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Personal perspective

Mapping the carbon reduction cycle: a personal retrospective

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Abstract

The photosynthetic carbon reduction cycle was elucidated through the use of $^{14}\text{CO}_2$ during photosynthesis to label metabolic intermediates. Mapping and proof of the cycle required identification of labeled metabolites, observation of changes in levels of labeled metabolites during transitions from light to dark and from high to low CO_2 levels, determination of intramolecular distribution of ^{14}C within the metabolites after a few seconds of photosynthesis with $^{14}\text{CO}_2$, and estimation of metabolite concentrations, used to calculate true free energy changes at each step in the cycle.

Prologue

Important scientific advances often result from the fortunate adaptation of new investigative techniques by scientists capable of interdisciplinary thinking. Such was the case when Ruben et al. (1939) commenced their studies of photosynthetic carbon fixation using radiocarbon. Real progress became possible with the discovery of carbon fourteen (^{14}C) with a long half life (Ruben and Kamen 1941). [For the story of the early work by Sam Ruben and others, as well as a detailed description of the identification of carbon fourteen labeled compounds and other advances in which Andrew Benson played such a prominent role, see his personal story (Benson 2002 a, b).]

This work was interrupted in 1943 by the untimely death of Sam Ruben (born 1913, died 1943) by a chemical accident. The studies resumed in 1945 when Earnest O. Lawrence, director of the University of California Radiation Laboratory invited Melvin Calvin (born 1911, died 1997) to be director of the Bioorganic Chemistry Group which was charged with investigating the use of radiocarbon as a tool in chemical and biochemical studies. Calvin selected Andrew

Benson to head a subgroup to perform studies in photosynthesis (see Benson 2000a for details).

My first awareness of the idea of using radioactive carbon dioxide in research on photosynthesis came while attending a freshman chemistry laboratory section at the University of California, Berkeley in 1940. Sam Ruben, a young member of the chemistry faculty, was our section leader. One morning, instead of the usual discussion of the scheduled laboratory experiment, he told us about his research. I was intrigued with the idea of using radiocarbon as a tool of study of photosynthesis. I never dreamed that I might someday have the chance to participate in this exciting work.

Such a possibility became even more remote when I left the University to serve three years as an officer in the United States Navy during World War II. When I returned to Berkeley, in 1946, I was at first granted admission for course work only. After a semester, however, Dean Wendell Latimer invited me to work towards a PhD degree and gave me a list of faculty in organic chemistry to interview. The first professor I visited was Melvin Calvin, and the first project mentioned was the study of photosynthesis using $^{14}\text{CO}_2$. Although I politely listened to other projects, my mind



Figure 1. Some members of the photosynthesis research group (about 1953). They are in front of Old Radiation Laboratory at UCB. This building was later demolished to make space for a new Department of Chemistry building, Hildebrand Hall. *Left to right:* Clinton Fuller, Robert Norris, Hans Kornberg, Alice Holtham, Melvin Calvin, J. Rodney Quayle, Malcolm Thain, James Bassham and Jaques Mayudon. Andrew Benson agrees that he probably took this picture, accounting for his absence from the picture.

was made up immediately, and I was allowed to join the photosynthesis group.

The Old Radiation Laboratory (ORL)

I was soon introduced to Andy Benson and other members of the photosynthesis group, and assigned a work bench in the laboratory. Our work area at first was small – the west end of the Old Radiation Laboratory (ORL), an old wooden two-story building where E. O. Lawrence had carried out some of his Nobel Prize winning work on the cyclotron. After a time, a larger room (formerly the home of Lawrence's cyclotron) became available and was similarly converted for our use.

This space enabled Professor Calvin to accept the visits of a number of outstanding young scientists from the USA and abroad. (Figure 1). They and their institutions were eager to receive training in the new tracer techniques being developed in Calvin's laboratory. Many of these visitors later had distinguished careers in the USA, Europe, and Japan.

The other end of ORL housed a carpenter shop, a glass-blowing shop, and a machine shop, which were

extremely valuable to our group. Although these shops served the rest of the Radiation Laboratory as well, they were very handily located for us, and helped greatly in the development of innovative equipment and methods.

Melvin Calvin and the bioorganic chemistry group

Melvin Calvin (Figure 2), with his boundless scientific enthusiasm and ability to transfer or apply information from one field to another made the laboratory a stimulating place to work. He had the knack of arousing new excitement and optimism in students and visitors even when they had become discouraged by what seemed to be slow progress. He could find importance in almost any experimental result and at once suggest new experiments.

Each Friday morning at 8 A.M., the entire Calvin group (including those engaged in other studies using radiocarbon) would meet for a very informal seminar. Everyone had to be prepared to report his/her data as there was no pre-announced speaker. Calvin would turn to someone who had not spoken for a while and



Figure 2. Melvin Calvin (ca 1970), courtesy of the Lawrence Berkeley National Laboratory Archives.

ask that person to present recent results. Anyone could interrupt at any time, and there were lively discussions. Although this meeting format may have initially put off some new participants, it resulted in a very productive exchange of ideas and information, and the science of the group benefited greatly.

We had the opportunity to apply a new and powerful technique to the study of photosynthesis. This was very exciting, and we young scientists worked long hours, often until late at night and weekends. When we did take a break, sometimes we would dash off to the Sierra Nevada for a rigorous weekend of mountain climbing (Figures 3 and 4).

Carbon fourteen labeling of four carbon acids

The early work summarized by Andy Benson in his historical perspective (Benson 2002a) showed the prominence of labeled four-carbon carboxylic acids as products of $^{14}\text{CO}_2$ fixation. Samples of labeled malate and succinate had been isolated. Once two dimensional paper chromatographic techniques (discussed below) were developed, malate was often seen to be an important product of $^{14}\text{CO}_2$ fixation, either during photosynthesis or in the dark after preillumination.

My first assignment was the chemical degradation of labeled succinic acid and malic acid to determine the intramolecular distribution of labeling of these four-carbon acids (Benson and Bassham 1948). This

involved working with rather small amounts of compounds, since the relatively small degree of labeling was diluted by whatever amount of carrier compound had to be added in order to have enough to perform chemistry and isolation of products. Andy Benson was experienced and skilled in the techniques of handling these small quantities of labeled compounds. I was much indebted to him for training in experimental methods.

Based partly on such evidence, Calvin and Benson (1948), in the first paper on ‘the path of carbon in photosynthesis’ (there would be eventually some 22 ‘path’ papers) proposed a possible sequence of reactions involving two carboxylation steps in which malate or succinate could be the products of the second carboxylation. A four-carbon acid was then presumed to split to give two 2-carbon compounds that could serve as receptors for the first carboxylation reaction. If one of the labeled four-carbon acids were indeed an intermediate in a regenerative cycle, then after a period of photosynthesis of several minutes, the label should be uniformly distributed throughout the molecule.

