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Personal perspective

Following the path of carbon in photosynthesis: a personal story

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Abstract

Chronological recognition of the intermediates and mechanisms involved in photosynthetic carbon dioxide fixation is delineated. Sam Ruben and Martin Kamen's development of application of radioactive carbon for the study of carbon dioxide fixation provided impetus and techniques for following the path of carbon in photosynthesis. Discovery The identity of the primary carboxylation enzyme and its identity with the major protein of photosynthetic tissues ('Fraction 1' protein of Sam Wildman) is reviewed. Memories are dimmed by sixty years of exciting discoveries exploration in newer fields [see Benson 2002 (Annu Rev Plant Biol 53: 1–25), for research and perspectives beyond the early Berkeley days].

Formaldehyde theory for CO_2 assimilation

As I was born (1917), Richard Willstätter and Arthur Stoll were recording their detailed investigations of chlorophyll's involvement in absorption of CO_2 and their search for photochemical production of formaldehyde (see Willstätter and Stoll [1918]: 'Untersuchungen über die Assimilation der Kohlensäure') in a forlorn volume of 448 pages of futile laboratory experiments. In contrast, their classic previous volume on the chemistry and structure of chlorophyll (Willstätter and Stoll 1913) is elegantly informative. The path of carbon in photosynthesis was, at that time, widely thought to involve the combination of carbon dioxide with chlorophyll, photoexcitation, and the production of formaldehyde (HCHO) followed by its polymerization to hexose, a carbohydrate (see below).

$$CO_2 \rightarrow HCOOH\ (CO) \rightarrow HCHO\ [\leftrightarrow (CH_2O)_6 \text{ etc.}$$

The concept of reaction of photoexcited chlorophyll with carbon dioxide remained in the minds of almost everyone concerned with the subject. For over 60 years, the formaldehyde theory allured chemists and physiologists. Its authoritative proponent, Adolph von Baeyer, and the absence of an equally feasible mechanism sustained it. Robert Emerson (1929), too, had devoted thought and experiments to the ideas of E.C.C. Baly (Baly et al. 1927; Baly and Hood 1929; see the book published in 1940), who had adopted the formaldehyde concept. Even as Melvin Calvin and I presented our early papers, the audiences were still contaminated with the magic of formaldehyde.

C^{11} experiments of Sam Ruben and Martin Kamen[1]

A hundred years of photosynthesis research had gone that far. Physicists and kineticists appreciated Willstätter's photochemical model, but it did not make any sense to Sam Ruben and Martin Kamen when they started looking at the carbon-11 (half life, 20 min) labeled products of brief photosynthesis during their 1938 experiments (Ruben et al. 1939). Fixation and reduction of carbon dioxide had to be a 'dark reaction' (Blackman 1905). Ruben and Kamen's search for identity of 'R' in R^1H +

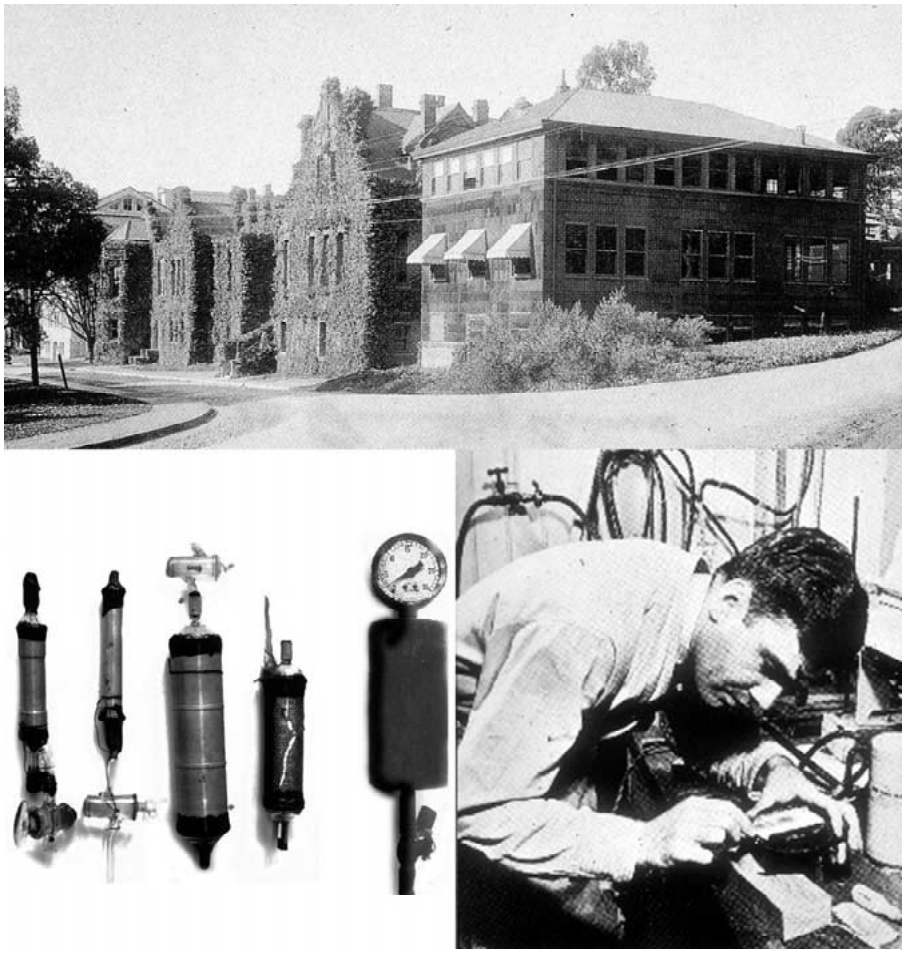


Figure 1. The era of Sam Ruben and Martin Kamen. *Top panel*: the 'Old Chemistry,' classic brick building, and the Chemistry Annex, 'The Rat House,' 1915 (see note 2). *Bottom right*: Martin Kamen, preparing boron oxide target for the 37-inch cyclotron bombardment with deuteron beam. *Bottom left*: Geiger–Müller counting tubes made by Sam Ruben. Also shown as the right most piece is the vacuum aspirator, made by Sam Ruben, and used by Martin Kamen for collection of gaseous products of boron oxide bombardment by deuterons in the 37-inch cyclotron.

$CO_2 \Longleftrightarrow$ RCOOH was based on their plausible evidence that the earliest product had to be a carboxylic acid. It was also based on rational energetics and their observation that the C-11 radio-labeled products precipitated with calcium or barium ions and liberated labeled carbon dioxide upon pyrolysis, thus indicating production of a carboxylic acid. Being physical chemists and following the thought of Fritz Lipmann and Herman Kalckar, they were aware of the thermodynamic aspects of metabolism. Even in 1942, Ruben, in particular, was concerned with the fact that carboxylations of known compounds would have an energy requirement of about 20 Kcal (see Ruben 1943; personal communication).

Prior to Ruben and Kamen, great progress and great polemics involved the nature of biological oxidation and reduction. The real giant was Heinrich Wieland (Witkop 1992), followed by René Wurmser, teacher of Hiroshi Tamiya. Wieland contended that dehydrogenation was the basis of biological oxidations. Wieland's nemesis, Otto Warburg, insisted that oxidation reactions must involve oxygen. However, the experiments and insights of David Keilin, Hans Krebs, and Albert Szent-Györgyi contributed reality. Cornelis B. van Niel and his students, following Wieland and Albert Jan Kluyver, became major collaborators with Sam Ruben and Martin Kamen (van Niel et al. 1942).

So, that is where I came in, as a new member of the Department of Chemistry faculty in Berkeley, June, 1942. My small (10 m^2) office-laboratory in 'The Rat House'[2] (see Figure 1, top panel) had just been vacated by Henry Taube. I cleaned up his glassware, but there were plenty of other future Nobel Prize winners around in case I should need further help. I

was immediately involved with frequent experiments with $C^{11}O_2$ produced by Martin Kamen's deuteron bombardment of boron targets in the 37-inch cyclotron (Figure 1, bottom right). Since it was the physicists' instrument, Kamen could use it only after their working hours, usually three times per week. Hence, his product was usually ready for his dash – the run from the Radiation Laboratory (later called ORL, the Old Radiation Laboratory) to the Rat House about 8 or 9 P.M.

With Ruben's meticulous preparation, all the counters (Figure 1, bottom left) and heaters were ON. The furnace with its silica tube of copper oxide was hot and ready for the mixture of CO_2 and CO from Kamen's target 'aspirator' (Figure 1, the right most piece in the bottom left panel) to be converted to pure $C^{11}O_2$ for the algae or plant experiments. Though Ruben and Kamen, with Zev Hassid, knew what they wanted to learn from the experiments, it seems now that, without considerable chemical transformations and separations, the fixation products would have been difficult to identify within the 2–5 h and the practical demise of the C^{11} radioactivity. Their early conclusion, based on pyrolysis of a barium salt precipitate, was that the new carbon-11 product contained a carboxyl group, the basis for their hypothesis, $CO_2 + \text{R-H} \Longleftrightarrow \text{R-COOH}$. With further experimentation and thought, Ruben and Kamen (1940) (see their photographs, Figure 2, top; Figure 2, bottom, shows Kamen, when he was unjustly accused by the Government) concluded that reduction of the carboxyl followed by re-carboxylation should be a 'cyclic regenerative process.'

