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Minireview

Along the trail from Fraction I protein to Rubisco (*rib**u**lose bis*phosphate *c*arboxylase-*o*xygenase)

Sam G. Wildman
Department of Cell, Molecular, and Developmental Biology, University of California, Los Angeles, CA 90024, USA; Address for all correspondence: 1024 Centinela Avenue, Santa Monica, CA 90403, USA (e-mail: swildman@ucla.edu; fax: +1-310-828-9347)

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Abstract

This historical minireview deals with events leading to the eventual discovery of Rubisco (ribulose bisphosphate carboxylase-oxygenase). This abundant leaf protein is not only responsible for the net fixation of CO_2 in all plants, but also causes the loss of carbon through *photorespiration*. The latter is a special 'problem' of the so-called 'C_3' plants. The protein was first called 'Fraction 1 protein' before it was recognized to be the same as Rubisco. Instead of reinventing words, text as needed has been freely used from three earlier publications (Wildman and Kwanyuen 1978; Wildman 1992, 1998)

At Ann Arbor: Chibnall's purification of leaf proteins

In 1940, a chance encounter with a recently arrived book at the University of Michigan Library inspired my interest in working on leaf proteins. That year, I was a graduate student in the Botany Department floundering around with an assigned research problem. The book was 'Protein Metabolism in Plants' by A.C. Chibnall (1939) of Imperial College, London. Only a small portion of Chibnall's book dealt with leaf proteins and that, for the most part, dealt with experiments conducted by his own laboratory group. What caught my eye was a new approach to removing nonprotein, nitrogen-containing contaminants prior to extraction of the leaf proteins.

Chibnall had observed that immersing an *intact* fleshy leaf (e.g., that of spinach) in ethyl ether for a brief instant was sufficient to destroy the semipermeability of the cell membranes and to allow a brown juice to escape by hand-squeezing the leaves. The brown juice was rich in nitrogen-containing substances, but almost devoid of proteins. The flaccid leaves would reimbibe distilled water whereupon more brown juice of a somewhat lighter shade could be removed by squeezing, and with several more repetitions of the imbibing-squeezing process, the end product would become an almost colorless liquid. The intact cell walls of the leaf had performed as ultrafilters. They allowed the ready passage of low molecular weight metabolites in and out of the cells while denying passage of higher molecular weight proteins. Chibnall could now obtain the proteins by macerating the pretreated leaves in a hand meat grinder, disrupting the cell walls, and allowing dispersion of the remaining cell contents into a salt-containing or a buffered solution. The liquid obtained was intensely green in color, but by judicious addition of ammonium sulfate, the green material could be preferentially flocculated and removed by filtration to leave an amber filtrate containing abundant protein. Adding acid to the amber solution to about pH 4.5 flocculated the protein. Adding more acid to about pH 3.5 redissolved the 'flocculum' as the protein passed through its isoelectric point. Alternatively, the flocculum would redissolve by increasing the pH to about 6. The flocculated

protein could be removed by filtration for analytical studies.

At Ann Arbor: colloboration with Solon Gordon

I recall having been so mesmerized by what Chibnall described that, with my own money, I bought some spinach (*Spinacea oleracea* L.) from a local market, borrowed a meat grinder, and proceeded to repeat Chibnall's experiment. When I reached the amber solution stage and added acid, the flocculum appeared**!** Adding more acid made it disappear, adding base made it reappear, adding more base made it disappear. I called to my laboratory partner, Solon A. Gordon, also a first-year graduate student, to come and watch as the flocculum was repeatedly made to appear and disappear. He was as surprised as I.