My results on the degradation of C-4 dicarboxylic acids at first suggested that the central atoms might be appreciably labeled. This was an experimental error, however, owing to the very low level of radioactivity in the sample and to variation in the ‘background’ count which is subtracted from the total count after many hours of counting. ORL was next door to the Crocker Laboratory where at the time a cyclotron was still operating. When it was running, our background count varied. Further work with more careful shielding of the Geiger counter showed that radioactivity was not spreading to the central positions fast enough for these compounds to be intermediates in the primary cycle.

Somewhat later, after the discovery of 3-phosphoglycerate (PGA) as the first product of carbon dioxide fixation during photosynthesis, malonate was employed as an inhibitor of the photosynthetic formation of labeled malate (Bassham et al. 1950). Though labeled malate was no longer formed there was no effect on the spread of label to positions two and three of PGA. This was further proof that there is no role for malate in photosynthetic carbon fixation in the algae and higher plants (barley, spinach, etc.) we employed. My thesis work on ‘The path of carbon in photosynthesis. The carboxylic acids’ and other contributions to the early work led to my PhD degree in 1949.

While it was not an intermediate in primary CO_2 fixation in the plants we used (which later came to be known as C-3 plants), malate has proved to be im-



Figure 3. On top of Mt. Whitney (about 1949). Left to right: Richard Lemmon, Will Siri, Andrew Benson, James A Bassham and Bert Tolbert. All except Siri were members of the Bio-Organic Chemistry Group.

portant in photosynthesis in C-4 plants such as maize and sugar cane. [For a full discussion of the history of the C-4 cycle in photosynthesizing C-4 plants, see M.D. Hatch (2003).] In ‘tropical grass plants,’ such as these, malate and oxaloacetate play important roles in transporting carbon into the bundle sheath cells where malate decarboxylation helps to maintain CO_2 level and thus reduces photorespiration (see W.L. Ogren, 2003, for a historical perspective on ‘Photorespiration’). This allows C-4 plants to increase the rate of photosynthesis at high light intensities where this rate would be more limited by photorespiration in C-3 plants.

Early experiments on shortterm photosynthesis with $^{14}\text{CO}_2$

An extensive description of the techniques used in these early experiments, also including plant culture and selection, photosynthesis with $^{14}\text{CO}_2$, analyses using paper chromatography, radioautography, and identification of labeled intermediates, may be found in the monograph by Bassham and Calvin (1957).

Both unicellular algae, usually either *Scenedesmus* sp. or *Chlorella pyrenoidosa*, and leaves of higher plants such as barley, soybean and spinach, were exposed to $^{14}\text{CO}_2$. Algae were by far the most often employed because of ease of handling and killing (to stop the reactions quickly, and the extraction of the labeled products).

In the early experiments, we poured a suspension of algal cells into a flat-sided circular glass vessel with a stopcock at the bottom and stoppered entry at the top. The laboratory glassblowers made these vessels for us and we called them ‘lollypops’ (for earlier vessels, see Benson 2002a). They were illuminated from each side by a bright photospot lamp. A glass tube through the top opening allowed for the illuminated algal suspension to be bubbled with air for about an hour to establish a high rate of photosynthesis.

We would then remove the bubbler, sometimes flush with N_2 , and quickly inject a solution of bicarbonate labeled with ^{14}C . The flask was immediately closed and shaken in the light. After the prescribed few seconds or longer, we opened the stopcock at the bottom, allowing the algae suspension to run into nearly boiling ethanol to give a final concentration of ~80% ethanol. We then concentrated the alcoholic sample of killed algae by vacuum evaporation of the ethanol and of much of the water. A slurry of labeled soluble compounds and insoluble material was obtained which was then ready to be analyzed by paper chromatography.

Adoption and adaptation of two-dimensional paper chromatography

While much was learned from 1946 to 1948 about techniques for exposing plants to $^{14}\text{CO}_2$ under various conditions, there had been limited progress made in analysis and identification of the labeled compounds.

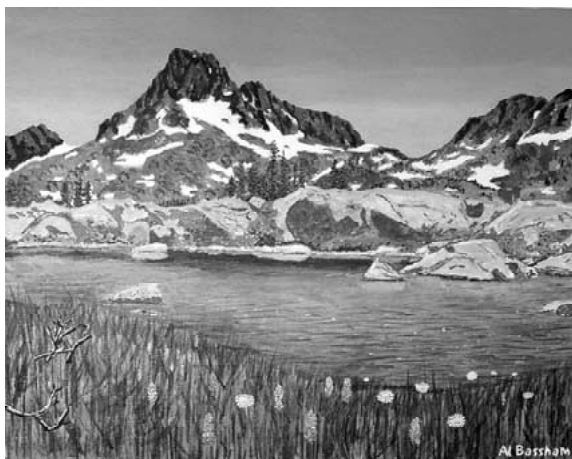


Figure 4. Painting by the author of Banner Peak. This peak, just south of Yosemite National Park, is in an area that was favored by the mountain climbers of the Old Radiation Laboratory. For a colour version of this figure, see section in the front of the issue.

This changed rapidly after the introduction of two-dimensional paper chromatography for separation of the compounds, followed by radioautography to locate the positions of the labeled compounds on the chromatograms (Benson et al. 1950). The Nobel Prize winning work of Archer Martin and Richard Singe (1941) had been adapted and used by Dent et al. (1947) for separation of amino acids from plant cells. This method was brought to ORL from Dent's laboratory by William Stepka. In ORL it was first used for the separation of labeled amino acids (Stepka et al. 1948).

Andy Benson ingeniously formulated new solvent mixtures for the effective separation of the strongly acidic compounds formed. These solvent mixtures were phenol, saturated with water in the first chromatographic direction, and butanol, propionic acid and water for the second (Benson et al. 1950). These solvents were not as terribly odiferous as the lutidine or collidine solvents formerly used. Nevertheless, some laboratory members who worked with the new solvents and then visited a cinema (after shedding their laboratory coats but not changing clothes) were asked to leave the theater owing to complaints from neighbors seated nearby.

