BOT 4503
5 Hours
Photosynthetic Carbon Metabolism

Objectives

1. Compare the energy required to reduce $NADP^+$ (from water) with that required for ATP synthesis. Compare #3, below.

2. Review glycolysis, with a particular focus on the steps leading to the reduction of NAD^+. Gain the perspective that the reductive step of the Calvin Cycle is a "reverse" of the glycolytic oxidative step.

3. Compare NAD to NADP. . . . where utilized, stoichiometry of water:NAD(P), &c.

4. Write out the carbon oxidation series. What is electronegativity (general terms)? Know all the given stoichiometries.

5. Know the broad outline of the energy-yielding pathways: glycolysis, TCA, OPPP.

6. Write an essay comparing the paper-chemistry route that we used in class (i.e., a *single* product) to the real situation *in vivo* where various products are siphoned off for different purposes.

7. Discuss the role of ^{14}C in the elucidation of the RPPP. Know alternative names for this pathway.

8. **Know** the steps of the RPPP from Ru 5-P to P-Gal. By "know," I mean names of intermediates, number of carbons, and special aspects of interconversions (e.g., ATP needed).

9. What does "EC," used in enzyme nomenclature, mean?

10. Name the isotopes of carbon. Which are radioactive? . . . half-lives? What does dpm mean? What does specific activity mean? What is the relationship between half-life and specific activity (@ 100 mol %)?

11. In broad terms only, draw the reaction of RuBP and CO_2 to form 3-PGA. . . . RuBP and O_2 to form 3-PGA and P-Gly.

12. Discuss the incorporation of CO_2 into organic form. Is the particular carbon of CO_2 reduced in the process? Is there an overall oxidation/reduction of carbon? Compare the average oxidation state of carbon in RuBP to that in PGA.

13. At which point is carbon reduced in the RPPP? Compare that step to the oxidative step of glycolysis.

14. Identify the energy-requiring steps of the RPPP.

15. **Know** the first two steps of the OPPP. Compare the rearrangement steps of the OPPP to the rearrangement steps of the RPPP (at a rather superficial level).

16. Compare the energy yield of the OPPP to the energy requirement of the RPPP. Repeat this process after we cover the PCOP (later in the notes). What is a futile cycle? Write an essay on the "requirement" to avoid futile cycling.

17. Briefly discuss the history of the discovery of light-activation of plant enzymes.

18. Give two reasons that the term "dark reactions" is a misnomer for the RPPP.

19. Describe how light activation and light deactivation of key enzymes of the RPPP and of the OPPP, respectively, prevent futile cycling. What is the molecular mechanism of the post-translational modification that effects the temporal separation of the RPPP and the OPPP? (Consider only that mechanism covered to this point; rubisco will be covered later; all mechanism have not been covered.)

20. Know in outline the steps of the PCOP. . . . number of carbons, nitrogen balance, energy-yielding, energy-requiring, toxin-producing, compartmentation, O_2-consuming, CO_2-yielding, direct fate of PGA formed, &c.

21. Do all photosynthetic organisms metabolize photosynthetically formed P-Gly in the same manner?

22. Overall, approximately how much oxygenation of RuBP occurs, compared with the carboxylation of RuBP?

23. Discuss the general role of α-keto acids in amino acid biosynthesis.

24. What does "quantum requirement" mean? What is the stoichiometry of the RPPP? Based on NADPH formation, what would be the theoretical quantum requirement for the reduction of CO_2? . . . considering also the required ATP? Give an approximate quantum requirement for C_3 photosynthesis that includes the "waste" associated with the operation of the PCOP.

25. Speculate on a "useful" function of the PCOP.

26. Give empirical reasons that support the fact that carboxylation of RuBP is the initial step of the RPPP. . . . that oxygenation of RuBP is the initial step of the PCOP.

27. Explain the biochemical basis for the observation that elevated [O_2] inhibits photosynthesis.

28. Explain the biochemical basis for the observation that there is a PIB.

29. Explain the biochemical basis for the CO_2-compensation point.

30. Discuss rubisco in terms of its abundance, its impact on nutrition, &c.

31. According to one view, limitations to CO_2 fixation under various conditions, say, high light and low [CO_2], can be ascribed to certain aspects of the RPPP. Discuss these conditions and identify the limitations.

32. Describe in broad terms the physical and genetic aspects of rubisco.

33. Using the modified form of the Michaelis-Menten equation that accounts for the competitive inhibition by O_2 of the carboxylation reaction of rubisco, discuss semiquantitatively how modification of one or another term would enhance or inhibit CO_2 fixation. (You do not need to commit this equation to memory.)

34. Discuss the rate of CO_2 diffusion in air and in water in the context of the peripheral arrangement of chloroplasts.

35. Discuss the abundance of rubisco in the chloroplast with respect to the assumptions made in the derivation of the Michaelis-Menten equation.

36. Discuss briefly the history of the discovery of rubisco and the initial difficulties in reconciling the kinetic properties of this enzyme with the kinetics of CO_2 fixation by the plant.

37. Describe the experiment that showed that activator CO_2 is not identical to substrate CO_2.

38. What is the active form of rubisco? How do changes in the chloroplast in light contribute to the activation of rubisco? Identify reasons that show that these conditions are insufficient for activating rubisco to the level that it achieves *in vivo*.

39. What is CA1P? How does it function? When does it accumulate in plants? . . . in all plants?

40. Discuss the several ways that rubisco is converted to an inactive form of the enzyme. Explain the role of rubisco activase in conversion of the inactive forms to the ECM form. Is rubisco activase a “traditional” enzyme? . . . does it require energy?

41. Speculate on how rubisco activase "senses" light?

42. Summarize photosynthetic characteristics of the three major groups of plants (C_3, C_4, CAM), with the focus on CO_2-compensation point, kranz anatomy, post-illumination burst of CO_2, rate of photosynthesis, discrimination against ^{13}C, water-use efficiency, nitrogen-use efficiency, &c.

43. Outline the C_4 pathway of photosynthesis and correlate the biochemistry to the attributes enumerated above.

44. Outline CAM. What are facultative CAM plants? Describe how you would determine whether a facultative CAM plant is in the CAM mode, now and historically.

45. How does the variation in carbon isotope ratios of C_3 plants provide insight into the water-stress history of the plant?

Lecture

The ultimate function of the leaf is to harvest light and convert that harvested energy into useful chemical energy. To provide the necessary perspective on the fixation and reduction of CO_2, a general outline of the several coordinated processes will be first provided.

Overhead: Photosynthesis

The initial step is the absorption of light, shown at the top. This process obviously occurs only in the light and high levels of light are required as it is the **energy** that is being harvested. (This requirement for high photon flux density is in contrast to some other light-mediated processes, which—like blue light sensing by guard cells—require only modest irradiance.) We have studied in some detail the harvesting of light. At present, we will only touch on the essentials. Light absorption occurs in the chloroplast membranes. The harvested energy is used to drive the "up-hill" extraction of electrons from water and "use" of the electrons to reduce the two-electron carrier $NADP^+$. (ΔG for this oxidation/reduction is ~53 kcal/mol, compared with ~7 kcal/mol for the hydrolysis of ATP). In this series of reactions ("photosynthetic electron transport 'chain'"), protons accumulate on one side of a membrane; the relaxation of this proton gradient is coupled to the phosphorylation of ADP. (On the next overhead, we will return to a discussion of oxidation and reduction reactions; meanwhile, I would ask you to review you CHM 1045/1046 notes.)

