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Historical corner

Memoir of a 1949 railway journey with photosynthetic bacteria

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Abstract

A serendipic observation at the Hopkins Marine Station of Stanford University in 1948 led to the discovery that anoxygenic photosynthetic bacteria can fix molecular nitrogen. To confirm the discovery, an unusual collaborative event was arranged between laboratories at Washington University (St. Louis) and the University of Wisconsin (Madison).

Introduction

On January 6, 1949, I performed experiments at Washington University that immediately and unexpectedly revealed that N_2 was not an inert molecule in the metabolism of *Rhodospirillum rubrum* . I quickly concluded it was highly likely that *R. rubrum* must be capable of using N_2 as a nitrogen source for growth. In 1949, only a few 'blue green algae' (cyanobacteria) and two kinds of free-living bacteria, *Azotobacter* and *Clostridium pastorianum*, were known to fix N_2 (Stephenson 1949). The nitrogen-fixing *Azotobacter* was isolated by Beijerinck in 1901 and during the succeeding half century, no new N_2 fixers had been discovered. From time to time, claims had been made by researchers for marginal N_2 fixation by various other bacteria, but when these were carefully examined by appropriate methods, they invariably proved to be erroneous (see Lindstrom 1951). Thus, the case for *R. rubrum* was bound to be viewed with skepticism...especially because I was a graduate student at the time.

I began graduate work with Max Delbrück at Vanderbilt University in 1942, but World War II interrupted my studies. I was offered a position in the Chemistry Division of the Manhattan Atomic Bomb Project, and spent the war years doing basic research on the chemistry and radiochemistry of uranium fission at the University of Chicago and later at Oak Ridge (Tenn.). A detailed account of the circumstances leading to my move to Washington University, where I became Martin Kamen's first graduate student, are detailed in Gest (1994a).

My thesis problem focused on the role of 'phosphate metabolism' in the photosynthesis of microscopic green algae (*Chlorella* and *Scenedesmus*). The aim was to obtain evidence for or against the hypothesis that light energy might be converted to chemical energy in the form of 'energy-rich' phosphate compounds such as ATP.

Summer at Pacific Grove, California

In 1947, I left St. Louis for the summer to go to the Hopkins Marine Station of Stanford University, where C.B. van Niel gave his famed microbiology course. I was one of nine students in the class. When I returned to St. Louis in September with a culture of *Rhodospirillum rubrum*, I was greatly enthused with the idea of working with van Niel's favorite organisms, the purple bacteria. Van Niel had spent years working on the 'non-sulfur purple and brown' photosynthetic bacteria, resulting in a monumental 118 page paper published in 1944 (van Niel 1944). I persuaded Kamen to abandon the algae, and the study of phosphate metabolism in photosynthesis proceeded using *R. rubrum* as the test organism.

I returned to van Niel's laboratory as a research student for the summer of 1948 with the aim of de-

Figure 1. Recipe for a growth medium for *R. rubrum*, written by Seymour Hutner and given to Howard Gest in 1948 (actual size).

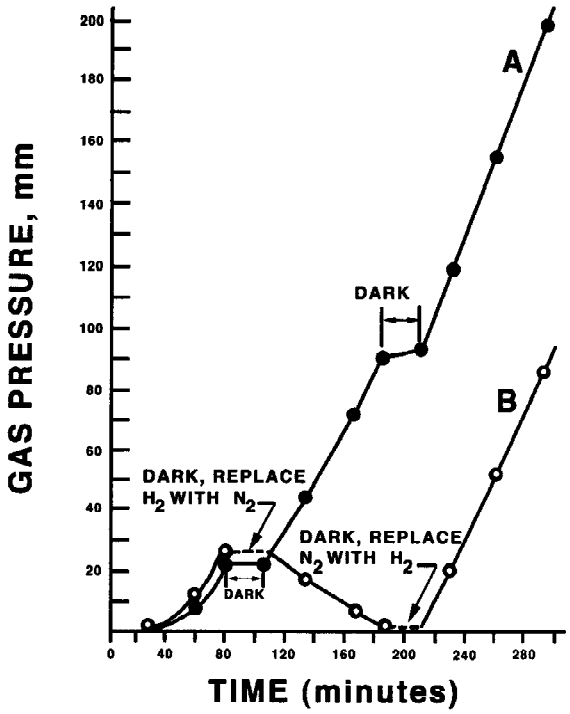

Figure 2. A typical experiment with *R. rubrum*, illustrating inhibition of light-dependent production of H_2 by N_2. Cells from H_2-producing cultures were washed and resuspended in 0.05 M phosphate buffer, pH 6.6. The initial gas atmosphere was 100% H_2, and 5 mg of DL-malate were added at zero time. The Warburg vessels contained 10% KOH in the center well, to absorb CO_2. In curve A, the gas phase was 100% H_2 throughout the experiment; in curve B, the gas phase was changed as indicated.