After chromatography of the algal slurry, we placed the dried paper chromatogram in contact with medical X-ray film in the dark and later developed the film to locate the positions of radioactive compounds. Superimposing the paper over the X-ray film on a light table, we outlined the positions of the radio-

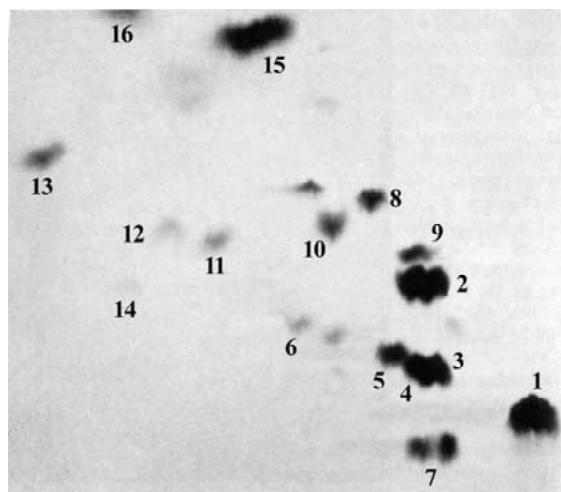


Figure 5. Radioautogram of a chromatogram made from extract of *Scenedesmus* after 60 seconds of photosynthesis with $^{14}\text{CO}_2$. (1) ribulose 1,5 bis phosphate (RuBP); (2) 3-phosphoglycerate (3PGA); (3) glucose-6 phosphate (G6P); (4) sedoheptulose-7-phosphate (S7P); (5) Fructose-6-phosphate (F6P); (6) Dihydroxyacetone phosphate (DHAP); (7) A compound yielding glucose after treatment with a phosphatase.; (8) Phosphoenolpyruvate (PEPA); (9) phosphoglycolate; (10) aspartate; (11) serine; (12) glycine; (13) alanine; (14) sucrose (barely visible at 60 seconds); (15) malate; (16) succinate. Other spots not here identified may have included other pentose phosphates and phosphoglyceraldehyde (Bassham and Calvin 1957).

active 'spots' (^{14}C -labeled compounds) on the paper for elution and subsequent analyses.

Labeled products of short term photosynthesis with $^{14}\text{CO}_2$

The radioautograph of algae exposed to $^{14}\text{CO}_2$ for one minute (Figure 5) reveals the presence of a number of compounds. The first chromatograms were of course not as good as this one, but they improved with practice, and soon information began to accumulate rapidly. We had much work to do, however, before the identities of these compounds were known. Prominently labeled intermediates included sugar phosphates, carboxylic acids (some as phosphates), and amino acids. All of the intermediate compounds of the photosynthetic carbon reduction cycle would in time be found to be among the phosphorylated derivatives of sugars and acids.

In the following sections, I will describe the identifications of these intermediates, and the several lines of investigations that led to the formulation and proof of the pathway of photosynthetic carbon dioxide fix-

ation and reduction to sugars. The final form of this pathway, Figure 6, may be helpful in following this discussion.

3-Phosphoglycerate, the first product of $^{14}\text{CO}_2$ fixation by photosynthesizing plants

One 'spot' in particular was by far the most prominently labeled compound after very short exposures (such as 5 s) of the illuminated plant to $^{14}\text{CO}_2$. Benson and Calvin (1948) eluted this compound from the paper chromatogram, purified it, and identified it as 3-phosphoglycerate (3PGA). Given its prominence after just seconds of photosynthesis, and the fact that a carboxylic acid was the expected product of a carboxylation reaction, 3PGA was at once suspected to be the product of the first step in the photosynthetic carbon fixation and reduction pathway. This hypothesis would be amply confirmed by other studies described below, such as changes in levels of labeled metabolites during transitions from light to dark and from high to low CO_2 concentration, and degradation of 3PGA that showed the ^{14}C label was in nearly all in the carboxyl carbon after 5 s, as would be expected of carboxylation.

Other products of short-term labeling during photosynthesis

The identifications of the rest of the various labeled sugar phosphates and acids were essential. The major part of this identification work was led by Andy Benson (1951) and Benson et al. (1951, 1952). The retrospective article by Benson (2002a) reviews this important work in more detail. The general approaches that were used are summarized as follows.

The compounds with attached phosphate groups move more slowly on the chromatograms due to their acidity and consequent greater distribution into the stationary water phase adsorbed on the paper. Sugar phosphates seemed likely candidates to be metabolic intermediates (based on knowledge of glycolysis).

Most of the slower moving compounds therefore were eluted from the paper and treated with a purified phosphatase (Polidase) to remove the phosphate groups. As expected, upon re-chromatography the free compounds moved faster. Various suspected sugars were added in small carrier amounts for the re-chromatography, and the dried paper was sprayed

with Tollen's reagent to find each added sugar. Coincidence between a color-developed spot on the chromatogram and the dark spot on the radioautograph provided identification of each compound. Although some sugar monophosphates overlapped on the original chromatogram, the free sugars moved further and separated.

Among sugar phosphates thus identified were the triose phosphates (glyceraldehyde-3-phosphate, or G3P and dihydroxyacetone phosphate or DHAP), and hexose phosphates (glucose-6-phosphate or G6P, fructose-6-phosphate or F6P, and fructose 1-6-bisphosphate or FBP). These compounds would of course be expected if the pathway from 3PGA to sugars followed a path similar to a reversal of glycolysis.

Cochromatography, after elution and addition of carrier amounts also served for identification of amino acids (ninhydrin spray) and carboxylic acids (indicator spray).

Sedoheptulose-7-phosphate

Among the labeled free sugars seen after treatment with Polidase and re-chromatography were two that did not co-chromatograph with any commonly known sugars. Andy Benson prepared uniformly labeled metabolites by allowing algae to photosynthesize for several minutes with $^{14}\text{CO}_2$ to allow for uniform labeling. After chromatography, elution, and treatment with Polidase of the several sugar phosphates in the area of G6P and F6P, he then isolated the free labeled sugars by re-chromatography, and eluted a labeled compound that did not coincide with the other commonly known sugars such as fructose, glucose and mannose.

I oxidized this presumed unknown sugar using a reagent that would give CO_2 from only the carbonyl carbon. To our surprise, only 14% of the ^{14}C in the original eluted sugar was in this carbonyl carbon. A second try produced the same result, and made us suspect that the compound was a seven carbon sugar, a heptose. Andy obtained a pure sample of sedoheptulose from Arnold Nordal in Norway. Co-chromatography of the authentic sugar with our labeled compound showed a coincidence of sprayed color spot on the paper with the radioactivity. (Benson et al. 1951). The labeled sugar phosphate therefore was identified as sedoheptulose-7-phosphate (S7P).