In retrospect, so many of the attempts at chemical identification of intermediates became inconclusive as a result of adsorption and absorption of small amounts of labeled products on large proteins, denatured by the practice of boiling the experimental material prior to separation of the 'extract.' Ruben's 1942 opinion (see Ruben 1943) that the initial CO_2 fixation was a reversible thermal (dark) fixation was consistent with his and Kamen's presumption that the dark fixation of CO_2 should best be done under anaerobic conditions in their effort to reduce the effects of respiratory metabolic exchange. Ruben sought evidence for this dark carboxylation. The fallacy derived from the erroneous concept that 'dark fixation of CO_2 in the absence of oxygen' could be related to photosynthetic carboxylation. This, unfortunately, was disastrous for all such concepts prior to 1948. If, as we know now, the dark fixation were to have followed pre-illumination in nitrogen or helium, the result would have been immediate production of the 'first products of photosynthesis.' Recently, Stanley Miller, Jim Cleaves, and I (unpublished) have explored the possibility of microreversal of the carboxylation of ribulose bisphosphate.

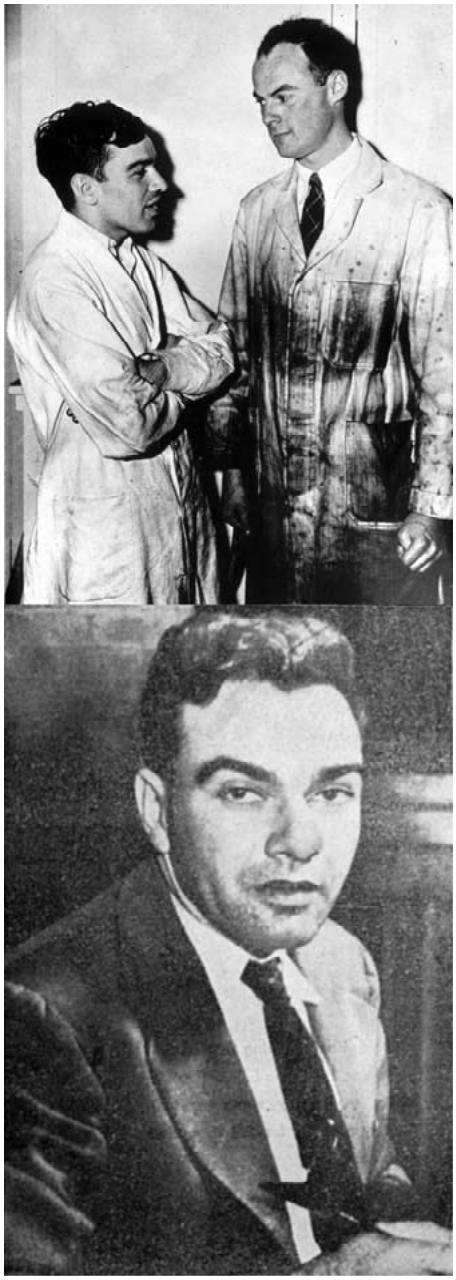


Figure 2. The era of Sam Ruben and Martin Kamen (continued). *Top*: Ruben and Kamen in their laboratory, ∼1940 (from a photograph hanging in the Lawrence Hall of Science at Berkeley, California). *Bottom*: 1945 Press photo when Martin Kamen was summoned to testify before the House Committee on Un-American Activities.

We found, as predicted by the extensive studies of George Lorimer (Lorimer et al. 1986), that the extent of such reversal was negligible.

The conclusion of Ruben and Kamen that the carboxylation product included a carboxyl group was correct but not related to that of phosphoglyceric acid (PGA), the first product of photosynthetic CO_2 fixation. (Hans Gaffron, Allan Brown and Bill Fager of the University of Chicago also erred in the 1950s from the presumption that adsorption was not important in decisions involving identity of first C^{14}-labeled products. With large molecules involved, adsorption was a serious and potentially misleading problem.)

The historical importance of the work of Sam Ruben and Martin Kamen was its profound impact upon contemporary biology and the ongoing development of modern biochemistry. It attracted widespread interest in and optimism for the future application of radioisotopes in study of metabolism. It certainly engendered interest of plant physiologists and those concerned with carbon dioxide uptake and metabolism.

Invention of the long-lived radioactive carbon, C^{14}

Ruben and Kamen were encouraged and supported by Ernest Lawrence in the quest for long-lived radioactive carbon. I call it 'invention' because they and Ernest Lawrence and many other nuclear physicists felt it must exist, a matter of 'faith.' They had tried all conceivable nuclear routes to C-14 until they finally succeeded; so it was more of an 'invention' than a 'discovery' (Ruben and Kamen 1941). The night that Kamen closed down the cyclotron (see Kamen 1985) after a 30-h bombardment of a dry film of Aquadag (an aqueous graphite suspension), trying to produce C-14 from its C-13, he left the crumbled graphite on Sam's desk to be assayed in the morning and stumbled home through the rain before dawn (February 27, 1940). Berkeley police were on the alert after a particularly awesome series of murders and, seeing a hunched and unkempt figure on the street, Martin was apprehended and taken to the police station for interrogation. A witness of the murder was called to identify the suspect. Failing to do so, Martin was released. Needless to say, the night of the discovery of long-lived radiocarbon was not a pleasant memory for Martin. In my view, Ruben and Kamen (1941) had certainly 'earned' a Nobel Prize with their invention of carbon-14 (Benson 1982).

Later, Ruben succeeded in isolating C^{14} oxides from the concentrated ammonium nitrate solution in the wall of stainless steel tanks in the cloud of slow neutrons surrounding the 60-inch Crocker Cyclotron just across the alley from the Old Radiation Laboratory and its 37-inch machine. An awful explosion of ammonium nitrate in New Jersey and the importance of the cyclotron's physical health (1942) led to the tanks being removed and C-14 production brought to a halt. Ruben and Kamen did no photosynthesis experiments with their newly found long-lived radioactive carbon. With its low energy beta emission and low specific activity, measurement was too tedious. The final C-11 experiments continued for a year. It is not clearly recognized that the many photosynthesis experiments performed by Ruben and Kamen (Ruben et al. 1939) during four years of hectic effort yielded no real information on the path of carbon in photosynthesis.

The early experiments on the path of carbon

The search for the product of 'dark fixation' proceeded when Ruben, almost completely involved in a 'classified' war gas defense project, handed me ALL of the $BaC^{14}O_3$ in the world to search for the first product of CO_2 fixation (Figure 3, top). With this $BaC^{14}O_3$, the search for the path of carbon in photosynthesis could begin. I carried out many dark fixations with the green alga *Chlorella* with this $C^{14}O_2$, following the Ruben and Kamen concept of reversible reaction with R-H to yield R-COOH.[3]

I could confirm Ruben and Kamen's conclusion that the product possessed a carboxyl group; it reacted with diazomethane. Its partition coefficient between water and ethyl acetate was 0.14. Three years later, in ORL, with daily helpful collaboration of Ed McMillan (Nobel Laureate 1951) I crystallized the dark fixation product and co-crystallized it with succinic acid to constant specific activity. (We knew later that succinic acid was not the product, as such, of CO_2 fixation.) Little did I realize that McMillan was involved in crystallizing salts of Neptunium at the same time. For further discussions, see Benson and Calvin (1947) and Calvin and Benson (1948).

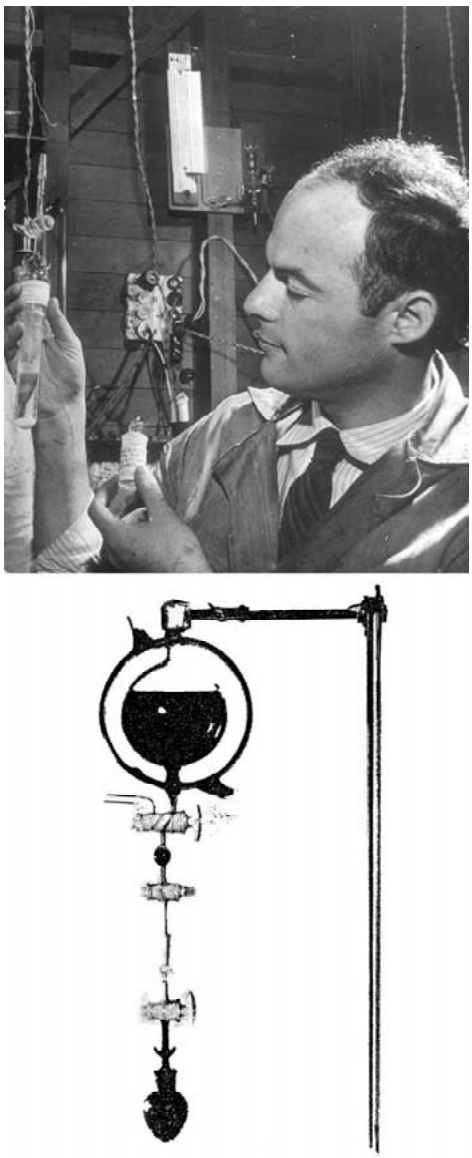


Figure 3. The era of Sam Ruben and Martin Kamen (continued). *Top*: Sam Ruben in the 'Rat House' holding a weighing bottle containing the then world's entire supply of Ba C^{14} O_3. *Bottom*: apparatus used to do pre-illuminated dark fixation experiments with algae.