vising new growth media for *R. rubrum*, suitable for experiments in which ^{32}P would be used as a tracer. Among the recipes I tested was a medium suggested to me by Seymour Hutner. It so happened that during the spring of 1948, I attended the national meeting of the Society of American Bacteriologists (now the American Society for Microbiology) and met Hutner in a hotel hallway. While we were chatting, he jotted down, from memory, the recipe of a growth medium that he had used for *R. rubrum* (see Figure 1).

At the Hopkins Marine Station, I was astonished to observe that in Hutner's medium, *R. rubrum* produced copius quantities of molecular hydrogen during anaerobic photoheterotrophic growth.

When I reported this at the daily tea-time séance that van Niel held with research students, 'The Microbe King' (so inscribed in Dutch on van Niel's tea cup) made remarks implying that I had surely contaminated my cultures with non-photosynthetic anaerobes known to produce H_2. In fact, my cultures were pure, and when I reported this several days later at tea-time, van Niel remained silent. During years of research with a variety of purple bacteria, he had never observed a phenomenon of this kind.

After returning to St. Louis in the fall, I conducted many manometric experiments (using the Warburg apparatus) aimed at demonstrating H_2 formation by illuminated 'resting' cells of *R. rubrum*, derived from H_2-producing growing cultures. In these trials, the cells were resuspended in a dilute solution of buffered mineral salts supplemented with malate, under anaerobic conditions established by using N_2 as the gas phase. In experiment after experiment, not a trace of H_2 was produced.

An experiment of desperation

During these discouraging experiments, I studied the research literature on metabolism of molecular hydrogen by microorganisms with diligence. Eventually, I was struck by the fact that *R. rubrum* appeared to behave exactly opposite to the fermentative heterotroph

Diplococcus glycinophilus in regard to H_2 metabolism. Since an atmosphere of 100% H_2 completely inhibited fermentative H_2 production by *D. glycinophilus* (Cardon and Barker 1947), I decided to test *R. rubrum* cells under an atmosphere of H_2. Surprisingly, for the first time, I observed a clear-cut production of H_2, dependent on light. Controls consisted of cells under an atmosphere of N_2 and as usual, these showed no H_2 formation (see Figure 2). I immediately tested cells under atmospheres of helium or argon, and observed abundant production of H_2. These were the experiments of January 6, 1949. Evidently, in *R. rubrum* there was an important interaction between N_2 and the system responsible for H_2 formation, and this indicated the likelihood that the bacterium had the capacity to fix N_2.

Tests for nitrogen fixation

We assumed that to validate N_2 fixation in *R. rubrum*, tests with ^{15}N would be required. The 'Mecca' of N_2 fixation studies at the time was the University of Wisconsin (Madison) where the laboratories of Perry Wilson (Microbiology) and Robert Burris (Biochemistry) were engaged in joint research projects. Kamen phoned Wilson with our news about *R. rubrum*, and Wilson was dubious, as expected. Nevertheless, it was agreed that we should conduct a joint test, in Madison. Since the Wisconsin scientists had no experience with photosynthetic bacteria, it was obvious that we would have to bring fresh, metabolically active, cultures to Madison.

R. rubrum *travels by train from St. Louis to Madison*

The only feasible way for us to go to Madison was by train, and it was clear that we would have to incubate our cultures under photosynthetic conditions en route. I have been unable to confirm the exact date of our trip, but it very likely was late in February 1949. At that time, several railroads ran Pullman sleeping cars from St. Louis to Chicago, leaving just before midnight for the 7 hour trip. In Chicago, we would transfer to another train, for the shorter journey to Madison. In preparation for our adventure, we arranged for a university carpenter to construct a portable wooden incubator box, with a hinged door on top. A socket for an incandescent light bulb was fixed on the bottom center, and bottle cultures could be secured at corners of the box. The sides of the box had many narrow slits to facilitate air circulation; this was important because the light bulb emitted considerable heat. I inoculated the bottles so as to have cultures at various stages of active photosynthetic growth when we arrived in Madison. Kamen and I took a taxi to the St. Louis train station, settled in a two-bed compartment, and plugged in the growth box.

