THE RELATIONSHIP BETWEEN PHOTOSYNTHESIS AND NITROGEN FIXATION*

BY E. S. LINDSTROM,† J. W. NEWTON AND P. W. WILSON

DEPARTMENT OF BACTERIOLOGY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN, MADISON

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In 1934 Fred and Wilson[1] called attention to a correlation between photosynthesis and nitrogen fixation in leguminous plants. Though based on a variety of experimental findings, the correlation was primarily an empirical observation since the biochemical methods available were not adequate for a direct test of the interdependence, if any, between them. Recently, we have undertaken such a direct test with two new experimental developments that promise greater hope for success than was possible with the relatively crude techniques of nearly 20 years ago. These are the use of N^{15} for detection of nitrogen assimilation and the discovery that photosynthetic bacteria fix nitrogen.[2, 3] The isotopic method provides a test with a sensitivity up to 100 times that of the conventional Kjeldahl method, and the photosynthetic bacteria furnish an agent that is somewhat more readily handled than legumes or nitrogen-fixing blue-green algae. Specifically, we have sought an answer to the questions: does nitrogen fixation occur in the absence of photosynthesis, and if so, what is the quantitative relationship in comparison with that in its presence.

Experimental.—The surface of the seeds of *Trifolium pratense* (strawberry red clover) were sterilized by immersion in 1:1000 $HgCl_2$. After six rinsings with sterile distilled water, they were germinated for 48 hours on moist filter paper in sterile Petri dishes, and then five to seven seedlings transferred to tubes (3 × 16 cm.) containing sterile quartz sand. The seedlings were covered with a thin layer of sand, watered with a nitrogen-free nutrient solution[4] and inoculated with a culture of *Rhizobium trifolium* 200. The culture tubes were topped with a standard taper joint that could be fitted on a gas manifold.[5] After 4 to 6 weeks' growth under controlled conditions, the plants were ready for use, the uninoculated controls showing typical symptoms of nitrogen starvation. The plants were either darkened by covering the tubes with lead foil, or the tops were removed at the sand level with curved surgical scissors. After a suitable period of pretreatment, these tubes plus the illuminated inoculated and uninoculated controls were placed on the manifold and gassed with an atmosphere containing excess $N_2{}^{15}$. After 1 to 3 days' incubation at 25°C., during which only the positive (inoculated) and the negative (uninoculated) controls were illuminated, the intact plants or the remaining roots were washed from the tubes and subjected to routine analyses for N^{15} content.

As controls for the effect of light on the uptake and incorporation of nitrogen independent of fixation, other tests were made in which $(N^{15}H_4)_2SO_4$ was added to the tubes of similarly predarkened plants. At the conclusion

