Technical Papers

Nitrogen Fixation by the Green and Purple Sulfur Bacteria[1]

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The relationship between hydrogenase, the enzyme specifically activating molecular hydrogen, and the nitrogen-fixing system in *Azotobacter* was established by Lee and Wilson in 1943 (*3*). This suggested that an organism that possesses hydrogenase may be a potential nitrogen-fixer. At that time representative genera of heterotrophic bacteria were tested using the sensitive isotopic technique, but the results were negative. Recently interest in this relationship has been renewed through the demonstration by Gest and Kamen (*1*) that photoproduction of H_2 by *Rhodospirillum rubrum*, a photosynthetic nonsulfur bacterium, was inhibited by molecular N_2. This unexpected analogue of the inhibition of nitrogen fixation in *Azotobacter* by H_2 (*5*) suggested that *Rhodospirillum* might fix nitrogen, a suggestion that was readily confirmed (*2*, *4*). Because of the importance of this discovery for a knowledge of the mechanism of biological nitrogen fixation, as well as its significance for the biogeochemistry of nitrogen, tests of other organisms known to contain hydrogenase are desirable. Of the untested hydrogenase-containing organisms (*4*), the purple sulfur and the green sulfur photosynthetic bacteria appeared to be the most likely candidates for hitherto unsuspected nitrogen-fixing ability. Through the courtesy of Mr. Helge Larsen and Professor C. B. van Niel, who kindly supplied us with representative cultures, we have recently examined this possibility.

The cultures tested were from two photosynthetic families: *Chromatium* sp., an uncharacterized species, isolated from marine mud, represented the purple sulfur bacteria; and *Chlorobacterium* sp., an organism known to carry out the reaction, $2\ H_2 + CO_2 \rightarrow (CH_2O) + H_2O$, represented the green sulfur bacteria. If a heavy inoculum of either of these organisms was added to a nitrogen-free medium containing minerals, bicarbonate, and a suitable hydrogen donor, profuse growth took place when the culture was incubated in the light under molecular nitrogen.

The inoculum for either culture was grown in a mineral medium containing NH_4Cl 1.0 g, KH_2PO_4 1.0 g, $MgCl_2 \cdot 5H_2O$ 0.2 g, NaCl 10.0 g, $NaHCO_3$ 2.0 g, $Na_2S_2O_3$ 1.3 g, $Na_2S \cdot 9H_2O$ 0.05 g, $FeCl_2 \cdot 6H_2O$ 0.5 mg, tap water 1 liter. The pH was adjusted to 7.4 for *Chlorobacterium* and to 8.5 for *Chromatium*. The level of NaCl was increased to 3% for *Chromatium*, and dl-malic acid was added to the medium to increase the speed of growth. An unwashed inoculum of 1.5 ml of a 4-day culture was added to 40 ml of a similar medium, except that it contained no added nitrogen. Nitrogen added in the inoculum and the tap water brought the initial concentration to about 15 μg N per ml. The cultures were incubated in artificial light under 0.8 atm of N_2. Controls analyzed at zero time were included, as well as controls that were incubated under H_2 rather than N_2. At the end of the incubation period, the nitrogen content was determined by a standard semimicro Kjeldahl method. Essentially the same general procedure was followed in the isotopic trials. The results are given in Table 1.

TABLE 1

NITROGEN FIXATION BY PHOTOSYNTHETIC BACTERIA

Experiment	Time, days	Total N in μg/ml		
		Control	Under H_2	Under N_2
		Chlorobacterium		
1	4	14, 14	17, 19, 22	30, 33, 32
2	3	12	14	23
	4	..	12	30
	5	12	14	32
3	5	14	14	24, 24, 31
	9	..	..	40, 36
		Chromatium		
4	5	10, 11, 11	11, 16	47, 56
	11	9	14, 14	59, 57
5	3	12	..	24
	4	..	15	32
	5	12	12	43
6	4	15, 16	..	37, 40

The final total nitrogen found in cultures of *Chlorobacterium* was about 35–40 μg/ml, independent of the initial nitrogen content or time of incubation. This limit was probably imposed by changes in the medium accompanying the photosynthetic reaction. Fixation by *Chromatium* showed no such definite restriction; if incubated for 2–3 weeks, fixation of 75–100 μg N/ml could be obtained. The results of the Kjeldahl trials were confirmed by the isotopic technique. When supplied an atmosphere containing 1.65 atom % excess of N_2^{15} *Chromatium* accumulated 0.442 atom % excess of N^{15} in one trial and 0.298% in a second. The corresponding figures for *Chlorobacterium* were 0.318% and 0.203%. Controls in N_2^{14} showed no change beyond the error of the experiment (about $\pm$ 0.03 atom %).

