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Paving the Path: A tribute to Sam Ruben, 1913 -1943

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Introduction

The well known Path of Carbon in Photosynthesis is part of biology but many of its beginnings are not. I offer here some anecdotes of the serendipity which led to my own involvement in that adventure (Benson, 2002) The powerful influence of Sam Ruben on studies of the path of carbon is reviewed.

University of California, Berkeley. My chemistry courses were punctuated with forays into anthropology, european history and engineering. Optical physics from Luis Alvarez (later, Nobel Laureate, N.L.) included his arrival for class one morning almost pale with fevered excitement. The word that Lise Meitner and Otto Hahn (N.L.) had reported nuclear fission was a cataclysmic shock in the Berkeley physics department. Of course, the fact that Europe was on the verge of conflict enhanced his apprehension. The optics lecture was dispensed with. I had been taking a superb course in European history from Professor Kerner who brought day-to-day tension to reality as Hitler took the Sudetenland and Austria. In the other corner of the campus I was enjoying courses in civil, mechanical, chemical and electrical engineering as well as fluid mechanics. As chemistry progressed, a few of us lab mates often snuck into the Thursday afternoon seminars under the cloud of smoke from the Philippine cigar of G.N. Lewis. I was making trinitrotriphenyl methane for Glenn Seaborg's research with Lewis on color of molecules. Melvin Calvin had just joined the faculty in 1937; his excellent questions often dominated discussions after the seminar.

Climbing at Caltech — 1939-1942. My applications to several schools were accepted. I chose Caltech because of its proximity to home and friends nearby. During my three years in Pasadena I followed all the carbohydrate chemistry courses delivered by Carl Niemann, student of Karl P. Link, University of Wisconsin. In the Niemann laboratory, directly above Linus Pauling's office, I enjoyed fellowship and advice from superb colleagues. Jim Mead, a year ahead of me, guided the technical aspects of my research on synthesis of fluorinated thyroxine analogs, a 20-step synthesis which started with a gallon of anethole (anise oil) oxidized in flames, within a flask of hot nitric acid and finished with two grams of difluorodiodothyronine. Jim was working on the structure of sphingosine which later became part of my thesis work on periodate and lead tetraacetate degradation of its vicinal amino glycol. This experience led directly to my subsequent work with Al Basasham on sedoheptulose and ribulose degradations. Jim Mead followed the discovery of the fatty acid alpha oxidase in avocado by Paul Stumpf with his recognition of the alpha oxidase enzyme in brain and nervous tissue. It is responsible for initiating degradation of phytanic acid from chlorophyll, thus avoiding the rare but debilitating "Refsum's Disorder".

The war in Europe was raging and I recall clearly, as does everyone, the news of December 7, 1941. Conscription had begun and decisions were considered. Our family supported conscientious objectors and sympathy with the tenets of the Society of Quakers. A small group of us at Caltech met frequently at noon hours with leadership of Robert (Bob) Emerson,

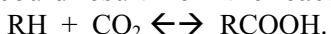
pioneer in photosynthesis and descendent of the distinguished Quaker family. I registered as a Conscientious Objector.

Managed from above. I had just finished describing my synthesis of fluoro-iodo-thyronines, sphingosine analogs, neurobiology of the sea scallop and discussion of my ten 'Propositions' before my Doctoral Committee in the afternoon of May 12, 1942 when Linus Pauling asked one more question: Andy, can you write on the board the differential equation for decay of a radioactive isotope? Well, I managed to get it down all right; but it was a surprise. I had absolutely no idea of why he asked that question; it had nothing to do with my doctoral thesis or training at Caltech! But, knowing his approach after several years in his classes, it was not unusual and proceeded to forget all about that incident. He had embarrassed me one evening in the lab, finding me balancing a twelve-liter flask of hot methanol on the edge of my sink, and suggested that it might not be necessary for me to go further in his quantum mechanics courses; I had not done at all well in the final exam.

In a week or so, I received a letter from Department Chairman Joel Hildebrand in Berkeley inviting me to join the faculty of the Department of Chemistry as Instructor. The salary would be \$2,000.00 per annum. That was nearly \$167 dollars a month! It was a stellar opportunity. So, why did Pauling ask me that question?