[Up to now, you probably have become familiar with NAD. NAD refers to either the oxidized form (NAD^+) or the reduced form (NADH). In glycolysis, NAD^+ is reduced; this reduction is coupled to the oxidation of the aldehyde (P-glyceraldehyde) to the acid (P-glyceric acid). In fermentation—when O_2 is absent—NADH is oxidized to NAD^+; this reaction is coupled to the reduction of pyruvate (C=O) to lactate (C-OH). In the TCA cycle, carbons are "oxidized off" the acid intermediates in the release of CO_2; NAD^+ reduction is coupled to some of these oxidation steps. In brief summary, a portion of the energy released during the oxidation of carbohydrate is "saved" as the energy required to reduce NAD^+ to NADH. As you recall, mitochondrial respiration transfers the two electrons from NADH (to yield NAD^+) to O_2, (to yield H_2O) with the concomitant phosphorylation of three ADPs. The foregoing should be familiar territory; as we make a shift in the focus of our studies, be alert to the fact that you may need to review BSC 2010 material, as we will have time only to provide a small jolt to the memory. Perhaps you have not encountered another important electron carrier, NADP. NADP (nicotinamide adenine dinucleotide **phosphate**) is very similar to NAD (nicotinamide adenine dinucleotide): both are two-electron carriers and it takes the same amount of energy to reduce each; in fact, these compounds are interconvertible (a light-activated NAD kinase phosphorylates NAD to form NADP when chloroplasts are illuminated). Whereas NAD is required in glycolysis and the TCA cycle (and other reactions), NADP is required in photosynthesis (and other reactions). As a broad generalization, you may say that the major fate of NADH is its oxidation to form 3 ATPs in respiration, whereas the major role of NADPH is to provide the reductant for assimilative processes. You may note that some enzymes that couple the oxidation or reduction of a substrate to NAD(P) are very specific for one or the other of these pyridine nucleotides (e.g., alcohol dehydrogenase), and that others will accept either (e.g., one form of malic enzyme).]

The stable chemical energy (NADPH, ATP) formed above is utilized in several ways. One important way is in the reduction of CO_2. This utilization consumes, say, 80% of the energy captured. To reduce CO_2 to carbohydrate ($(CH_2O)_n$) is a 4-electron reduction. {Throughout, our focus will be on reduction to the level of carbohydrate, but please keep in mind that this is certainly one, but not the exclusive, outcome. Carbon can be reduced more (to the level of lipid) or less (to the level of a keto acid, e.g., glyoxylate, which is a precursor to amino acid.}. At the very beginning, it is useful to remember the stoichiometries: extraction of four electrons from two waters results in the evolution of one molecular oxygen; reduction of carbon dioxide to carbohydrate is a four-electron reduction; two NADPHs are formed with the evolution of one molecular oxygen; two NADPHs are oxidized in the reduction of one

molecule of carbon dioxide to carbohydrate; one molecule of oxygen is evolved for each carbon dioxide that is reduced to carbohydrate. Other major uses of the captured light energy are in the assimilation of nitrogen (20%) and sulfur (1%). Separate lectures will be devoted to these last two topics.

Overhead: Carbon Oxidation Series

Before we delve into photosynthetic carbon metabolism, we will review. Dissimilar covalently bonded atoms do not share electrons equally. Oxygen is very electronegative; carbon, less so; hydrogen, even less so. Thus, in a carbon-oxygen bond, the oxygen "draws" electrons away from the carbon. In a carbon-hydrogen bond, the electrons are weakly asymmetrically distributed toward the carbon. In essence, the carbon atom in carbon dioxide is fully oxidized because all valence electrons are shared with oxygen. As the difference in electronegativity is large, C-O bonds are polar. At the other end of the spectrum, the carbon atom in methane is fully reduced because this atom is bonded only with hydrogen. As the difference in electronegativity is small, C-H bonds are non-polar. Using oxygen as the electron acceptor, the oxidation of methane is spontaneous, with the release of a large quantity of heat. You should refresh your memory of the carbon oxidation series shown on the overhead. In general, our particular focus will be on the reduction of carbon dioxide to the level of carbohydrate.

The bottom of the overhead provides a brief review of several basic carbon pathways. The top three [bracketed by red] are energy-yielding pathways Glycolysis is your responsibility to review: a hexose is metabolized to 2 3-carbon acids, viz. pyruvate. The tricarboxylic acid cycle (TCA cycle) is also your responsibility to review from BSC 2010: for each turn of the cycle, an acetyl moiety is introduced and two carbon dioxide molecules are evolved. The third pathway—the oxidative pentose phosphate pathway (OPPP, aka the pentose-phosphate shunt) —accounts for about one/fourth as much carbon flow as the previous two. Like glycolysis and the TCA, the OPPP provides carbon skeletons for the biosynthesis of other required cell constituents; the five-carbon sugar ribose, in particular, is the product of the oxidation of hexose by the OPPP. Although you may not have encountered the OPPP before in your course-work here at FSU, it will be your responsibility to learn the essentials from your text. We will often discuss comparatively the reduction of carbon dioxide in photosynthesis and the oxidation of carbon in the pathways aforementioned.

The Reductive Pentose Phosphate Pathway (RPPP, aka Photosynthetic Carbon Reduction Pathway, aka Calvin cycle (after Mel Calvin, in whose lab at UC-Berkeley this pathway was elucidated following the availability of ^{14}C; aka Benson-Calvin cycle (to recognize the contributions of a senior postdoctoral associate, A. A. "Andy" Benson), and on occasion, the Calvin-Benson-Bassham cycle (to recognize also the contributions of James "Al" Bassham, another member of the research team) is the pathway by which inorganic carbon is fixed photosynthetically. At the expense of redundancy, this pathway will be our major focus for the next few hours.

Overhead: The reductive pentose phosphate pathway

This overhead is the first of several paper-chemistry exercises that we will cover in the remainder of the course. It is important to recognize that this rendition is only one way to balance the carbon flow. There is a somewhat different version in your textbook. You may wish to compare this version to that in the text. In any case, this version results in the "production" of one phosphorylated hexose from six CO_2 molecules. This "profit" is convenient to use because a phosphorylated hexose is an "entry point" for the synthesis of starch. You need to keep in mind, however, that these chemicals exist as pools In the general, overall sense, one product is not passed off to the next enzyme in the pathway, although this so-called metabolite tunneling is thought to facilitate flux through portions of some biochemical sequences. (This phenomenon may be exhibited with some enzyme complexes of the RPPP, but we will not discuss it.) A main point is that, e.g., ribose—an intermediate in the pathway—may be used for nucleic acid biosynthesis, and not used for the regeneration of RuBP. Given these guidelines for the interpretation of the overhead, we will begin with the reaction that incorporates CO_2, namely the reaction that is catalyzed by the enzyme, RuBP carboxylase. As we will discuss in the not-too-distant future, this enzyme catalyzes the incorporation of O_2 into organic form (a statement that will be slightly modified later). The full trivial name of this enzyme is RuBP carboxylase/oxygenase, or, most commonly, simply, rubisco. We will generally use this latter name, as it is easier to say.

[As a relevant aside, we will not use the formal name of any of the enzymes that we will study because they are too bulky; the formal names (e.g., 3-phospho-D-glycerate carboxy-lyase [dimerizing]) are followed (or preceded) by a number, e.g., EC 4.1.1.39. EC is an abbreviation for "Enzyme Commission." Naming formally enzymes is, however, a necessary exercise, just as Latin binomials are necessary for

unambiguous identification of organisms. In several instances, different enzymes catalyze similar or the same reactions. An example is the enzyme that catalyzes one step in the RPPP. Triose-phosphate dehydrogenase, as you will learn, catalyzes a step that is the "reverse" of the almost identical step of glycolysis. Simply referring to that enzyme as we did in the last reference would not permit its distinction from that of glycolysis. Just as common names can be useful if it is insured that all parties understand, trivial names of enzymes can also be useful.]

Let us start with the introduction of CO_2; this is both a convenient place to start an explanation, and it was, of course, the place that biochemists started in their elucidation of the pathway. Feeding $^{14}CO_2$ to illuminated photosynthetic cells resulted in its incorporation into organic form.

[There are two ways to introduce an isotope into a metabolic pathway. One—called "steady-state" labeling—is to feed continuously the isotope. In this method, the concentration of the isotope is constant over the labeling period, which usually is long enough to reach some kind of equilibrium. The other extreme prototype for introducing a label is to "pulse-label." In this method, the organism is exposed only briefly to the isotope of interest, and then the incubation continues under the prelabeling condition.]