From the evidence in this and previous papers (*2*, *4*), it seems that the ability to fix atmospheric nitrogen is widespread among the photosynthetic bacteria, positive findings having been established for representatives of the three families *Athiorhodaceae*, *Thiorhodaceae*, and *Chlorobacteriaceae*. The discovery is noteworthy for several reasons, chief of which is that the initial test was

[1] Supported in part by grants from the Rockefeller Foundation and from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

not an empirical choice but was suggested by a consideration of the specific function of H_2 in the mechanism of biological nitrogen fixation—certainly a significant example of the role of theory in research. Likewise worthy of mention is that these photosynthetic organisms are the first bacteria to be unequivocally established as nitrogen-fixers since the discovery of *Azotobacter*, and that they are the anaerobic analogue of the blue-green algae as *Clostridium* is to *Azotobacter*. One member, *Rhodospirillum*, also possesses the distinction of having its nitrogen-fixing ability first demonstrated by the use of an isotope, then confirmed with Kjeldahl analyses (*2*, *4*), a reversal of the usual procedure, but one that seems appropriate for this atomic age.[2]

References

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[1] Since this manuscript was submitted, Duchow and Douglas *J. Bact.*, 1949, **58**, 409) have described a new genus of photoheterotrophic bacteria, *Rhodomicrobium vannielii*. Although related biochemically to the *Athiorhodaceae*, its unusual morphology and mode of cell division suggest that it should not be included among the *Eubacteriales*; pending further investigation they suggest it be placed in a provisional appendix to the *Schizomycetes*. Through the courtesy of Prof. Douglas we obtained a strain of this interesting organism for test of nitrogen fixation. Positive fixation has been obtained in both Kjeldahl and isotopic experiments.

Windowless, Flow Type, Proportional Counter for Counting C^{14} [1]

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The need in this laboratory for better counting of $BaCO_3$ samples with low activity has led to the design, construction, and use of a flow type, windowless, proportional counter (*1*, *2*, *4*).

The advantages to be gained by using proportional counting rather than Geiger-Müller counting are: (1) a higher maximum counting speed and no dead time corrections; (2) less sensitivity of the counter to gas contamination; (3) indefinitely long counter life; and (4) the opportunity to use pulse discrimination to reduce the background.

Operation in the proportional region necessitates the use of an amplifier of voltage gain between 100 and 1,000. This places more stringent conditions on the insulation, shielding, and line isolation of the circuits than is the case with Geiger-Müller counting.

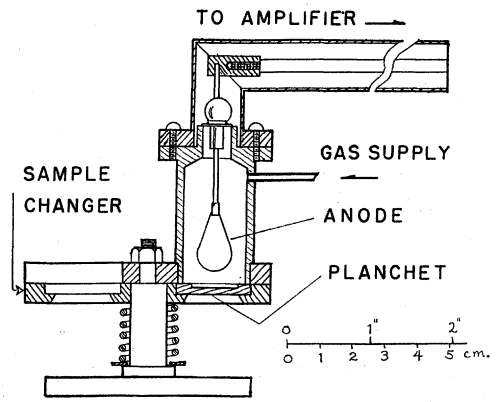

FIG. 1. Section of flow counter and sample changer.

The entire counting apparatus consists of (1) the counter, (2) the sample changer, (3) the electronic unit, and (4) the gas supply tank and connections.

The gas used is argon plus 5% CO_2 and is purchased already mixed. The gas flow is controlled by a needle valve with an extended handle and two stops, one for the flush position and one for steady flow. From the valve the gas is piped to an oil bubbler and thence to the counter. The gas can escape around the planchet and through the hole below.

The electronic unit is a Model 162 amplifier-scaler made by Nuclear Instrument and Development Laboratories. The amplifier has a maximum gain of 400 and the scaler will pass .40-v pulses. The counter is coupled directly into the amplifier input by a short piece of coaxial line. With this arrangement no preamplifier is required and the interference picked up in connections is minimized. An isolation transformer in the 110-v supply is necessary to prevent line interference.

A section of the counter and the sample changer is shown in Fig. 1. The counter has a ¾-in. diam brass cathode and an anode consisting of a loop of .002-in.-diam tungsten wire. The loop is 9 mm by 17 mm and reaches to within 5 mm of the bottom of the planchet. The planchets used are of stainless steel and have a recess 9/16 in. in diam and .037 in. deep. Measurements of the asymmetry of sensitivity of this counter have been made. For a small spot source placed on a planchet near the edge of the recess, the rate of counting with the source in the plane of the loop is 10% more than at 90° from this position. For a reasonably uniform sample, therefore, the asymmetry is negligible.

The sample changer has a rotating disk as shown in Fig. 1, with two holes for planchets and a spring, not shown, which positions the planchets in the counter by catching in each of two notches in the rim of the disk.

Ten seconds has been found to be an adequate time for flushing the counter. If flushing is inadequate, the counting rate is depressed until the air has had a chance to clear out. Thus a method of checking on the presence

[1] This work was supported in part by the Office of Naval Research, the U. S. Atomic Energy Commission, and the American Cancer Society. The author wishes to thank D. Steinberg for help with the preparation of the $BaCO_3$ samples, the Harvard Cyclotron group for providing C^{11}, and Elizabeth White for counting the samples.