Our mentor was the auxin physiologist Prof. Felix Gustafson. Sol had already been taught the *Avena* bio-assay technique and was using it for his assigned project. Based on flimsy evidence, speculations had been advanced that auxin became attached to proteins during extension growth of plant cells. I suggested to Sol that Chibnall's new method offered the opportunity to investigate which, if any, of the leaf proteins auxin might be attached. I proposed to isolate the leaf proteins and treat them with proteolytic enzymes as a way of releasing the 'bound' auxin, if Sol would extract the digests and test them for auxin activity by the *Avena* bio-assay. He agreed to this arrangement and, in short order, we found that auxin could indeed be released from leaf proteins by crystalline proteolytic enzymes. Moreover, two different protein fractions could be obtained from Chibnall's solution of 'cytoplasmic' proteins. More auxin was released from one of the protein fractions than the other. Auxin was also released from the 'chloroplastic' material but in lesser amount than the 'cytoplasmic' proteins (Wildman and Gordon 1942). Sol and I also found that ca. 10 times more auxin was released by heating the leaf proteins in weak base than was released by proteolytic enzyme activity. We became suspicious that tryptophan released during alkaline degradation of the leaf proteins may have been transformed by oxidative decarboxylation into indoleacetic acid, also known as heteroauxin. Tryptophan alone in warm, weak alkali was found to produce heteroauxin (Gordon and Wildman 1943).

Loss of our notebook

Sol and I were excited by what we were finding but were soon to be thwarted by two obstacles. We had both been entering our experimental results into a single lab notebook that was kept on a laboratory counter. Nearly a year's experimental data covering many pages had accumulated in that notebook. In discussing our results with our fellow students, faculty, and Prof. Gustafson's occasional visiting firemen, we were accustomed to showing them the raw data straight out of that notebook until, one morning, the notebook was found missing. We searched high and low with the aid of friends, the janitor and whoever else would listen to our plight, but to no avail. Neither Sol nor I had ever taken it out of the laboratory. We could never completely divorce ourselves from the thought that someone had deliberately taken our notebook and destroyed its precious (to us) contents. The next six months were spent repeating the previous experiments, as best we could remember them, always wondering if the 'cursed' notebook would appear as suddenly as it had disappeared. It never did!

Two PhDs out of one joint research project

The second obstacle was how to get two PhD theses out of a completely joint effort. Prof. Gustafson was pretty adamant in his view that the Graduate School would never allow a degree to be granted unless the research had been performed entirely by one individual. The United States of America was now engaged in World War II, and both Sol and I were subject to the draft. Fortunately, Prof. H.H. Bartlett, Head of the Botany Department, listened to what Sol and I had accomplished research-wise and came to a different opinion. He thought that the work should be divided. The proteolytic enzyme release of auxin from the leaf protein fraction should be used for my thesis. Sol should use the alkali auxin release from protein and the tryptophan transformation by alkali to heteroauxin for his thesis. Mister Bartlett, so called because he had been too busy to have acquired more than a Master's degree, made a special trip to make sure that his proposed plan for our salvation would be approved by the Dean of the Horace Rackham Graduate School.

The above experience instilled within me an abiding interest in leaf proteins that remains to this day. Learning how to isolate Chibnall's leaf proteins and separate them into different components indeed paved the way to the ultimate recognition of Rubisco as being

the world's most abundant single species of soluble protein.

At Pasadena with James Bonner: Fraction 1 protein

I arrived at Pasadena (California Institute of Technology, CalTech) shortly before the Battle of the Bulge in December 1944. The success of the Allies against the German counterattack appeared to herald the end of World War II in Europe. On this account, James Bonner, with whom I was to work, felt we could now devote our attention to fundamental auxin problems. We would pursue my previous work by concentrating on the further separation of the proteins within Chibnall's soluble 'cytoplasmic' protein fraction.

