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Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts

(legumes/energy)

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ABSTRACT **Nitrogenase-dependent hydrogen evolution from detached legume nodules and from reaction mixtures containing cell-free nitrogenase has been well established, but the overall effect of hydrogen evolution on the efficiency of nitrogen fixation *in vivo* has not been critically assessed. This paper describes a survey which revealed that hydrogen evolution is a general phenomenon associated with nitrogen fixation by many nodulated nitrogen-fixing symbionts. An evaluation of the magnitude of energy loss in terms of the efficiency of electron transfer to nitrogen, via nitrogenase, in excised nodules suggested that hydrogen production may severely reduce nitrogen fixation in many legumes where photosynthate supply is a factor limiting fixation. With most symbionts, including soybeans, only 40–60% of the electron flow to nitrogenase was transferred to nitrogen. The remainder was lost through hydrogen evolution. *In situ* measurements of hydrogen evolution and acetylene reduction by nodulated soybeans confirmed the results obtained with excised nodules. In an atmosphere of air, a major portion of the total electron flux available for the reduction of atmospheric nitrogen by either excised nodules or intact nodulated plants was utilized in the production of hydrogen gas. Some nonleguminous symbionts, such as *Alnus rubra*, and a few legumes (i.e., *Vigna sinensis*) apparently have evolved mechanisms of minimizing net hydrogen production, thus increasing their efficiency of electron transfer to nitrogen. Our results indicate that the extent of hydrogen evolution during nitrogen reduction is a major factor affecting the efficiency of nitrogen fixation by many agronomically important legumes.**

The increasing world population and depletion of fossil fuel supplies have stimulated renewed interest in methods of increasing agricultural productivity while minimizing the consumption of fossil fuels. A major factor limiting agricultural production is nitrogen fertilizer, the synthesis of which consumes major quantities of energy. Approximately 3% (600×10^9 cubic feet; 16.8×10^9 meter^3) of the natural gas consumed in the United States in 1973 was used for the synthesis of 17×10^6 U.S. tons (153×10^8 kg) of anhydrous ammonia (1, 2). About 10×10^6 U.S. tons of synthetic ammonia were used for nitrogen fertilizer to supply a portion of an annual agricultural nitrogen demand of about 18×10^6 U.S. tons (1, 2). A large part of the remaining need for nitrogen in agriculture was supplied by nitrogen-fixing organisms, such as legumes, which utilize photosynthetically stored solar energy to reduce atmospheric nitrogen to ammonia. Because the biological nitrogen-fixing process is not dependent upon nonrenewable energy resources, its use in agriculture should be maximized. Factors limiting biological nitrogen fixation therefore deserve thorough investigation (3, 4).

One characteristic of all cell-free nitrogenase preparations that might limit nitrogen fixation is the release of hydrogen gas concomitant with nitrogen reduction (5–11). During this process, which occurs at substantial rates even when nitrogen is saturating (11, 12), about four molecules of ATP are hydrolyzed per H_2 produced (13–15). Although H_2 evolution has been observed from detached nodules of some legumes (16–20), it has been suggested that no net ATP-dependent production of hydrogen via nitrogenase occurs *in vivo* (5). This question, however, has not been adequately resolved. Under conditions where photosynthate is limiting, hydrogen production by nitrogenase could decrease nitrogen fixation (21). At present the generality of H_2 evolution by nodulated nitrogen-fixing symbionts is unknown and the quantitative relationship of H_2 production to the efficiency of the nitrogen-fixing process has not been critically assessed. An examination of these questions is reported in this paper.

MATERIALS AND METHODS

Plant materials were either collected from the field or cultured in a greenhouse or growth chamber. Unless otherwise specified (Table 1), the plants were infected with naturally occurring endophytes. Random samples of nodules were collected from vigorous mature plants after 5–10 hr of exposure to light. Nodules were excised with a small segment (2–3 cm) of root, and precautions were taken to keep nodules moist without excessive wetting. Cell-free extracts of soybean nodules were prepared according to methods of Klucas *et al.* (22). Gases were obtained from National Cylinder Gas Co. (Portland, Oreg.) or from Matheson Gas Products (Newark, Calif.).

