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Personal perspective

Affixing the O to Rubisco: discovering the source of photorespiratory glycolate and its regulation

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Abstract

The source of glycolate in photorespiration and its control, a particularly active and controversial research topic in the 1970s, was resolved in large part by several discoveries and observations described here. George Bowes discovered that the key carboxylation enzyme Rubisco (ribulosebisphosphate carboxylase/oxygenase) is competitively inhibited by O_2 and that O_2 substitutes for CO_2 in the initial 'dark' reaction of photosynthesis to yield glycolate-P, the substrate for photorespiration. William Laing derived an equation from basic enzyme kinetics that describes the CO_2, O_2, and temperature dependence of photosynthesis, photorespiration, and the CO_2 compensation point in C_3 plants. Jerome Servaites established that photosynthesis cannot be increased by inhibiting the photorespiratory pathway prior to the release of photorespiratory CO_2, andDouglas Jordan discovered substantial natural variation in the Rubisco oxygenase/carboxylase ratio. A mutant *Arabidopsis* plant with defective glycolate-P phosphatase, isolated by Chris Somerville, definitively established the role of O_2 and Rubisco in providing photorespiratory glycolate. Selection techniques to isolate photorespiration-deficient plants were devised by Jack Widholm and by Somerville, but no plants with reduced photorespiration were found. Somerville's approach, directed mutagenesis of *Arabidopsis* plants, was subsequently successful in the isolation of numerous other classes of mutants and revolutionized the science of plant biology.

Introduction

This article relates a personal odyssey through what might be described as the photorespiration wars of the 1970s, a decade of highly contentious research that began with intense interest in the oxygen inhibition of CO_2 fixation and stimulation of CO_2 release in the light observed in many plants, and the consequential loss of photosynthetic productivity. By the end of the decade, plants created by directed mutagenesis had resolved these questions with finality while at the same time launching the *Arabidopsis* revolution that has transformed the face of plant biological research. Much of the noteworthy research on this topic was carried out in my laboratory by a succession of outstanding graduate students and postdoctoral associates, who are largely responsible for this remarkable chronicle. The discussion below will recount my recollection of the path of these experiments and, where germane, the logic or serendipity that went into them. More detailed reviews of the early history of photorespiration and of the research described here can be found elsewhere (Chollet and Ogren 1975; Ogren and Chollet 1982; Ogren 1984). Another account describing photorespiration research in the 1970s was written by Israel Zelitch (2001).

Richard Hageman

Upon completing requirements for the PhD degree in the Chemistry Department at Wayne State University under David Krogmann in autumn 1965, I was hired by the US Department of Agriculture (USDA) at their laboratory in Urbana, Illinois, to improve soybean photosynthesis. At that time I knew little about the ways of agriculture or agricultural research. After floundering in the laboratory for a year or so, Dick Hageman, an agronomy professor at the University of Illinois, kindly took me aside and described his research on variation in levels of nitrate reductase and other enzymes in corn, and how the concept of rate-limiting reactions could be used to improve crop performance (Hageman et al. 1967). From these discussions it became clear that one approach to improving soybean photosynthesis would be to determine if varietal differences existed and, if so, to incorporate higher rates into existing elite performing varieties. To this end Paul Curtis, a graduate student in my laboratory, assayed CO_2 fixation in several soybean varieties and indeed found significant differences when expressed in the standard units of that time, mg $CO_2 dm^{-2}$ h^{-1} (Curtis et al. 1969). At about the same time, experiments in Dick Shibles' laboratory found similar differences (Dornhoff and Shibles 1970). Shibles' group also expressed the data as specific leaf weight (mg CO_2 (leaf dry weight)$^{-1}$ h^{-1}), but in this calculation the differences became much smaller.

Infrared gas analyzers (IRGAs) became widely available during the 1960s, so photosynthesis rates of many crop plants were beginning to be measured. The results for most species were similar to that found for soybean, namely, significant differences in photosynthesis rate when expressed on a leaf area basis but small differences when expressed on a weight basis. The latter observation, a strong correlation between specific leaf weight and photosynthesis rate, led many researchers to conclude that measurements of specific leaf weight could replace time-consuming IRGA assays as an estimate of photosynthesis rate. However, I concluded that leaf photosynthesis assay differences occurred because higher rate varieties were 'thicker' and that they simply had more photosynthesis machinery per unit area, with little or no difference in fundamental photosynthetic efficiency. Thus, while higher specific leaf weight led to higher rates in assay cuvettes, where there is excess light, in the field there would be little advantage because essentially all available light is absorbed by the crop canopy, and so the efficiency of assimilating CO_2 under these conditions would vary little (Ogren 1976). Thus, it became evident to me that there were no significant varietal differences in soybean photosynthesis and the only way to substantially improve soybean CO_2 fixation was to create a more efficient photosynthetic process.