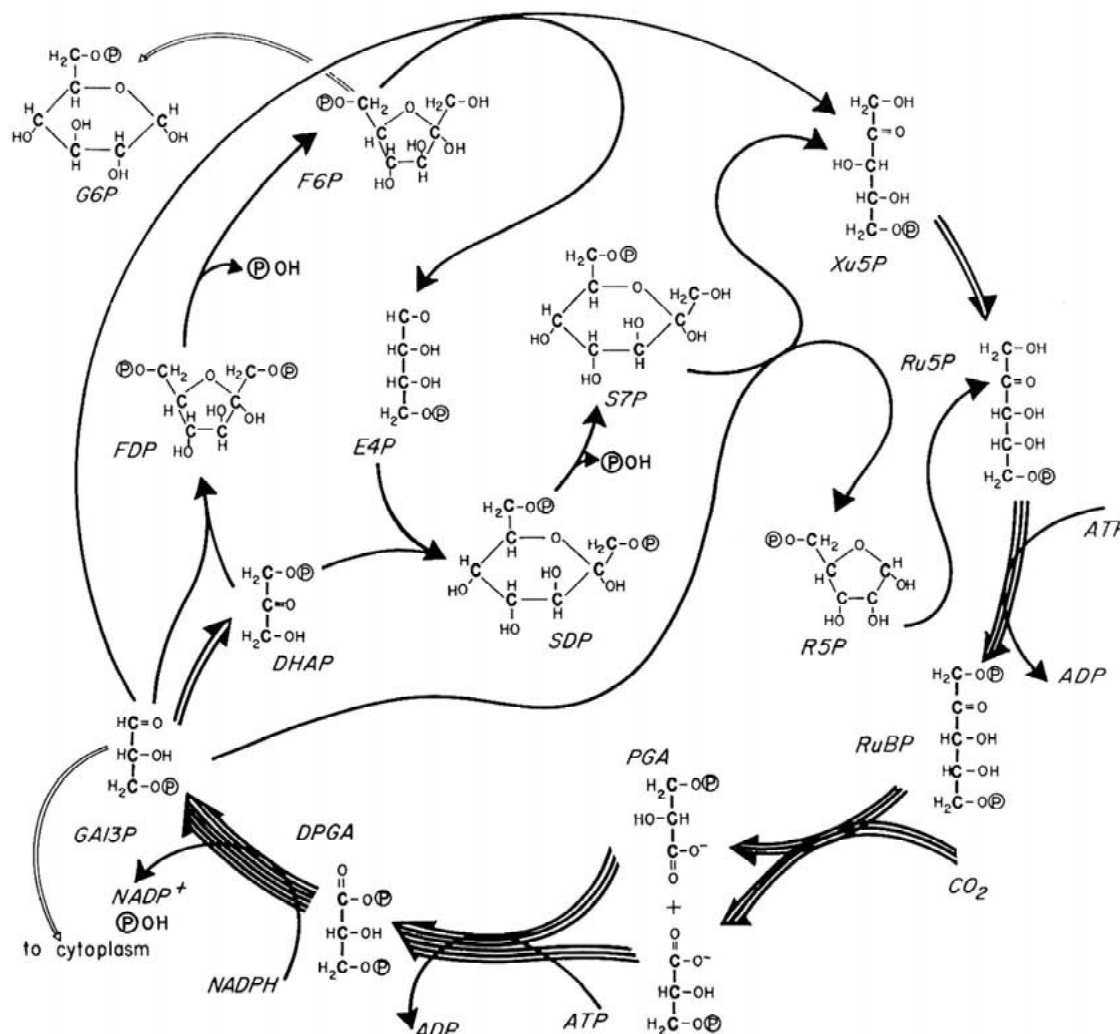


Figure 6. The photosynthetic carbon reduction cycle (PCR cycle) (from Bassham 1979). The number of times per complete cycle each reaction occurs is indicated by the number of lines per arrow. In the text, abbreviations for modern names are used: RuDP = RuBP, SDP = SBP, FDP = FBP. The circled P represents PO₃H⁻.

Ribulose-1,5-bisphosphate

The radioautographs showed a very dark spot near the origin. After it was eluted and phosphatased it ran farther than the hexoses. Andy Benson did an ingenious double labeling experiment using both ¹⁴CO₂ and ³²P-labelled phosphate – again allowing time for photosynthesis sufficient to completely label the photosynthetic intermediates. He found that the ratio of ³²P to ¹⁴C was two to five as expected for a pentose diphosphate. The eluted, Polydased, and rerun labeled sugar co-chromatographed with ribulose. Thus the original compound was ribulose-1,5-bisphosphate

(RuBP) (Benson 1951). During those early days we called it ribulose-1,5-diphosphate (RuDP).

Other sugar phosphates

From the sugar monophosphate area, smaller amounts of ribulose, xylulose and ribose were later seen, indicating that ribulose-5-phosphate (Ru5P), ribose-5-phosphate (R5P), and xylulose-5-phosphate (Xu5P) were labeled in our experiments. Some of these pentose phosphates as well as the triose and tetrose phosphates occur in very small concentrations and often did not appear as visible spots on the radioauto-

grams. For measurements of concentrations we later had to increase and maintain the levels of $^{14}\text{CO}_2$ employed (see section on steady-state photosynthesis, below).

Transition kinetics and the carboxylation reaction

Some of the strongest evidence for the nature of the reactant and the product in the carboxylation of the photosynthetic carbon reduction (PCR)¹ cycle came from studies of the changes in labeled intermediates during transitions from one condition to another. Methods were developed for maintaining a constant level of CO_2 and $^{14}\text{CO}_2$ during photosynthesis for a long enough time to completely label intermediates of the PCR cycle. We would take samples of algae from time to time to measure the extent of labeling. Then we would change a condition, and rapidly take samples over a minute or so to examine the effects of the change. Each sample would be analyzed for ^{14}C content of each labeled compound.

In the first such experiment, the light to dark transition was examined (Calvin and Massini 1952). These transient changes as seen in a later study (Bassham et al. 1956) are shown in Figure 7. When the light was turned off, the level of RuBP declined rapidly to zero, while the level of 3-PGA rose. These changes provide a compelling argument for two conclusions (1). Cofactors generated by light are required for the formation of RuBP but not for its utilization in an subsequent reaction (2). Light-generated cofactors are required for transformation of 3PGA to sugars but not for the reaction which forms 3PGA.

Since sugar phosphates were clearly intermediate compounds in any regenerative cycle, it seemed fairly obvious that their formation from 3PGA by an approximate reversal of steps in glycolysis would require an electron carrier such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) and (for energy input) adenosine triphosphate (ATP). Both of these might be generated directly or indirectly by light reactions. Also, the formation of RuBP from other sugar phosphates might reasonably be expected to require ATP for the action of a phosphoribulokinase.

The above conclusions could be tested by another experiment in which (after steady-state photosynthesis was achieved) the levels of $^{12}\text{CO}_2$ and $^{14}\text{CO}_2$ were drastically reduced and later restored (Wilson and Calvin 1955). Lowering the carbon dioxide levels results in a rapid spike in labeled RuBP and a some-

what slower, but then significant drop in labeled PGA (Figure 8). Restoration of the level of carbon dioxide results in an immediate drop in labeled RuBP and immediate rise in labeled PGA. A few seconds later, there are secondary 'waves' of labeling, as the renewed flow of carbon passes through the cycle.

Two molecules of 3-phospho-glyceric acid (3PGA) from each carboxylation of Ribulose bis phosphate (RuBP)

Taken together, these two experiments provided strong evidence that the PCR cycle requires the conversion of RuBP and CO_2 to 3PGA. Very careful steady state experiments were designed allowing for rapid sampling during the transition from light to dark (Bassham et al. 1954, 1956). From 0 to 4 s of darkness the rate of increase in labeled 3PGA corresponded to two molecules of 3PGA being formed for every molecule of $^{14}\text{CO}_2$ taken up during the preceding steady state photosynthesis. This ratio lasted nearly until 10 s of dark, after which it fell off rapidly as the pool of accumulated RuBP was used up. The breakup of the putative six carbon addition product of RuBP and CO_2 thus seemed to result in the formation of *two* molecules of 3PGA, at least when the light was turned off. This has not precluded suggestions from time to time that in the light the products might be different.