Resolution of protein mixtures was more art than science in those days. A common practice was to keep adding a saturated solution of ammonium sulfate to a solution of proteins until a precipitate appeared and then collect that precipitate before adding more ammonium sulfate to the supernatant in the hope of producing another precipitate. In the case of the soluble proteins extracted from spinach leaves, a copious precipitate appeared at approximately 35% saturation. After collecting this precipitate, no further precipitate was obtained even when the solution reached 50% saturation. Instead, the protein remaining in the supernatant at 35% saturation was collected by transferring the liquid to Visking cellulose tubing, allowing the ammonium sulfate to dialyze away in the presence of a neutral buffer surrounding the tubes. Dialysis resulted in a simultaneous large dilution of the protein. By hanging the tubes in front of air flowing from an electric fan, water rather quickly escaped from the tubes and evaporated, while low molecular weight constituents collected on the outside of the tubes where they could be washed away. To keep track of things, the protein that precipitated at 35% saturation was called '*Fraction 1*.' The protein that did not precipitate but could be collected by evaporative concentration was called '*Fraction 2*.'

In contrast to the paucity of equipment at the University of Michigan applicable to protein research, Caltech was a Mecca housing a variety of the most sophisticated kinds of equipment. Headed by Linus Pauling, still 5 years away from his monumental publication on the α-helix, the Chemistry Division was heavily engaged in the wartime development of substitutes for human blood proteins. The Gates and Crellin Laboratories of the Chemistry Division were only a few steps from the lab that I was occupying in the Kerckhoff Building of the Biology Division. This is the same building where Robert Emerson and William Arnold had earlier done their classical experiment on 'The Photosynthetic Unit' in 1932 (see Emerson and Arnold 1932a, b). Shortly after my arrival, I had made friends with George Feigen and Prof. Dan Campbell working on blood substitutes under Pauling's sponsorship. They introduced me to an apparatus for freeze-drying large volumes of protein solutions by lyophilization using a vessel constructed by the Crellin glass-blowing shop. A spare unit was made available for lyophilizing leaf proteins, which greatly facilitated collection and preservation of large quantities of cytoplasmic proteins prior to further purification.

The Tiselius apparatus

Another important friend was Stanley Swingle, a recent PhD under Pauling's tutelage. Stan had supervised design and construction, and only recently put into operation a moving-boundary electrophoresis apparatus constructed by the Crellin machine shop. The instrument followed the design of the Swedish investigator, Arne Tiselius, who would receive the Nobel Prize for his invention. The device had the capacity to identify and quantify the amount of different proteins in blood that differed only slightly in electrical charge. Whereas an analysis required only a few milliliters of protein solution, the apparatus was so large that it had to be housed in the basement of the Crellin Laboratory, where space was available to accommodate an optical bench of about 30 feet in length. The idea – in simple terms – was to place protein contained in buffered solution in a U-tube and impose an electrical current to cause migration of the charged protein molecules. The different proteins would begin to separate from each other according to their difference in electrical charge. The boundaries of the different proteins could be visualized by passing collimated light through the solution where the light would be refracted to different degrees depending on the number of boundaries. The refracted light was focused on a photographic plate and recorded as a Schlieren pattern where the individual proteins were registered as Gaussian peaks.

Stan Swingle had made the U-tubes himself by fusing together optical-grade panes of quartz. The cells were constructed in three parts. A complicated mechanical device made it possible to move the top and bottom parts so that the protein solution could be

loaded into one arm of the middle part and continue through the bottom part constituting the 'U.' Sharp boundaries separated the protein solution from the buffer reservoir and electrodes in the top and from the buffer contained in the opposite arm of the middle part when electrophoresis commenced. The cell was placed in a water bath mounted on a massive concrete pillar. The bath was maintained at 4 °C, the maximum density of water to minimize diffusion of the protein boundaries. The water bath had two quartz portholes to allow light to pass through the protein solution and reach a concave mirror 15 feet away which reflected it back on another path through the portholes to the photographic recording device located 30 feet from the mirror. The mirrors and windows had been made in the renowned Caltech Astronomy shops.