Forrester, Krotkov and Nelson 1966

In looking for potential photosynthesis inefficiencies to remedy, photorespiration quickly came to the fore. I was particularly intrigued by papers from the laboratory of Gleb Krotkov (Forrester et al. 1966; Tregunna et al. 1966), in which he and his colleagues demonstrated an ordered relationship between CO_2 fixation and O_2 concentration and between O_2 and the CO_2 compensation point. One of these papers was devoted to soybean (Forrester et al. 1966), and I spent the better part of a year analyzing the gas exchange data in biochemical and physiological terms. Krotkov's laboratory reduced their gas exchange analyses to a simple equation:

$$\begin{aligned} \mathrm{APS} = \mathrm{TPS} - \mathrm{PR} &= \mathrm{CE}([\mathrm{CO_2}] - [\mathrm{CO_2}]_{\mathrm{cp}}) \\ &= \mathrm{CE}[\mathrm{CO_2}] - \mathrm{CE}[\mathrm{CO_2}]_{\mathrm{cp}}, \end{aligned} \quad (1)$$

where APS is the measured photosynthesis rate, TPS is the true photosynthesis rate, PR is the rate of photorespiration, CE is a term they referred to as carboxylation efficiency, and $[CO_2]_{cp}$ is the CO_2 compensation point. As a function of O_2, Equation (1) could be expressed (Ogren and Bowes 1970) as:

$$\mathrm{APS} = \mathrm{CE_o}\, e^{-(k_1[O_2])}[\mathrm{CO_2}] - \mathrm{CE_o}\, e^{-(k_1[O_2])} k_2[\mathrm{O_2}], \quad (2)$$

where CE_o is CE in the absence of O_2, $-k_1$ is the slope of a plot of ln CE against O_2 concentration, and k_2 is the slope of $[CO_2]_{cp}$ against $[O_2]$. The primary conclusions I drew from these equations, which held true over a range of CO_2 and O_2 concentrations, were that the rates of photosynthesis and photorespiration were positively correlated, and, since the $[CO_2]_{cp}$ was a linear function of $[O_2]$, *the rates of photosynthesis and photorespiration were limited by a common enzyme*. Further, the O_2 inhibition of photosynthesis in soybean as expressed in Equation (2) indicated two separate O_2 effects: an inhibition of the term CE and a stimulation of photorespiration. Thus Equation (2) suggested that a single rate-limiting step in photosynthetic carbon fixation was inhibited by O_2 and also regulated the synthesis of photorespiratory substrate.

In retrospect, the positive relationship between photosynthesis and photorespiration is readily apparent, but at the time a prominent worker in the photorespiration field, Israel Zelitch, was publishing data suggesting that higher rates of net photosynthesis in certain tobacco varieties were due to lower photorespiration rates in these cultivars (Zelitch and Day 1968). However, perhaps the only point of agreement among photorespiration workers was that the $[CO_2]_{cp}$ represented the balance between photosynthesis and photorespiration, and many measurements in my laboratory and in the literature showed that basically all photorespiratory species exhibited a $[CO_2]_{cp}$ of 40 ppm at 21% O_2 and 25 °C. The extraordinary constant nature of the $[CO_2]_{cp}$, regardless of photosynthesis rate or C_3 species, could not be explained by any means other than a common, unbreakable, direct positive link between photosynthesis and photorespiration. The linear dependence of $[CO_2]_{cp}$ on O_2 concentration is a second truly remarkable biological occurrence. Neither observation would hold if photosynthesis and photorespiration were independently regulated. These insights provided us with a clear experimental focus as well as an impenetrable intellectual fortress from the various attacks on our theories over the next few years.

Whence photorespiratory glycolate?

Glycolate is an early product of photosynthesis, and Zelitch suggested glycolate metabolism was involved in photorespiration after inhibiting the peroxisomal enzyme glycolate oxidase (Zelitch 1966). Zelitch (1965) considered that glycolate arose from a direct condensation of CO_2 though no such pathway has ever been observed. Tolbert (1971) believed that glycolate arose from glycolate-P hydrolysis, based on the presence of glycolate-P phosphatase in leaves (Richardson and Tolbert 1961), though he did not know the source of glycolate-P. A third theory held that glycolate arose from light-mediated oxidation of the transketolase intermediate in the photosynthetic carbon reduction cycle (Coombs and Whittingham 1966). Tolbert and his colleagues described the catabolism of glycolate to CO_2 and serine, and the return of serine to the carbon reduction cycle in the late 1960s (Tolbert 1971), so the sole remaining unknown piece of the photorespiratory pathway puzzle at the beginning of the 1970s was the route of glycolate synthesis.

