best-documented of these mechanisms (238), but it is clearly not the only such energy consuming cycle. The thioredoxin system operates against a constant decay to O_2, the synthesis and degradation of CA-1-P most probably consume energy, and it is proposed that the activation of Rubisco by "Rubisco activase" involves a direct ATP-dependent reaction acting in opposition to the passive decay of activated Rubisco. These cycles will have metabolic "costs" that presumably must be offset by improved energetic efficiency of the photosynthetic process. The superimposition of these complex regulatory processes upon existing allosteric mechanisms is one of the remarkable features of the photosynthetic system. Studies of the characteristics of these mechanisms, which may serve principally to modify the feedback processes in photosynthesis, seem important to understanding the high energetic efficiency of the system.

Studies of the regulation of photosynthesis have tended to focus at the enzyme level, and this has helped us enormously to understand the responses of enzymes to their "local" conditions. Recent progress in understanding the factors controlling Rubisco under conditions of steady-state photosynthesis in vivo have been given considerable attention in this review. Nevertheless, these studies by themselves provide no understanding of the rationale, in

terms of the overall performance of the system, for the observed responses. This can only come from viewing these responses in the larger context of the system of photosynthetic reactions.

The structure of the photosynthetic system is remarkable because the two major input pathways—one involving the transduction of quantum energy and the other the supply of electron acceptors principally by carboxylation or oxygenation of RuBP—are essentially irreversible. These pathways must therefore be subject to a feedback regulation that maintains the stoichiometric balance required for steady-state photosynthesis. We have examined two such regulatory sequences as well as a third that permits the distal reactions involved in sucrose synthesis to feed back on both of these initial steps of photosynthesis. Our knowledge of these sequences is still at a rudimentary level, and much work is still required—particularly regarding the molecular details of the mechanisms that regulate Rubisco and the electron transport system. Other key regulatory problems have not been addressed in detail here. One of the most important involves the mechanisms that maintain the pools of PCR cycle intermediates in the face of rapid fluctuations in the rate of CO_2 fixations.

Finally, we have only touched on the problems associated with understanding the basis for differences in photosynthetic rate. This must be dealt with if we are to develop rational strategies for improving photosynthetic performance by plants or to examine questions of optimization of the photosynthetic system to different environments. It is our opinion that this requires a quantitative approach, whereas qualitative or comparative approaches have been most frequently used. The methods of control analysis (131) and metabolic systems analysis (210) have been widely used in other areas of biology and appear to be well suited for application to quantitative analysis of the marginal control of photosynthesis by co-limiting factors.

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