Acetylene (C_2H_2) reduction assays of excised nodules were performed as described previously (23). Evolution of hydrogen from excised nodules or cell-free nodule extracts was recorded continuously by use of an amperometric method that utilized a hydrogen sensitive electrode (YSI 4004, Yellow Springs Instrument Co., Yellow Springs, Ohio) (24). The electrode cuvette was obtained from Gilson Medical Electronics (Gilson, Mich.) and a model 602 electrometer from Keithley Instruments (Cleveland, Ohio). The electrode was sensitized and calibrated with 1% H_2:99% argon. The problem of signal instability reported by Wang *et al.* (24) was alleviated by continuously maintaining a potential of +0.6 V across the electrode. Hydrogen evolution and acetylene reduction assays were conducted on soybeans *in situ* in Saran bags (25). Hydrogen, oxygen, and nitrogen concentrations were determined with a modified Carle 8515 gas chromatograph (Carle Instruments, Fullerton, Calif.) equipped with dual columns (⅛ inch × 2.75 feet Porapak Q in series with ⅛ inch × 10 feet Molecular Sieve 5A, 40–60 mesh). Argon (Ar) was used as a carrier at a flow rate of 2 ml/min. The column temperature was 75°. The signal from the chromatograph was amplified with an operational amplifier (McKee-Pederson Instruments, Danville, Calif.). Other experimental details are listed in the legends.

Table 1. A survey of the magnitude of hydrogen evolution from nodules of nitrogen-fixing symbionts

Species	Inoculant	No. samples	H_2 evolution*		C_2H_2 reduction*	Relative efficiency ‡	
			Air	Ar†		$1-\frac{H_2\ (air)}{H_2\ (Ar)}$†	$1-\frac{H_2\ (air)}{C_2H_2}$
Legumes							
Alfalfa (*Medicago sativa*)	Commercial §	5	7.16	16.36	15.08	0.54 ± 0.04	0.51 ± 0.04
Alfalfa (*M. sativa*)	Native	3	3.38	6.98	7.16	0.51 ± 0.03	0.53 ± 0.03
Sweet clover (*Melilotus alba* and *M. officinalis*)	Native	4	4.00	10.22	9.84	0.59 ± 0.03	0.59 ± 0.03
Strawberry clover (*Trifolium fragiferum*)	Commercial	3	4.57	11.13	10.03	0.59 ± 0.01	0.55 ± 0.04
Alsike clover (*T. hybridum*)	Commercial	9	2.66	5.77	8.87	0.50 ± 0.04	0.68 ± 0.04
White clover (*T. repens*)	Commercial	3	3.84	7.52	6.94	0.50 ± 0.02	0.45 ± 0.04
Red clover (*T. pratense*)	Commercial	3	2.03	4.11	4.00	0.47 ± 0.08	0.49 ± 0.01
Subterranean clover (*T. subterraneum*)	Commercial	3	1.65	2.79	2.11	0.40 ± 0.05	0.20 ± 0.06
Subterranean clover (*T. subterraneum*)	Commercial	3	3.76	8.58	7.73	0.56 ± 0.02	0.41 ± 0.18
Birdsfoot trefoil (*Lotus corniculatus*)	Native	4	2.43	6.09	4.38	0.61 ± 0.03	0.44 ± 0.14
Lupine (*Lupinus sp.*)	Native	3	1.59	3.83	4.96	0.58 ± 0.05	0.69 ± 0.03
Soybean (*Glycine max*)	Commercial	6	3.78	9.19	7.31	0.57 ± 0.03	0.52 ± 0.07
Garden pea (*Pisum sativum*)	*R. leguminosarum* 128 C54	5	6.35	12.57	17.37	0.46 ± 0.04	0.63 ± 0.04
Garden pea (*Pisum sativum*)	*R. leguminosarum* 128 C75	8	6.74	12.99	13.61	0.45 ± 0.03	0.46 ± 0.07
Common vetch (*Vicia sativa*)	Native	4	0.65	1.25	1.03	0.46 ± 0.04	0.36 ± 0.03
Common vetch (*Vicia sativa*)	*R. leguminosarum* 128 C54	1	5.37	11.86	13.54	0.55	0.60
Scotch broom (*Cytisis scoparius*)	Native	5	0.53	1.09	2.24	0.52 ± 0.04	0.78 ± 0.05
Cowpea (*Vigna sinensis*)	*R. 32H1*	2	0.03	10.55	14.26	0.99 ± 0.003	0.99 ± 0.003
Mung bean (*Phaseolus aureus*)	*R. 32H1*	3	1.88	6.35	12.33	0.73 ± 0.06	0.82 ± 0.08
Non-legumes							
Red alder (*Alnus rubra*)	Native	6	0.03	0.53	6.96	0.94 ± 0.01	0.99 ± 0.001
Purshia tridentata	Native	6	0.03	0.03	1.2	0.88 ± 0.05	0.97 ± 0.01
Russian olive (*Elaeagnus angustifolia*)	Native	8	0.36	2.9	14.8	0.87 ± 0.05	0.97 ± 0.01
Ceanothus velutinus	Native	3	0.38	0.83	1.84	0.33 ± 0.23	0.77 ± 0.04
Myrica californica	Native	4	0.001	0.12	1.49	0.98 ± 0.006	0.99 ± 0.0003