George Bowes

Work in Olle Björkman's laboratory showed a high correlation between carboxylase activity and photosynthesis rate (Björkman 1968), and so I became interested in examining the properties of this enzyme which might also be crucial in determining the photosynthesis rate in soybean. In 1968 George Bowes accepted a postdoctoral position under Dick Hageman, but after George arrived in Urbana, Dick ascertained that George was more interested in photosynthesis (Figure 1) than in nitrogen metabolism, and so agreed that he should work in my laboratory. In George's doctoral research at the University of London he examined physiological properties of plant response to light, and he had come to Urbana to obtain expertise in biochemistry. Björkman's experiments included measuring the response of leaf photosynthesis to O_2, so in one aspect of George's research he examined CO_2 incorporation catalyzed by Ribulosebisphosphate (RuBP) carboxylase under nitrogen and O_2, and included experiments where more than one gas phase was used, namely, in some instances a N_2 flush was followed by O_2 prior to assay, and vice versa. (For an account of the discovery of the enzymeRubisco, initially called fraction 1 protein, see Wildman 2002).

Initial experiments showed that O_2 had no effect on $^{14}CO_2$ incorporation. However, on one fateful day in December 1969, George's data showed a reversible inhibition by O_2. Carboxylase contains many sulfhdryl groups, so the enzyme was presumed to be sensitive to oxidation. To prevent this supposed oxidation, standard assay mixtures always contained a reduced thiol reagent. In the late 1960s, reduced glutathione was the reagent of choice because it was the only commonly available reagent. For our assays, reduced glutathione was prepared in batch and then frozen in several small test tubes for use in individual experiments. In examining the glutathione used this particular day, George determined that it had sat in the freezer too long and became oxidized. However, we were puzzled by the restoration of activity when an O_2 flush was followed by assay under N_2. After a talking about it for a short time, it dawned on us that the diminished activity observed under O_2 compared to N_2 was not the result of an oxidized, denatured enzyme, but that O_2 was an inhibitor of carboxylase activity. Shortly after this realization, and from deducing the product of RuBP oxidation along with our understanding of Krotkov's equation, we reasoned that O_2 also lopped off the top two carbons of ribulose bisphosphate via