C-11 phosgene and Ruben's accident

Ruben had visited the army's Dugway Proving Ground where tests with goats indicated that phosgene exposure induced lung edema and that the fluid affected other goats in a similar manner. Having been exposed to the immunological studies of Dan Campbell and Linus Pauling at Caltech, my theory was that phosgene, a double acid chloride, could couple proteins or so alter the conformation of one protein to yield a novel antigenic protein and consequent lung edema. So I developed rapid (30 min) phosgene synthesis from the $C^{11}O_2$ (20 min half life) from Martin Kamen and the 37-inch cyclotron (Ruben and Benson 1945).

Ruben's technique with our phosgene-exposed rat was to drop the victim into the Waring Blendor to produce a protein preparation for measurement of its C-11. For Sam's own phosgene experiments, I had made small steel bombs with valves that I could fill with phosgene (bp 8 °C) from the ancient Kahlbaum ampoules having laid in sawdust in back of the chemical store room for perhaps 20 years. With no chemical hood in the Rat House, but only a large open window, I did this carefully with ice-cooled ampoules and vacuum transfer to the steel bombs. (I still insist that all chemistry students should recognize the odor of phosgene.)

Finally, it became impossible for Wendell Latimer to extend my teaching contract and I left Berkeley in July, 1943 for Civilian Public Service in the Nevada mountains, fighting forest fires, building dams, and logging for Forest Service construction projects.[4] A few weeks after I had departed, Ruben's phosgene supply became exhausted and, with his broken arm in a sling, he tried to transfer liquid phosgene. Too impatient to cool the ampoule in ice-salt, he immersed it in liquid air; the aged soft glass ampoule cracked, releasing the deadly liquid into the boiling liquid air, splattering it all over his wool sweater from which it was impossible to escape. Sam carried the boiling cauldron outside, for safety of the building's occupants, and lay down on the lawn. One of his student assistants was hospitalized and Sam succumbed in a day as his lungs filled with fluid. That was September 28, 1943. Sam was almost 30 years old. He had sent me a thoughtful letter just the week before. This great tragedy of science left the Path of Carbon without its real leader and Sam's family without the future it deserved (Johnston 2002, see chapter on 'Sam'; also see Maruo and Akazawa 1997).

The early C^{14} experiments at the ORL, Old Radiation Laboratory (official name after 1945)

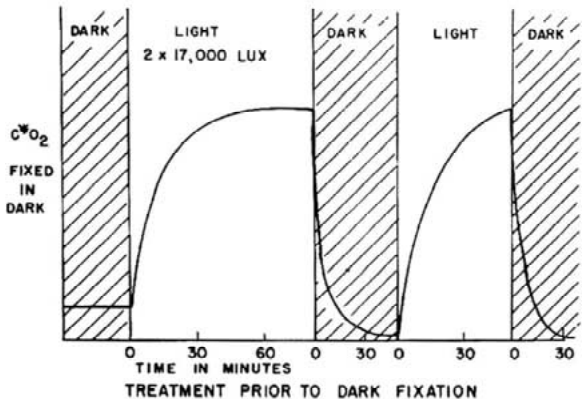
World War II was over. In 1945, Ernest Lawrence recruited Melvin Calvin to continue the photosynthesis work in the University of California Radiation Laboratory (UCRL) and Melvin invited me to begin it as Director of the Photosynthesis Laboratory, a section

of his Bio-Organic Laboratory of UCRL. Lawrence provided a portion of the space in the 'old radiation laboratory' since its cyclotron was to be retired and removed to the University of California at Los Angeles (UCLA). Though the 37-inch cyclotron was well known, I had never seen it. The facility had been secured during the war when I arrived from Caltech in June 1942. Now a large west side room, adjacent to that of the venerable 37-inch cyclotron, was to be our photosynthesis laboratory.[5]

Preillumination-enhanced dark fixation

To dispel the contention that CO_2, adsorbed in chlorophyll, was photochemically reduced, I illuminated Chlorella under helium, and passed an aliquot portion into a black flask containing C^{14}-bicarbonate (Figure 3, bottom). Clearly, 'preillumination' enhanced 'dark fixation' (Benson et al. 1949; Calvin, 1949). Blackman (1905) had documented indirect evidence for the reality of a 'dark reaction' by his ingenious assembly of kinetic evidence for enhanced carbon dioxide reduction after high light intensity illumination. My initial rate of dark uptake of $C^{14}O_2$ approached that of steady state photosynthesis and was 100 times the rate of dark respiratory exchange. When the preillumination ceased, the dark fixation decayed to the 'dark respiration' level (Figure 4, top) (Benson et al. 1949). I observed sucrose synthesis in the dark! After 10 min of preillumination with CO_2-free helium flushing, *Scenedesmus* cells produced 42% of the fixed C^{14} in phosphoglycerate (PGA) during 1 min of dark fixation. During 30 s of photosynthesis, 27% of the fixed C-14 was phosphoglycerate. After 60 s of photosynthesis, 20% was found in phosphoglycerate (Benson and Calvin 1948). The middle and bottom of Figure 4 show radioautograms after 10 s and 30 s illumination of *Scenedesmus* cells. Clearly, the first product is phosphoglycerate, and sugar phosphates appear later.

Fifteen years later, Al Bassham and Kirk (1963; see Bassham 1964) performed a much more definitive experiment for which they plotted the kinetics of formation of labeled products of $C^{14}O_2$ dark fixation following steady state photosynthesis. The rate of malic acid synthesis was relatively low compared with that of PGA and the sugar phosphates. After 15 s, the amount of labeled PGA diminished as it was converted to other compounds, including phosphopyruvate, which may have enhanced the yield of labeled



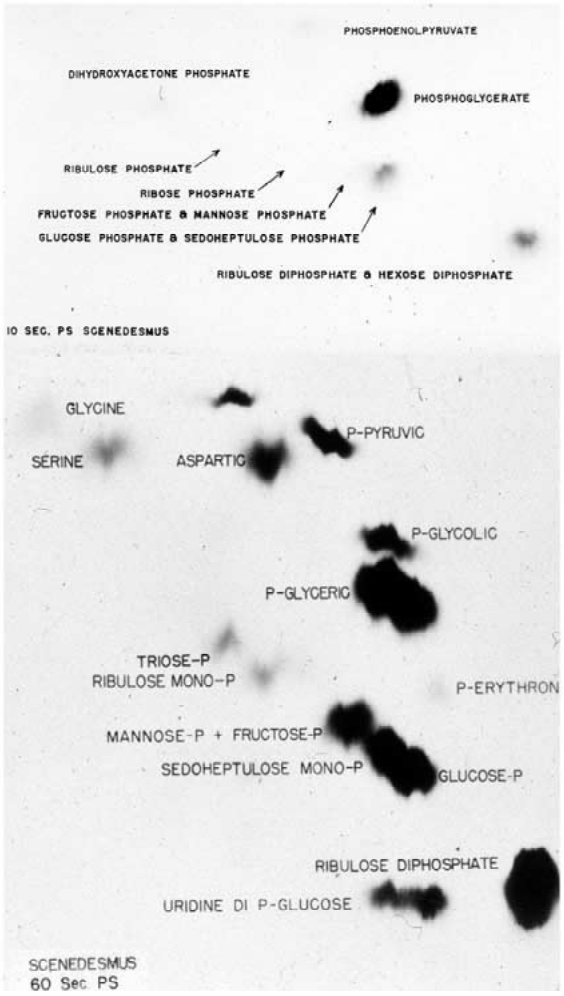


Figure 4. Early C-14 experiments. *Top*: C-14 O_2 dark fixation following pre-illumination. *Middle*: a 10-s radio-autogram showing that the major product is phosphoglycerate in *Scendesmus* cells. *Bottom*: a 60-s radio-autogram showing that the major products are sugar phosphates in *Scendesmus* cells.

malic acid in the kinetic experiments of Benson et al. (1952).

Otto Warburg had been taking advantage of the 'preillumination' phenomenon to convince skeptics that the quantum requirement of photosynthesis was as low as 1.0, creating and maintaining a 10-year polemic with the Urbana–Chicago–Madison groups who insisted that eight (or more) quanta were required to yield one oxygen. (For a recent discussion on the quantum requirement controversy, see Govindjee, 1999.) Clearly, by adjusting light/dark periods, the slower kinetics of enzymatic accumulation of intermediates and the far more rapid photochemical processes could alter the apparent quantum yield measurements. I felt that Warburg should have understood this, but doubt if he really did.

3-Phosphoglyceric acid: the first product

With very brief fixations of $C^{14}O_2$, I observed one predominant product in my first rather inadequate paper chromatograms. Convinced that my poorly-resolved product could actually be a first product of CO_2 fixation, I eluted it from the paper (Calvin et al. 1950). Calvin and I studied its behavior on an anion exchange resin column and found that it bound much more tightly than ordinary sugar phosphates. We deduced from this that it must have not one but two anionic binding sites. Upon acid hydrolysis of its phosphate ester group and conversion to a p-bromophenacyl ester in the presence of 112 mg of carrier glyceric acid from an ancient Kahlbaum bottle in Mr Ray's Chemical Storeroom, the product was crystallized (Benson et al. 1949). A number of successive recrystallizations failed to alter the specific activity of the crystalline product, thus establishing the radioactive product's identity as glyceric acid. Hence the first product of CO_2 fixation was 3-PGA (Benson and Calvin 1948; Benson et al. 1949).