[There are several isotopes of carbon. An element, recall, is defined by the number of protons in the nucleus. Isotopes of an element differ only in the atomic number. The garden-variety of carbon is ^{12}C, which accounts for approx. 99% of atmospheric carbon dioxide. The second stable isotope of carbon is ^{13}C; its natural abundance is approximately 1% of atmospheric carbon dioxide. The most common radioactive isotope of carbon is ^{14}C; this isotope is a relatively weak β-emitter and has a very long half-life (the length of time required for one-half of the nuclei to disintegrate) of >5000 years. Having a long half-life and being relatively safe, ^{14}C is commonly used. The other radioactive isotope of carbon is ^{11}C. This isotope has a very short half-life (~20 min); as it is a strong emitter, it has the advantage of permitting the researcher to study its spatial movements from outside the organism. Finally, in the use of different isotopes, one needs to express quantitatively the enrichment with respect to a particular isotope. For this purpose, the concept of "specific activity" is used. An example expression is xxx dpm mol^{-1} where dpm is an abbreviation for disintegrations (of atoms) per minute. Alternatively, one might say xxx Curies mol^{-1}, where Curie is a count of the number of dpm (3.7×10^{10} dpm). As there is a characteristic half-life, there is a characteristic specific activity for a particular isotope. (The shorter the half-life, the higher the specific activity of the isotope.) For ^{14}C, the specific activity is ~60 Ci mol^{-1}. That value obtains for carbon that is 100% ^{14}C. Even if experimental carbon is all ^{14}C, the ^{14}C is diluted as it moves through a metabolic

pathway. The extent to which the isotope is reduced in specific activity can provide insight into the reaction pathway, the pool sizes of the intermediates, and physical compartmentation.]

Returning to the major point, we note that Calvin and his associates fed $^{14}CO_2$ to the green alga *Chlorella*. They used very short time periods so that the ^{14}C would have time only to move into the first part of the pathway.) Following the brief exposure to the isotope, the algae were quickly killed (to stop further metabolism of the ^{14}C) and then the extracts were subjected to paper chromatography, a method of choice then of separating various chemicals in an extract. A spot corresponding to the migration position of 3-P-glyceric acid (PGA) was radioactive, as determined by exposure of the chromatogram to X-ray film. (This last technique is called autoradiography, and it is one of the most common techniques used in a modern biochemistry and molecular biology laboratory.) PGA is a 3-carbon compound, and the first guess was that the precursor was a 2-carbon compound to which the ^{14}C was added. That simple guess was wrong; the precursor turned out to the 5-carbon sugar, ribulose-1,5-bisphosphate (RuBP). CO_2 attacks the #2 carbon of RuBP, and there is a transient unstable 6-carbon intermediate, which falls apart to yield two molecules of 3-PGA. Obviously, only one of these two identical product molecules is radioactive, so, at a minimum, the specific activity of the carboxyl functional group on 3-PGA is dropped to one-half the value of the fed CO_2, even if all the 3-PGA present had immediately been formed from carboxylation of RuBP. In steps that are essentially the reverse of glycolysis, the PGA is reduced to P-glyceraldehyde (P-Gal). Commit these steps to memory.

There is only one point in the Calvin cycle in which carbon reduction occurs, viz., the step in which PGA is reduced to P-Gal; the electron donor is NADPH. Similarly, there is only one step in which carbon oxidation occurs in glycolysis: at the oxidation of P-Gal to PGA (in two steps in glycolysis and the Calvin cycle); the electron acceptor is NAD^+. Consider these facts: CO_2 is fully oxidized and RuBP is at the oxidation state of carbohydrate. In the fixation of CO_2, the #3 carbon of RuBP is oxidized from the level of alcohol to the level of acid. (This approximation serves us O.K.) On the other hand, the CO_2-carbon is reduced from the level of carbon dioxide to the level of acid. In summary, the oxidation levels of various carbons are changed during the fixation of CO_2, but there is no overall reduction or oxidation. (As a matter of interest, an earlier name for the enzyme was carboxydismutase.)

As mentioned, the version of the paper chemistry that I have drawn out is based on a stoichiometry of 6 CO_2s reacting with 6 RuBPs to yield 12 PGAs. (Please note that the circled Arabic numerals associated with the metabolites is the total number in the scheme. The uncircled numbers by the small

arrows indicate the number reacting along a certain pathway.) 12 ATPs phosphorylate the 12 PGAs and the 12 diphospho-glyceric acids are reduced by 12 NADPHs. Now, back up one step, to the formation of RuBP. That reaction requires 6 ATPs (to generate the 6 RuPPs). The preceding enumerated steps account for the overall energetic requirements of the Calvin cycle: 1 CO_2: 3 ATP:2 NADPH.

Although you will not be asked to recall the individual steps of the rearrangement reactions of the Calvin cycle, you should "walk" through the scheme once or twice. In summary, the 12 P-Gals (12 X 3 = 36 carbons) are rearranged into 6 RuBPs (6 X 5 = 30 carbons). The carbons not accounted for in this summation comprise a hexose, which, as mentioned, is a precursor for starch biosynthesis. Starch, without credible exception in higher plants, is stored within the plastid. Later, we will discuss how the carbon that is used for sucrose biosynthesis is exported from the chloroplast.

In the introduction to this lecture series, I asked you to familiarize yourself with the Oxidative Pentose Phosphate Pathway (OPPP). In this pathway, a hexose is oxidized, thus:

(1) Glc 6-P + $NADP^+$ → 6-P gluconate + NADPH {X6}

(2) 6-P gluconate + NADP+ →→ Ru 5-P +NADPH + CO_2 {X6}

(3) Ru 5-P → (rearrangement)→ Glc 6-P {6 X 5-C Ru 5-P to 5 X 6-C Glc 6-P}

In essence, a 6-carbon carbohydrate is twice oxidized to yield a CO_2 and a 5-carbon carbohydrate. At the expense of redundancy, the oxidation from the level of carbohydrate to CO_2 is a 4-electron oxidation. As shown, the two-electron acceptor $NADP^+$ is utilized twice. The third process shown above for the OPPP shares many steps with the RPPP. With the exception of the regeneration step for RuBP and for the carboxylation step of RuBP, the other metabolite interconversions of the RPPP are not unique.

The result of the OPPP is to oxidize carbohydrate to CO_2 with a stoichiometry of 1 $(CH_2O)n$:2 $NADP^+$. The result of the RPPP is to reduce CO_2 to carbohydrate with a stoichiometry of 1 CO_2: 2 NADPH:3 ATP. Obviously, if these cycles were to function simultaneously in the plastid, 3 ATPs would

be lost for each turn of the two cycles. The simultaneous uptake of CO_2 and reduction to carbohydrate and the oxidation of that carbohydrate back to CO_2 is an example of a futile cycle. A futile cycle is a "pointless" energy sink. Mechanisms have evolved to avoid futile cycling. Several steps of the RPPP are light activated. The Calvin cycle is sometimes referred to as the "dark reactions," this is a misnomer because the Calvin cycle has an indirect requirement for light (provision of NADPH and ATP) and because many of the enzymes are only active in an illuminated organism. I have shown these light-activated enzymes by an open arrow (○⇒) on the overhead. The Zieglers of Munich Tech Univ. discovered this phenomenon of light activation in the late 60's; it was a serendipitous discovery. In brief, woman Dr. Ziegler was working in the laboratory of man Prof. Dr. Ziegler. She was measuring the activities of $NADP^+$-requiring triose-P dehydrogenase (aka 3-P glyceraldehyde dehydrogenase). Her experimental values were inexplicably irreproducible, until she realized that the higher values came from plants that had been illuminated. The activation of triose-P dehydrogenase is modest (say 3x), but certain others, particularly Ru 5-P kinase (aka phosphoribulokinase, which catalyzes the formation of RuBP), are "off" in dark tissues or "on" in illuminated tissues. The activation of the phosphatases (2 steps, which see), Ru 5-P kinase, and, in part, triose-P dehydrogenase is a result of a reversible post-translational modification, i.e., the reduction of a disulfide bridge (R-S-S-R) to the sufhydryls (R-SH plus HS-R). The reductant is formed in the photosynthetic electron transport chain. Thus, harvesting of light by chloroplasts not only provides the NADPH and ATP required for the RPPP, but the harvested light provides a signal that activates the RPPP. (Refer back to the unit on photosynthetic electron transport if needed.) Turning off the RPPP in darkness avoids the energy drain, but it is only one-half of the story. The OPPP is turned off in illuminated tissues. The branch-point enzyme that leads into the pathway (see # 1, above), namely Glc 6-P dehydrogenase, is deactivated in illuminated tissues. The deactivation of this enzyme results from the same reversible post-translational modification and utilization of the same reductant as that which activated the RPPP.