With Sam Ruben. Sixty years later, it dawned on me that Linus Pauling at Caltech and Wendell Latimer, Dean of the College of Chemistry, had contrived to provide support in organic chemistry for a young and rising star in the Berkeley faculty, Chemistry Instructor Sam Ruben who was on the verge of being advanced to Assistant Professor. So, when I arrived on July 1, 1942, Latimer directed me to the Rat House where Sam welcomed me to a small Office/Laboratory and mass of dirty glassware freshly deserted by Henry Taube (N.L.,1983). Sam introduced me to the Warburg Apparatus in the dingy lab upstairs lighted by bare clear light bulbs. He handed me his copy of *Manometric Methods* by Burris, Stauffer and Umbreit and explained that he needed to know more about the ratios of photosynthesis to respiration in his *Chlorella*. Needless to say, I did not realize that Bob Burris must have been but a few years my senior, nor that his son, John would later be earning his doctorate in my laboratory, nor that his student, Nathan Edward Tolbert (1997) would become a close friend and colleague who later would convince the world that photorespiration exists in spite of my refusal to consider it very remarkable.

Ruben et al. (1939) had observed reversible fixation of $C^{11}O_2$ forming a water-extractable product possessing a carboxyl group. They had deduced from their early fixation experiments that the product(s) of dark fixation could result from the reaction



van Niel et al.(1942) had pointed out that in bacterial photosynthesis no O_2 is produced and that bacteria must have access to a reducing agent to provide hydrogen for the reduction of CO_2 . No oxidizing agents present in living things are powerful enough to dehydrogenate water except for the photochemical reaction centers of photosynthetic organisms. Sam Ruben had many distinguished colleagues -- H.A. Barker, W.Z. Hassid, M.B. Allen, M. Randall, R. Overstreet, T.C. Broyer, S.F. Carson and C.B. van Niel. With Martin Kamen, Ruben and his students published over thirty papers from 1939 to 1942, a phenomenal record.

At each step of these developments, Ruben and Kamen considered cyclic processes for the regeneration of the CO_2 acceptor, R-H. With the imposing influence of phosphorylation processes being developed by Herman Kalckar and by Fritz Lipmann, Sam Ruben increasingly leaned toward the concept of the high energy phosphate bond participating in the process. At this point, I arrived in the Rat House and was immediately influenced by Sam's enthusiasm expressed in *Photosynthesis and Phosphorylation* (Ruben, 1943). Sam was influenced by the exciting developments from Otto Warburg and handed me copies of the latest papers from Berlin. Needless to say, I had no idea that in a few years I would be with

Otto Warburg in his laboratory in Dahlem. Or, that Warburg would later send me a check for \$1000.00 to attend, with Clint Fuller, his lecture in Strassbourg, 1963.

I immediately joined Sam and his students in many late night experiments with $C^{11}O_2$. Physicists dominated their cyclotron's time. So Martin Kamen could only use it at night. Fortunately deuteron bombardment of Martin's boron targets were not time-consuming and experiments with C^{11} began about 8 pm. Each was meticulously planned and orchestrated by Sam. I recall none which failed because of inadequate planning. On two occasions, Martin called just before the experiment, "Cyc's sick, Sam". A disappointment, but an opportunity for us to go home early! In the lab, Sam revealed little of his family life. A poignant biography of Sam Ruben and his family (Harold S. Johnston, 2002) will provide insight to the character and innate 'drive' of a remarkable man. Never was there any friction involved in working with Sam Ruben. With little children to manage it was not at all easy for Helena, trapped by children from a promising career in chemistry, or for Sam. He was a gentle human who drove himself but did not demand the same of his colleagues, though he certainly appreciated their efforts with good humor. Charlie Rice was "Rice Crispies" and Mary Belle Allen was "Madam Curie". These were the last of a long series of such experiments (Ruben et al., 1939, 1942). Though long-lived radiocarbon was 'invented' by Ruben and Kamen (1941), they had not applied it for photosynthesis study; its specific activity was too low and its measurement too tedious. Soon it was 1943 and Sam's efforts were directed towards a meteorological effort (war gas defense). Martin Kamen, too, was engaged by the local shipyard (Kamen, 1985). At this point Sam gave me ALL of the $BaC^{14}O_3$ in the world to follow the Path of Carbon. Coupled with an appointment in the finest chemistry department in the country, such a golden opportunity now seems near to unbelievable. At the time, I was too embedded in the problems at hand to realize where I trod on the path of carbon being paved, its ultimate end still over the horizon.