The great polemic: the Chicago group

Apprehensions over the validity of radiotracer research and its relation to the widely recognized observations and concepts of Ruben and Kamen developed until the 1947 meeting of the American Association for Advancement of Science in Chicago on December 26, 1947. A great crowd had assembled to hear statements from two sides. Melvin Calvin and I had taken a train trip to Chicago (not very common these days). Melvin wanted time to plan in his remarkable memory, his lecture for the symposium. Calvin and I presented the Berkeley point of view (Benson 1949; Calvin 1949) while Brown, Fager, and Gaffron (1948) presented the Chicago view. Melvin presented the then current concept of the Path of Carbon while I presented the identification of phosphoglycerate as primary product of CO_2 fixation. Physicist Farrington Daniels of the University of Wisconsin mediated the contentions. There was no question in my mind that our observations were real and our conclusions correct. I thought that was the end of the polemic, though aftershocks of the occasion permeated statements published in the proceedings of the meeting (Franck and Loomis 1949). However, it finally was a Waterloo for the Midwest contingent and, after their cautious capitulation (Fager 1949), we could continue along 'The Path.'

Soon, Fager and Rosenberg (1950) and Fager et al. (1950) published their own isolation and identification of phosphoglyceric acid, PGA. Bill Fager proceeded to earn a degree in ecology at Oxford University and later became a respected Professor of marine ecology at the Scripps Institution of Oceanography.

The tension, though, claimed its toll. Melvin was struck by a coronary infarct during a budget review meeting. With Jack Goffman in the adjacent laboratory, we knew about high density lipoprotein (HDL) and low density lipoprotein (LDL) and diet before anyone else and, with Genevieve Calvin's determined dietary regime, Melvin was restored to health and enjoyed a long productive life.

Malic acid, the 'first' product in sugarcane: experiments by the Hawaii Group

George O. Burr, formerly of the University of Minnesota, and then Director of the Hawaiian Sugar Planters Experiment Station laboratories, visited us at ORL several times with a problem, namely not finding any phosphoglycerate in early $C^{14}O_2$ photosynthesis by sugar cane leaves. He and Hugo Kortshak could only find C^{14}-malate. My later experiments with Kortshak and Constance Hartt in 1957 failed as well because of inadequacy of my ethanol extraction of labeled products. They had found malate and related dicarboxylic acids, but very little phosphoglycerate. (I now wonder if I had used methanol for extraction, as developed by Al Bassham after I left ORL, it might have been possible to get the expected results. Methanol specifically weakens the cell membrane structure,

Figure 5. Top: Constance Hartt with Andrew Benson (Honolulu, 1957). *Bottom*: from left to right: Shinichi Kawaguchi, Andrew Benson and Melvin Calvin (1950).

allowing influx of toxicants and ultimate loss of membrane integrity and extraction of soluble components.) Burr had reported the C^{14}-malic acid accumulation to us in 1949 and 1950. It interested us, but we had no sugarcane for an experiment. Experiments by Burr and column analyses by Kortshak (Burr et al. 1957, and personal communications) later led to delineation of the C-4 pathway by M.D. Hatch and C.R. Slack (see M.D. Hatch, this issue).

Far prior to the involvement of Burr and Kortshak, however, Constance E. Hartt (see photograph of Hartt, Figure 5, top; Figure 5, bottom, shows Calvin and Kawaguchi, mentioned later) of their Experiment Station had been searching for the mechanism of sucrose production in sugar cane leaves since 1932. With very logical and persistent studies of the effects of a wide variety of inhibitors of steps in the glycolytic sequence (Hartt 1943), she developed evidence for the activity of triose phosphate dehydrogenase – clear evidence for involvement of PGA in the production of sucrose. She concluded in 1944:

> The theory that malic acid with its dehydrogenase may aid the formation of sucrose was tested by supplying malic acid with and without glucose to detached blades. Malic acid alone decreased the loss in total sugars and sucrose and may have substituted for sugar as a source of energy. Malic acid alone made no sucrose, but when given with glucose increased the gains in total sugars and sucrose and increased the synthetic efficiency. These results are in accord with the theory that malic acid aids in the synthesis of sucrose.

Further, she developed evidence that malic acid dehydrogenase is likewise involved. Here, Constance Hartt's (1944) conclusion preceded recognition of the C-4 pathway of photosynthesis by almost two decades. The fact that her work had not been recognized by others appears to have stemmed from the travel restrictions within her quite isolated organization. When I visited her and Hugo Kortshak's laboratories in 1957, she informed me of her early work and her experiments with $C^{14}O_2$ fixation by illuminated sugar cane leaves. Her interest in botany and contemporary biochemistry as well as the plants of Hawaii was as boundless as her patience with the countless sucrose analyses upon which she had based her conclusions.

The use of paper chromatography: the solvents

Bill Stepka brought paper chromatography from Rochester, where it had been established by C.E. Dent (Dent et al. 1947). Two-dimensional paper chromatography effectively separated amino acids, sugars, and other groups of compounds. Their solvents included noxious and sickening lutidine and collidine as well as phenol; each required separation of the organic phase from the 'water phase' before use. The physicists in offices near our Chromatography Room on the second floor of ORL were so sensitive to such odors that several were taken to Cowell Hospital for treatment.

I formulated a phenol-water solvent by using my distilled phenol with 40% of its weight of water. This gave a water-saturated phenol solution at room temperature. I selected propionic acid for addition to n-butanol for acidification and for enhancing the water content of the 'organic phase' of our solvent (Benson et al. 1950). Knowing the necessary amounts of water, butanol, and propionic acid, I prepared two solutions, one of water and butanol and the other of water and propionic acid, such that equal volumes of the two solutions would yield a solution identical

with the 'water-saturated organic phase' of that system. This was simple, it avoided esterification and allowed chromatographic separation of a host of components, from lipids to sugar diphosphates. I discarded the English convention for placement of the 'origin' on the paper. There are eight possibilities; I chose to use the Cartesian coordinates convention for 'x' and 'y' values of the Rf (distance traveled relative to that of the 'solvent front') measurements. Paper chromatographic separations are, in effect, gradient elutions. The solvent loses water to the paper as it travels and becomes more 'organic' in composition. Thus, the sugar phosphates separated with the water-rich solvent and later the phospholipids and pigments separated in the more organic, less aqueous, solvent. I demonstrated this by analyzing the solvent composition as it traveled on the paper.

Paper chromatographic separations are the result of 'partition' between the moving organic phase and the stationary aqueous phase. The partition coefficient for each substance and solvent system is unique; it was the basis of my unpublished 1943 study of the products of dark $C^{14}O_2$ fixation. I recall two of Glenn Seaborg's 1939 seminars on his work defining the actinide series. There it was clear that partition between two immiscible solvents can provide valid information, even when only a few atoms are involved.

A primary attribute of our application of two dimensional paper chromatograpy (Benson et al. 1950; Benson 1977) was the practice of applying an aliquot portion of the 'total extract' of the plants labeled in the experiment. Others had complicated interpretation of their work by chromatographing several kinds of extracts separately. It is absolutely essential that the whole assembly of products be examined in the chromatogram. Nothing can escape. The insoluble proteins and polymers then remain on the origin. They could be measured and treated chemically or enzymatically for further resolution and identification. (My students may recall that I urged them to carry a sharp pocket knife – for cutting out and eluting radioactive spots from the chromatogram.)

'Standardyes' for chromatographic Rf orientation were selected from Mr. Ray's fabulous Chemical Storeroom, on the basis of their chemical structures and estimated relative water/solvent solubilities, for cochromatography with labeled compounds. The mobilities of Tropeolin (Orange II) and Ponceau-4R (red) were reasonably reliable for comparison with C^{14} compounds, the Rf of Tropeolin being the most useful. Hence, one could judge the position of a labeled compound by virtue of its mobility relative to those of the 'standardyes.' Further, the solvent could be permitted to travel much farther, past the paper's edge, without loss of relative coordinates of separated compounds remaining on the paper sheet.