Stan generously allowed me and my talented laboratory assistant, Jean M. Campbell, to use his Tiselius apparatus to examine the proteins extracted from spinach leaves. The result was very intriguing. Without ammonium sulfate fractionation, the cytoplasmic proteins migrated as if 70% of their content consisted of a single protein. When the ammonium sulfate cut labeled Fraction 1 was tested, it migrated as a single, electrophoretically homogeneous component. Furthermore, the minimal spreading of the boundary during electrophoresis suggested the protein to be of high molecular weight (Wildman and Bonner 1947). Jean extended this observation to include the electrophoretic behavior of cytoplasmic proteins extracted from numerous other species of plants. *Caltech did not accept women as students in its hallowed halls in those days. So an arrangement was made for Jean to work for her PhD at UCLA, but to utilize the Tiselius instrument at Caltech for her thesis research.* She also made the puzzling discovery that no distinct Fraction 1 was seen in the Schlieren patterns of cytoplasmic proteins isolated from maize (*Zea mays* L.) leaves. The explanation of this puzzling observation had to await the discovery of the C-4 photosynthetic pathway by M.D. Hatch and C.R. Slack many years hence. Only then would it become clear that in the C-4 case, the amount of Fraction 1 needed for its unique enzymatic activity was greatly reduced in relation to other cytoplasmic enzymes required for operation of the C-4 pathway (see M.D. Hatch, this issue, for the history of the discovery of C-4 photosynthesis).

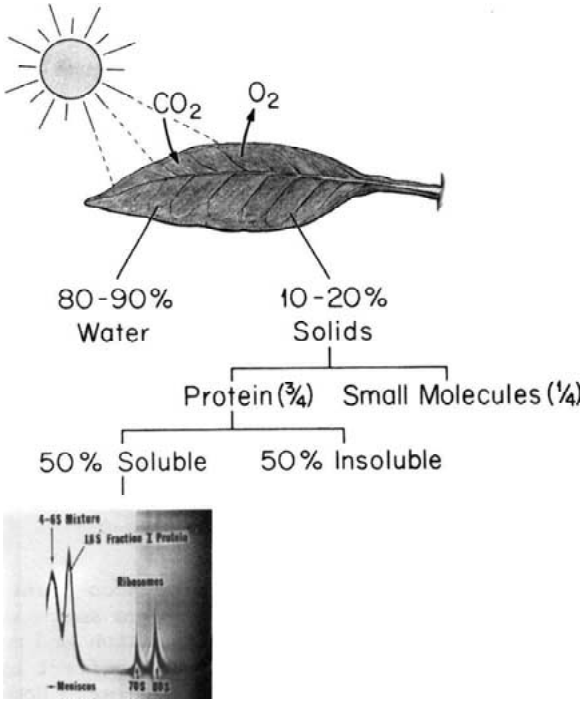
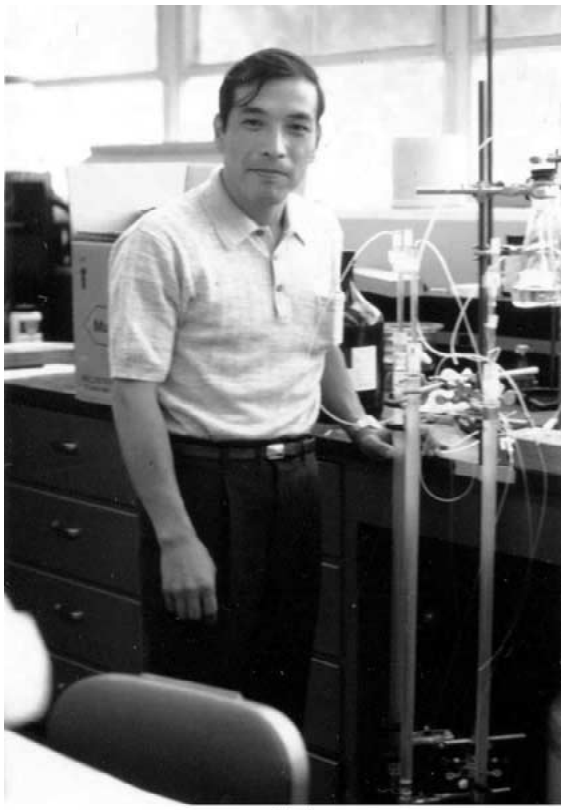