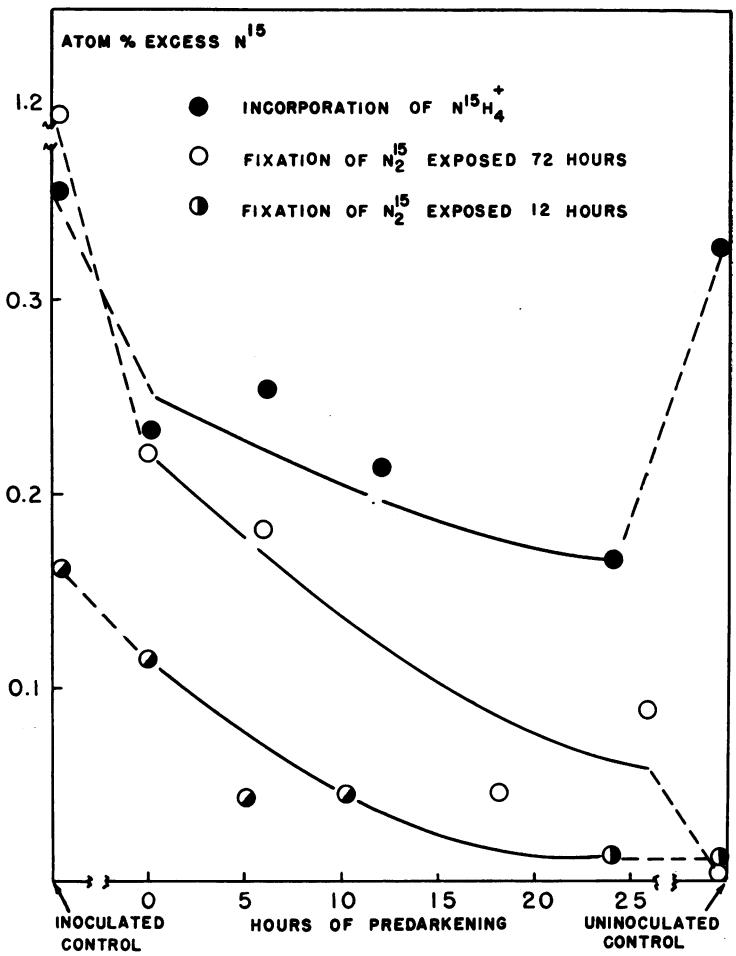

FIGURE 1

The effect of predarkening on the fixation and incorporation of N^{15} by *Trifolium pratense*. The control plants were treated in the light, the experimental plants in the dark. Five plants per treatment. N_2 supplied was 32 atom per cent excess N^{15} for fixation experiments. For incorporation experiments 5 mg. $(NH_4)_2SO_4$-N (32 atom per cent excess N^{15}) supplied per five plants.

of these experiments, the plants were washed from the tubes, rinsed six times in distilled water, and analyzed for N^{15} content. To determine NH_4^+ *uptake*, the N^{15} content of the plants was determined without further

treatment. To determine NH_4^+ *incorporation*, the plants were hydrolyzed with 6 *N* HCl in closed tubes in the autoclave (120°C.) for five hours, the hydrolysate made alkaline and steam distilled to remove the ammonia and amide nitrogen, and the residuum analyzed for N^{15}. As a control for removal of all NH_4^+-N in the hydrolysate, 1 mg. of $N^{14}H_4^+-N$ was distilled over after the primary distillation was complete. Analysis of this distillate for N^{15} confirmed complete removal.

Rhodospirillum rubrum S1 were grown anaerobically in the light at 25°C. for 4 to 6 days in a medium of: DL-malic acid, 3.5 g.; L-glutamic acid·HCl, 5.0 g.; sodium citrate·$2H_2O$, 0.8 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; K_2HPO_4,

TABLE 1

NITROGEN FIXATION BY DECAPITATED OR PREDARKENED *Trifolium pratense*

TREATMENT	ATOM PER CENT EXCESS N^{15}	µG N FIXED/7 PLANTS (CALCULATED)
Experiment I[a]		
Inoculated—always in light	+0.93	27
	+1.40	49
Inoculated—decapitated at time of exposure to N^{15}	+0.580	23
	+0.166	6
Inoculated—predarkened for 6 hours and for the course the experiment	+0.477	21
Uninoculated—always in the light	+0.005	..
Experiment II[b]		
Inoculated—always in the light	+0.826	28
Inoculated—decapitated at time of exposure to N^{15}	+0.208	7
	+0.120	4
Inoculated—predarkened for 2 hours and the course of the experiment	+0.254	10
Uninoculated—always in the light	+0.005	..

[a] 7 plants per treatment. 0.4 atm. of 70% N_2 (28.5 atom per cent excess N^{15}), 20% O_2, 10% CO_2. Incubated 36 hours at 25°C.

[b] 7 plants per treatment. 0.3 atm of 75% N_2 (32 atom per cent excess N^{15}), 25% O_2. Incubated 48 hours at 25° C.

1.0 g.; $CaSO_4$, 0.1 g.; Difco yeast extract, 0.5 g.; distilled water, 1 liter; pH adjusted to 7.0 with 6 *N* NaOH before autoclaving. The cells from 10 ml. of this medium were centrifuged and resuspended in 25 ml. of medium of K_2HPO_4, 1.2 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; $CaSO_4$, 0.1 g.; DL-malic acid, 3.5 g.; biotin, 20 µg.; MO, 0.01 mg.; distilled water, 1 liter; pH adjusted to 7.0 with 6 *N* NaOH before autoclaving. Such cultures were incubated in the light and dark in atmospheres containing N_2^{15} at 30°C. for 3 to 5 days.

Results.—The results of the plant experiments are summarized in figure 1. Any quantitative comparisons of the fixation by predarkened plants should exclude the value of the light control since this depends on the length of the experiment.