Strangers appear: two unknown sugars appear in $C^{14}O_2$ photosynthesis products of *Rhodospirillum rubrum*

Probably the first example of 'radiochromatographic exploration' involved the characterization of two radioactive compounds occurring in Clint Fuller's *R. rubrum* extracts and, to lesser extent, among the products of algae and plants. Their surprising appearance was tantalizing. After preparing hundreds of radiograms from our two-dimensional paper chromatograms, the usual pattern of compounds and their relative amounts had become very familiar. But, in this case, two radioactive spots just jumped out at us, strangers among a well known group of compounds. It must have been the result of phosphatase activities liberated in preparation of the bacterial extracts (Fuller 1998). I eluted our phosphate ester compounds and hydrolyzed them with Polidase; the same two new compounds appeared, with the usual glucose, fructose, and triose. Soon it was clear that both were sugars, being neutral and hydroxylated. But they were neither hexoses nor known pentoses. Frantically, I chromatographed one of the radioactive products with ribose, arabinose, and xylose; close, but not the same. The other unknown, lying between glucose and fructose, was even more confusing. A clue developed when I heated it briefly in acid. A new, much farther moving product appeared. I called it 'UH+'. It seemed like a year before we knew the answer, though I was proceeding with identification of both unknowns at the same time. With Al Bassham's able collaboration, I made uniformly labeled unknowns by several minutes of photosynthesis in $C^{14}O_2$ for the purpose. Al oxidized them with lead tetra-acetate. He found 14% of the C^{14} in barium carbonate. *'That's unheard-of Al, try again.'* And he found it again (Benson et al. 1951). It was hard to believe – seven carbons! We looked in the books and, sure enough, sedoheptulose had been isolated by F.B. LaForge and C.S. Hudson (1917). The most recent paper on the subject was by Arnold Nordal, at the University of Oslo. I wrote to Nordal asking for a sample of his sedoheptulose; he sent beautiful crystals of sedoheptulose and its acid-dehydrated

product, sedoheptulosan, as well as instructions for preparation and use of his orcinol spray reagent for specific detection of these compounds on the paper chromatogram. The discovery of sedoheptulose and its phosphate ester's involvement in the path of carbon in photosynthesis was complete (Benson 1951, 1981, 1987a, b; Benson et al. 1951, 1952a).

The question of how the sedoheptulose evolved was clarified by the then ongoing work of Smyrniotis and Horecker (1956). Their discovery of transketolase, the enzyme that transfers the two-carbon moiety from C-1 and C-2 of fructose-6-phosphate to an aldehyde receptor, coupled with the fact that the C-1 and C-2 of sedoheptulose possessed identical configuration, now made it possible to assemble part of the final photosynthetic carbon reduction cycle.

The other new unknown, just beyond alanine in our chromatograms, yielded its identity slowly. I reduced it with hydrogen over platinic oxide, Adam's catalyst, to its glycitol, which co-chromatographed with the glycitol prepared from ribose. Further, I labeled its precursor, in the hexose diphosphate area of our chromatograms with P-32 and measured the ratio of P^{32} to C^{14} in the compound. The result was a ratio of 2:5. So, finally the unknown pentose in the sugar diphosphate area was a pentulose diphosphate. It could only be a diphosphate of ribulose, or xylulose. That led to a search for the two authentic ketoses for comparison with the unknown product. After a long episode of silver nitrate (Tollens' Reagent) spraying, I could identify the unique pale brown colors of the two pentuloses that I had synthesized by epimerization of arabinose and xylose by hot pyridine treatment. I found that the C-14-labeled unknown pentulose co-chromatographed with my synthetic ribulose and only a little of it matched my synthetic xylulose. At last it was possible to write a letter to the editor of the *Journal of American Chemical Society* (JACS) entitled 'Identification of ribulose diphosphate in photosynthetic carbon metabolism.' I did not understand why Calvin changed the title to 'Ribulose in photosynthesis' and, without his customary authorship, the note went off to the journal (Benson 1951).

The five carbon sugar: ribulose diphosphate

Identification of ribulose diphosphate (later called ribulose bisphosphate) involved a variety of synthetic and chemical procedures. Ribulose, being a labile sugar, not commercially available, was synthesized from arabinose by epimerization in pyridine. Being a ketose like xylulose, prepared similarly from xylose, it gave a uniquely colored product when sprayed with the Tollens' Reagent, allowing chromatographic identification of ribulose. Ribulose mono- and diphosphate esters had been hydrolyzed easily with our phosphatase preparation called 'Polidase S' to which Nathan E. Tolbert (Tolbert 1997) had introduced us. Operations involving storage and re-chromatography of RuDP invariably led to production of appreciable amounts of PGA and phosphoglycolic acid, which were easily recognized by their chromatographic properties. This property was reported in the 1951 paper (Benson 1951) describing identification of ribulose diphosphate. C^{14}-labeled ribulose diphosphate was found to be oxidized by air in diethylamine solution or on anion exchange resins to give phosphoglycerate and phosphoglycolate as major products. I wrote, 'These were identified after phosphatase hydrolysis as glyceric and glycolic acids. An examination of the kinetics of formation of ribulose diphosphate from $C^{14}O_2$ during steady state photosynthesis and a discussion of its importance as a C_2 donor in the cycle for regeneration of the CO_2-acceptors will be published.' This led to the cycle (see Figure 6) reported by Bassham et al. (1954).

Clearly, reaction of oxygen with ribulose diphosphate was a result of the labile nature of its enol form. As a consequence, I failed to get excited when Nathan E. Tolbert, T. John Andrews, and George H Lorimer (Andrews et al. 1973; also see Tolbert 1997) reported an 'oxygenase' function of the carboxylation enzyme that we had termed 'carboxydismutase.' Also, it was the reason for accumulation of glycolic acid during photosynthesis in the presence of air (Benson and Calvin 1950a, b). Tolbert, however, constructed an important C_2 cycle and recognized peroxisomes and their metabolic conversions of the phosphoglycolate.

Hiroshi Tamiya and photorespiration

In 1949, Tamiya and Huzisige had proposed that O_2 and CO_2 competed for a common site, although knowledge of biochemistry of CO_2 fixation was not available to them. Our 1950 publications reporting recognition of glycolic acid accumulation as indicator of oxygen involvement in its formation attracted the interest of Hiroshi Tamiya, a leading biologist of postwar Japan. He had been involved in planning C^{11} photosynthesis experiments with the cyclotron of Japan's

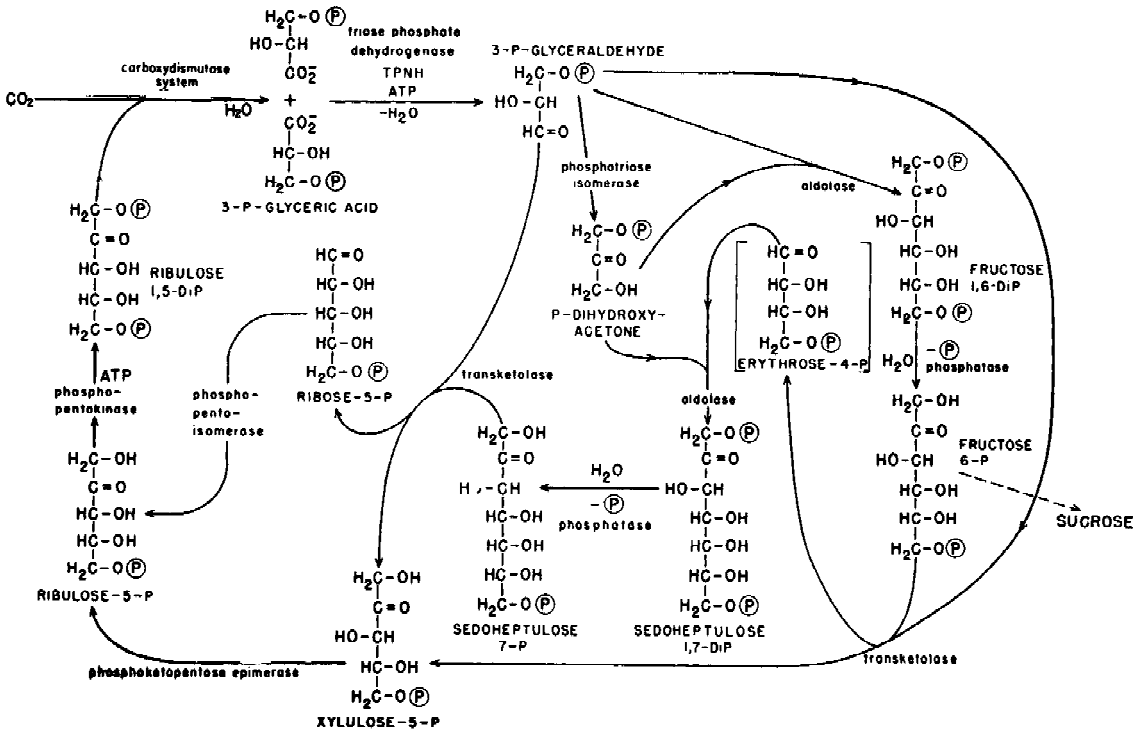


Figure 6. Photosynthetic carbon reduction cycle (*source*: Bassham et al. 1954).

leading nuclear physicist Yoshio Nishina, a friend of Ernest Lawrence in Berkeley. Tamiya, trained with René Wurmser in Paris and fluent in French and English, became a major guide and interpreter for the scientific representatives of the Allied Supreme Command after the World War II. A Massachusetts Institute of Technology (MIT) physicist, Harry Kelly, had been selected for this role and immediately recruited a loyal group of younger distinguished physical scientists, including nuclear physicists Yoshio Nishina, and Ryokichi Sagane (who had worked with Niels Bohr and Ernest Lawrence), and biologist Hiroshi Tamiya. Tamiya's planned photosynthesis research with one of Nishina's cyclotrons was terminated by a misjudged edict from the Supreme Command when Japan's five cyclotrons were destroyed. Reconstruction of Japanese science and reorganization of the governmental Institute of Physical and Chemical Research (RIKEN), resulted from the tireless efforts of Harry Kelly (Yoshikawa and Kauffman 1994) and later those of Bowen C. Dees of the Supreme Command in Tokyo. During this period, several groups of distinguished American scientists visited Japan and were guided by Hiroshi Tamiya.