The next morning, in Chicago, we transferred to a first-class lounge coach in a train stopping in Madison. The only available electrical outlet in the coach was at one end, near floor level. We attached the light cord of the box and opened the lid to permit better air circulation, and the box was now emitting a rosy red glow. Passengers walking through the coach glanced at the box suspiciously. Kamen (1986) later noted "The sight of two strangely intense, wide-eyed young men hovering anxiously over a box from which emanated a red glow must have been alarming."

In Madison, we were met by a delegation, and we proceeded directly to Wilson's laboratory to set up experiments. Cells were centrifuged down and resuspended in a nitrogen-free solution containing the appropriate supplements. Suspensions were incubated anaerobically in special flasks under an atmosphere of $^{15}N_2$, and shaken in a constant temperature water bath with illumination from incandescent light bulbs. The plan was to determine how much ^{15}N was fixed in the cells after overnight incubation.

The plans of mice and men often go awry

As soon as we arrived at the laboratory the next morning, I looked at the cultures in the waterbath and was immmediately taken aback. Instead of the rich reddish purple color of the usual cultures, they were a chalky pink. My heart sank; something was seriously wrong. This was confirmed by a microscopic examination; all the cells were non-motile and obviously dead. The bleached appearance of the cultures indicated to me that the pH probably was off, and a test of one of the suspensions, with pH paper, showed that it was very acidic. Kamen, trying to snatch victory from the jaws of defeat, asked me if it was possible there was something wrong with the pH paper! Explanation of the problem soon emerged. The graduate student assigned to prepare the resuspension fluid had forgotten to neutralize it! Despite great disappointment, Kamen and I tried to put a good face on the situation. What to do? We decided to proceed with the ^{15}N assays anyway. Remarkably, despite all, the assays showed definite ^{15}N incorporation into the cells – obviously far from optimal – but sufficient to leave no doubt that *R. rubrum* fixed N_2. The excitement was consid-

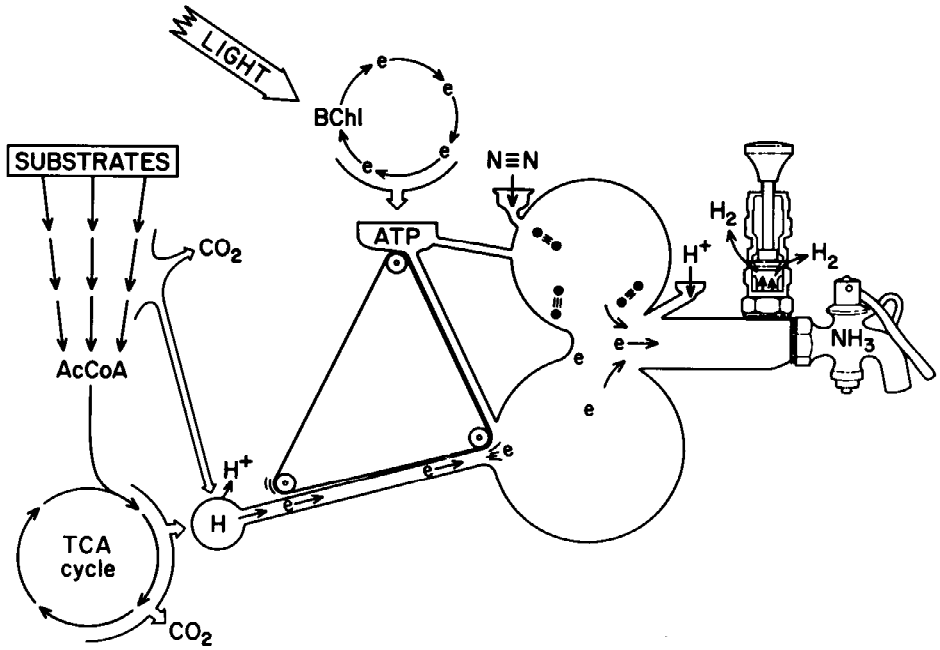

Figure 3. A representation of metabolic conversions occurring during photoproduction of H_2 by photosynthetic bacteria (see text). If N_2 is present, H_2 is not produced and reducing power is used instead for reduction of N_2 to ammonia.