As mentioned several times, rubisco catalyzes not only the carboxylation of RuBP, but it also catalyzes the oxygenation of RuBP. This is an unusual, if not unique, situation, whereby an enzyme forms alternative products from the same substrate. Thus, there is not only a photosynthetic carbon reduction pathway (RPPP), but there is also a photosynthetic carbon oxidation pathway (PCOP), as shown on the following overhead:

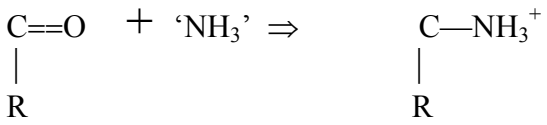
Overhead: Photosynthetic carbon oxidation pathway

Observe that there are several physical compartments, as delineated by the solid lines. We will start at the top, in the chloroplast. The RPPP is shown in abbreviated form—just enough to show its relationship to the PCOP. As I will show in more detail later, oxygenation of RuBP yields two product molecules: the "bottom" three carbons form the familiar 3 PGA and the "top" two carbons form the 2-carbon compound, phospho-glycolate (P-gly). Thus, oxygenation results in the conversion of one 5-carbon carbohydrate to one 3-carbon acid and one 2-carbon acid. To reveal the obvious, oxygenation is an energy loss. Speaking teleologically, the 'challenge to the plant' is to recover from this loss as efficiently as possible. On the PGA side, it is rather simple--PGA, being formed in the chloroplast as it is, is routed into the RPPP. Metabolism of P-gly is rather more complicated.

[About 30 % of the carbon fixed in the RPPP winds up in the PCOP; thus, this process is not trivial—either from the standpoint of agronomy or the physiology of the plant. Since there is such a huge amount of P-gly formed, the plant 'has no choice' except to recover it. Not all photosynthetic organisms have this pathway, however. Certain simple organisms that live in water simply excrete the glycolate, and others have an alternative means of metabolizing it. These alternative means are beyond the scope of this course.]

The PCOP is concerned with the metabolism of P-gly, which is dephosphorylated in the chloroplast by a specific phosphatase. The resulting glycolate is transported out of the chloroplast and into the cytosol, from whence to the peroxisome. This organelle, recall, is one type of microbody. (The other type is the glyoxysome, which is involved with the mobilization of fats, their conversion to carbohydrate; both types of microbodies house H_2O_2-forming reactions and abundant catalase, an enzyme that is exceedingly efficient at degrading the peroxide.) In the peroxisome, the glycolate is oxidized to glyoxylate, an α-keto acid. The electron acceptor is O_2 and, as implied in the preceding, the reduced product is peroxide, which is converted to water and O_2. An important principle is that the peroxide-forming reaction is housed in the peroxide. Thus, formed peroxide, a very reactive species, is quickly neutralized.

α-Keto acids occupy an important position in the synthesis of amino acids:



The transamination reaction shown above normally uses glutamate ('NH_2') as the amine donor; the other product, not shown, is α-keto glutarate, or, as it is known by the more modern name, 2-oxy glutarate.

Returning to the pathway: the product of glycolate oxidation, without energy conservation, is glyoxylate, the 2-carbon α-keto acid. Glyoxylate is transaminated to form glycine, the simplest amino acid. Glycine—'carrying' carbon and nitrogen—is transported out of the peroxisome and into the cytosol, where it may be used either for protein synthesis or for transport into the third photosynthetic organelle, the plant mitochondrion. In the mitochondrion, two glycines (2 X 2 carbons=4 carbons) are converted to one serine (1 x 3 carbons) + 1 CO_2. Although we will not belabor the steps involved in the conversion of 2 glycines to serine plus carbon dioxide, overall, there is no energy conservation.

Serine is transported out of the mitochondrion and into the cytosol. Its fate may be for synthesis of protein there, or for further metabolism via the PCOP, which we will follow. The transport of serine out of the mitochondrion plus the evolution of a CO_2 is tantamount to the export of four carbons, providing balance for the two required glycines. (Overall, we are not keeping track of the stoichiometry through the PCOP, but on the point of C and N balance, do pay attention.) Transport of serine also removes one of the two N atoms imported as glycine. The other N atom is transported as glutamate out of the mitochondrion to the peroxisome. At this point, the mitochondrion is balanced with respect to C and N, but the peroxisome is "short" one atom of N. Transport of the serine into the peroxisome accounts for that N atom.

Inside the peroxisome, the serine loses the amine (by a transaminase reaction), and β-hydroxypyruvate is formed. With a requirement for reduced pyridine nucleotide, β-hydroxypyruvate is reduced to glycerate (reduction of the carbonyl ["aldehyde"] to the alcohol). This step is catalyzed by the peroxisome-specific enzyme, β-hydroxypyruvate reductase. (In other words, if you questioned whether a microbody should be classified as a peroxisome or a glyoxysome, assay for this marker enzyme should yield a definitive answer.) (As another relevant aside, this enzyme will also act as a glycerate dehydrogenase, i.e., it will also catalyze the "back" reaction. The enzyme will also catalyze the interconversion of glycolate & glyoxylate. However, the K_M for these last reactions is so high that in vivo,

reaction with glycolate or glyoxylate is thought not to occur—were it to, energy conservation would take place and production of H_2O_2 would be avoided.

At the expense of an ATP, glycerate is converted to PGA, which can enter the RPPP.

In broad summary, the oxygenation of RuBP itself results in energy loss (reduction level). Furthermore, additional oxidation, which is not coupled to a physiological electron acceptor, occurs. C is lost as CO_2. Energy, both ATP and NADPH, is required to recover—even at the level of acid, 3/4s of the carbons formed as glycolate.

Let us return now to the subject of energy required to reduce CO_2 to $(CH_2O)n$. In our assessment in the Calvin Cycle, we "established" the stoichiometry of 1 CO_2:3 ATP:2 NADPH. That stoichiometry was, of course, based only on the theoretical requirement of the RPPP. Any carbon that is diverted through the PCOP will raise the energy requirement of fixing CO_2. Raising the energy requirement implies that the number of photons absorbed by the light harvesting apparatus will be increased. Extraction of each electron from water requires two photo-acts. Thus, a 4-electron reduction using photosynthetically formed NADPH would require the absorption of 8 photons. (On this basis alone, we would say that the quantum requirement for CO_2 reduction is 8.) As mentioned, however, 3 ATPs are also required for each CO_2 reduced in the RPPP. Synthesis of these ATPs will raise the theoretical quantum requirement to 9 photons per CO_2. Because of the energy waste[1] associated with the PCOP, a more typical quantum requirement would be 12, as Sharkey has calculated. Obviously, less active oxygenation of RuBP (e.g., high $[CO_2]_{internal}$ associated with well watered plants) would result in a smaller quantum requirement. Since electrons are shuttled between H_2O and CO_2 or O_2 and $(CH_2O)n$, the stoichiometry between O_2 and CO_2 will still be unity.

Overhead: Rubisco reactions

[1]Because of the speculative nature of this comment, I will put it in a footnote. We do not **know** why the PCOP evolved. Some say that nature could not devise an enzyme that would accept CO_2 but not O_2. That statement may hold for gaseous CO_2, the substrate for rubisco. However, it is certainly not true generally of enzymes that carboxylate. An example is the ubiquitous plant enzyme PEPC. This activity, recall, uses HCO_3^- to carboxylate PEP without an oxygenation reaction. Tolbert always promoted the idea that the PCOP is a clutch. Sometimes, the light-harvesting apparatus is endangered because it can absorb more energy than can be utilized by the RPPP. Tolbert reasoned that the higher quantum requirement imposed by the PCOP could be a protective mechanism.

This overhead provides a convenient backdrop for a few comments concerning the origin of glycolate and the relative carboxylation vs. oxygenation reactions. (We will discuss later the activation and deactivation of rubisco; at the moment, suffice it to allow that there is no known (or suspected) modulator or activation mechanism that causes the reaction to shift more toward the utilization of CO_2 or O_2.) In the carboxylation reaction, all atoms of CO_2 are incorporated as the # 1 carboxyl of one of the formed 3-PGAs. Note that there is an unstable 6-carbon intermediate; we will refer to this compound later. In the oxygenation reaction, one of the atoms is incorporated into the carboxyl functional group of P-glycolate and the other is released to the medium as a hydroxyl ion.