Fixation of nitrogen can take place in the dark though at decreasing rates as the period of predarkening is increased. The incorporation of externally applied $N^{15}H_4^+$ into the protein N of the plants is also affected by predarkening though to a lesser extent. In an experiment to determine the effect of predarkening on the uptake of $N^{15}H_4^+$, the following data were obtained: 24 hours predarkening, 3.25 atom per cent excess N^{15}; 10 hours, 1.61; 5 hours, 2.83; 0 hour, 2.45; light control, 4.30 atom per cent excess N^{15}. From these data it can be seen that the uptake of NH_4^+ is even less light dependent. Table 1 presents data to show that plants decapitated before exposure to N_2^{15} can also fix nitrogen.

In an experiment with *R. rubrum*, the cultures were incubated for 72 hours under 0.2 atm. of a gas mixture of the following composition: 75% N_2 (32 atom per cent excess N^{15}) and 25% O_2. The culture incubated in the light assayed 9.78 atom per cent excess N^{15}; the one in the dark assayed 0.104. Therefore *R. rubrum* will fix nitrogen aerobically in the dark at a rate of about 1% that in the light. This agrees well with the level of fixation reported by Gest, Kamen, and Bregoff[2] for anaerobic dark fixation.

Discussion.—Nitrogen fixation is dependent on photosynthesis both in *T. pratense* and *R. rubrum*, but the available data permit only suggestions of possible mechanisms of dependence. Since both organisms utilize NH_4^+-N in the dark, it is not lack of demand for fixed nitrogen that is limiting fixation to an equilibrium value. The relationship is probably that of a photosynthetic intermediate and/or a direct product of photosynthesis necessary for nitrogen fixation.

From the data it is evident that predarkening the clover plants for 24 hours will completely stop nitrogen fixation. In contrast, *Rhodospirillum rubrum* grown heterotropically and aerobically in the dark and then exposed to N_2^{15} in the dark fixes nitrogen at the same level as do photosynthetically *grown* cells exposed to N_2^{15} *in the dark*. Thus, the bacteria appear to have a non-photosynthetic mechanism that can supply at a low level whatever is necessary for nitrogen fixation. Possibly some form of reducing power created through photosynthesis is necessary for fixation and to a less extent for incorporation of nitrogen. It should be noted that fixation falls off in darkened *R. rubrum* much more rapidly than in darkened *T. pratense*. The low value for dark fixation by *R. rubrum* has been consistently observed and has defied our attempts to increase it.

Summary.—Both *Rhodospirillum rubrum* and inoculated plants of *Trifolium pratense* assimilated molecular nitrogen in the dark under aerobic conditions, though at a rate much slower than in the light. Although a definite interdependence between nitrogen fixation and photosynthesis exists in both organisms, dark fixation was of much less magnitude in *R. rubrum*.

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† Present address: Institute for Enzyme Research, University of Wisconsin, Madison.

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*OXIDATION-REDUCTION POTENTIALS OF THE DIPHOSPHOPYRIDINE NUCLEOTIDE SYSTEM**

BY F. LEE RODKEY AND ERIC G. BALL

DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, BOSTON

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Since the discovery of diphosphopyridine nucleotide (DPN) and its implication in biological oxidative mechanisms, many attempts have been made to estimate the oxidation-reduction potential of the system formed by this compound and its reduction product. The majority of the potential values reported in the literature are calculated from data on the equilibrium constants for reactions involving DPN and various enzyme-substrate systems.[4, 6, 8, 15] Such calculations require a knowledge of the oxidation-reduction potential or free energy change for the substrate system which in some cases is not too accurately known. Moreover in many cases the equilibrium constants for these reactions are of a magnitude which is not favorable to their exact determination. The only report of direct measurement of the oxidation-reduction potential of this system is the colorimetric technique used by Ball and Ramsdell.[2, 3] In view of the above considerations, it is not surprising that the values reported for the E_0' of the DPN system at pH 7.0 range from -0.26 to -0.35 volt.

It is our purpose to present in this paper what is believed to be the first potentiometric determination of the oxidation-reduction potential of the DPN system.

Experimental.—Apparatus: The apparatus used has been previously described.[14] Gold-plated platinum electrodes were used with a saturated calomel electrode as reference half cell. The potential of the saturated calomel half cell was established by measurement against the hydrogen electrode in standard buffer solutions of the type suggested by Hitchcock