Formulation of the mechanism of the carboxylation reaction

A crucial question was how the two molecules of 3PGA might arise from RuBP and CO_2 . Consider the requirements: (1) The carboxylation reaction has to be energetically possible despite the generally unfavorable energetics of carboxylations of sugar phosphates. (2) The reaction leads to two identical products – not what you might expect from carboxylation of a sugar phosphate.

An ingenious solution to the question soon occurred to Melvin Calvin. He had long been interested in mechanisms of organic reactions and had co-authored a widely read book on the subject (Branch and Calvin 1941). I recall him saying that the answer to the carboxylation mechanism came to him as he was stopped in traffic waiting for a stoplight to change (see Calvin 1989).

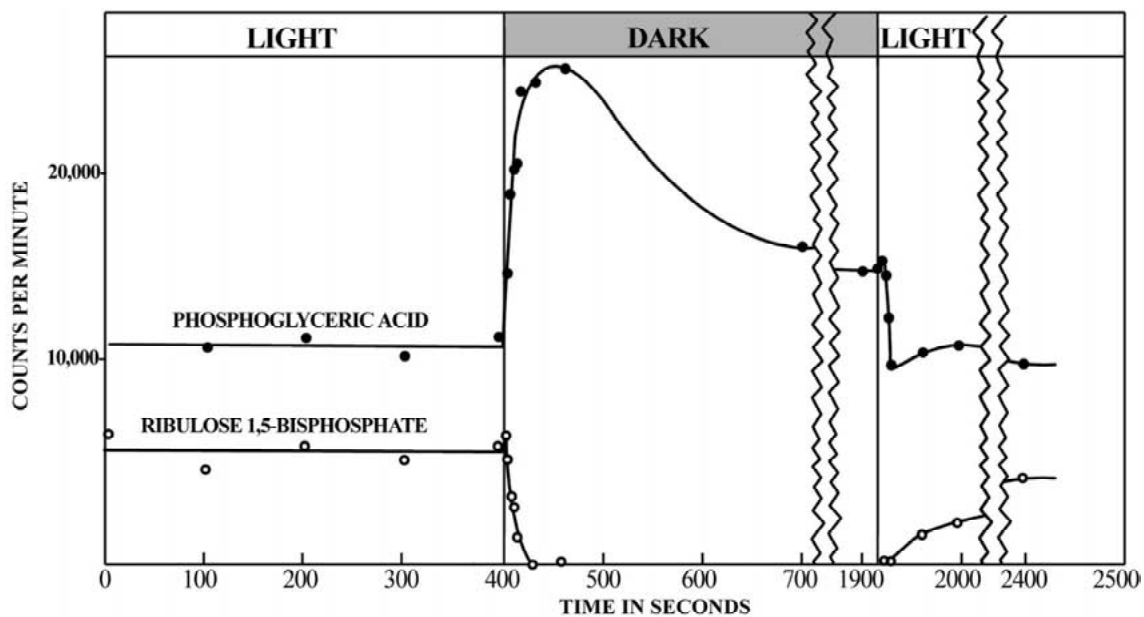


Figure 7. Changes in levels of labeled RuBP and 3PGA after transitions between light and dark (Bassham et al. 1956).

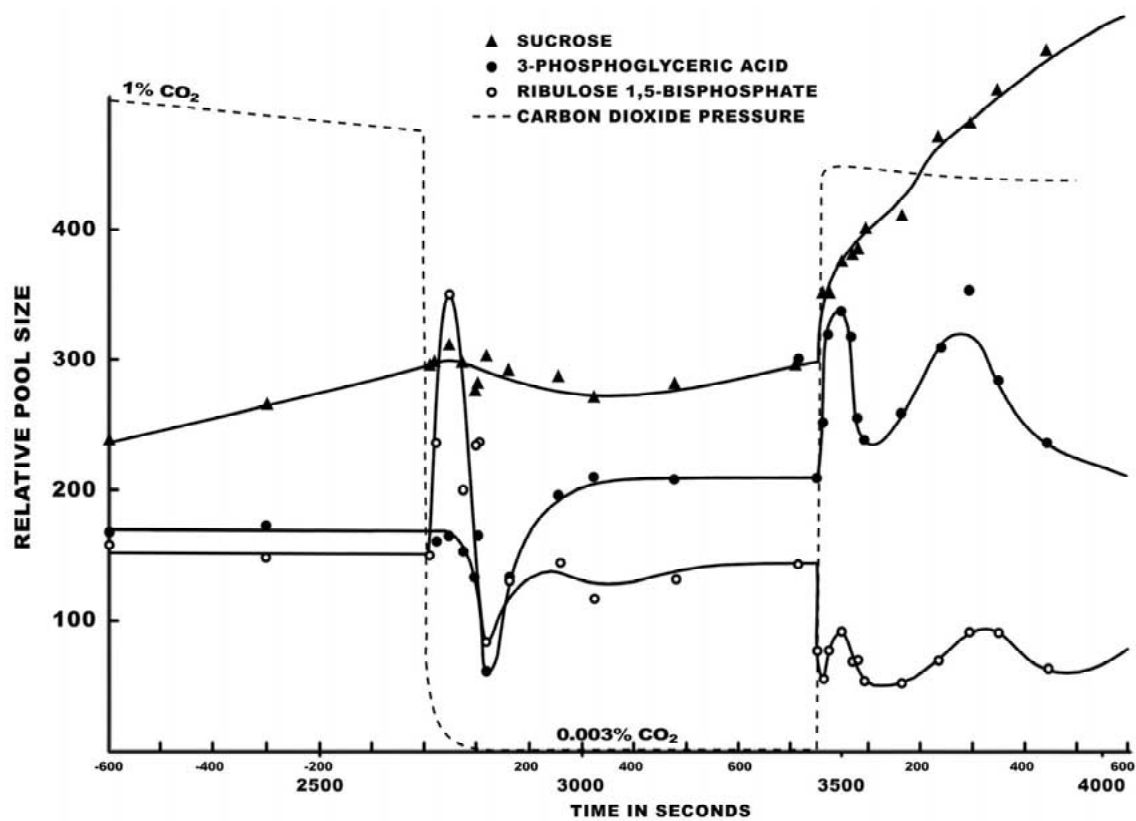


Figure 8. Changes in levels of labeled RuBP and 3PGA after transitions between levels of CO_2 . From high to low carbon dioxide pressures (Wilson and Calvin 1955).

