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Minireview

Nitrogen fixation by photosynthetic bacteria

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Abstract

In 1949, Howard Gest and Martin Kamen published two brief papers in *Science* that changed our perceptions about the metabolic capabilities of photosynthetic bacteria. Their discovery of photoproduction of hydrogen and the ability of *Rhodospirillum rubrum* to fix nitrogen led to a greater understanding of both processes.

The discovery of nitrogen fixation by photosynthetic bacteria

In two brief yet remarkable papers in *Science* in 1949, Howard Gest and Martin Kamen described their observations on the photoproduction of hydrogen gas and the fixation of nitrogen by the phototrophic bacterium *Rhodospirillum rubrum* (Gest and Kamen 1949; Gest et al. 1949). The nonsulfur purple photosynthetic bacteria are easily cultured, and their use as experimental organisms had been popularized by Cornelis B.VanNiel, Hans Gaffron, and others, but the knowledge of their metabolism of gases other than CO_2 remained unreported until this ground-breaking work. Today, it is known that almost all phototrophs among the proteobacteria fix nitrogen and manifest hydrogenase activity, and this knowledge derives from the initial experiments performed in Kamen's lab in the late 1940s.

Gest was Kamen's first graduate student, a newly-appointed chemistry professor at Washington University. They were investigating *Rsp. rubrum* metabolism under the 'inert' atmospheres of argon, helium, or nitrogen, which turned out to be critical for their observations. Also critical was the use of glutamate or aspartate as the nitrogen source for growth of the cultures – as they quickly discovered, growth on ammonium-containing medium eliminated the evolution of H_2 gas. Using classical Warburg manometric techniques, they also observed that the cells cultured on malate-glutamate medium evolved copious amounts of H_2 under a gas phase of argon, helium, or even H_2, but that under N_2, no H_2 evolution was observed. The single figure included in the first manuscript provided evidence that the H_2 evolution was light-dependent (Figure 1). The observation that *Rsp. rubrum* was not inert to N_2 led to the hypothesis that the phototrophs were capable of N_2 fixation, a property that had been described for *Clostridia* in the 1890s by S. Winogradsky (Winogradsky 1893) and subsequently for a few other genera of bacteria, but not for the phototrophs.

The subject of the second *Science* paper was testing the hypothesis that N_2 fixation occurred in *Rsp. rubrum*, and the work involved produced one of the field's amusing anecdotes. While Gest and Kamen had convincing growth studies and manometric evidence for the uptake of N_2 by cultures of *Rsp. rubrum*, the definitive method for confirmation of the presence of nitrogenase activity at that time was the use of the stable isotope ^{15}N. The most trusted practitioners of ^{15}N measurement by isotope ratio mass spectrometry

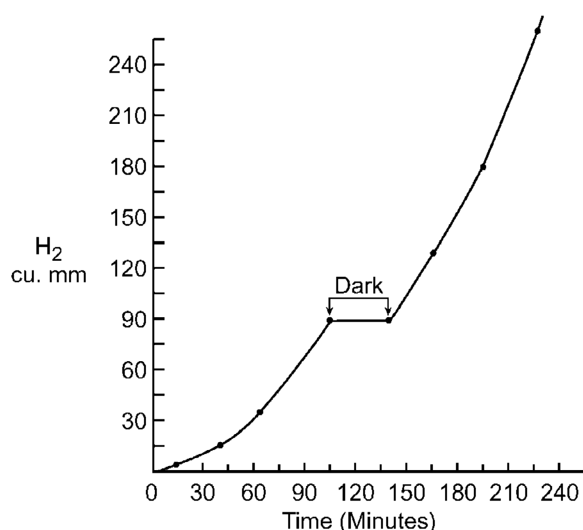

Figure 1. Photochemical production of H_2 by *Rhodospirillum rubrum* (SI): 50 $mm^3$of washed cells was suspended in 2 ml M/20 phosphate buffer (pH 6.6) and 5 mg of D,L-malate was tipped in at zero time. The center well contained 0.2 ml of 10% KOH; the gas space was filled with 100% helium. (Reprinted with permission from H. Gest and M. Kamen, Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*, Science 109: 558–559. ©American Association for the Advancement of Science, 1949.)

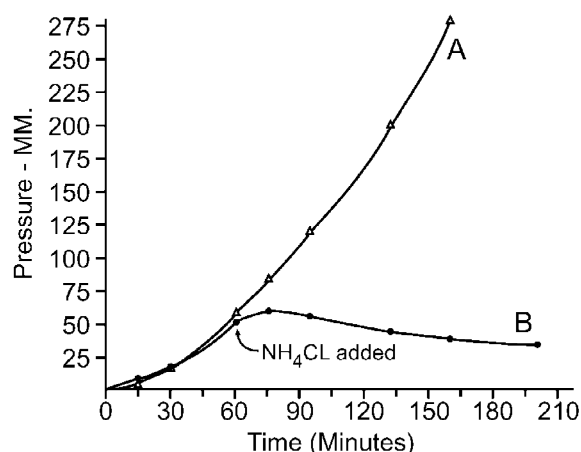

Figure 2. Effect of NH_4Cl on photoproduction of H_2 from malate by *Rhodospirillum rubrum* (SI). Washed cells (60 c mm) were suspended in 2 ml of 0.05 M phosphate buffer (pH 6.6); 5 mg of *dl*-malate tipped in at zero time; 0.2 ml. of 10% KOH in center well; 100% He in the gas space. At 60 min, 1 mg of NH_4Cl was tipped into the vessel in Curve B. Curve A shows photoproduction of H_2 in controls (no NH_4Cl added). (Reprinted with permission from H. Gest, M.D. Kamen, and H.M. Bregoff (1950) Studies on the metabolism of photosynthetic bacteria: photoproduction of hydrogen and nitrogen fixation by *Rhodospirillum rubrum*, J Biol Chem 182: 153–170.)