* Mean rates of H_2 evolution and C_2H_2 reduction are presented (μmol/hr per g fresh weight). C_2H_2 assays were conducted on nodules after measurements of H_2 evolution.
† 79.96% Ar:20% O_2:0.04% CO_2.
‡ Values for relative efficiency are expressed as means ± SEM.
§ Commercial inocula were obtained from The Nitragin Co. (Milwaukee, Wisc.).

RESULTS

Utilizing the amperometric technique, hydrogen evolution from reactions containing crude extracts and purified components of nitrogenase from soybean root nodules was continuously monitored. Because changes in the rate of ATP-dependent hydrogen production (nitrogenase activity) can be observed rapidly, the amperometric technique has many advantages for biochemical studies of the mechanism and regulation of nitrogenase. The only other continuous nitrogenase assay follows spectrophotometrically the oxidation of dithionite in the presence of a reducible substrate (26). Characterization of the requirements for hydrogen production in nitrogenase catalyzed reactions indicated that protons

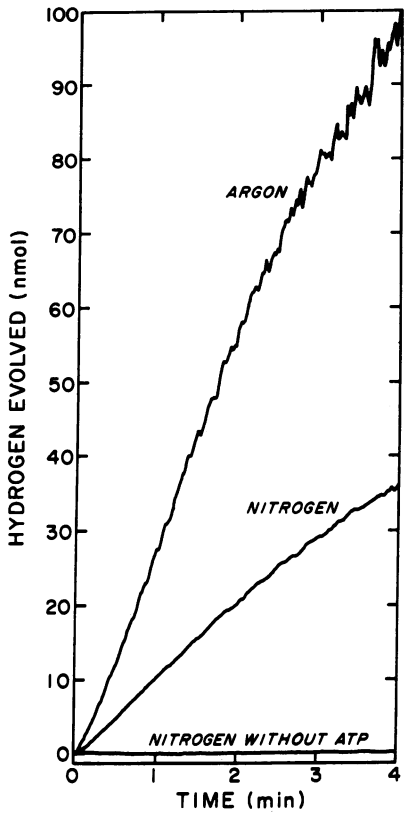

FIG. 1. Continuous amperometric measurements of hydrogen evolution from reactions containing nitrogenase components from soybean root nodules. The complete assay mixture contained 50 μmol of creatine phosphate, 7.5 μmol of Na_2ATP, 0.2 mg of creatine phosphokinase, 20 μmol of $Na_2S_2O_4$, 10 μmol of $MgCl_2$, and 37.5 μmol of {TES [N tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid} buffer at pH 7.5 in a final volume of 1.5 ml. The assay temperature was 30°. Reactions were initiated by the addition of 10 μl of purified molybdenum-iron protein (15 mg/ml) and saturating amount (50 μl) of partially purified iron protein (6 mg/ml). Recorder tracings are shown for reactions under nitrogen or argon and also for a reaction without ATP carried out under nitrogen. Tracings similar to that recorded when ATP was omitted were obtained when either dithionite or enzyme was omitted or when oxygen was added to the reaction mixture.