Table 1. Fixation of $^{15}N_2$ by *R. rubrum*

Vessel contents	Atom% excess ^{15}N in cells
Illuminated cells	3.14
Cells in darkness	0.189
Boiled cells	0.008

erable, and immediately persuaded Wilson and Burris to undertake studies with photosynthetic bacteria.

^{15}N tests in St. Louis

As soon as we returned to Washington University, we decided to do the ^{15}N experiments ourselves. We set up a vacuum line to generate $^{15}N_2$ from isotopically-labeled ammonium nitrate, and the gas was introduced into three Warburg vessels attached to the line. Two of the vessels contained H_2-producing cells of *R. rubrum* resuspended in a buffered solution that included malate as a carbon and electron source. The third vessel was a replicate control containing boiled cells. After 6 days of incubation, the cells were separated from the suspension, and analyzed for cellular ^{15}N content, with the results shown above (Kamen and Gest 1949).

Thus, light-dependent N_2 fixation was clearly confirmed. Our results were published in two short, back-to-back papers in Science (Gest and Kamen 1949; Kamen and Gest 1949), and resulted in a dramatic news article by famed science writer William L. Laurence in the June 25 issue of *The New York Times* (p. 15). The headlines were: NEW CLUE IS FOUND TO PHOTOSYNTHESIS...Sunlight, for First Time, Is Caught in Vital Act of Generating Hydrogen: "This [research] provides evidence for the first time that there exists a nitrogen-fixing system in photosynthesizing bacteria, and that there is a direct connection between the mechanism of the liberation of hydrogen by sunlight and the building up of the nitrogen-containing proteins. This correlation between the action of sunlight in the liberation of hydrogen and the fixing of nitrogen in the building up of proteins has been looked for for years without success."

One of our Science papers noted that the ^{15}N uptake data reflected a net fixation of nitrogen. In fact, in a number of experiments I was able to show significant growth of *R. rubrum* on N_2 as the primary or sole nitrogen source. Later experiments revealed that rapid growth on N_2 could be obtained by ensuring strongly reducing conditions in the medium (Gest et al. 1956).

During April 1949, Kamen was away from St. Louis at a meeting and wrote me in a letter dated April 20: "Burris brought some more data with him to the meeting....and it appears that Wisconsin has gone into the *Rhodospirillum* business." The letter also said: "I had a session with [Hans] Krebs. After initial indifference he warmed up and although he had no suggestions he showed a keen appreciation of the phenomena and now wants these bacteria too. So please wrap up our Deluxe model stab culture (a couple of samples) with complete instructions like you sent [David] Rittenberg and mail them to him- -Dr. H.A. Krebs, c/o Rockefeller Foundation). He is sailing May 5. So the bacteria should get to NY by May 3."

Studies in Madison after our visit

Wilson and Burris quickly made plans to study N_2 fixation by photosynthetic bacteria. At the time of our visit to Madison, Eugene Lindstrom was one of Wilson's graduate students. In a 1985 letter to me, Lindstrom (then at Penn State University) recalled that after our visit to Madison, Wilson met with his graduate students: "Wilson said that one of us would have to drop what we were doing and work on this problem. He lined us all up, and we each had to give a status report on our research and it was obvious that my research was going much more slowly than Shirley Tove's, Gene Rosenblum's, or Stan Martin's. So I was dragged kicking and screaming into studying photosynthetic bacteria."

Shirley Tove was the '^{15}N specialist' graduate student liason with the Burris lab, and was also directly involved in setting up our experiments at Madison. I had hoped that the PhD theses of Lindstrom and Tove (Lindstrom 1951; Tove 1950) would include some details of our joint experiment in Madison, but while writing this paper I found that neither had a single word about our visit! Kamen (1986) gave a brief account of the trip in the revised paperback edition of his autobiography.