were R. H. Burris, Perry Wilson, and members of their labs at the University of Wisconsin at Madison (Wilson et al. 1943). Gest and Kamen set up cultures on malate-glutamate medium to allow the N_2 fixation system to develop. They gave one set of the cultures to Mildred Cohn at Washington University for analysis with her collaborators, D. Rittenberg and J. Sucher at Columbia University, and they set off for Madison (via Chicago) on the overnight train with a second set. A portable incubation box, fitted with an incandescent light bulb, was used to keep the cultures growing and illuminated during the trip so that they would be active upon arrival in Madison. According to one oral account of the Madison trip, the only electrical outlet on the train was in the men's room. While the price of success in science is often a night or two of sleep, rarely does it involve the loss of a night's sleep and standing guard over the experimental cultures in the men's room of the overnight train. [Howard Gest has written his own version of this trip (Gest 1999) and must be considered the authority, as he was there.]

Robert Burris and Perry Wilson met Gest and Kamen at the railway station and whisked them to the lab, where Burris had the then new Consolidated A.O.Van Nier isotope ratio mass spectrometer ready and waiting. (Note: that instrument is still functional in the basement of the old wing of Biochemistry at the University of Wisconsin.) Both Cohn's and Burris' results were positive for incorporation of ^{15}N into the bacterial cells, providing incontrovertible evidence for N_2 fixation. The resulting paper recorded three very significant results: first, *Rsp. rubrum* was capable of N_2 fixation; second, this fixation was light-dependent, the first report that light energy could be used to drive nitrogen fixation; and third, small amounts of ammonium added to the culture inhibited the incorporation of $^{15}N_2$, demonstrating the ability of *Rsp. rubrum* to 'switch off' its nitrogenase activity. The first paper also contained a very significant speculation – that 'this hydrogen evolution is linked to nitrogen fixation and perhaps even to a nitrogenase system.' This speculation was subsequently confirmed when William Bulen and coworkers demonstrated that isolated nitrogenase was indeed able to catalyze the reduction of protons to H_2 in addition to the reduction of N_2 to ammonium (Burns and Bulen 1965).

Newer results

A subsequent paper, published in the *Journal of Biological Chemistry* by Gest, Kamen, and Herta Bregoff,

elaborated on the initial studies and demonstrated the rapidity with which ammonium or darkness caused the cultures of *Rsp. rubrum* to lose the ability to photoproduce H_2 (Gest et al. 1950; Figure 2). Several decades later, the molecular basis of this observation was ascribed to the reversible ADP-ribosylation of the dinitrogenase reductase protein of the nitrogenase complex (Ludden and Roberts 1995; Roberts et al. 1998).

L.E. Mortenson and coworkers first described methods for the preparation of cell-free extracts of *Clostridia* that were capable of N_2 reduction, but the activity in cell-free extracts of phototrophs was very inconsistent (see Carnahan et al. 1960). This problem was partially solved by Burns and Bulen, who found that addition of high concentrations of Mg^{2+} to the assay mixture resulted in higher nitrogenase activities (Burns and Bulen 1966). T.O. Munson and Burris further improved the consistency of preparations by using cells grown under carefully controlled, N-limited continuous culture. An observation resulting from Munson and Burris' work was that it was not necessary for N_2 to be present to obtain nitrogenase activity; that is, N_2 is not a required inducer of the nitrogenase complex (Munson and Burris 1969).

These various observations regarding cell-free nitrogenase activity in *Rsp. rubrum* were placed in context when it was realized that the nitrogenase enzyme in cell extracts required activation by a membrane-bound 'activating factor' independently discovered by the Burris group and by S. Nordlund and H. Baltscheffsky in Stockholm (Ludden and Burris 1976; Nordlund et al. 1977). Nitrogenase was inactive because the cells were of necessity placed in the dark during cell harvesting and, as Gest and Kamen had originally noted, *in vivo* nitrogenase activity was lost during darkness.

Subsequent work has elaborated the complicated mechanism by which the cells accomplish the regulation of gas metabolism first noted in Gest and Kamen's simple manometric experiments. As noted above, nitrogenase is subject to reversible ADP-ribosylation, and this modification occurs on the surface of dinitrogenase reductase where it docks with dinitrogenase in the course of electron transfer. Recent results of Yaoping Zhang make it apparent that this ADP-ribosylation process is itself controlled by elements of the central nitrogen and carbon regulatory systems (Zhang et al. 2000, 2001). Yet other recent results from Robert Tabita's laboratory provide support for Howard Gest's long-controversial proposal that one role of nitrogenase is to provide a vent for excess reductant in the phototrophs (Tabita 1995).

The papers of Gest and Kamen merit occasional, if not regular, re-reading because so many gems of ideas were presented therein – perhaps an overnight train ride with our cultures would do us all some good.

Acknowledgments

The authors appreciate the willingness of those who have gone before, especially Bob Burris, Howard Gest and Bill Bulen, to share not only the data, but also the stories that make up the science that we all pursue. This paper was edited by H. Gest. A recent photograph of M. Kamen and H. Gest appears in the Editorial by Govindjee and H. Gest, this issue.

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