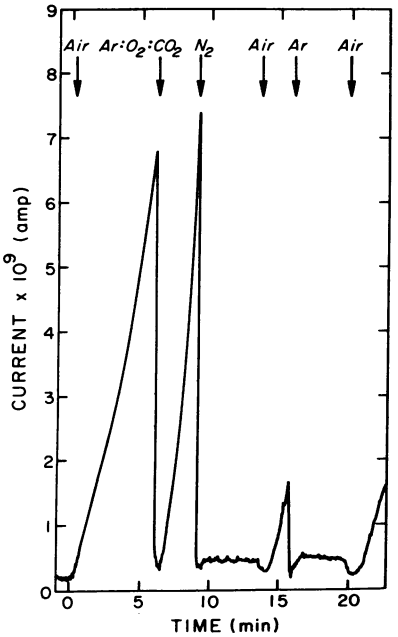

FIG. 2. The effect of different gases on the amperometric measurement of hydrogen evolution from intact soybean nodules. Soybean nodules (0.256 g) were excised with a small segment of root (2–3 cm) and placed in the electrode chamber at zero time. Hydrogen evolution was followed continuously. The chamber was flushed for 30 sec with the gases or mixtures of gases as indicated (↓). After each flushing the electrode chamber was sealed by placing water in the capillary outlet. After measurement of hydrogen evolution, the nodules were assayed by the acetylene reduction technique. The rate of hydrogen evolution in air, in a mixture of 79.96% Ar:20% O_2:0.04% CO_2, and the rate of acetylene reduction in air were as follows: 2.8, 8.1, and 8.3 μmol/hr per g fresh weight, respectively. The relative efficiency was 0.66.

were reduced by nitrogenase and that hydrogen evolution was dependent upon an ATP-generating system, dithionite, and nitrogenase components. The rate of hydrogen evolution increased in an atmosphere of argon under conditions in which the iron-molybdenum protein was saturated with the iron protein. The ratio of hydrogen evolution in argon to that in nitrogen was 3.3, although this ratio varied with component ratio. The addition of oxygen to the reaction mixture eliminated further production of hydrogen. Linear rates of hydrogen evolution were obtained for three to four minutes (Fig. 1).

The amperometric technique was adapted to measure hydrogen production from excised root nodules. Thus, the technique served as a tool to assess the magnitude of hydrogen evolution from intact nodules and to rapidly record the effects of changes in the gaseous environment on hydrogen production. A typical amperometric tracing of hydrogen evolution from excised soybean root nodules is represented in Fig. 2. The production of hydrogen was oxygen dependent, and the rate of hydrogen formation increased in an atmosphere in which nitrogen was replaced with argon. This behavior is consistent with the assumption that hydrogen is evolved from nitrogenase rather than a classical hydrogenase (16, 27, 28).

According to Hardy and others, the rate of hydrogen evolution in the absence of other reducible substrates should represent the total electron flux to the nitrogenase system (5). Thus, the rate of nitrogen reduction in intact nodules should be equivalent to the difference between the rates of hydrogen evolution in a mixture of Ar, O_2, and CO_2, and the rate in air. Likewise, in the presence of saturating amounts of acetylene, no hydrogen is formed and the total electron flow to nitrogenase presumably is used to reduce acetylene (20, 25). Assuming there is no utilization of hydrogen formed by nitrogenase through a classical hydrogenase, the rate of acetylene reduction should equal the rate of hydrogen evolution in $Ar:O_2:CO_2$. Consequently, the relative efficiency of electron transfer to nitrogen via nitrogenase may be defined in the following manner:

$$\text{Relative efficiency} = 1 - \left[\frac{\text{rate of } H_2 \text{ evolution in air}}{\text{rate of } H_2 \text{ evolution in } Ar:O_2:CO_2 \text{ or rate of } C_2H_2 \text{ reduction}}\right]$$

Using H_2 evolution and C_2H_2 reduction measurements to calculate the relative efficiency of electron transfer to nitrogen via nitrogenase, the significance of hydrogen production from nodulated nitrogen-fixing symbionts was surveyed (Table 1).

Generally, the relative efficiencies of electron transfer to nitrogen ranged from 0.40 to 0.60 for legumes, except cowpeas and mung beans that were inoculated with *Rhizobium*

Table 2. *In situ* measurements of hydrogen evolution from soybeans

No.	Treatment*	Rate of H_2 evolution†	Rate of C_2H_4 production†
1	Incubated in air	8	0
2	Incubated in air with C_2H_2‡	0	13
3	Incubated in 80% Ar: 20% O_2	13	0
4	Incubated in 80% Ar: 20% O_2 + C_2H_2‡	0	12

* Soybeans (4-weeks-old) grown in greenhouse in 8-inch pots filled with Perlite. Plants were sealed in Saran bags after 7 hr of exposure to light (1600 footcandles; 17,600 lux) and then exposed to indicated gas mixtures.