With the $C^{14}O_2$ I carried out a series of dark fixations, following Sam and Martin's concept of reversible reaction with R-H to yield R-COOH. I did many dark $C^{14}O_2$ fixations with *Chlorella*. To reduce respiratory exchange, they were done in nitrogen, not a good idea. Counting C^{14} was a chore and difficult to achieve reproducibility. Sam had some Libby 'Screen Wall' counters for it. But they required repeated assembly with deKhotinsky compound. I designed them with standard taper joints which could easily be opened and re-used. Samples were dried on the interior surfaces of glass cylinders which could be slid over, or away from, the screen wall of the G.-M. tube. It was refilled on the vac line with fresh counting gas and the counting voltage plateau determined. 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 anhydride in pyridine for hydroxyl groups. Evidence for reaction was estimated from behavior of the activity's partition between water and ether or water and ethyl acetate. I could confirm Ruben and Kamen's conclusion that the product possessed a carboxyl group; the partition coefficient between water and ethyl acetate was 0.14. That product of dark fixation finally crystallized and, with the experienced assistance of Ed McMillan (N.L., 1951), I showed it was succinate- C^{14} (Benson, 1996).

Phosgene and Ruben's Accident Sam had visited the army's Dugway Proving Ground where tests with goats indicated that phosgene exposure induced lung edema and that the fluid was similarly antigenic to other goats. Having been exposed to the immunological studies of Dan Campbell and Linus Pauling, my theory was that phosgene, a double acid chloride, could couple two proteins or so alter the conformation of one protein to yield a novel antigenic protein. So I developed rapid phosgene synthesis from the 20-minute $C^{11}O_2$ from Martin Kamen and the 37-inch cyclotron. Sam's technique with the phosgene-exposed rat was to drop the victim into the Waring Blender to produce a protein preparation for measurement of

its C-11. For Sam's own phosgene experiments I had fitted small steel bombs with valves so I could fill them with phosgene from the ancient Kahlbaum ampoules lying in sawdust in back of the chemical store room. Not having a chemical hood I did this carefully with ice-cooled ampoules and vacuum transfer to the steel bombs. I still insist that all students should recognize the odor of phosgene.

Finally it became impossible for Wendell Latimer to extend my teaching contract and I left for Civilian Public Service in the Nevada mountains, fighting forest fires, building dams, and logging for Forest Service construction projects.

Later, when I had departed, Sam's phosgene supply became exhausted and he tried to transfer liquid phosgene (b.p. 8 °C.) with his broken arm in a sling. 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 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. 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. (Harold Johnston, "Sam" 2002.)

Associate Director, Photosynthesis Laboratory, ORL 1946 --1954 In May 1946 came the invitation from Melvin Calvin to join his bio-organic group as Associate Director of the yet non-existent photosynthesis laboratory. It was to be a separate branch of his Bio-Organic Group and in the Old Radiation Laboratory, ORL, the birthplace of the nuclear age and home of Ernest Lawrence's 37" Cyclotron in the center of the old maple-floored well-built wooden building. Though still functioning, the cyclotron was scheduled for transfer to UCLA's physics department and we occupied the long room just west of the central cyclotron area. Its floor was orange-yellow with uranium residues and somewhat radioactive. This problem was easily solved by a cover of low-cost brown linoleum.

Paper chromatography. Paper chromatographic separations are the result of partition between the moving organic phase and the stationary aqueous phase. As the solvent progresses through paper, its composition changes as does the partition coefficient. The partition coefficient for each substance and solvent system is unique and was the basis of the partition experiments with my C-14 photosynthesis and dark fixation products in 1942-43. I recall clearly two of Glenn Seaborg's 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. An important attribute of our application of two dimensional paper chromatography (Benson et al., 1950) was the practice of applying an aliquot portion of the 'total extract' of the plants labeled in the experiment. It is absolutely essential that the whole assembly of products be examined in the chromatogram. Nothing can escape.