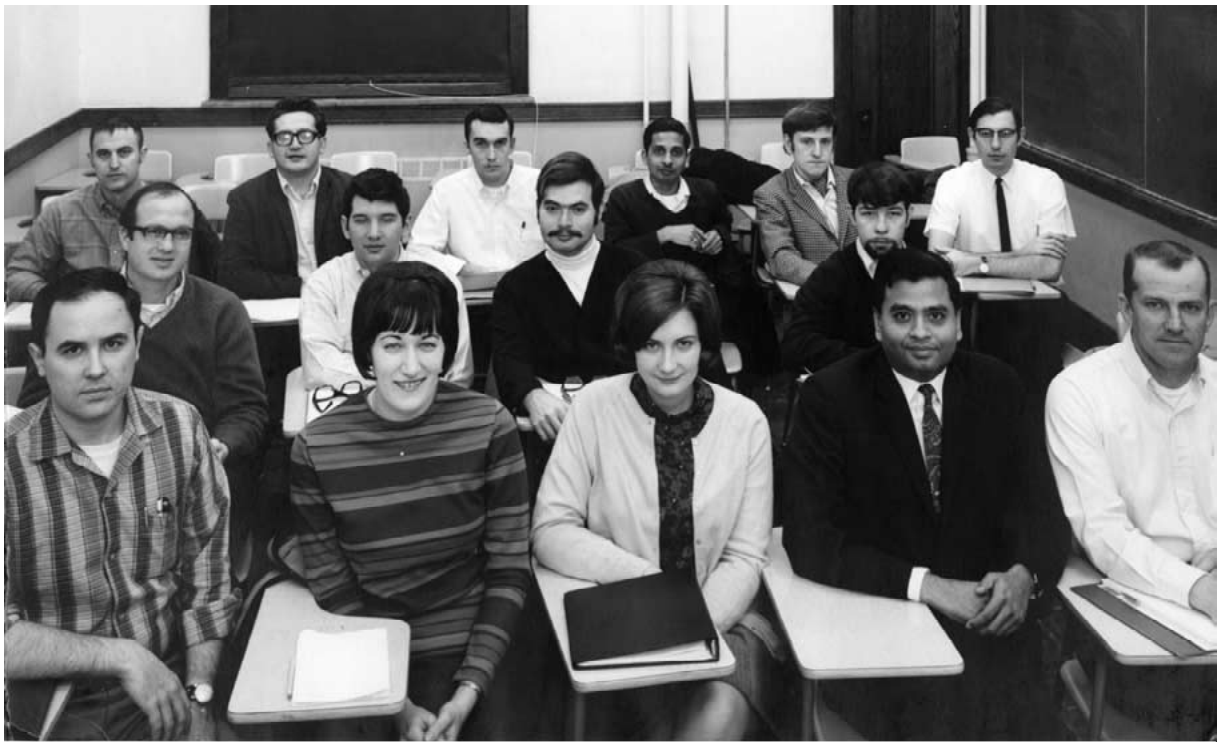

Figure 1. Participants in a photosynthesis course taught at the University of Illinois in 1969 by William Ogren (back row, right) and Govindjee (front row, second from right). George Bowes (back row, second from right) informally audited the course. Students in the class who became prominent photosynthesis researchers are Alan Stemler (middle row, left), Raymond Chollet (middle row, second from left), and Prasanna Mohanty (back row, third from right).

the carboxylase to yield glycolate-P, the source of glycolate in photorespiration. We had not observed O_2 inhibition in the presence of reduced glutathione in the early experiments because reduced glutathione is autoxidizable, and its oxidation had consumed the O_2 in the reaction mixtures.

George determined that O_2 was a competitive inhibitor of CO_2 fixation by carboxylase and measured the $K_m(CO_2)$ and $K_i(O_2)$ (Bowes and Ogren 1970). He then devised an assay for glycolate-P formation but in several experiments was not able to demonstrate the reaction. We decided to write up what we had and sent it off to *Science* for publication, but the paper was rejected. When the manuscript was returned, we thought that if we could show glycolate-P synthesis we would have better success in publishing the data. Several efforts by George again failed to show oxygenase activity but we decided to try once more to publish the O_2 inhibition data, this time sending it to *Nature*. To our great delight, *Nature* published the paper in March 1971 (Ogren and Bowes 1971), and followed it with an editorial article describing our work and putting it into the context of the current state of photosynthetic carbon research (Anonymous 1971), which was undergoing a robust renaissance at the time because of the recent discovery of C_4 photosynthesis (see Hatch 2002) and a concurrent strong interest in photorespiration. On seeing these articles in print we were again inspired to look for glycolate-P, and George made one more effort to produce it from RuBP in the presence of carboxylase and O_2. This attempt, in early May 1971, was successful, and a paper describing these experiments was sent off for publication in August (Bowes et al. 1971). From that time forward, our laboratory never failed to detect oxygenase activity.

Personal encounters of the diaphanous kind

There is always more to a science story than is found in the published record, and this certainly holds true for Rubisco and photorespiration. Some comments on the background of this topic have already been published (Somerville 2001a, b; Lorimer 2001). I am able to extend and, in certain cases, clarify these published

remarks. In May 1970, about six months after Bowes discovered O_2 inhibition of carboxylase activity, I attended a photosynthesis meeting in Gatlinburg. Ed Tolbert was there along with John Andrews, who had recently joined Ed's laboratory as a postdoctoral researcher. Our discussions turned to photorespiration, and I mentioned George Bowes's data and our belief that glycolate arose from O_2 substitution for CO_2 in the RuBP carboxylase reaction. This suggestion was met with hearty chuckles, and it was sardonically pointed out to me that oxygenases utilizing molecular O_2 required a reductant or metal cofactor, and, since there was no cofactor associated with carboxylase activity, such a reaction was not possible. In retrospect I was rescued from my considerable naïveté by this errant dogmatism that a cofactor, specifically a reductant such as reduced ferredoxin or $NADPH_2$, was necessarily required for O_2-dependent glycolate-P synthesis (Andrews et al. 1971). Consequently, '*in vivo*, neither ribulose 1,5-diphosphate nor any phosphorylated pentose is subject to a four-electron oxidation by molecular oxygen to yield phosphoglycerate and phosphoglycolate or glycolate' (Andrews et al. 1971). Had I been taken seriously, someone else might have been invited to write this historical article on glycolate-P synthesis in photorespiration. I did not think much about this encounter at the time, as my most vivid memory upon leaving the meeting was a hike to the summit of Clingman's Dome with a group of conference participants that included Robert Hill. Another strong recollection from the meeting is that of our esteemed co-editor Govindjee, himself an earnest young man at the time, energizing the conference by engaging photophosphorylation guru Daniel Arnon in vigorous debate over the proper formulation of the two photosystems (see Pearlstein 1971; these proceedings honored William Arnold, C. Stacy French, Eugene Rabinowitch, Hans Gaffron and Larry Blinks).