† Values are mean rates (μmol/hr per g fresh weight) of four replicates, with the exception of treatment 3, where one replicate was excluded as a result of leakage. Results are based upon linear rates between 2 and 10 hr.

‡ C_2H_2 concentration was approximately 0.1 atm (10^4 Pa).

32H1. All of the nodulated non-legumes apparently were well coupled, as indicated by relative efficiencies approaching unity in most cases. The high efficiencies of some symbionts (i.e., the non-legumes) may be the result of hydrogen utilization through a classical hydrogenase such as reported by Dixon (18, 19). This conclusion is supported by the observation that the rates of C_2H_2 reduction by nodules from non-legumes were significantly higher than corresponding rates of H_2 evolution in argon. Apparently this is the case for red alder (*Alnus rubra*), but not for cowpeas inoculated with *Rhizobium* 32H1. Nodules of red alder exhibited hydrogen uptake under conditions in which H_2 evolution was blocked with C_2H_2, whereas no uptake was observed for the relatively efficient cowpea nodules. Some evidence of variation in efficiencies of different strains of *Rhizobium* on a given legume cultivar was observed, but further investigation of this point is necessary. From these experiments (Table 1) it is evident that electron transfer to nitrogen via nitrogenase in excised nodules of a variety of legumes is inefficiently coupled and that the loss of energy through H_2 evolution is a major factor influencing the efficiency of nitrogen fixation in nodulated legumes.

To test the possibility that the relative efficiency of electron transfer was influenced by excision of the nodules, an experiment was conducted in which hydrogen evolution from nodulated soybeans was measured *in situ* (Table 2). As postulated, the rate of hydrogen production in $Ar:O_2:CO_2$ was approximately equivalent to the rate of acetylene reduction and no evolution of hydrogen was detectable in the presence of saturating concentrations of acetylene. The most significant findings were that hydrogen was produced by nodulated soybeans *in situ* in air, and the magnitude of hydrogen evolution accounted for a significant fraction (60%) of the electron flow available for nitrogen reduction. These values, which were obtained by direct measurement of hydrogen production by gas chromatographic analysis, were consistent with results of amperometric measurements of hydrogen evolution from excised nodules.

There are reports (15, 29) that the extent of hydrogen evolution *in vitro* varies with energy charge and that energy charge in nodules was affected by the rate of photosynthesis (30). It was considered necessary, therefore, to measure hydrogen production by intact nodulated soybean cultures under conditions where shoots were exposed to light and only the root systems exposed to C_2H_2. Time courses for hydrogen evolution and C_2H_2 reduction for replicated pots of soybeans are shown in Fig. 3. The relative efficiency of electron transfer to nitrogen was 0.56. These results confirm the conclusion that H_2 evolution from nitrogenase constitutes a significant loss of the energy available for nitrogen fixation *in situ* by cultures of soybeans in an atmosphere of air. Further investigations need to be conducted under field conditions where microbial utilization of H_2 in the soil might occur.