Pattern recognition Alex Wilson, as Dean in New Zealand's University of Waikato, hoped to measure genius in students. He realized that there are at least two types of genius — those capable in mathematical concepts and those capable in pattern recognition. The first category includes the peoples from Asia and the Middle East, strong in numerical and business transaction capabilities. The second includes the artists with their strengths in recognition of color or structural relationships. The real geniuses in pattern recognition, Alex said, are the Australian Aboriginal Black Trackers whose capabilities for tracing animals or criminals over a boulder patch are well documented. Their powers for detecting, observing and storing relationships are phenomenal. Such was the recognition of novel information revealed in the hundreds of two-dimensional paper chromatograms accumulating in my studies of $^3\text{H-H}_2\text{O}$, $^{14}\text{CO}_2$, ^{35}S , ^{32}P , ^{74}As , ^{75}Se and ^{125}Sb metabolism.

Norway 1951-1952 Melvin Calvin and his wife Genevieve and her Norwegian mother visited Norway after his 1949 coronary infarction and a year of recovery, during which I wrote our review (Benson and Calvin, 1950). At the agricultural college, Professor Lindemann induced him to send a colleague to Norway for establishment of a laboratory of radioisotope 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.

Invitation from Otto Warburg Being in Europe, I was invited to present a paper (Benson, 1952), a comprehensive review of the path of carbon, including the carboxylation of C-2 from ribulose diphosphate to yield PGA in a meeting of the Bunsengesellschaft für physikalische Chemie in Lindau, a distinguished group of photochemists which included Otto Warburg. He was most interested in our results and an opportunity to recruit our support for his heretic contentions that four quanta were required for fixation of CO₂ and production of O₂. He invited me to Berlin to observe how he grew his special *Chlorella* and made his measurements. I appreciated Warburg's arguments, though they seemed 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, 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 Helsingør. Warburg peered through an iron gate into the darkness below, "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 broiled.

The Cyclic Path. Ruben's concept of using C¹⁴O₂ to follow kinetically its fixation and reduction was simple and logical. I set up a system for rapid collection of algal samples from 5 sec. to 10 min photosynthesis and Shinichi Kawaguchi and I prepared the series of two-dimensional paper chromatograms and their radioautographs. The kinetic curves clearly showed that two carboxylation products, phosphoglycerate and malate appeared linear from time zero. The more complex transketolase interactions of sedoheptulose and ribulose are elegantly reviewed by Al Bassham and Clint Fuller; they need not be repeated. The earlier preillumination experiments provided evidence that a CO₂ acceptor molecule, in considerable quantity, was included in the observed collection of compounds on the chromatograms. Ribulose diphosphate was the most likely receptor capable of rapidly producing so much phosphoglycerate. I knew it was relatively unstable, producing phosphoglycolate and phosphoglycerate on standing, an indication of considerable enolization, which soon became the basis for interpreting the mechanism of carbon dioxide fixation (Bassham et al. 1954). Quantum conversion. **Enthusiasm for Melvin Calvin's proposed thiocetic acid theory captivated the laboratory for two years (Fuller, 1999 p.12). However, I continued with demonstration of the enzyme-catalyzed carboxylation of ribulose diphosphate with Fuller and Rod Quayle.**

Carboxydismutase. Finally, Jacques Mayaudon, with a Belgian Fellowship from Louvain, and I proceeded to isolate the enzyme, now known as Rubisco. For several years I had frequently visited Sam Wildman at Caltech, learning of his procedure for isolating Fraction I Protein. While I isolated ribulose diphosphate for my carboxylase assays with C¹⁴O₂, Jacques deftly concentrated the spinach enzyme activity. Soon, it dawned on me that his ammonium sulfate precipitation concentrations were exactly those of Sam Wildman for Fraction I Protein (Singer et al. 1952, Wildman, 1998). The carboxylase and 'Wildman Fraction I Protein' were identical (Dorner et al. 1957)! I consider it my most exciting adventure in ORL. I wrote a note describing the discovery with many Sam Wildman Fraction I references for publication and submitted it to internal Radiation Laboratory review before

leaving, with pride in my accomplishments, for Pennsylvania State University on January 1, 1955 (Calvin, 1955 p.215; Mayaudon 1957).

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