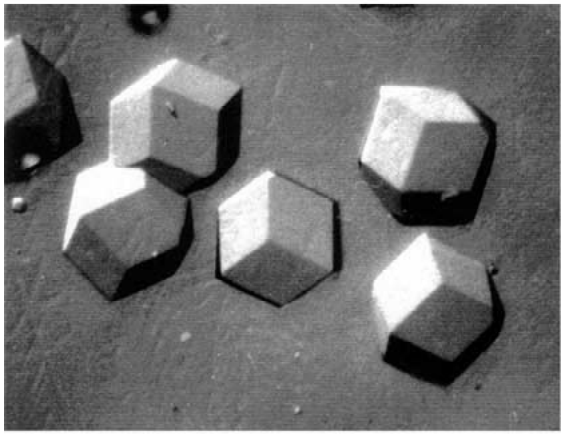
Figure 1. Diagram to show the gross composition of constituents of leaves and the ratio of insoluble to soluble proteins. *Source*: Figure 1 from Wildman and Kwanyuen (1978). Courtesy of Prachuab Kwanyuen.

The Svedberg analytical centrifuge

In line with Pauling's insistence on having available the most up-to-date instruments for protein characterization, the Chemistry Division shops had begun construction of an analytical centrifuge shortly before the United States became engaged in World War II. This instrument had also been invented in Sweden by Teodor Svedberg, who had previously received the Nobel Prize for another discovery and was located at the same institution as Tiselius. Exotic metals were required for some of its parts; consequently, construction remained in limbo until V-J (Victory) Day when the metals again became available. Stan Swingle supervised the construction before its lengthy interruption. By the time it was resumed, Swingle had left Caltech for another position. When work resumed on the analytical centrifuge, Dr S. Jonathan Singer had become a postdoctoral associate of Linus Pauling and supervised further construction. The idea behind this instrument was to have a rotor spinning in a high vacuum chamber at speeds greater than 50 000 revolutions per minute, thereby creating gravitational fields of sufficient intensity to sediment proteins. The ro-



(A)



(B)

Figure 2. (A) Nobumaro Kawashima who discovered methods to crystallize Fraction 1-protein. He is shown here with Sephadex columns in the Plant Physiology Building at the University of California at Los Angeles (UCLA). (B) Photograph of the crystals of the fraction 1 protein by the author. Courtesy of N. Kawashima.

tor, made of special metals to withstand such extreme gravitational forces, was oval in shape. Two holes, ca. 1.5 inch in diameter, were drilled on opposite sides

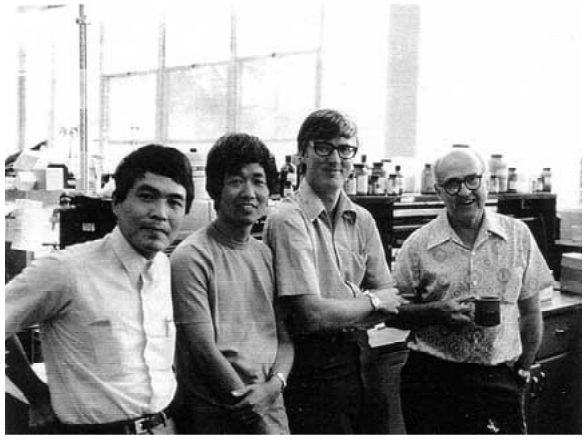


Figure 3. The author (extreme right) with his coworkers (from left to right): Katsuhiro Sakano (worked on a simplified method to obtain crystalline fraction 1 protein in large scale; now at the National Institute of Agrobiology Research, Tsukuba, Ibaraki, Japan); Shain-dow Kung (did electrofocussing studies to unravel composition of fraction 1 protein; now at the Biology Department, The Hong Kong University of Science & Technolgy, Kowloon, Honkong); Bentley Atchison (studied chloroplast structure, chloroplast DNA, etc.; now at Forensic Laboratory of the Melbourne Police Department, Melbourne, Australia), and the author. Courtesy of K. Sakano.