Tamiya's plans for C^{11} research in photosynthesis were dashed by the destruction of Nishina's cyclotrons; however, he proceeded by examining kinetic evidence for interaction of oxygen with the intermediates of photosynthetic carbon dioxide fixation. In 1952, he and Nobuko Tamiya came to Berkeley, where he hoped to apply C^{14} techniques for recognition of 'photorespiration.' He and I did several experiments with algal suspensions and re-examined our information on the production of glycolate during photosynthesis in air and in carbon dioxide nitrogen mixtures. Though our conclusions were not exciting, I believe that Tamiya's quest was satisfying.

From that time, Hiroshi Tamiya became a 'scientific foster-father' for me. He sent his brilliant and richly cultured student, Kazuo Shibata, son of the famous artist Seiho Takeuchi, to our lab in ORL. Kazuo was involved in classic works, then and in years following. He was loved by all, and I considered him one of my finest friends. Later, Tamiya rescued my career by sending Bunji Maruo to collaborate with me in very successful discoveries after I relocated at Penn State in 1955. Hiroshi Tamiya was a leading figure in Japan and internationally in photosynthesis and all biology (Atusi Takamiya 1990).

Shinichi Kawaguchi and the kinetics of C^{14} incorporation

With paper chromatographic techniques vastly improved, it was time to look at the kinetics of C^{14} incorporation in the many compounds resolved in our chromatograms. Dr Shinichi Kawaguchi, Assistant Professor of Physical Chemistry in Osaka City University, the first Japanese scientist in our laboratory in ORL, arrived in 1949 (see Figure 5, bottom) for a photograph of Calvin, Benson and Kawaguchi); he was an ideal collaborator for study of kinetics. With a relatively large (100 ml) illumination chamber (dubbed a 'lollipop' by colleagues and Melvin), I added up to a millicurie of $NaHC^{14}O_3$ at $t = 0$ to a one cc suspension of *Scenedesmus* cells in 100 ml of water in steady-state photosynthesis and, with continued rapid manual agitation, took samples at 5, 10, 30, 60 s, 5 and 10 min. This series was not the only one. Perhaps five had been done, each requiring repetition because of inadequate chromatography or some other uncertainty. Finally, a complete set of paper chromatograms from each of the samples from the sixth time series was prepared. The data were plotted revealing the anticipated immediate labeling of PGA, and also very rapid labeling of malic acid. This indicated that the phosphoenolpyruvate (PEP) carboxylase system is operative.

Dephosphorylation by phosphatase-catalyzed hydrolysis

Since strong acid hydrolysis can destroy or damage many sugar phosphates, my colleague Nathan Tolbert recommended enzymic hydrolysis and the fungal phosphatase Polidase S. Phosphate esters, eluted from paper chromatograms were treated over night with Polidase solution and the product(s) rechromatographed for identification of the sugar or other products. Since each of the pertinent sugar phosphate esters were not unequivocally resolved, we eluted each of them, treated with Polidase, chromatographed the hydrolysate, prepared the radioautographs and counted the freed sugar products with large window Geiger–Müller counters. The maximum or asymptote of each curve related to the relative concentration of each of the intermediates (Benson et al. 1952b). We were not impressed with the value of trying to interpret the kinetics of labeling of the many other products. Sadly, Shinichi Kawaguchi, diagnosed with tuberculosis, was accosted by the local medical staff and summarily sent back to Osaka. We had many enjoyable reunions in subsequent years, during which time Shinichi served as President of the Japanese Chemical Society. Later, with more specific conditions and objectives, kinetic experiments by Al Bassham, Peter Massini and by Alex Wilson revealed information on factors influencing specific steps in the path (Calvin and Massini 1952; Wilson and Calvin 1955; Bassham and Calvin 1957; Bassham 1964).

Visits abroad

Norway 1951–1952

Melvin Calvin, his wife Genevieve, and her Norwegian mother visited Norway after his 1949 coronary infarction and recovery, during which I wrote our review for *Annual Reviews of Plant Physiology* (Benson and Calvin 1950b; also see Benson and Calvin 1950a). At the agricultural college, Professor Lindemann induced Calvin to send a colleague to Norway for establishment of a laboratory of radio-isotope applications in agriculture. This resulted in my appointment as Fulbright visiting professor and delightful experiences for me and my family. This also began our friendship with pharmacognosy Professor Arnold Nordal and his family. Nordal had provided me with his pure sedoheptulose and knowledge of its chemical properties. Later we collaborated in ORL and Scripps Institution of Oceanography. Several excellent students came from Norway.

Invitation from Otto Warburg

Being in Europe, I was invited to present a comprehensive review of the path of carbon, including the carboxylation of C_2 from ribulose diphosphate to yield PGA (Benson 1952), in Lindau before a meeting of the Bunsengesellschaft für physikalische Chemie, a distinguished group of photochemists, which included Otto Warburg. Sam Ruben had earlier introduced me to Warburg's work, his algae, and his manometry. Warburg was most interested in our results and an opportunity to recruit our support for his contentions that four quanta were required for fixation of CO_2 and production of O_2. He invited me to Berlin to observe how he grew his special *Chlorella* and made his measurements. It was a delightfully impressive experience to have lunch with him and Herr Heiss in their home.

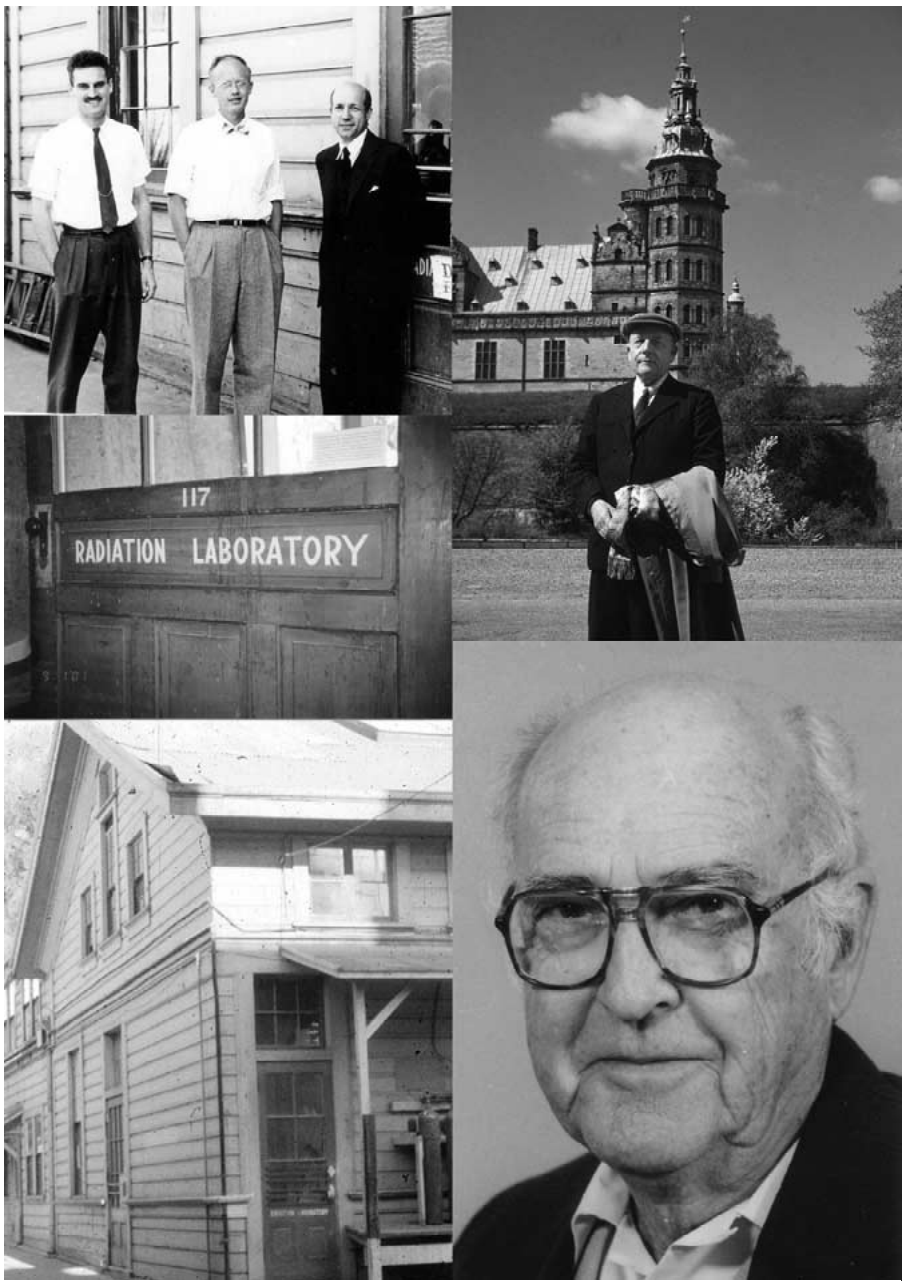


Figure 7. Top, left: Jacques Mayaudon, Andrew Benson, and Melvin Calvin, 1954, outside entrance to 'Old Radiation Laboratory.' The door behind Calvin was displayed in an exhibition on 'The early history of the nuclear age.' National Museum of Science and Industry, Washington, DC, 1995. There was a sign 'Do not Enter' on this door; it is through this door that all laboratory staff entered the building. *Middle, left*: a photo close up of the main door that was by the Guard house of ORL. It is now exhibited in the exhibition area of Lawrence Berkeley Lab, at UC Berkeley. This photo was taken in 2001 by Govindjee. *Bottom, left*: portion of the radiation laboratory. *Top, right*: Otto Warburg at Helsingör. *Bottom, right*: a portrait of Samuel Wildman, who isolated 'fraction 1 protein' that was later shown to be the carboxylation enzyme, Rubisco.