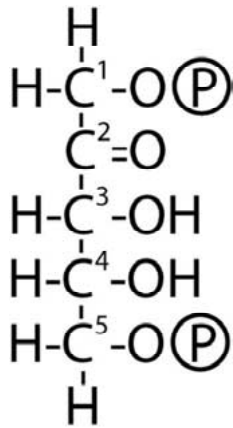


Figure 9. Ribulose-1,5-bisphosphate (RuBP). The circled P represents PO_3H^- . The carbon atoms are numbered 1 to 5 as in the discussion.

RuBP, shown in Figure 9, can exist only in the straight-chain form, unlike several other sugar phosphates of the cycle which exist mostly in cyclic form. In order for the addition of carbon dioxide to RuBP to produce two molecules of equal oxidation state, the following events have to occur on the enzyme-substrate complex. This listing is not intended to suggest any order – most likely it all occurs more or less concurrently. A bond must form between CO_2 and C-2 of RuBP, while electrons are transferred from C-3, oxidizing it to a carboxyl group ($-\text{COOH}$). This produces one 3PGA molecule from what had been C-3, C-4, and C-5 of the RuBP and a second 3PGA

molecule containing C-1, C-2 plus the new carboxyl group.

The CO_2 bonded to C-2 becomes the carboxyl group of the second 3PGA molecule. Also required is the reduction of the keto group on C-2 of the original RuBP to a hydroxyl group. It might be that the enol form of RuBP, with a double bond between C-1 and C-2, is reduced by the two electrons that were obtained by the oxidation of C-3 of RuDP. This at least is how I thought of the reaction a half century ago. For current views about this key enzyme, see review by Spreitzer and Salvucci (2002).

Glycolate and phosphoglycolate

Glycolate and phosphoglycolate were observed as labeled products of photosynthesis with $^{14}\text{CO}_2$, especially if the cells were deprived of CO_2 (Benson and Calvin 1950). When we administered ^{14}C -labelled glycolate to illuminated *Scenedesmus* cells, products similar to those formed during photosynthesis with $^{14}\text{CO}_2$. In the dark, serine and glycine were the principal products (Schou et al. 1950).

There is a competition between O_2 with CO_2 for the reaction with RuBP. Oxygenation of RuBP in place of carboxylation produces phosphoglycolate and one molecule of 3PGA (Bassham and Kirk 1962). This oxygenation of RuBP initiates the process of photores-



Figure 10. The Calvin Laboratory at the University of California at Berkeley. This laboratory was completed in 1963.

piration (for a review, see Tolbert 1997; also see Ogren 2002).

The enzyme of the carboxylation reaction

In ORL, Quayle et al. (1954) first reported the enzymatic carboxylation of RuBP. When unlabeled RuBP and $^{14}\text{CO}_2$ were added to a cell-free extract of *Chlorella pyrenoidosa*, 3PGA labeled only in the carboxyl carbon position was formed. The authors of this report named the enzyme that would catalyze this two-part reaction *carboxydismutase*. While this name has not been accepted, it indicates the dual functions (carboxylation and intramolecular electron transfer) of the enzyme better than the name *ribulose-1,5-bisphosphate carboxylase* which has come to be the accepted name.

Weissbach et al. (1954) described fixation of CO_2 by spinach extracts with pentose phosphate. Further work on this enzyme by the ORL group appeared later (Mayoudon 1957; Benson 2000a). It was subsequently established that *ribulose-1,5-bisphosphate carboxylase* was identical with 'fraction-1-protein' also called *Rubisco* (*ribulose bisphosphate carboxylase oxygenase*); see Wildman 1998, 2002; Benson 2002a; Ogren, this issue.

From 3PGA to RuBP: evidence from studies of intramolecular labeling after a few seconds of photosynthesis with $^{14}\text{CO}_2$

Formulation of a detailed proposal for a regenerative PCR cycle involving not only 3PGA and RuBP required us to learn about the intramolecular distribution of ^{14}C in the 3PGA and sugar phosphates labeled after few seconds exposure. By looking at unequal labeling within each possible intermediate compound, and comparing the patterns from one intermediate to another, we could discern possible paths for conversions from one intermediate to another.

For this purpose we had to degrade chemically not only 3PGA, but also various hexoses, pentoses and sedoheptulose by procedures which would allow us to isolate and measure ^{14}C -label in specific positions within the molecules. We then used this information to draft proposals for specific transformations between the intermediates and eventually to construct a complete cycle (Bassham et al. 1954).

We used both algae and soybean leaves. I modified the technique with algae from that used earlier. We illuminated an algae suspension in a thin transparent

chamber, while bubbling the suspension with CO_2 in air. A pump was used to force the suspension from this vessel through a length of transparent plastic tubing, folded so that the entire length was illuminated. Using a hyperdermic syringe and needle, we injected a solution of $\text{NaH}^{14}\text{CO}_3$ into the tubing through which the algae suspension was flowing. The injections were made at flow points predetermined to be from 0.4 s up to 20 s from the bottom end of the tubing. From that end, the algal suspension poured into boiling methanol for nearly instantaneous killing and stopping of all further metabolic steps.

For soybean leaves, we used another kind of illuminated transparent chamber through which flowed CO_2 in air. We switched to a flow of $^{14}\text{CO}_2$ seconds before removing a detachable face of the vessel, snatching the leaves, and immersing them in boiling methanol (all in less than a second). While this method was somewhat cruder than the algal experiments, we were subsequently pleased to obtain similar results for algae and leaves.

We isolated sugar phosphates and PGA by chromatography, removed the phosphate groups and, if necessary, did a second chromatography. After elution of a given intermediate, we added a small amount of carrier compound, then performed the chemical degradation. This work involved a large effort by competent technicians and by me. In particular, Lorel D. Kay contributed many of the fine results we obtained. It was characteristic of Melvin Calvin's generous recognition of all who contributed that all were listed among the authors of our definitive paper (Bassham et al. 1954) that described all of the essential evidence for the proposed PCR cycle.

Intramolecular distribution of ^{14}C in intermediate compounds

As an example of this work, consider just the 5.4-s point from the algae experiment. The distributions of label (somewhat simplified) follow. For 3PGA the carboxyl carbon was heavily labeled whereas the other two carbon positions had very small, but equal label. Fructose was heavily and equally labeled in the central positions (C-3 and C-4) while the other positions (C-1, C-2, C-5, and C-6) had small but equal labels. Sedoheptulose had heavy and roughly equal labels in its three middle positions (C-3, C-4, and C-5) but small but equal labels in its end positions (C-1, C-2, C-6, and C-7). Finally, ribulose had the heaviest label in its

central position (C-3). There was also significant label in C-1, and C-2, and some lesser label in C-4 and C-5.