In January 1971 David Canvin invited me to participate in a photorespiration symposium during the annual meeting of the Canadian Society of Plant Physiologists, and I agreed to do so. At the meeting, held in Toronto in mid-June, I presented the carboxylase O_2 inhibition and glycolate-P synthesis data (Ogren 1971) to an audience that included Tolbert, who was also on the program, Andrews, and a graduate student in Tolbert's laboratory, George Lorimer. After my lecture Andrews and Lorimer approached and told me that while listening to my talk they had devised an improved assay for oxygenase activity, utilizing ^{14}C-labeled RuBP, and asked for our reaction conditions, which I gave to them. Several weeks later in August, at about the time Tolbert's group submitted for publication their paper asserting that molecular oxygen did not produce glycolate-P from RuBP by O_2 substitution for CO_2 in the carboxylase reaction nor by direct oxidation of any other Calvin–Benson–Bassham cycle intermediate (Andrews et al. 1971; for a history of the cycle, see Benson 2002; and Bassham 2003), I received a phone call from Andrews stating they were unable to reproduce RuBP oxygenase activity, and he pointedly asked whether I provided complete assay details. I assured him the assay protocol was complete, and offered to send him soybean enzyme that Bowes had used in successful oxygenase experiments (Figure 2), enzyme left behind when George moved to a position in the Carnegie Institution of Washington at Stanford. John replied that he would rather drive down to Urbana from East Lansing and pick up the enzyme so he could keep it on ice during transit. A couple days later he arrived, accompanied by Lorimer. With a boxful of paper charts showing chromatographic separation of carboxylase reaction products, they argued to me and Raymond Chollet, a graduate student in my laboratory at the time, that glycerate was the only product present in reaction mixtures following phosphatase treatment whether or not O_2 was present during assay. I told them to take Bowes' enzyme back to East Lansing and try again. A couple weeks later Andrews called to say that they confirmed our observation of glycolate-P synthesis with the soybean enzyme, and subsequently found activity in spinach carboxylase (Lorimer et al. 1972). Receipt of our soybean Rubisco was acknowledged by Andrews et al. (1973), but its critical role in facilitating their experiments was not disclosed.

William Laing

In January 1972, William Laing, a postgraduate scientist on leave from the Department of Scientific and Industrial Research in Palmerston North, New Zealand, arrived in Urbana to pursue his doctoral degree. An intellectually brilliant individual, Bill is particularly skilled in mathematical analysis. In talking with him about the carboxylase and photorespiration, I told him I understood mechanistically how O_2 inhibited CO_2 fixation and stimulated glycolate synthesis through carboxylase, but it was not clear to me how the kinetic constants of one enzyme regulated both photosynthesis and photorespiration, as

Figure 2. George Bowes assaying RuBP oxygenase activity at Urbana in 1971. Glycolate-P production was measured colorimetrically after enzymatic and chemical conversion to glyoxylate phenylhydrazone.

implied by Krotkov's term carboxylation efficiency (Equation (2)). When Bill's verbal explanation failed to enlighten me, he left and returned shortly with a set of equations, derived from fundamental enzyme kinetics in the presence of competitive inhibitors, that immediately clarified the picture (Laing et al. 1974),

$$v_c = V_c K_o C/(K_c K_o + K_c O + K_o C) \quad (3)$$

$$v_o = V_o K_c O/(K_c K_o + K_c O + K_o C) \quad (4)$$

where v_c is the rate of CO_2 fixation, v_o the rate of O_2 fixation, K_c and K_o the K_m values for CO_2 and O_2, and C and O are the CO_2 and O_2 concentrations. These kinetic parameters of Rubisco, describing the rates of carboyxlation (photosynthesis) and oxygenation (photorespiration), correspond to Krotkov's term CE in Equation (1). Solving Equations (3) and (4) at the $[CO_2]_{cp}$ shows that $[CO_2]_{cp}$ is indeed a linear function of oxygen concentration and can be expressed as:

$$[CO_2]_{cp} = t(V_o K_c/V_c K_o)[O_2] \quad (5)$$

where t is the fraction of glycolate carbon released as CO_2 in photorespiration, shown by Tolbert's group to be 0.25 (Tolbert 1973) and $V_o K_c/V_c K_o$ is the inverse of a term we later defined as the Rubisco substrate specificity factor (Jordan and Ogren 1981b). Calculating $[CO_2]_{cp}$ from experimentally determined kinetic constants gives the observed CO_2 compensation point, verifying the validity of the Equation (5).