That afternoon I walked down the street to the quiet Dahlem Museum and into a small room where I found myself alone – with 'Nefertiti.' That thrilling experience still brings out the goose bumps. I appreciated Warburg's arguments, though they seemed easily interpretable on the basis of my own experiments with pre-illuminated algae. Later, with the Linderstrom-Langs at the Carlsberg Laboratory in Copenhagen, I

presented a seminar (April 1952), again with Otto Warburg in the audience (he was visiting Denmark to see his allergy physician). On a beautiful afternoon, I drove him and Herman Kalckar to 'Hamlet's Castle' at Helsingör (see a photograph of Warburg, Figure 7, top right). Warburg peered through an iron gate into the darkness below and said, 'Ach, that's a perfect place for that Midwest Gang.' This, of course, followed Warburg's stay in Urbana where the polemic over quantum requirement of photosynthesis had become heated and ended with hardly significant agreement.

The carboxylation and the carboxylase

Ribulose diphosphate carboxylation

A search for the receptor of carbon dioxide in photosynthesis would naturally involve illumination of algae or other plants in the absence of carbon dioxide. The receptor molecule should, of course, accumulate to some extent. Surprisingly, this experiment was not done before 1951 when ribulose diphosphate was identified (Benson, 1951). An experiment reported by Calvin and Massini (1952) clearly reported results of such experiment without pointing out the significance of the information. Illumination of C^{14}-labeled algae was ceased and the several labeled components separated and measured. An immediate increase of phosphoglyceric acid occurred concommitantly with an apparently identical decrease in ribulose diphosphate. This clearly revealed the function of ribulose diphosphate as carbon dioxide receptor. (I did not notice recognition of this relationship in the text of the publication or in the publication of Weissbach et al. 1957.)

With the carboxylation receptor and products recognized (Bassham et al. 1954; Bassham and Calvin 1957; Benson 1987a, b, 1990), Gérard Milhaud returned to Paris (1954) and his post in Institut Pasteur, down the hall from Claude Monod and the office of visiting scientists, Arthur B. Pardee and Bernard L. Horecker. With collaboration of C.B. van Niel, Milhaud was growing cultures of *Thiobacillus denitrificans* anaerobically. With $C^{14}O_2$ as substrate, he and his collaborators recognized the carbon reduction cycle of green plants, and photosynthetic bacteria as the same process for chemoautotrophic bacterial biosynthesis as well (Aubert et al. 1955; cf. Stoppani et al. 1955).

After the cycle seemed to be established: a side story on the demise of the thioctic acid hypothesis

I felt that the next important objective must be the carboxylating enzyme, and gave it my highest priority. I present here, however, a story that is not connected with the 'path of carbon,' but is of historical significance in understanding how Science moves. It is the story of thioctic acid (Benson 1996). At the time, Melvin Calvin was still more concerned with his exciting thioctic acid theory and its demonstration (Barltrop et al. 1954; Calvin 1954). For two years, it seemed like an exciting adventure, and it was entirely Melvin's idea. His work in photochemistry of organic molecules with Michael Polanyi and Gilbert N. Lewis prepared him well for work on photochemistry of photosynthesis; this was widely recognized.

It was the most exciting idea that I and many of my colleagues experienced. Melvin Calvin's 'Thioctic acid mechanism of photosynthesis,' a superb concatenation of information, ideas, and experimental evidence appeared to fit with all we knew of photochemical energy conversion in the chloroplast. It developed at the time thioctic acid (lipoic acid) and its function had just been discovered. It is a yellow compound, with absorption at 330 nm, capable of accepting energy from an excited chlorophyll molecule. The absorption of energy by thioctic acid seemed plausible. The product, a dithiyl radical [R-S$^{\bullet}$ $^{\bullet}$S-R], was consistent with the plethora of sulfur radicals detected in photosynthetic tissues with the then-novel EPR spectrometers. John Barltrop, who had come from Oxford University's chemistry department, proceeded to develop experimental support for the theory. He and Calvin collected convincing evidence for the reaction of such radicals with water or alcohols. For this work, they had received from my former Caltech student, John Brockman, a collection of synthetic thioctic acid and a number of its analogs. Thus, it was hypothesized, that photolysis of the strained disulfide ring in water could yield both R-SH and R-SOH, a sulfenic acid, on the same thioctic molecule, one, a reducing agent, and the other, a sulfur analog of an alkyl hydroperoxide capable of yielding oxygen. The energy of the quantum absorbed by chlorophyll then might yield the essential requirements for photosynthesis.

Finally, Barltrop and Calvin tested the hypothesis in *Scenedesmus* treated with added thioctic acid; oxygen production increased 50%. The plausibilty of the theory was elegantly developed in over 40 pages of ensuing publications documenting the experimental

evidence. Had Nature overlooked this opportunity it would have made a mistake, it seemed. The quality of the research was superb, as one can appreciate from the meticulous publications. For two years the whole effort was exhilarating. It was truly a Nobel idea.

The high point of this saga was Melvin's lecture in 1954 at the American Association of Advancement of Science (AAAS) meeting in Berkeley. Beginning with his usual hesitant manner and leading to a magnificent crescendo of convincing evidence for the mechanism of the quantum conversion of photosynthesis, the audience was totally impressed. The great C. B. van Niel jumped from his seat in the front row with tears in his eyes to congratulate Melvin. It must have seemed a consummation of his own decades of thought and effort dedicated to understanding photosynthesis.

The final proof lay in identification of thioctic acid in the chloroplast, but the assay was tedious and required microbiological experience. I grew some *Chlorella* in sulfate-S^{35}, chromatographed the extract, and prepared the radio-autograph of my paper chromatogram. With Melvin and the others standing around the great white table, I laid the film on the paper. There was a huge black spot, right in the position we expected for thioctic acid. Melvin's eyes just about dropped out onto the film. It was a breathtaking moment. The S^{35} radioactivity had to be proved to be thioctic acid. Clint Fuller with a new PhD from the Stanford laboratory of Ed Tatum, had been recruited a year earlier to study bacterial photosynthesis and was now conscripted for the sensitive microbiological assay (see Fuller 1999). Try as he would, Clint and his *Streptococcus fecælis* bacterial assay could not detect a trace of thioctic acid! One by one, the evidence for the several critical steps weakened and the thioctic theory quietly evaporated. The massive effort, the elegant chemistry and photochemistry produced impressive publications, which no longer attract attention. *Yes, the theory was in ashes but we should see a 'take home lesson' in this saga. One can survive a failed effort; even one which had involved many man-years of work and excitement* (Benson et al. 1959; Benson 1995). Melvin's faith in the theory dimmed only slowly. He included its tenets in Path XXI, the photosynthetic cycle. He even included the theory and its evidence later in his lecture on the Path of Carbon in the Biochemical Congress in Brussels in the spring of 1955 (Calvin 1956).

The primary carboxylation reaction: search for carboxylase

I was off on a search for the carboxylase. I knew how its activity could most easily be assayed; I knew how to prepare the substrate, ribulose diphosphate, in sufficient quantity. It would be the world's supply of the pure compound with which we could assay enzymatic carboxylation using $C^{14}O_2$ and measuring fixed radioactivty which would be in the phosphoglycerate produced. I enlisted Clint Fuller and Rod Quayle, who had been only minimally associated with the Thioctic Acid project. Both of them brilliant, fun, and wonderful friends, and it was a delight to work with them. Fuller was experienced in breaking cells for enzyme preparation; Quayle was a superb chemist and scholar in all realms. I would supply the RuDP substrate; I had done it many times before and was confident in its purity and the results. Preparation of the RuDP was easy for me. I collected extracts of *Scenedesmus* cells that had been quickly extracted under conditions of maximal RuDP content. Cells doing photosynthesis in optimal CO_2 concentration (4%) were flushed with nitrogen and then killed in hot ethanol. I prepared stripes of the extract on our large sheets of Whatman No. 4 paper with small samples of C-14 RuDP at the ends to establish the location of the desired product. Here, and later, I was making dozens of such chromatograms of algal extracts, eluting the pure RuDP for the assay substrate. Fuller would make the sonicated cell-free preparation and Quayle would measure the C-14 in the product fixed by the vital solution. We had a ball; the carboxylation worked well. Our manuscript written, Melvin's name was added; there was no alternative. It was submitted for internal University of California Radiation Laboratory (UCRL) review in May, 1954 and published in the Journal of the American Chemical Society on July 5, 1954 (Quayle et al. 1954).

The next step toward the carboxylase went well – up to the publication: exciting months in the laboratory with Jacques Mayaudon (1954)

The disappointment of the demise of the Thioctic Theory probably left Melvin's ultimate objective in disarray. I continued my ongoing project with Jacques Mayaudon, which I considered extremely important – to follow the previous successes with isolation of the enzyme processing most of the CO_2 fixation on earth.