of the long axis of the rotor. One hole was to accommodate a counterbalance, the other to allow precise positioning of a cell in the perpendicular direction with two quartz windows enclosing the top and bottom of the cell. The cell, itself of intricate assembly and disassembly, contained the protein for analysis, protecting it from evaporating into the vacuum. At the bottom and top of the vacuum chamber, quartz windows were placed so that UV light from outside the chamber could be passed through the cell spinning within the rotor and out of the top window and thence to a Schlieren optical system for visual analysis and photographic recording. The rotor was spinning so fast that there was no seeming interruption of the light by the opaque counterweight. The protein boundaries could be observed as they moved as a perpendicular plane sedimenting towards the outside of the rotor. The rotors had a propensity to blowup with devastating effects. Thus, the centrifuge was surrounded by reinforced concrete walls with access ports to conduct the light from the outside, through the cell in the rotor, and return it to the outside where visualization and photographic records of the Schlieren patterns could be made. The system was located in Crellin nearby the Tiselius instrument.

Four years transpired from the time Fraction 1 had been seen in the Tiselius apparatus until the Caltech

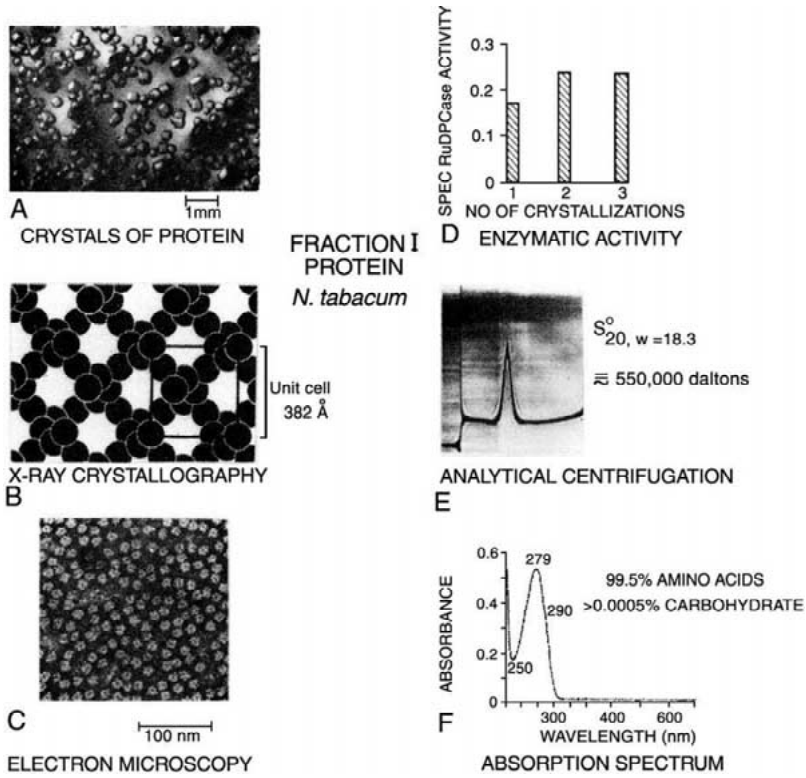


Figure 4. Properties of crystalline Fraction I protein of tobacco leaves. *Source*: Figure 2 from Wildman and Kwanyuen (1978). Courtesy of Prachuab Kwanyuen.