Given the large effects of temperature on the relationship between photosynthesis and photorespiration, particularly on the CO_2 compensation point, it was relatively easy to provide additional supporting evidence that these equations were correct (Laing et al. 1974). We did, however, first have to overcome a serious assay dilemma. CO_2, in addition to being a substrate for carboxylase, also activates the enzyme. This effect, first observed in Melvin Calvin's laboratory (Pon et al. 1963), was at the time under scrutiny by a few people including Bill (Laing et al. 1975). The assay problem we faced was that CO_2 is a competitive inhibitor of oxygenase activity yet its presence was required for the enzyme to be active. We decided the only way the CO_2/O_2 interaction could be accurately assessed was to measure both activities simultaneously in the same reaction mixture, so an appropriate assay procedure was developed. A series of assays at different temperatures showed that the kinetic parameters of the enzyme changed in a manner quantitatively identical to the temperature dependence of the relationship between photosynthesis and photorespiration *in vivo* (Laing et al. 1974). Furthermore, from these kinetic parameters we were able to establish the stoichiometry between carboyxlase and oxygenase activities in C_3 plants, which, at atmospheric CO_2 and O_2 concentrations at 25 °C, is approximately one oxygenation per four carboxylations. This stoichiometry accounted for the observed increase in CO_2 fixation upon changing the atmospheric composition at air-levels of CO_2 from 21% O_2 to 2% O_2, the rate of CO_2 evolution

into CO_2-free air, the extent of O_2 inhibition of photosynthesis and rate of photorespiration as determined by Canvin's technically superb $^{12}CO_2/^{14}CO_2$ dual gas exchange assay (Ludwig and Canvin 1971), and, as mentioned above, the effect of temperature on the CO_2 compensation point.

Bill Laing's derivation and subsequent experimental verification of the kinetic relationship between photosynthesis and photorespiration as functions of CO_2 and O_2 concentrations and temperature is a spectacular contribution. His equation is the basis of dynamic photosynthesis models, and no description of whole plant environmental response is complete without it. This most elegant analysis resolved a complex, fundamentally important biological question, providing a solution as close to mathematical perfection as one can get in a living system.

Jerome Servaites

Following Laing's work, two approaches to reducing photorespiration rate were apparent to me. First, since the ratio of photorespiration to photosynthesis is a function of the kinetic constants of Rubisco, altering Rubisco so that the ratio of oxygenation to carboxylation was less than 1:4 under standard atmospheric conditions would reduce photorespiration. Second, compounds that inhibit steps in the photorespiratory pathway prior to CO_2 release were suggested to increase net CO_2 uptake by preventing photorespiratory CO_2 evolution. Such compounds were isonicotinyl hydrazide (INH), which inhibits the conversion of glycine to serine and CO_2 (Pritchard et al. 1963), and α-hydroxypyridinemethanesulfonic acid (α-HPMS), which inhibits glycolate oxidase (Zelitch 1966). In addition to pursuing experiments with Jack Widholm to alter Rubisco (described below), I wanted to determine if photosynthesis could be increased by chemically inhibiting the photorespiration pathway.

In the summer of 1974, Jerry Servaites had finished the requirements for a MS degree with Don Geiger at the University of Dayton and was seeking a suitable laboratory to pursue a PhD degree. He was familiar with the photorespiration literature and, following some discussion of the topic, he became particularly interested in examining the chemical inhibition approach to reducing photorespiration and decided to join my laboratory. His initial progress was slow, as I wanted these experiments to be done with soybean cells and fractionation of the soybean leaf into intact, photosynthetically active cells was exceedingly difficult. After several attempts to isolate active cells, Jerry designed an ingenious apparatus that stirred leaf pieces in the presence of specific pectinase enzymes and collected active cells at a site removed from the stirring action (Servaites and Ogren 1977a). In establishing the photosynthetic characteristics of the isolated cells, Jerry found that kinetic constants of the cells, such as K_m and K_i for CO_2 and O_2, and the temperature dependence of these constants, were nearly identical to those measured by Bill Laing with purified Rubisco (Servaites and Ogren 1977a, b). These experiments provided further evidence for our premise that photosynthesis and photorespiration *in situ* were determined by the kinetic constants of Rubisco.

With photosynthetically active soybean cells in hand, Jerry was able to determine the effect of glycolate pathway inhibitors on photosynthesis. His initial experiments demonstrated that α-HPMS poisoned photosynthesis in addition to inhibiting glycolate oxidation (Servaites and Ogren 1977b), so Jerry synthesized a more specific glycolate oxidase inhibitor, butyl 2-hydroxy-3-butynoate (BHB). The results with BHB and INH were identical: photosynthesis was not affected at 2% O_2, conditions of negligible photorespiration, but at 21% O_2 these chemicals greatly increased the inhibition of CO_2 uptake over the inhibition normally seen in air. Thus it became abundantly evident that chemical inhibition of the photorespiratory pathway past the point of glycolate-P synthesis was not a viable approach to increasing photosynthesis. Rather, it is essential that the carbon remaining after glycine decarboxylation be returned to the photosynthesis cycle so as to maintain a concentration of photosynthesis intermediates sufficient to support the steady state rate of CO_2 fixation. From Jerry's unequivocal experiments we concluded that the only way to reduce photorespiration was to find a way to decrease the rate of RuBP oxygenation relative to the rate of carboyxlation.