As we speak today, there is no question that rubisco catalyzes the initial reaction of the RPPP and the PCOP. It is nevertheless useful to review the evidence that supports our knowledge. (Regardless how well entrenched an idea, question authority; the exercise does a mind good.) This first set of observations provides support for the fact that rubisco catalyzes the primary reaction of the RPPP: (1) 3-PGA is the compound most heavily labeled after a pulse of $^{14}CO_2$. At early times ($\leq$ 1 sec), almost all the label is in the C-1 position. (2) Usually, there are reciprocal pool-size changes for [RuBP] and [PGA]. When the light comes on, PGA concentration increases. (This metabolite is a positive effector of the rate-limiting enzyme in starch biosynthesis, a point that we will return to.) We will also discuss transport into and out of the chloroplast; now, simple acknowledge that the triose-Ps permeate the plastid envelope. When triose-P is fed to isolated chloroplasts along with an inhibitor of photophosphorylation, CO_2 fixation stops. Since only RuBP requires ATP to be made from triose-P, this observation indicates that RuBP is necessary for CO_2 fixation. (3) As we discussed early on, atmospheric CO_2 comprises two stable isotopes, viz. ^{12}C and ^{13}C. Although we use isotopes as "perfect" tracers, they are not. As an immediate example, ^2H_2O vapor will not diffuse as quickly as regular water vapor, simply because of the mass differences. (For a refresher, return to the lectures on gas exchange and study the section that dealt with the relative rates of effusion of CO_2 and H_2O.) Chemical reactions, including those that are enzymically facilitated, may discriminate against one or another isotope. This fact means that possibly various metabolites along a pathway will be enriched with or depleted of a particular isotope if all isotopes are equally available. "Normal" plants (hereafter, C_3 plants), those that do not have an auxiliary pathway, discriminate against atmospheric ^{13}C.

$$\text{Discrimination} \equiv \delta\ 13C‰ \equiv [(\text{Sample mass ratio})/(\text{Standard mass ratio}) - 1] \times 1000$$

Using the typical standard (a limestone from PeeDee, Carolina), the atmospheric $\delta^{13}C = -6.7‰$ (read: minus six point seven mils). Carbohydrates of C_3 plants have a $\delta^{13}C$ in the range of $-28‰$[2], which is the same as the discrimination by rubisco. This concordance is strongly suggestive of rubisco's service as the port through which CO_2 is converted to organic form. (4) Oxygen is an inhibitor of photosynthesis by plants (the Warburg O_2 effect, reported without explanation in 1943); CO_2 utilization by rubisco is inhibited by O_2, as first reported by George Bowes and Bill Ogren (in 1971). This second set of observations provides support for the fact that rubisco catalyzes the primary reaction of the PCOP: (1) There is a specific chloroplastic P-glycolate phosphatase, which would account for the rapid appearance of glycolate as a photorespiratory substrate. Treatment of isolated chloroplasts with Fl^-, an inhibitor of phosphatases, caused P-glycolate to accumulate. Moreover, high-CO_2-requiring mutants of *Arabidopsis* lacking in P-glycolate phosphatase accumulated P-glycolate in normal O_2/CO_2 air. Furthermore, glycolate accumulated in wild-type plants that were treated with a glycolate oxidase inhibitor. (2) The uncoupler FCCP prevented the oxygenation of RuBP by chloroplasts that were fed triose-P. Recall that triose-P moves across the chloroplast envelope. This was a particularly important observation because there was one camp that held to the belief that oxidation of the two-carbon fragment off the transketolase complex was the source of photorespiratory glycolate. Because all the rearrangement reactions require no energy (ATP), the transketolase complex would be formed even with the uncoupler present. As mentioned, RuBP alone requires ATP from the triose-P step "forward." (3) Lorimer and Tolbert and others showed that molecular oxygen was incorporated into glycolate as predicted in planta. (4) CO_2 inhibits glycolate synthesis, just as O_2 inhibited CO_2 fixation. (5) Light dependency of the rate of photorespiration (and of photosynthesis) can be explained by the energetic requirement of regenerating the acceptor molecule, RuBP. (6) As mentioned, the quantum requirement for CO_2 fixation can be increased by increasing the ratio O_2/CO_2.

How can the explained biochemical observations be tied to the behavior of the plant? (It is exceedingly important, regardless of the tools employed and the concepts toyed with that we remember

[2]In well watered plants that have widely open stomata, the leaf intercellular CO_2 composition is similar to that of the atmosphere and the discrimination against ^{13}C is expressed most. In water-insufficient plants, the stomata are narrow and the internal CO_2 concentration is low. In this situation, the lowered CO_2 concentration has been depleted somewhat of ^{12}C (because it has been used preferentially by rubisco); said alternatively, the CO_2 "available" to rubisco

here, now, tomorrow . . . that our goal is to learn how plants work. Anything else is superfluous.) First, as already mentioned, oxygen is an inhibitor of photosynthesis. We have described how that is a manifestation of the oxygenase activity of rubisco. Second, years ago (mid 50's?), it was reported that immediately following a light→dark transition, the plant evolved a "burst" of CO_2. One would have expected that in the light there would be net uptake of CO_2 (say, 300 μmol mg chl^{-1} hr^{-1}) and a comparable rate of O_2 evolution. In darkness, one would have expected that there would be a net evolution of CO_2 (say, 30 μmol CO_2 mg chl^{-1} hr^{-1}), which could be accounted for by "dark" respiration (glycolysis and the TCA). Those expectations obtain. The unexpected observation was that for ~1 min after steady-state photosynthesis in moderate to strong light, there was a so-called Post-Illumination Burst (PIB) of CO_2. We can now handily explain this phenomenon: As mentioned earlier, physiological perturbations—and turning on or off photosynthesis is a huge one—result in alterations of chemical pool sizes of intermediates in a pathway. (Recall that PGA increased in concentration at the expense of RuBP on a dark→light transition.) During photosynthesis, the metabolites "upstream" of the photorespiratory-CO_2-evolution step (i.e., 2 x glycine conversion to 1 x serine plus 1 CO_2, which occurs in the mitochondrion) increase in concentration. On transition to darkness, rubisco is rapidly deactivated, and no more CO_2 is fixed photosynthetically. Net photosynthesis—which is what we can measure noninvasively—is a sum or dark respiration + photorespiration + CO_2 fixation. For present purposes, consider dark respiration invariant[3]. Thus, the PIB is a manifestation of the facts that CO_2 fixation stops abruptly, whereas photorespiration continues briefly, until pathway intermediates "burn out." Third, as we have repeatedly mentioned and will elaborate on, CO_2 is a competitive inhibitor of the oxygenase activity of rubisco and O_2 is a competitive inhibitor of the carboxylase activity of rubisco. Competitive means that O_2 and CO_2 compete for the same active site on rubisco. Raising the $[O_2]$ while holding the $[CO_2]$ steady will favor oxygenation; raising the $[CO_2]$ while holding the $[O_2]$ steady will favor carboxylation. Under particular conditions (e.g., of temperature, pH, &c.), there is a particular K_M for O_2 and a particular K_M for CO_2. With the whole plant, at some concentrations of O_2 and CO_2, carboxylation and oxygenation of RuBP are balanced such that CO_2 is evolved (primarily by photorespiration, but also by dark respiration) at the same rate as CO_2 is taken up. In other words, there is no net exchange of CO_2. This special $[CO_2]$ is called the CO_2-compensation point.

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is enriched with respect to ^{13}C. Thus, water-stressed plants will show somewhat less apparent discrimination, and a range of values for C_3 plants is found.

Typically, placing a C_3 leaf in a closed, illuminated chamber will result in the [CO_2] dropping to a steady state concentration of ca. 60 ppm.