Svedberg analytical centrifuge was launched on its maiden voyage with spinach cytoplasmic proteins as the first passengers on board. Jon Singer was disposed to reserve judgment on the significance of homogeneity of proteins ascertained by electrophoresis. He was therefore surprised to see a Schlieren pattern develop showing a large molecular weight component separating far ahead of the remaining proteins (see Figure 1, from Wildman and Kwanyuen 1978). Moreover, the fast-moving component appeared to comprise approximately 50% of the total cytoplasmic proteins. When Fraction 1, prepared by ammonium sulfate precipitation, was examined by analytical centrifugation, its migration as a single, fast-moving component confirmed the electrophoretic evidence favoring its homogeneity. Moreover, the 18 S (S for Svedberg units) protein had a calculated molecular mass close to 600 000 daltons. Being homogeneous by the two most rigorous analytical tests then available at Caltech, Fraction 1 was dignified by a new name: *Fraction 1 protein* (Singer et al. 1952). However, a prolonged period would go by before it became obvious that this protein catalyzed the first step in photosynthetic CO_2 fixation. In the meantime, an opportunity arose in 1950 for me to become a member of the Department of Botany faculty at the University of California at Los Angeles (UCLA). One of the inducements was that the University would finance the expensive purchase of Tiselius and Svedberg instruments which had only recently become commercially available.

The Calvin–Benson studies

By 1954, Melvin Calvin and Andrew A. Benson and associates had identified each one of the many steps in the cyclic pathway of photosynthetic CO_2 fixation (Bassham et al. 1954; Bassham and Calvin 1957; see perspectives by Calvin 1989; Hatch 1994; Fuller 1999; Benson, this issue). This Noble-Prize-winning achievement provided impetus for Bernard L. Horecker and associates and Arthur Weissbach and associates in 1956 to search for an enzyme activity that would cause the combination of carbon dioxide with ribulose-1,5-diphosphate [RuDP (now called bisphosphate)] followed by dismutation into two molecules of 3-phospho-glyceric acid (3-PGA), the first

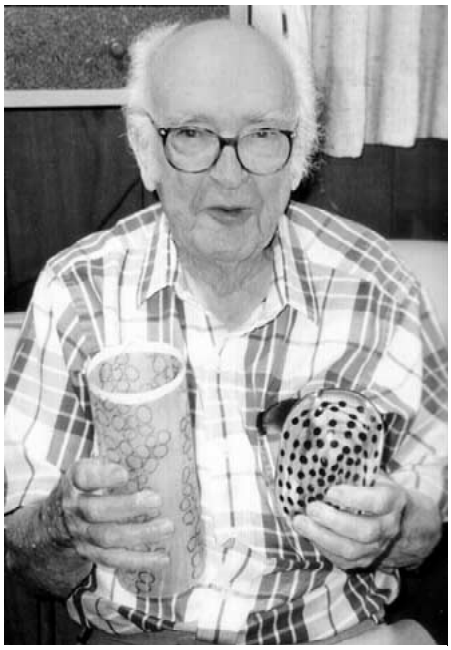


Figure 5. The author in August, 2001. Photo was taken by Govindjee during his visit to my home when I lectured him on my unusual ideas of arrangement and origin of grana in higher plant chloroplasts.

step in the Calvin–Benson Cycle. They chose spinach leaves for their source of enzyme with obliging results (Weissbach et al. 1956). They succeeded in purifying a carboxylation enzyme that formed 3-PGA from RuDP and CO_2 with a sedimentation constant of 18S! Not much time passed before my associates, Robert Dorner and Albert Kahn, seized on the idea that the carboxylation enzyme and Fraction 1 protein in spinach leaves had to be one and the same (Dorner et al. 1957). (Also see Benson, this issue, for his earlier contributions, that remained unpublished, in showing that Fraction 1 protein and what was then called carboxydismutase were one and the same protein.)