Douglas Jordan

Doug Jordan came to my laboratory from the Oklahoma University in 1978 with a keen interest in biochemistry and so he decided to examine more closely the kinetic and regulatory properties of Rubisco. I spent the summer of 1980 in Washington, DC, assisting David Krogmann, who was at the time Chief Scientist in the USDA Competitive Research Grants

Office (now known as the USDA/NRI Competitive Grants Program). (For the scientific life of Krogmann, see his perspective in Krogmann, 2000.) While in Washington I received a telephone call from Doug saying that he assayed Rubisco from *Chlamydomonas*, an organism that had recently been introduced into my laboratory by Robert Spreitzer, and found the CO_2/O_2 specificity to be reproducibly less than the specificity for soybean and spinach enzymes. I suggested he repeat the experiment with another batch of enzyme and send me the data, which he did forthwith. The assay Doug had developed, based on $^{14}CO_2$ and [1-^{3}H]RuBP (Jordan and Ogren 1981a) was both accurate and sensitive, and there was no doubt *Chlamydomonas* Rubisco specificity was less than found in C_3 plant enzymes. Although increased Rubisco specificity in higher plants was our Holy Grail, prior observations in my laboratory and elsewhere that the CO_2 compensation point was virtually identical for all C_3 plants had conditioned me to expect no naturally occurring variation.

To determine if additional differences occurred in the oxygenase/carboxylase ratio, Doug collected Rubisco from diverse classes of photosynthetic organisms and found substrate specificity variation of nearly an order of magnitude, from a value of 9 in the L_2 form of Rubisco in *Rhodospirillum rubrum* to 48 in two cyanobacteria species, 62 in two green algae species, and 80 in higher plants (Jordan and Ogren 1981b). Oxygenase activity in the *R. rubrum* enzyme is so high that, if it were the carboxylating enzyme in a C_3 plant, the CO_2 compensation point would be greater that the atmospheric CO_2 concentration, so no photosynthesis could occur. Doug's splendid experiments clearly established the potential to improve Rubisco and reduce photorespiration, and demonstrated that while photorespiration may be inevitable (Lorimer and Andrews 1973), it is not immutable.

Jack Widholm

In 1967 Jack Widholm, a scientist at International Minerals and Chemical Corporation, located in a northwest Chicago suburb, wrote to USDA headquarters requesting support for a project he had conceived to screen for plants with reduced photorespiration. The USDA did not fund the project, but his letter was forwarded to me. I wrote to him about his proposal and learned that he was about to begin a position at the University of Illinois, and so we agreed to pursue the idea jointly when he arrived at Urbana, in May 1968. Jack's scheme was based on the differences in CO_2 compensation points between photorespiratory and nonphotorespiratory plants. He reasoned that if photorespiratory plants, soybean for example, were placed in an air-tight box with a nonphotorespiratory plant, such as corn, upon illumination the CO_2 would reach a concentration between the two compensation points, leading to the death of the photorespiratory plant but survival of the nonphotorespiratory plant. After Jack came to Urbana we quickly verified the validity of his theory, demonstrating that all plants survived for an extended period at CO_2 concentrations above their compensation point, and that those parameters which speeded photorespiration, such as temperature and O_2 concentration, also increased the rate of senescence of photorespiratory plants held below their CO_2 compensation points (Widholm and Ogren 1969).

After successfully demonstrating that the procedure could select for reduced photorespiration, we decided to search for such plants. We undertook two major projects. First, Shibles' laboratory reported large variations in oat CO_2 compensation point (Criswell and Shibles 1971). Since I presumed that the compensation point directly reflected the Rubisco carboxylase/oxygenase ratio, these data suggested cultivar differences in oat photosynthesis/photorespiration ratios. To examine this possibility, we requested and received seeds of more than 5000 oat cultivars in the USDA collection. To screen the oats, we constructed a controlled environmental chamber that fit over one of three 7-foot by 15-foot sand bases, and planted one-quarter of the area with corn and the remainder with oats (Figure 3). Since it took about four days to germinate the plants, a week to grow the plants to a stage where they could be screened, and four to five days to kill the photorespiring plants, the three bases allowed us to screen plants continually. The ratio of the two species in the chamber provided a CO_2 concentration of about 25 ppm, approximately one-half of the CO_2 compensation concentration at the operating temperature (30 °C). After several weeks of screening we did not find any survivors in the 5000 cultivars. Occasionally a few plants would survive the screening period, so the CO_2 compensation point would be measured directly. In all cases (about 100 oat cultivars were assayed individually) we found the CO_2 compensation points to be identical, 40 ppm CO_2 in 21% O_2 at 25 °C. Thus we concluded that there were in fact no varietal

Figure 3. William Ogren (left) and Jack Widholm inspecting soybeans after screening in the photorespiration chamber in the early 1970s. The airtight chamber can be seen on another base in the background.

differences in the oat photosynthesis/photorespiration ratio.