Such distributions of label supported the following conclusions: (1). 3PGA is reduced to triose phosphates (Gal3P and DHAP) that condense to form fructose bis phosphate, FBP. The 3PGA carboxyl carbons become the two central carbons of FBP. (2). The C-3, C-4, C-5 labeling of sedoheptulose bis phosphate (SBP), then called SDP, results from condensation of DHAP labeled in C-1 with erythrose-4 phosphate (E4P) labeled equally in C-1 and C-2. E4P labeled in this way would be derived from the FBP positions C-3, C-4 (strongly labeled), C-5, and C-6 (small label), after phosphate removal (to give F6P) and splitting off of carbons C-1 and C-2 of F6P. (3). The carbons C-1 and C-2 from F6P when added to Gal3P gives a molecule of Xu5P labeled mostly in the central, C-3, position. (4). A similar removal of C-1 and C-2 from S7P (from the SBP) and transfer to Gal3P gives a second Xu5P labeled predominantly in C-3. (5). The remainder of the S7P, carbons C-3, C-4, C-5, C-6 and C-7, becomes R5P, labeled in C-1, C-2, and C-3.

When the two molecules of Xu5P, each labeled mainly in C-3, and the molecule of R5P labeled in C-1, C-2, and C-3 all are converted to RuBP, the resulting pool of RuBP should be labeled mostly on C-3 (labeled in its three precursors) but with significant label also in C-1 and C-2 from the R5P.

The ratio of label between C-1, C-2, and C-3 of RuBP was not, 1:1:3, but actually about 1:1:7. Recall that this was after only 5.4 seconds of photosynthesis so that the specific ratio of $^{14}\text{C}/^{12}\text{C}$ is quite small. The rate of formation of R5P is only half that of the rate of formation of Xu5P, and (as measured later) the pool of R5P is larger than that of Xu5P. Therefore, the level of labeled carbon flowing out of the R5P pool is less than that coming from Xu5P into the RuBP pool. Other complexities also result from relative flow rates and pool sizes, but nevertheless, the data from all the experiments were as expected from the formulation of pathways just outlined.

Formulation of the photosynthetic carbon reduction (PCR) cycle

These results enabled us to sketch out a pathway whereby 3PGA formed by the carboxylation of RuBP could be reduced to trioses and hexoses. These in turn could be converted to first heptose and then pentoses, all three of which ended up as RuBP. (Bassham et al.

1954) We had already reached these conclusions about the general form of the regenerative cycle when evidence appeared from other laboratories (Horecker et al. 1953; Racker et al. 1953) providing knowledge of the plant enzymes, particularly *transketolase*, that would be required for the conversions from triose and hexose phosphates to RuBP. The appearance of enzymatic evidence for the postulated reactions as well as more complete characterization of the carboxylation enzyme (discussed above) was most timely.

The photosynthetic carbon reduction cycle formulated in 1953 has proved over time to be correct. A later depiction with molecular structures and arrows to indicate number of times each reaction occurs per cycle was shown earlier in Figure 6 (Bassham 1979). A complete cycle uses three moles each of RuBP and CO_2 , and produces six moles of 3PGA. These molecules are reduced to six moles of triose phosphate. Five of these moles of triose phosphate are required to regenerate the required three moles of RuBP. The remaining mole of triose phosphate can be used for biosynthesis of sucrose and other compounds required by the plant cells, either inside the chloroplast or after export to the cytoplasm. Some of the three carbon product can also be removed as 3PGA for subsequent conversion to such compounds as alanine and serine, for example.

The energy and the electrons for a complete turn of the cycle comes from cofactors generated by the light reactions of photosynthesis. A complete cycle, reducing three moles of carbon dioxide to one mole of triose phosphate, requires six moles of ATP and six moles of NADPH for the reduction of six moles of 3PGA, and three more moles of ATP for the formation of three moles of RuBP from Ru5P.

Enzymes of the photosynthetic carbon reduction (PCR) cycle

Except for contributions to the discovery of the carboxylation enzyme of the cycle by workers in ORL (Quayle et al. 1957; Mayoudon et al. 1957), the isolation and characterization of enzymes needed for the complete cycle were the work of other laboratories, and I will not review them here. An early listing of known enzymes required may be found in Calvin and Bassham (1962). For a more complete review, see Latzko and Kelly (1979).

Determining the energetics of the PCR cycle

When formulating the PCR cycle, it was important to determine the free energy change for each step, especially the presumed difficult carboxylation step (Bassham and Calvin 1957). There are two parts to such a determination. (1) Calculation of the standard free energies of formation of each intermediate compound, based on published values and reasonable assumptions. (2) Determination of actual concentrations of intermediates and required cofactors and calculation of the actual free energies of formation of the intermediate compounds at those concentrations.

I started with the calculations published by Burton and Krebs (1953) for reactions of glycolysis and for ATP hydrolysis, and the calculations for phosphopyridine nucleotide reduction, by Burton and Wilson (1953). Some chemically reasonable assumptions were made to calculate free energies of formation (ΔG s) of other sugar phosphates of the photosynthetic carbon reduction cycle that are not involved in glycolysis. A 'physiological' standard free energy of formation, ΔG , at 10^{-2} M, aqueous was calculated for each intermediate. These free energies were expressed as $P - X$, where P represented the energy of the phosphate group and X represented the rest of the molecule. For example, the ΔG for 3PGA was expressed as $P - 156.9$ kcal/mol while ΔG for RuBP was given as $2P - 170.1$ kcal/mol.

The actual free energy change ($\Delta G'$) for a step in the cycle depends on the ΔG s of the reactants and products corrected to the concentrations at the reaction site. These concentrations were determined by establishing steady-state conditions under which levels of CO_2 and $^{14}\text{CO}_2$ were maintained constant during photosynthesis until all intermediate compounds were labeled in all carbon atoms to the same specific $^{14}\text{C}/^{12}\text{C}$ ratio as the carbon dioxide gas. After the samples from the experiments have been analyzed and the amount of ^{14}C in each compound determined by radioactivity counting, the amounts of carbon in each compound can be calculated by dividing its ^{14}C by the $^{14}\text{C}/^{12}\text{C}$ ratio of the carbon dioxide.

Further assumptions have to be made about the actual volume of the chloroplast stroma where these reactions are occurring to obtain concentrations. These assumptions can, however, be somewhat arbitrary since an error in the assumption will effect reactants and products equally for $\Delta G'$ calculations. An exception is the carboxylation reaction, where *two* molecules of 3PGA are formed for each RuBP and CO_2

used, but it is not a serious problem, since an error would have only a small effect on the outcome of the calculation.

As mentioned above, triose, tetrose and pentose monophates generally occur in low concentrations.

Under physiological conditions during photosynthesis, the carboxylation reaction is strongly driven in terms of energy, since the $\Delta G'$ is around 9–10 kcal/mol. This large energy release for this key step is of great importance, since carbon dioxide has to compete with oxygen for the reaction with RuBP. The other major points of energy release are the phosphatase reactions in which FBP and SBP are converted to the respective monophosphates (around 7 kcal/mol), and the *phosphoribulokinase* reaction in which Ru5P and ATP are converted to RuBP and ADP (4 kcal/mol). Bassham and Krause (1969) proposed that these steps also may be sites of light–dark regulation, that is, points at which enzyme activity might be turned off in the dark to allow for the efficient operation of the oxidative pentose phosphate cycle.