Interest in tobacco mosaic virus had led my laboratory to experiments with the tobacco plant (*Nicotiana tabacum* L.) also for Fraction 1 protein research. Dr Nobumaro Kawashima, on study leave from the Japan Tobacco Monopoly Corporation, had come to my laboratory in 1968. There still existed nagging doubts about the ultimate degree of homogeneity of Fraction 1 protein. Could an unseen contaminant perform the carboxylation catalysis instead of a protein of such monstrous size and amount and at a pathetically low 'turnover'? In an offhand way, I suggested to my new associate how nice it would be if Fraction 1 protein could be crystallized as an aid in resolving the issue of whether it was, in fact, the carboxylation enzyme. Little did I imagine that a spine-tingling message would soon be heard on my home telephone one Saturday afternoon. Still impeded by the vagaries of the English language, Nobumaro had entrusted a student to convey the message that, 'Dr Kawashima thought he was seeing crystals of Fraction 1 protein by naked eye, but please, could you come and examine them by microscope?' You better bet your britches I could! And sure enough, there were beautiful, many-sided, nearly transparent, crystals of all sizes to be seen by microscopy (Kawashima and Wildman 1971; see Figure 2 for a photograph of Kawahima and the crystals). They were immediately photographed should they prove difficult or impossible to obtain again. Happily, the latter was not the case. However, I was disturbed that the crystals were not birefringent and immediately sought advice from Prof. David Eisenberg of the Chemistry Department and Molecular Biology Institute at UCLA. He reassured Kawashima and me that not all protein crystals were birefringent. While studying the crystals under low power magnification, he poked one of them with a needle to see it almost immediately disappear. This strange behavior tweaked his interest sufficiently to enlist his laboratory's associates in ascertaining the X-ray crystallographic structure of Fraction 1 protein. The strange behavior arose because Kawashima's crystals contained 80% water! The Eisenberg group had to find another crystal form with much less water before the atomic structure could be realized.

It was some time after Nobumaro had to resume his duties in Japan that Pak Hoo Chan, Katsuhiro Sakano, and Shalini Singh developed a simple procedure for large scale preparation of crystalline Fraction 1 protein from tobacco leaves (Chan et al. 1972; Figure 3 shows a lab photograph of Sakano, Shain-dow Kung, Bentley Atchison and Sam Wildman). The simple procedure provided the means whereby Fraction 1 protein was repeatedly recrystallized without reduction in ribulose-1,5-bisphosphate carboxylase specific activity and proved to possess carboxylation activity as an inherent part of its structure (see Figure 4, reprinted from Wildman and Kwanyuen 1978).

The name of the enzyme – upon which all life based on light-dependent, carbon fixation depends – kept evolving from 1956 until 1979. It was variously called carboxydismutase, ribulose diphosphate or RuDP carboxylase, ribulose bisphosphate, or RuBP carboxylase until the final absurdity – 3-phospho-

D-glycerate carboxylase (dimerizing) EC 4.1.1.39. What a jawbreaker to use at the lecture podium! Then the carboxylation enzyme was found to *oxygenate* ribulose-1,5-diphosphate (Bowes et al. 1971), necessitating a further change in name to ribulose-1,5-bisphosphate carboxylase-oxygenase. *Lord knows where it might have ended if David Eisenberg had not called it Rubisco as a joke while delivering a talk at my retirement symposium in July 1979*. He explained the acronym as 'Ru' standing for *r*ib*u*lose, and the following five letters serving for '*bis*-*c*arboxylase-*o*xygenase.' If one were to substitute 'Na' for 'Ru,' he pointed out that it would call to mind a retiree now devoted to promoting the public acceptance of colorless, odorless, tasteless, and highly nutritious crystalline tobacco Fraction 1 protein as a food (Wildman and Kwanyuen 1978). It would also be an intimation of where it might someday be found ready to eat!

The world's most abundant protein seemed to me to deserve a very special common name to set it apart from other enzymes with common names such as pepsin, trypsin, papain, urease, and catalase. When David said, 'RUBISCO,' I knew instinctively that a worthy successor to Fraction 1 protein had at last come down like manna from heaven. I used Rubisco in my next paper. To my immense satisfaction, David's new name quickly caught on.

I end this article with a fond memory of the visit by Govindjee and his wife Rajni Govindjee to our home in California in August, 2001. At his suggestion I have included here a photograph of me that he then took (see Figure 5).

Acknowledgments

I thank Govindjee and Raymond Chollet for taking three of my published articles, editing them, and adding references to produce this historical minireview. Figures and photographs are joint efforts of Chollet, Govindjee, and Prachaub Kwanyuen.

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