NITROGEN FIXATION BY PHOTOSYNTHETIC BACTERIA[1]

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The results from a series of studies concerned with the influence of molecular hydrogen on biological nitrogen fixation (Wilson, 1940) led to the suggestion that hydrogenase, the enzyme activating H_2, was associated in some way with the nitrogen-fixing enzyme system. Although obscure in the symbiotic combination of leguminous plant and *Rhizobium* (Wilson, Burris, and Coffee, 1943), the association was so noteworthy with *Azotobacter* that it suggested that any organism possessing hydrogenase might be a potential nitrogen fixer. At that time we tested three organisms using N^{15} as a tracer: *Escherichia coli; Proteus vulgaris*, Hoberman's (1942) strain that catalyzes the hydrogen-exchange reaction; and *Scenedesmus*, Gaffron's (1944) strain that, with appropriate treatment, switches from photosynthesis to photoreduction. This limited survey revealed no new nitrogen-fixing agents, but recently Gest and Kamen (1949*a*,*b*) and Kamen and Gest (1949) have provided a most interesting example in *Rhodospirillum rubrum*, a nonsulfur purple bacterium. Because of the significance of their findings, we undertook to check their more important observations. On their invitation[2] we first made a joint experiment testing the ability of a heavy suspension of the organism to fix N_2^{15}. The results were so clear-cut that it appeared likely that more conventional procedures using growing cultures on a low-nitrogen medium with a light inoculum and analysis by the Kjeldahl method would readily detect fixation by this organism. Independent tests in our three laboratories have confirmed this prediction.

The results from typical experiments are summarized in table 1. The medium consisted of KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; DL-malic acid, 3.5 g; Difco yeast extract, 0.2 g; biotin, 20 μg; trace metals (plus Mo) according to Hutner (1946); distilled water to 1 liter after the pH was brought to 7 with 6 N KOH. The inoculum was 2 per cent by volume of a culture grown on a similar medium (0.8 g sodium citrate added and the quantity of yeast extract doubled). Incubation was from 3 to 6 days at 25 C. Light was from a 200-watt mazda bulb about 30 inches from the cultures. Accompanying growth of the organism was an increase in alkalinity, but the pH seldom exceeded 8.0 at harvest. Little or no growth was noted anaerobically in the dark; aerobically, both in dark and light, growth was definite but apparently restricted to the combined nitrogen

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of the medium. Fixation thus apparently accompanies photoreduction (anaerobic, light); at least heterotrophic fixation (aerobic, dark) was insufficient to be detected with the Kjeldahl method. With N_2^{15}, evidence of a small though significant fixation has been observed; this point will require further testing.

TABLE 1

Nitrogen fixation and photoreduction by Rhodospirillum rubrum

CONDITIONS OF INCUBATION	EXPERIMENT I	EXPERIMENT II	EXPERIMENT III
Time in days	4	5	4
Anaerobic, light	77*	88†	77
Anaerobic, dark	22	22	20
Aerobic, light	22	25	18
Aerobic, dark	22	21	17
Uninoculated control	20	21	20

In experiment III, 0.05 M lactate were used instead of malate.

* All data are final total N in micrograms per ml.

† 101 micrograms per ml after 6 days.

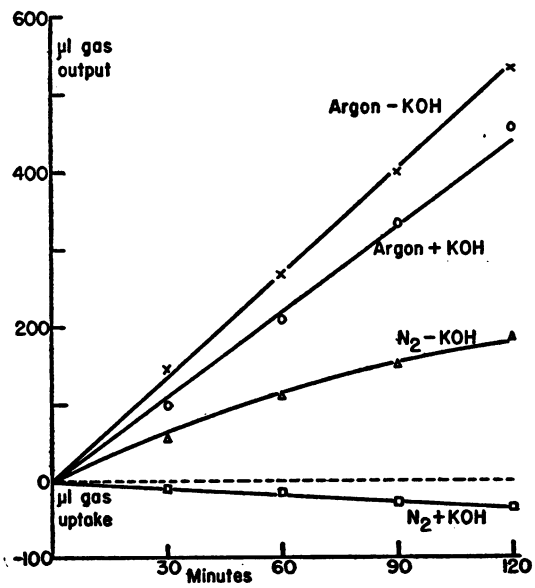

Figure 1. Suppression by molecular nitrogen of hydrogen evolution from *Rhodospirillum rubrum*. Forty-eight-hour culture of organism washed and suspended in pH 6.6 M/20 phosphate buffer; 2 ml of this suspension (1.24 mg cell N) plus 5 mg neutralized malic acid in 0.2 ml solution and 0.15 ml 20 per cent KOH (when used) placed in each Warburg flask. Flasks flushed with argon or nitrogen and gas exchange measured at 30 C under mazda lamps at light intensity of approximately 100 foot-candles.

One of the most significant observations made by Kamen and Gest was the inhibition of hydrogen evolution in *Rhodospirillum* by molecular nitrogen—the counterpart of hydrogen inhibition of nitrogen fixation. This critical observation likewise has been confirmed, as shown by the data in figure 1. In the presence of

N_2 the hydrogen evolution observed under argon was entirely suppressed. The gas uptake observed in an N_2 atmosphere in the presence of KOH probably arises from nitrogen fixation; during the 2-hour period such an uptake would constitute a 3.5 per cent increase in the cellular nitrogen present. The gas evolved from the growing culture before it was harvested for the manometric test formed an explosive mixture with O_2. The washed cells had a Q_{H_2} (N) of 185.

The close agreement of our experiments with those reported by Kamen and Gest is in itself noteworthy, since ability to duplicate results has scarcely been characteristic of the research on biological nitrogen fixation. Of greater importance is that the findings emphasize again that organisms known to contain hydrogenase, e.g., *Thiorhodaceae*, *Vibrio desulfuricans*, *Acetobacter peroxydans*, and certain strains of the luminous bacteria should be surveyed for the possible occurrence of hitherto undetected nitrogen fixers.

SUMMARY

The observations of Gest and Kamen that *Rhodospirillum rubrum* fixes molecular nitrogen and that the fixation is associated with photoreduction and the hydrogen metabolism of the organism have been confirmed. The results suggest that other organisms possessing hydrogenase should be examined for nitrogen fixation.

ADDENDUM TO PROOF

Of the untested hydrogenase-containing organisms, representatives of the *Thiorhodaceae*, because of their close relationship to *Rhodospirillum*, appear to be the most likely ones able to fix N_2. Professor C. B. van Niel kindly furnished us with strains of two genera to test this crucial point: *Chromatium*, a purple sulfur bacterium; and *Chlorobacterium*, a green sulfur bacterium. While this manuscript was in press results from Kjeldahl trials provided definite evidence that nitrogen fixation among the photosynthetic bacteria is not limited to the *Athiorhodaceae*. The results from a typical experiment were:

Chromatium:	refrigerated controls, 12 μg/ml
	in H_2—15 (4 days); 12 (5 days)
	in N_2—32 (4 days); 43 (5 days)
Chlorobacterium:	refrigerated controls, 12
	in H_2—12 (4 days); 14 (5 days)
	in N_2—30 (4 days); 32 (5 days)

Experiments with isotopic N_2^{15} are now in progress.

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