The second major screening project was an attempt to create soybean plants with reduced photorespiration by mutagenesis. Soybeans were either bombarded with neutrons or gamma rays by the Oak Ridge National Laboratory, or treated with ethyl methanesulfonate at Urbana, and the M2 generations were produced. We again planted corn in one-quarter of the chamber, and soybeans in the remaining area. The number of individual soybean plants per chamber was about 1500. We began screening in 1970, examining approximately 350 000 M2 soybean plants. During this period no survivors were found and in 1975 we decided, with reluctance and disappointment, to terminate the experiment.

Christopher Somerville

In early 1978, I received a letter from Chris Somerville at the University of Alberta stating that he had read with interest the paper by Jack and me describing the photorespiration screen (Widholm and Ogren 1969), and that he had devised a procedure to accomplish the same end by directed mutagenesis. Chris outlined a two-step procedure, in which he would first turn photorespiration into a lethal process by selecting mutants with defects in the pathway, and then use the mutant plants to select for viable second-site revertants. The rationale for revertant survival was that such plants would possess reduced photorespiration activity. I was very much intrigued by this approach and in late summer of that year, at my eager invitation, Chris and his wife Shauna arrived in Urbana.

The initial selection scheme was a simple one to execute, seeking M2 plants that died in air but survived at high CO_2. This scheme was based on the premise that lethal concentrations of metabolites would accumulate in plants with defects in the photorespiratory pathway in air, but, at high CO_2, RuBP oxygenase activity would be suppressed and, since little if any carbon would enter the pathway, photorespiration defects would be inconsequential and the plants would grow normally. Chris chose to do these experiments with *Arabidopsis* plants, at the time being studied by a handful of geneticists but unused in physiological or biochemical experiments because of their small size. [A detailed account of the genesis of the Somervilles' application of molecular biology techniques to plants has been published (Pennisi 2000).] Within a matter of weeks Chris had isolated 40 plants with the desired phenotype, and by the end of the year began to characterize strains derived from these plants.

The first measurement to characterize mutant plants was kinetic analysis of photosynthetic CO_2 gas exchange. The basic pattern was the same for all mutants. In 21% O_2, CO_2 uptake began upon illumination, followed by a saturation of rate and then various rates of decline in photosynthesis. At 2% O_2,

photosynthesis in the mutant plants was generally sustained at a high rate. One mutant strain exhibited particularly clean kinetics in air, showing a peak of photosynthesis after about 5 min of illumination in air followed by a decline to zero after about 10 min. Chris decided to examine this particular mutant strain first and found, after feeding $^{14}CO_2$, that little glycine or serine was synthesized and that large amounts of carbon accumulated in glycolate-P (Somerville and Ogren 1979). When the mutant was treated with the glycolate oxidase inhibitor BHB, glycolate-P, not glycolate, accumulated. Also in the mutant, there was no evolution of CO_2 into CO_2-free air in the light, unlike wild-type plants. Finally, this strain was found to be greatly deficient in the enzyme glycolate-P phosphatase. These experiments, together with absolute cosegregation of phenotype and enzyme deficiency in the F2 generation, conclusively demonstrated that glycolate-P was the sole precursor to photorespiratory glycolate and CO_2, closing debate on the source of glycolate in photorespiration.

Having successfully turned photorespiration into a lethal process, Chris then attempted to isolate photorespiration-free revertants. About 500 000 M2 plants of the glycolate-P phosphatase mutant strain, and a like number of seeds of a strain deficient in serine-glyoxylate aminotransferase, were screened and several viable plants were found. Examination of these plants revealed that true reversion had occurred in all cases, and the original genetic lesion had been repaired. To reduce the background rate of viability, Chris created a strain deficient in both enzymes and, with help from Shauna, screened about 15 million M2 plants of the double mutant. No viable revertants were recovered.

Conclusion

Although we did not reach the ultimate goal of increasing photosynthesis by reducing photorespiration, we did identify a potentially successful approach, namely, decreasing the Rubisco oxygenase/carboxylase activity ratio. I remain confident that someone will accomplish this objective in the future. An important biological problem, initiation and regulation of photorespiration, was definitively elucidated. Finally, the plant biology community gained a powerful new genetic approach for resolving fundamental questions in most if not all facets of plant science. Given the astonishing impact that *Arabidopsis* has had on the course of plant biology research over the past two decades (see, for example, the June 2001 issue of *Plant Physiology*), I am pleased that these techniques were first applied to the ultimate resolution of the photorespiratory pathway. In addition to the scientific advances described above, the personal development and joy of discovery I witnessed in my laboratory as this story progressed supplied the finishing touch to an exceedingly rewarding decade-long journey.

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