Within months of our visit, Lindstrom, Burris and Wilson published a paper in the Journal of Bacteriology (Lindstrom et al. 1949) entitled 'Nitrogen fixation by photosynthetic bacteria', which described their confirmation of our basic findings with *Rhodospirillum rubrum*. In a letter to me dated November 11, 1949, Burris wrote: "Last week we made a run with *R. rubrum* under $^{15}N_2$ for 30 minutes. The cells have been hydrolyzed and if their ^{15}N content is high enough we will separate the amino acids from them and determine the ^{15}N distribution....I don't know exactly what Perry's group has done. Lindstrom checked the fixation aerobically and anaerobically, light and dark. Then he started testing other organisms and found fixation by the purple and green sulfur bacteria." Lindstrom had written to Helge Larsen, who was working with green photosynthetic bacteria on his PhD thesis with van Niel at the time, requesting cultures of *Chlorobium* and *Chromatium*. Lindstrom informed Larsen that these organisms also could grow on N_2 as the sole nitrogen source. However, many years later, Lindstrom wrote me that "van Niel didn't believe us until he saw them growing in our incubator room".

Eventually, it became clear that with rare exception, the more than 80 species of photosynthetic bacteria are active N_2 fixers. Exploiting this fundamental fact, our laboratory at Indiana University developed a highly selective enrichment culture procedure for isolating photosynthetic bacteria from natural sources (Gest et al. 1985). Use of the procedure revealed the existence of a number of new species and a hitherto unknown family of green photosynthetic bacteria, the heliobacteria (Gest 1994a, b).

The serendipic 'scenario'

The key facts leading to discovery of 'photohydrogen' production and nitrogen fixation by photosynthetic bacteria were:

a) ammonium salts, typically used as nitrogen sources in lab media, repress synthesis as well as activity of the nitrogenase complex. Hutner's medium contained only glutamate as the sole N source, and the enzyme complex was, consequently, derepressed;
b) in the absence of nitrogen gas, all nitrogenases show the alternative activity of reducing protons to hydrogen gas. Experiments with *R. rubrum*, in fact, gave the first indications that both the utilization of nitrogen gas and the production of hydrogen were catalyzed by the same enzyme system (i.e. nitrogenase; Ormerod and Gest 1962); and
c) in the presence of N_2, the formation of H_2 is completely inhibited, as might be expected; reduction of N_2 to ammonia becomes a predominant metabolic process.

Our later studies on the light-dependent evolution of H_2 by nitrogenase in photosynthetic bacteria led to the concept that this activity is a physiological reg-

ulatory device that helps balance bioenergetics with biosynthetic activity (Gest 1994; Hillmer and Gest 1977a, b). Figure 3 gives an overall view of the flow of carbon, electrons, and ATP in cells during photoheterotrophic growth. The double-bulbed device represents the nitrogenase complex. In the absence of ammonia, the nitrogenase is derepressed, and when N_2 is absent, the enzyme complex functions as a regulatory H_2-evolving catalyst. With glutamate as the nitrogen source, the supplies of ATP from photophosphorylation and electrons from organic substrates presumably are in excess relative to biosynthetic activity. Under these conditions, protons are reduced yielding H_2, shown in the figure as being discarded via a 'H_2 safety valve'. If N_2 becomes available, H_2 production stops because ATP and the electron supply are used for the production of ammonia, which in turn is rapidly consumed for the synthesis of amino acids and other nitrogenous compounds (see further discussion in Gest 1999).

In retrospect

It has been very satisfying to witness the evolution, through more than four decades, of a serendipic observation on light-dependent H_2 production by growing cultures of *R. rubrum* to sophisticated investigations on the biochemistry of regulation of nitrogenase activity. Paul Ludden at the University of Wisconsin (Madison) has been particularly prominent in pursuing the molecular bases of the observations reported in the 1949 Science papers. An excellent review by Ludden and Gary Roberts, published in 1989, traces the progress of the research, especially in respect to regulation of activity of the nitrogenase complex by reversible ADP ribosylation of dinitrogenase reductase. They concluded: "Much remains to be learned about the integration of ADP ribosylation mechanisms into metabolism, and the *R. rubrum* nitrogenase system provides an excellent experimental system for the investigation of such regulation." Ludden and Roberts noted that ADP ribosylation was first discovered as a mechanism of action of the diphtheria toxin, and they anticpated that "ADP ribosylation will be found throughout nature".

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