We will resume our focus on rubisco: its physical properties, genetics, and kinetic properties. As they say, a river is deep or a river is wide—it will not be both. Thus, we have our choice; we could touch on many many different plant enzymes and learn one or another superficial fact about each. I have chosen, instead, to provide overviews of several important pathways and focus only on one enzyme, rubisco. Why choose this enzyme? The overwhelming bulk of all CO_2 fixed on earth passes through this enzyme (There are two other pathways used only by certain respective bacteria.) Rubisco is very abundant—it is touted as the most abundant enzyme on earth. It may even be the most abundant protein on earth, its competitors for this recognition being one class of cell-wall protein and the connective protein, collagen. It is even one of the most abundant biomolecules (cf. cellulose, collagen and lignin). Nominally 25% of leaf soluble protein is rubisco, making it a significant source of nutrition. The concentration of rubisco in the chloroplast is about 50% of total protein; it is more concentrated in the stroma than protein in a protein crystal. The genetics of rubisco is complex. The regulation of rubisco is multifaceted. Last, but certainly not least, in vitro activity of rubisco is the least of any of the RPPP enzymes, suggesting that it may be limiting the rate of photosynthesis. Indeed, rubisco is thought to be the major limitation to whole-plant photosynthesis under the conditions of high light and low [CO_2][4]. In sum, to cite Bob Spreitzer, "Rubisco is destined to become the best model in which genetic and biochemical methods are intimately combined to probe the structure/function relationships of enzyme catalysis."

Our remarks will be restricted to rubisco of plants, or to rubisco that resembles that of plants. Because of the wide array of photosynthetic organisms, it is consistent with expectation that quite a number of differences would be found, e.g., in the exact number of copies in the genome, or the number of amino-acid residues, or the structure of the genome, &c. In the end, these differences do not matter a great deal. The similarities are the more substantial.

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[3]As far as I am aware, there is no consensus on the issue of whether dark respiration proceeds unimpeded in light. There are many studies on the subject, but it appears to be a question, the kind of which is difficult to answer generally and unequivocally.

[4]Under low light and high [CO_2], the regeneration of RuBP is limiting, whereas under high light and high [CO_2], the utilization of triose-P ("rearrangement") is limiting. This last condition is characterized by the absence of a response to increased [CO_2].

Rubisco holoenzyme comprises 8 large subunits (rbcL, to take the liberty of referring to the peptide by the name used for the corresponding gene) and 8 small subunits (rbcS, ...same liberty). The large subunits have a mass of ca. 55 KD, and are encoded for by the chloroplast, as first inferred by Sam Wildman who showed that it was inherited uniparently in 1972. (As a matter of perspective, that year was only 9 years after the demonstration of a unique chloroplast DNA species, but was 63 years after the first report of non-Mendelian inheritance.) Qualifications excepted, each chloroplast chromosome has a single copy of rbcL[5]. At the cellular level, therefore, the chloroplast genome is highly polyploid, which is a conservative force in evolution. The rbcS genes comprise a nuclear-encoded family of two to ten members in a given species. The gene product contains a transit-peptide, has a mass of about 15 KD (preprocessed mass of ~20KD), requires ATP for transport into the chloroplast. Assembly in the chloroplast into the 550,000 Mr holoenzyme requires molecular chaperones (similar to heat-shock proteins). The large subunit is catalytic, and it is believed that the small subunit is regulatory. Whereas the rbcL genes have high sequence homology, particularly at the active site, the rbcS are more divergent. Differential expression of the rbcS during development suggests the potential for influencing the catalysis of the holoenzyme. As a measure of interest Genbank has sequences for beaucoup 35 rbcL genes rbcS genes. Because there is variation among taxa concerning the carboxylation/oxygenation ratio—with higher plants having the highest (see below)--there is optimism that a "better" rubisco may be engineered, but I am not aware of any practical application of this information.

An equation similar to the familiar Michaelis-Menten equation has been derived to account for the competitive inhibition of carboxylation by O_2:

$$v_{carboxylation} = (V_{max} \times [CO_2])/([CO_2] + K_M\ carb(1+ [O_2]/K_M\ oxy))$$

You are not required to remember this equation but we will use it as a basis for discussion. A related equation that provides the ratio of carboxylation: oxygenation incorporates the V_{max} for

[5] http://www.clivias.com/Articles/Article016.htm: "The really surprising thing about the chloroplast DNA is the large number of copies which are present: up to 300 in a mature plastid. Since an average of 160 chloroplasts are present in a mesophyll cell of the mature leaf of a cereal such as wheat, this means that there may be as many as 48 000 chloroplast 'chromosomes' per mesophyll cell. The reason for this enormous redundancy of genetic information is unknown."

oxygenation also. This so-called specificity factor is relatively low for rubisco of photosynthetic bacteria, and about 5x higher for rubisco of higher plants. The given equation formalizes what we have said: increase of oxygen concentration decreases carboxylation; decrease of K_M for O_2 decreases carboxylation; increase of $[CO_2]$ increases carboxylation. Thought invokes other constraints: relative solubilities (which we will not go into) obviously affect the gaseous concentration around the rubisco active site; diffusion—which for CO_2 is about 10^4x slower in water than in air—may be a significant limitation to photosynthesis. However, does a Michaelian analysis hold? It seems to, or at least be an unlikely coindence: a v vs. S plot for whole-leaf photosynthesis (v≡rate of CO_2 uptake; S≡$[CO_2]$) gives a hyperbolic plot. I cannot say that I have the final answer on this one either, but there is a major theoretical objection to a Michaelian-Menten analysis. The derivation of the equation held the assumption that the enzyme concentration is very low, << the [S]. As I mentioned earlier, rubisco is very concentrated in the stroma: ~4 mM active sites!! The [RuBP] is ≈ 0.2 - 0.4 mM, which is to be compared with a K_M (RuBP) ≈30 μM. A simple calculation (forget O_2 for the moment) would allow you to predict that rubisco would be more-or-less saturated with respect to RuBP, and, therefore, the carboxylation would be running at full velocity. On the other hand, logic dictates that rubisco can only achieve $V_{max}/2$ only if half the active sites are occupied, which cannot be true if the [RuBP] is 10x less than the active site concentration. In addition to this major conceptual infidelity[6], there are other "inconveniences," e.g., it is known that the chloroplast proteins non-specifically bind a number of organic anions, including RuBP, so the effective concentration of this metabolite is bound (sorry for the pun) to be less than the measured concentration, which was extracted at low pH and relatively high ionic strength. The interactions within the chloroplast are complicated—elevation of one metabolite could out-compete another ligand for the same site, and increase its effective concentration. Another question concerns the form of RuBP used by the enzyme (probably the tetravalent anion).

There are a number of factors that affect the relative rates of carboxylation and oxygenation, such as source of enzyme, as mentioned, and conditions that otherwise affect the relative stabilities of the two transition states, such as temperature. However, there is no known mechanism in the plant that will differentially activate the carboxylase and not the oxygenase, and vice versa[7]. All the factors discussed

[6]The foregoing implies that the RuBP concentration is a major limitation to photosynthesis. Recently, the [RuBP]s I cited have been called into question. If [RuBP] is high, the argument that I gave falls apart.

[7]A major effort in the mid-70's to early 80's in the U.S. was to discover a chemical that would shut off the oxygenase. Many chemical powerhouses such as Union Carbide and Colgate jumped into the fray. In the end—in the U.S.—hope was lost that the search would result in a reliable, safe, general growth regulator based on this chemistry. There are, of course, many other very profitable plant growth regulators that form a basis for the agrichem industry.

below activate both activities of rubisco. First, let us get a historical perspective. Bonner and Wildman first reported the protein that we now know as rubisco in 1947. Very soon, a problem became apparent: the K_MCO_2 was much higher for the enzyme than for the value for CO_2 calculated from whole-leaf photosynthesis. Actually, those experiments led to the counter-intuitive "conclusion" that rubisco could not be the CO_2-fixing enzyme. Every generation of scientists, I suppose, suffers from some prejudices. Without attribution, it was (and perhaps still is) held that "one should not waste clean thoughts on a dirty enzyme." In the push to purify for the sake of purity, kinetic properties of rubisco were altered. Fortunately, Dick Jensen and Jim Bahr, his postdoc, decided to work with a dirty enzyme: they quickly assayed rubisco from freshly lysed photosynthesizing chloroplasts—before the enzyme had time to be altered kinetically. In one fell swoop, an objection to the role of rubisco was removed, as contemporaries thought it would be. The push, then, was on to learn how to convert the high-K_M form (purified) to the low K_M-form (physiological). The path has been fraught with difficulties (such as contaminants in the commercial preparations of the substrate), unexpected findings (such as the importance of the order of addition of the substrates); in short, we have not removed to nirvana, but a great deal of progress has been made. A real clue surfaced when it was discovered that incubation of rubisco with high [CO_2] and high [Mg] activated the enzyme and incubation of rubisco under non-catalytic conditions (e.g., no CO_2) with the substrate RuBP inactivated it. An elegant, simple experiment showed that the activator CO_2 binds tightly with an allosteric site, that it is not exchangeable with the substrate CO_2.