Thermodynamics requires that every step in the cycle has to have an energy release, and this requirement was found to be satisfied after the techniques for steady-state photosynthesis studies (described below) had been perfected to allow for the accurate measurement of all of the metabolites (Bassham and Krause 1969; Bassham 1979).

Under the steady-state conditions of photosynthesis we used, the total energy supplied by nine moles of ATP and the oxidation of six moles of NADPH (one complete turn of the PCR cycle, with three carboxylations) comes to -427 kcal. The energy stored by the conversion of three moles of carbon dioxide and water to a mole of oxygen and a half mole of glucose is $+354$ kcal. The difference of 73 kcal is also the sum of the energies released by all the reactions in one complete turn of the cycle plus the conversion of a mole of triose phosphate to 1/2 mole of glucose.

Oxford

I spent the academic year of 1956–1957 at Oxford where I had the great honor and pleasure of working in the laboratory of Sir Hans Krebs. He was interested as was I in measuring steady-state levels of reduced and oxidized pyridine nucleotides. I worked with one of his expert assistants Reg Hems developing methods of measurement of these cofactors in quickly killed animal tissues. This was more difficult than we expected,

and the work was completed at the Oxford laboratory after I returned to Berkeley (Bassham et al. 1959).

I would later return to determinations of NADPH/NADP⁺ in connection with studies of regulation of plant enzymes (Lendzian and Bassham 1975).

Steady-state photosynthesis.

The light–dark and high–low carbon dioxide experiments described earlier had been performed under steady-state conditions but were limited to some extent in length of experiment and ease of sampling. Beginning in 1959, my very able assistant, Martha Kirk and I developed a more sophisticated steady-state apparatus (Bassham and Kirk 1960). Features of this equipment included continuous gas monitoring of ¹⁴C, CO₂ and O₂, automatic pH control of the algae suspension, large gas reservoir, pump, and a solenoid-operated sampling valve so that samples could be taken with the push of a button. Using this apparatus we made more accurate determinations of the pool sizes of PCR cycle intermediates, as already discussed. We also measured rates of carbon dioxide fixation into the PCR cycle intermediates and into secondary products such as sucrose and amino acids (Smith et al. 1961; Bassham and Kirk 1964).

In 1963, the Bioorganic group, now the Chemical Biodynamics Division of the Lawrence Radiation Laboratory moved into the new Calvin laboratory (Figure 10), and we built an even finer steady-state apparatus. Over the years, many more studies of photosynthetic carbon fixation and its regulation, products, etc. were carried out using this apparatus, but these are beyond the scope of this retrospective article. One other facet of the PCR cycle operation in chloroplasts should be mentioned, however.

The PCR cycle in isolated spinach chloroplasts

Much important work on photosynthetic reactions by isolated chloroplasts had been done in many laboratories, including those of Daniel Arnon, and of David Walker, by the late 1960s, but rates of carbon dioxide fixation comparable to those of intact cells had not been achieved without the addition of carbon cycle intermediates to the suspending medium. In our laboratory, Dick Jensen and I were able to demonstrate rates of carbon dioxide by isolated spinach chloroplasts up to two thirds those of the leaves from which they were

obtained, without the addition of PCR cycle intermediates (Jensen and Bassham 1966). Both before and after that date, many studies of photosynthesis by isolated chloroplasts were performed by David Walker and associates, and a retrospective review of that work is available (Walker 2003). Dick Jensen moved to the University of Arizona and continued his studies of the isolated chloroplasts (Jensen 1979).

Concluding remarks

The photosynthetic carbon reduction cycle, (PCR cycle) was mapped and proven by 1953 in the Old Radiation Laboratory at University of California at Berkeley, UCB. The key steps in the mapping and proof were: (1) The identification of the labeled metabolites formed by photosynthesis with ¹⁴CO₂. (2) Identification of the light-driven steps by following changes in metabolite levels during transitions from light to dark. (3) Identification of the carboxylation step by changes in metabolite levels during the transition from high to low CO₂. (4) Formulation of the carboxylation reaction and demonstration of its enzyme, now called *ribulose-1,5 biphosphate carboxylase oxygenase* in plant extracts. (5) Determining the pathway of the cycle from the product of carboxylation (3PGA) to the substrate for the carboxylation, RuBP, by isolation and chemical degradation of labeled metabolites and using that data to choose among the various known and concurrently discovered metabolic steps in green plant cells. Subsequent proof that each step of the PCR cycle was energetically possible came from measurement of actual metabolite levels during photosynthesis, and use of these levels to calculate a negative free energy change for each step.

The photosynthetic carbon reduction cycle is a somewhat complex pathway involving the reduction of 3PGA to triose and hexose sugar phosphates by reactions similar to reversal of glycolysis, the conversion of five triose phosphate molecules to three pentose phosphate molecules as RuBP via branching pathways, and the carboxylation of RuBP to give two molecules of 3PGA. The energy and reducing power for the cycle is supplied from the light reactions of photosynthesis as six NADPH and nine ATP molecules per cycle. Only a few of the steps in this cycle involve pentose phosphates, and I prefer the name photosynthetic carbon reduction cycle to the name reductive pentose phosphate cycle.

By 1949, work was underway in several laboratories on various aspects of photosynthetic carbon metabolism and related processes, and during the following decades the use of radiocarbon as a tracer became very popular. An excellent compendium of the work by many of these investigators may be found in 'Photosynthesis II. Photosynthetic carbon metabolism and related processes' (Gibbs and Latzko 1979). Although I have briefly alluded to some of these important reports, my article is mainly a retrospective of our work at Berkeley in that period and is in no sense intended to be a review of the field.

Acknowledgments

I am indeed thankful to Govindjee for convincing me to write this retrospective, despite my reluctance some 16 years after retirement and since writing my last scientific article. Looking back at the early part of my career in science, I realize how fortunate I was to have been associated with the inspirational teacher and eminent interdisciplinarian Melvin Calvin. I owe many thanks to Andy Benson for his instruction in special laboratory techniques required for the early work I have described and for the privilege of working with him, as well as for an introduction to mountain climbing. While I have mentioned in the article my appreciation of certain other coworkers, I owe much to technicians, graduate students and visiting scientists far too numerous to list. This manuscript was edited by Govindjee.

Note

¹In this paper, J.A. Bassham refers to the carbon reduction cycle as PCR cycle. Although it is scientifically the best name, uninitiated students may confuse it with the PCR (polymerase chain reaction) cycle used in molecular biology research. This cycle is also known by many authors as the Calvin cycle. However, in recognition of the pioneering contributions of Andy Benson and Al Bassham, it is also known as the Calvin-Benson cycle or Calvin-Benson-Bassham cycle – Govindjee, Editor.

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