Jacques Mayaudon came to the ORL with a Fellowship of a Belgium Foundation, 1954–1955. (The

project, which Calvin, asked Jacques to develop in Donner Laboratory proved less than stimulating to him, and he came to me hoping to work on photosynthesis, since our lab had discovered most of the important aspects of the path of carbon in photosynthesis by that time.) I was anxious to follow the carboxylation process by demonstrating and, hopefully, isolating the enzyme responsible for the process which Calvin had properly called 'carboxydismutase'. Jacques Mayaudon was an ideal collaborator (see a photograph of Mayaudon, Benson and Calvin, Figure 7, top right, in front of our entrance door to 'ORL') Figure 7 (middle, left) shows the main door; and Figure 7 (bottom, left) shows a portion of the radiation lab building.

Very fortunately I had frequently visited Sam Wildman (see his photograph, Figure 7, bottom right; and also see S.G. Wildman, this issue) at Caltech in Pasadena, where I had earned my doctorate in chemistry and where my wife's family resided. I followed, from the very beginning, Wildman's exciting work (with James Bonner) in isolating and characterizing the major protein of leaves, which they had named 'fraction I protein.' It is the predominant protein in phototosynthetic tissues, but neither Wildman nor his associates recognized its function as the critical enzyme in the path of carbon in photosynthesis at that time (see Wildman 1998).

Jacques Mayaudon was familiar with ammonium sulfate precipitation for purification of proteins and their characterization based upon the required ammonium sulfate concentration for their precipitation. We wanted to use spinach for the enzyme source, but it was not available in Berkeley that time of the year, so we used 'New Zealand Spinach' which was just as suitable for our purpose. Jacques homogenized the leaves and precipitated the solubilized protein with increasing concentrations of ammonium sulfate. We worked day and night, running dozens of large stripe chromatograms for isolation of the substrate. Jacques was grinding spinach and precipitating, centrifuging and re-dissolving and re-precipitating. He was a delightful and enthusiastic colleague; we enjoyed working together. In an enthusiastic mood, he once exclaimed, 'If Science was a woman, I would be a great lover.' At each step, we assayed the enzyme activity with my ribulose diphosphate and $C^{14}O_2$. Melvin Calvin had been totally disinterested in this sort of project, but he discerned that we were frantically working long hours and eight days a week. Soon it dawned on me that Jacques was finding that the enzyme activity was being concentrated by the same sequence and ammonium sulfate concentrations that Sam Wildman had found for his fraction I protein. We had made an important discovery. It was exciting for me, even more so than my most important discoveries. Sam Wildman recalled (22 July 2001, in conversation with Govindjee) that I had telephoned the news in 1954 but also knew that nothing had been published rapidly. I had typed a brief manuscript describing our discovery and including extensive reference to Wildman's 'fraction I protein'; it was in the format for a Letter to the Editor of the Journal of the American Chemical Society, where many of our earlier works had been published. Being a government laboratory, it was required that publications pass through an 'in-house-review'; I submitted the manuscript to Melvin Calvin. The results of our tremendous efforts could have been published in 1954, but first appeared in print late in 1957 (Mayaudon et al. 1957) with no mention of the fraction I protein. Possibly Melvin did not recognize its importance – since he was unfamiliar with and disinterested in the work of Sam Wildman at Caltech. I left the laboratory at the end of 1954 and was unable to follow the work. Jacques continued in 1955, masterfully documenting our discovery. Identification of the fraction I protein with the carboxydismutase protein appeared in print in 1957 (Mayaudon 1957).

B.L. Horecker and colleagues purified ribulose diphosphate carboxylase in 1954 (Horecker et al. 1954), but did not recognize its identity with the Fraction 1 protein. In Sam Wildman's laboratory, Robert Dorner and Albert Kahn (Dorner et al. 1957) recognized the apparent identity of the fraction 1 protein and the carboxylation protein with its 18S sedimentation constant observed by Weissbach et al. (1956). Only later did P.H. Chan et al. (1972) (Andrews et al. 1973) unequivocally establish the identity of the fraction 1 protein and ribulose diphosphate carboxylase. One may be further enlightened by reading Melvin Calvin's (1992) autobiography, a volume elegantly entitled 'Following the Trail of Light.'

Thus ends the rich tapestry of my part of the Path of Carbon in Photosynthesis, including its few 'dropped stitches.'

Subsequent activities: 1955–2002

The easiest path for me is to refer the reader to Benson (2002) for my research activities in other fields, and to list here some of these later excitements. (1) Discovery and identification of a major membrane phospholipid,

phosphatidylglycerol, an important component of bacterial membranes and of all algae and green leaves (with Bunji Maruo). (2) Discovery and identification of the sulfolipid of plants, probably the best detergent molecule in nature (with Helmut Daniel). (3) Development of the study of sulfocarbohydrate metabolism. (4) Development of neutron activation paper chromatographic analysis. (5) Recognition of wax ester as a major marine nutritional energy source and its role in providing for survival of marine animals (with Judd C. Nevenzel and Richard F. Lee). (6) Discovery of the intermediates of arsenic metabolism in aquatic plants and the unique arsenolipid produced by such plants (with Bob Cooney). (7) Discovery that the highest concentration of arsenic, known to accumulate in living organisms, is in the kidney of the giant clams of the Great Barrier Reef, Australia (with Roger E. Summons). (8) Discovery of related stibnolipids in algae. (9) Further, with Gérard Milhaud, I utilized spawning salmon as a model for study of the degenerative process of aging in humans. (10) We recognized the importance of calcium regulation for the salmon. (11) With Arthur M. Nonomura, we discovered methanol stimulation of plant growth and productivity of agricultural crops. (See Benson, 2002, for additional information.)

A tribute: Paul Saltman, a fleeting spirit in photosynthesis

I end this article by paying a tribute to Paul Saltman. Throughout the period of development of the path of carbon in photosynthesis, there appeared wonderful fleeting spirits which lighted the path with their brilliant exuberance and spirit of supportive camaraderie. Such a spirit was Paul Saltman. With brilliance in elocution to match his striking physique, Paul provided wit and often sage criticism based on his experience with the finest teachers as an undergraduate and graduate student at Caltech. An All-American in sports and an avid researcher in cell-free photosynthesis (Saltman et al. 1956) and phosphorylation, his contributions to cohesiveness of the workers in the field enlightened us all. Often the goat of clever collegiate pranks (such as being handed fake telegrams reporting that his NSF Grant was terminated while in the midst of delivering his paper at national meetings), Paul became a leader in providing cohesiveness and wonderful humor among those involved in following the path of carbon. He showed us time and again that science is fun. Paul went on to a great career in teaching and administration which provided international impetus for good science teaching. He touched more lives with his desire to improve teaching and appreciation of science education than any of us had realized during our exploration of the path of carbon in photosynthesis.

Acknowledgments

I am thankful to Govindjee for pestering me for 13 years to write this perspective. He goaded me by e-mail, and during August–November 2001, he provided information, and his excellent editorial skills that led to production of this version of this perspective. I also thank him and Howard Gest for visiting me in my laboratory during August on a NSF grant (SES 00-92507) to provide an impetus to finish this story. I am also grateful to James (Al) Bassham, Martin (Marty) Gibbs and R. Clinton (Clint) Fuller for reading my story and for providing suggestions to improve my perspective, not all of which were incorporated here. My wife Dee has provided incentive to 'finish this story and get on with life.'

Notes

[1]This article will use C^{11} and C^{14}, which was the convention at the time. Further, it is superior from a diadactic standpoint, i.e. in speaking, to the current convention, i.e. ^{11}C and ^{14}C.

[2]The Rat House, officially the Chemistry Annex, was a shingled wooden structure well built in 1915; it was termed Rat House because of a previous use for biological experiments and its population of escapees. Only its single classroom was finished with a plastered surface. The laboratories were unfinished and readily adaptable for experimental wiring and other construction.

[3]To reduce the respiratory exchange, the experiments were done under nitrogen. Sam was apprehensive about problems resulting from respiratory exchange. Counting C^{14} was a chore and difficult to achieve reproducibility. He used Libby Screen Wall Counters to measure the activity, but they required repeated assembly with deKhotinsky sealing compound for each sample. I made new ones with standard taper joints, which could easily be opened and reused. Samples were dried on the interior surfaces of glass cylinders, which could slide over or away from the screen wall of the Geiger–Müller counter. It was filled on the high-vacuum line with counting gas and its voltage plateau determined each time. With the low activities, the work was tedious. To follow the chemical reactivity of the unknown product(s), I converted the fixed activity to derivatives in an endeavor to discern their structure, diazomethane for carboxyl groups and acetic anhyhdride in pyridine for hydroxyl groups. Evidence for reaction was estimated from behavior of the activity in partition between water and ether or water and ethyl acetate.

[4]While a chemistry graduate student at Caltech, Pasadena, I had registered as a Conscientious Objector with my Pasadena Draft Board. A small group of students met frequently out on the playing

field at noon hours with Bob Emerson, from a distinguished Quaker family, who provided support for our convictions.

[5]The floors were yellow with uranium salts and had to be covered with cheap linoleum. I designed the two chemical benches with excellent over-the-bench fluorescent lighting and large clean porcelain sinks, a far cry from those in the old brick chemistry building. With the glass shop, carpenter shop and machine shops next door, it was a superb place to begin.

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