Overhead: Distinct activator and substrate CO_2 species

As shown, rubisco was incubated with $^{14}CO_2$ and Mg^{2+}. After the incubation, the rubisco mixture was injected into a solution that contained enough RuBP to permit several turnovers and a large molar excess of $^{12}CO_2$. The enzyme was then rapidly purified and assayed for radioactivity. If the activator CO_2 were also the substrate CO_2, the bound ^{14}C would be incorporated into the PGA product, and the assayed protein after the experiment would not be radioactive. On the other hand, if the activator CO_2 were bound at a site different from the active site, ^{12}C would be incorporated into PGA because of the large molar excess of that isotope, and the assayed protein after the experiment would be radioactive. Empirically, the CO_2 bound to the enzyme had a specific activity of 30-60x that of the medium, indicating that this binding was not quickly exchangeable and that the binding was not dislodged as the enzyme cycled. We now know that the active form of rubisco is the ECM complex (enzyme that has an activator CO_2 reacted with a lysine

("carbamylation") that coordinates a metal, which completes the formation of the active site). The CO_2-binding described above is spontaneous, but in planta the $[CO_2]$s are not high enough to drive the activation, activation levels that are achieved in vitro are less than those that obtain in vivo, light levels required for activation of rubisco are much less than the levels required to modify the chemical environment of rubisco sufficient for light activation, and light activation was not dependent on $[CO_2]$. For these reasons, we had to abandon the simple notion that light-driven ionic changes in the chloroplast stroma (increase in $[Mg^{2+}]$ and decrease in $[H^+]$) during light were sufficient to drive non-enzymic carbamylation of rubisco.

Meanwhile, Servaites and Seeman independently discovered an endogenous rubisco inhibitor that accumulates nocturnally. The inhibitor, 2-carboxyarabinitol 1-P (CA1P), is an analog of the unstable transition state intermediate 2-carboxy-3-keto-arabinitol bisphosphate. A great deal of discussion centers on the importance of this natural inhibitor. In some plants, it does seem to be important, accumulating in sufficient quantities at night. In other plants, it appears not to be important. Personally, I place more value on positive results, so I am inclined to think that CA1P will be demonstrated to be of general importance, but it is premature to present it that way in an unreserved fashion.

Chris Sommerville, Archie Portis, and Bill Ogren reported on a mutant *Arabidopsis* that lacked rubisco activation *in vivo*. That seminal observation led to the discovery of a new protein, rubisco activase[8], hereafter simply activase.

Overhead: Summary of rubisco deactivation and activation by activase.

(overhead courtesy of M. Salvucci, 2002)

The active form of rubisco is shown on the right. This form of the enzyme is carbamylated at an allosteric site, as shown, and can carry out carboxylation by the addition of another CO_2 and RuBP. The active form converts to the inactive (high-K_M form) by loss of the CO_2 (center panel). This inactive form of rubisco can bind to RuBP to form a "dead" enzyme. The active form of the enzyme as a third fate—it may bind to XuBP, which is a misfire product of the main reaction, (I.e., instead of carboxylating RuBP, sometimes rubisco isomerizes it to XuBP. In brief, activase, which requires an ATP, binds to the "dead"

[8]Those of you with a molecular bent may take pleasure in the knowledge that activase was the first example of a plant mRNA to undergo alternative spicing to form two protein products from a single message.

rubisco and causes the release of the sugar phosphate. The resulting inactive rubisco can then be carbamylated to form the active for of the enzyme.

Regulation of rubisco activation is not so simple as the preceding would allow you to infer. As mentioned, the stromal chemical environment is altered as photosynthesis is initiated. The pH goes from about 7 to about 8, and the [Mg^{2+}] increases. These changes enhance carbamylation and Mg-coordination. In addition, rubisco activity is higher at the higher pH. A number of phosphorylated compounds interact with rubisco is complicated ways—some effects are observed within minutes, some take hours. Some (e.g., RuBP itself) bind biphasically. In any case, from this overview of one enzyme, you will better appreciate the wondrous complexity and fascination of plant life!

We are now making a major shift in our focus, being "finished" with rubisco and the RPPP *per se*. Over the years, a number of different disjointed observations suggested that photosynthesis was not the same in all plants. As a relevant example, 19th-century German anatomists recognized that some species exhibited so-called kranz anatomy. (Kranz is a German word that means something like "wreath" or "halo.") In "normal" plants, the bundle sheath is more-or-less devoid of chloroplasts; chloroplasts are more-or-less restricted to the mesophyll cells. In species with kranz anatomy, however, the bundle sheath cells also have large and well-developed chloroplasts. As another relevant example, it has been well known for a long time that some plants yield extracts that are more acidic at one time over the diel cycle than at another. In the mid-to-late 60's, these observations began to make sense. A worker at a Hawaiian sugar-cane research station found that the first product of photosynthesis was not PGA in sugar cane. Hatch and Slack in Australia picked up this observation, as did Clanton Black at the University of Georgia, and in addition to "normal" C_3 plants, a second photosynthetic group of plants, C_4 plants, were identified. These plants include many plants of agronomic importance, such as the crop plant maize, the turf and pasture grass Bermuda, the weed crab grass. Although we first thought that C_4 plants might be only tropical grasses, we know now that many different types of plants exhibit C_4 photosynthesis, which is polyphyletic, having arisen even several times within a single family. At about the same time, another photosynthetic group was identified. Today, these plants are known as CAM plants; CAM is an acronym for crassulacean acid metabolism. The name implies that this type of photosynthesis is restricted to the family Crassulaceae, but it is not. CAM plants also include many agronomically important species: the crop plant pineapple (not to mention the Central American century plant, *Agava tequilana*), ornamental succulents like Christmas cactus, and the weed Spanish moss. In a reversal of the strategy used to

introduce you to photosynthesis, let us examine how these different biochemical pathways are manifested physiologically.

Overhead: Distinguishing characteristics of three groups of higher plants

The first column summarizes some photosynthetic characteristics of C_3 plants—characteristics that we have already covered and provided a biochemical explanation for. These established characteristics provide a comparative basis for examination of the second and third groups. Recall that the CO_2-compensation point is the "break-even" CO_2 concentration, and that it is a result of the balancing of the oxygenase and carboxylase activities of rubisco. As you note, the compensation point is somewhat higher at elevated temperatures, which favor the oxygenase reaction, compared with the carboxylation reaction. Photorespiration, as measured by the PIB, is active in C_3 plants, which do not have kranz anatomy. Light saturation for C_3 plants occurs at nominally 35% of full sun (summer Florida sun = 2000 μmol m^{-2} s^{-1}). These plants have a moderate rate of photosynthesis (15 -35 mg CO_2 dm^{-2} h^{-1}), discriminate against ^{13}C, and lose a great deal of water in the process of acquiring CO_2 for photosynthetic reduction (>500 g H_2O gm dry $mass^{-1}$). In contrast, C_4 plants (which have kranz anatomy) have a low CO_2-compensation point, which is consistent with the low or not-detectable level of photorespiration. As you infer, these preceding three facts are different manifestations of the same phenomenon, namely that the oxygenase activity of rubisco is virtually "silent" in C_4 plants. The rate of photosynthesis is high in C_4 plants (40 - 80 mg CO_2 dm^{-2} h^{-1}); the optimum temperature is high, and light saturates at about full-sun. These plants are water-use efficient, and the discrimination against ^{13}C is low. The following overhead will condense a great deal of hard work that resulted in our understanding of the physiological phenomenon on a biochemical basis: C_4 photosynthesis comprises the "standard" Calvin-Benson cycle, which is localized in bundle sheath cells. In addition, mesophyll cells have abundant PEPC, which does not have an oxygenase activity and which has high affinity with CO_2. PEPC, in essence, traps CO_2, which is then "pumped" into the bundle sheath cells.

Overhead: Summary outline of C_4 photosynthesis.

A CO_2 diffuses into the leaf through stomata and into the cytosol (a photosynthetic compartment), where it is hydrated to form HCO_3^-, which is the substrate for PEPC. PEPC has an exceedingly low K_M for HCO_3^-, which means that there is little dependence on $[CO_2]_{air}$. Thus, leaf intercellular space $[CO_2]$ is low,

which provides a large driving force for CO_2 diffusion into the leaf. Consequently, the stomata may be narrow, which also increase the resistance for H_2O loss. These facts explain the high water-use efficiency of C_4 plants. C_4 plants discriminate only modestly against ^{13}C because the initial "trapping enzyme," PEPC--unlike rubisco--does not discriminate against ^{13}C. The product of the PEPC carboxylation is OAA, which is unstable, and quickly converted to the 4-carbon organic anion malate[9]. (Thus, C_4 plants are so-called because malate is the first detectable product of photosynthesis.) Malate diffuses out of the mesophyll cell, to the bundle sheath cell, where it is decarboxylated to release CO_2 near rubisco[10]. This "CO_2-pumping mechanism" has been estimated to raise the $[CO_2]$ in the bundle sheath chloroplast to about 0.25 - 0.30 %, about an order of magnitude higher than the consonant value in C_3 plants. Thus, the ratio O_2/CO_2 in C_4 plants is approximately 100, whereas it is 1000 in the C_3 chloroplast. These facts have an important implication: rubisco activity is shifted in a major way to the carboxylase activity. The little oxygenation that occurs yields P-glycolate in the usual way, and it is metabolized in the usual way, with the photorespiratory release of CO_2. This CO_2 is not observed, however, as a PIB because it trapped by the mesophyll cells—PEPC has a very low K_M for HCO_3^-—as it would exit the leaf to be detected. The 3-carbon fragment resulting from the decarboxylation of malate diffuses back to the bundle sheath cell, where it again serves as the substrate for PEPC. At the risk of oversimplification, C_4 photosynthesis is a spatial separation of photosynthesis; "one" 3-carbon compound is cycled between the mesophyll, where it "picks up" a CO_2, and the bundle sheath cell, where the CO_2 is released for photosynthetic reduction. C_4 photosynthesis (as well as CAM, which will be discussed in the next section) is not a different pathway of photosynthesis, it is an "add-on" or auxiliary pathway "attached to" the (regular) RPPP.

As alluded to, the energetic requirements for the C_4 pathway are higher than for the C_3 pathway. (This requirement is provided as an explanation for the fact that C_4 plants do not compete well with C_3 plants in a light-limited situation, such as shade.) As also mentioned, there are three different routes by which the C_4 pathway is implemented; these do not have exactly the same energy requirements. As a generality, the stoichiometry for C_4 photosynthesis is 1 CO_2:5 ATP:2 NADPH. The quantum requirement

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[9]OAA, the familiar 4-carbon α-keto acid of the TCA, may instead be transaminated to form the amino acid aspartate. Conceptually, the result is the same, so we will not dicker about the subtle differences.

[10]Again, there are subtle differences is how decarboxylation may be achieved. It may be catalyzed in the mitochondrion by an NAD-specific enzyme, or it may be decarboxylated by one of two different enzymes in other cellular compartments. The exact method may change the theoretical energy requirements somewhat, but the more important consideration is that all C_4 plants have a higher energy requirement for CO_2 fixation **and** for maintenance of the pathway structure.

in C_4 plants for reduction of one CO_2 is about 15 photons. (Recall that C_3 plants have a requirement of 8 (for $NADP^+$ reduction), 9 (if the ATP requirement is included), and about 12 (to account for "average" operation of the PCOP). What are the advantages of C_4 photosynthesis, what with the higher energy requirement, and the genetic, protein and structural "baggage" associated with the pathway? First, as temperature increases, the oxygenase activity of rubisco increases relative to the carboxylase activity. As you know, increased operation of the PCOP increases the quantum requirement, so that at temperatures over, say, 30° C, the quantum requirement for C_3 photosynthesis is actually higher that that of C_4 plants. This observation is probably one contributing reason why C_4 plants are not found in the north, mostly being restricted to tropical and subtropical areas. Second, as we have discussed, C_4 plants are more water-use efficient. They do particularly well in disturbed sites, such as ditches, row middles, lawns. Third, C_4 plants are nitrogen-use efficient. After water, N is usually the most limiting resource for terrestrial plants: it is at low availability in the soil, and it is expensive energetically to reduce to level of amine, as it is in protein. The major reason for the higher N-use efficiency of C_4 plants is because they contain 3-6x less rubisco (which is, say, 25% of the protein of a C_3 leaf), and consequently they have an overall lower nitrogen content. In brief, C_4 plants have a lower N content on an area basis (~150 mmol N m^{-2}) than do C_3 plants (~225 mmol N m^{-2}) and they photosynthesize faster on this same basis (say, 25 vs. 60 mg CO_2 dm^{-2} h^{-1}). Per unit of dry mass accumulated, then, C_4 plants do with about 4x less N!

Overhead: return to Distinguishing Characteristics....

Because of the parculiarities of CAM, some of the kinds of comparisons that were used with C_3 vs. C_4 plants can not be logically applied. Whereas C_4 photosynthesis represented a histochemical compartmentation of photosynthesis, with the mesophyll cells being put to the task of trapping CO_2 and the bundle sheath cells being adapted to conduct the normal RPPP at elevated CO_2 concentration, CAM is, instead, a temporal separation of the auxiliary C_4-type pathway. Stomates are open at night. CO_2 enters the leaf, and is fixed into the prototypical organic acid, malic acid, by a CAM version of PEPC. Malic acid represents a storage form of CO_2. The accumulation of malic acid in the vacuole is the reason that extracts of CAM plants are more acid at the end of the dark period—this diurnal fluctuation in acidity is the hallmark of CAM photosynthesis. In the light period, the stomates close. CO_2 is released from malate (again, by one of the three ways used by C_4 plants). The massive release of CO_2 in the leaf intercellular spaces—which represent a closed system because the stomates are closed—builds up the internal [CO_2] to

an incredible 2%. Under these conditions, there is no photorespiration (remember that in all cases, the RPPP and the PCOP only operate in the light because requirement for ATP and NADPH). . . . and if there were photorespiration, it could not be detectable by the standard assay, viz., the PIB. Similarly, the CO_2-compensation point, interpreted as a manifestation of the oxygenase/carboxylase activities has no meaning. If we consider it literally, however, the value is low, ~10 ppm. CAM plants do not have an enrichment of ^{12}C because the initial step of CO_2 incorporation is by PEPC, which does not, recall, discriminate against ^{13}C. Water-use efficiency is very high because stomates are open at night when the driving force for water exit is low; this high water-use efficiency is the advantage of CAM.

The preceding account provides an explanation for the daily cycling of malic acid, with the focus on the "trapped" CO_2. There is also a corresponding diurnal fluctuation of starch, which is broken down by glycolysis to PEP, the carboxylation substrate, during the dark period. At the end of the light period, starch concentration is high, because of the contribution by the RPPP, but more importantly, by the recycling (gluconeogenesis) of the 3-carbon fragment that resulted from CO_2 release into starch.

Finally, CAM plants may be obligate (as a species, the plant is either CAM or is not CAM) or facultative (i.e., it operates as a normal energy-efficient and relatively rapidly photosynthesizing C_3 plant until it is water- or salt-stressed, at which point, it develops the CAM pathway). On examination, it is easy to determine whether a facultative CAM plant is in the CAM mode or not (does it have high PEPC activity? . . . does it show a diurnal fluctuation of acidity? . . . does the starch content have a remarkable diurnal pattern that matches the expectation of providing carbon skeletons for malate synthesis? . . . do the stomates open at night or during the day?) It is also straightforward to determine the extent to which the facultative plant has been operating in the CAM mode over its growth history: the carbon in the plant will be more C_3-like (with respect to $^{13}C/^{12}C$) if most of the carbon accumulated as a result of C_3 photosynthesis or more C_4-like if most of the carbon accumulated as a result of C_4 photosynthesis.