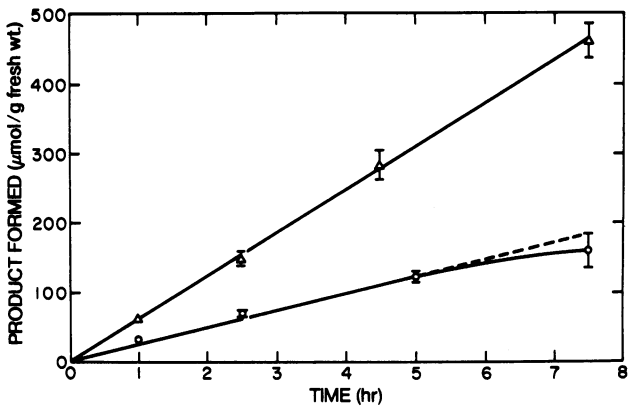

FIG. 3. *In situ* measurement of hydrogen evolution and acetylene reduction by soybeans. Pots containing 4-week-old soybean plants were placed in Saran bags. The bags were sealed around the stems and around a sampling tube in each bag with Plasticine. The nodulated roots were exposed to an atmosphere of either air or air containing about 0.1 atm of C_2H_2. During an 8-hr incubation at 28°, plants were exposed to light with an intensity of approximately 1600 foot candles (17,600 lux). Gas samples were removed for gas chromatographic analyses of hydrogen evolution from plants exposed to air and ethylene produced from plants exposed to C_2H_2. Mean rates of product formed ±SEM for replicates are shown for H_2 (○) and ethylene (△). The dashed line in the lower curve is an extrapolation of an initial linear rate.

DISCUSSION

The evolution of H_2 from reaction mixtures containing cell-free nitrogenase has been thoroughly documented, but the generality of occurrence and the significance of hydrogen evolution *in vivo* have not been ascertained (5). This work establishes the occurrence of hydrogen production in a wide variety of nodulated nitrogen-fixing symbionts and provides a quantitative estimate of the proportion of the total electron flow to nitrogenase that is diverted for proton reduction. The apparent failure of many researchers to take into account the magnitude of nitrogenase catalyzed hydrogen production may have contributed to the wide ranges in C_2H_2 to N_2 ratios and ATP per electron pair ratios reported in the literature (5, 13–15, 31, 32). Conversion of rates of acetylene reduction to rates of nitrogen fixation based on an assumed ratio of 3 mol of C_2H_2 per N_2 obviously may be subject to considerable error. For example, in our experiments where C_2H_2 completely suppressed hydrogen evolution and approximately half of the total electron flux to nitrogenase in air was lost as hydrogen, the expected C_2H_2 to N_2 ratio would be 6 to 1 rather than 3 to 1. In order to obtain valid estimates of nitrogen fixation using acetylene reduction values, conversion factors must be determined under experimental conditions, as emphasized by Burris (25).

The 40–60% loss of energy from most legumes through hydrogen evolution constitutes a reduction in the efficiency of nitrogen fixation comparable to the decrease in photosyn-

thetic efficiency resulting from photorespiration (33). Photosynthate supply appears to be the most important factor limiting nitrogen fixation in field-grown soybeans (21). According to Minchin and Pate (34), 12% of the total photosynthate produced in peas is used for nodule respiration. Assuming energy supply limits nitrogen fixation by agricultural legumes and the relative efficiency of electron transfer to nitrogenase is about 0.5, then conservation of the energy lost as hydrogen could theoretically double the estimated 12.8×10^6 U.S. tons of nitrogen fixed annually by legumes in the U.S. (5). Alternatively, if some factor other than photosynthate supply controls the amount of nitrogen fixed, then the reduction of energy consumption for hydrogen evolution via nitrogenase presumably would increase yield of dry matter.

Based on our experimentally determined rates of hydrogen evolution by nodulated soybeans, we have estimated that the hydrogen produced annually by 50×10^6 acres of soybeans in the U.S. is equivalent in energy to 300×10^9 cubic feet of natural gas (5). The possibility of "harnessing" the solar energy released as hydrogen gas from nodulated plants should be considered (35, 36). This approach, however, may not be feasible because of the technical problems in trapping, purifying, and concentrating hydrogen gas evolved from nodules.

The efficiency of nitrogen fixation may be improved by selecting nitrogen-fixing symbionts that efficiently utilize the energy provided by photosynthesis for the reduction of nitrogen to ammonia. In our laboratory attempts are being made to select combinations of *Rhizobium* strains and legume cultivars that produce nodules with minimal rates of net hydrogen evolution. Our initial results and the observations of others (19, 37) suggest that this is feasible. In the event that satisfactory naturally occurring efficient symbionts cannot be obtained, then use of techniques of genetic and chemical manipulation may be necessary.

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1. Council for Agricultural Science and Technology Report (November 1973) *Energy in Agriculture* (Ames, Iowa).
2. U.S. Department of Agriculture Economic Research Service (1974) *Fertilizer Situation* (Washington, D.C.).
3. Hardy, R. W. F. & Havelka, U. D. (1975) *Science* **188**, 633–643.
4. Evans, H. J., ed. (1975) *Enhancing Biological Nitrogen Fixation* (Division of Biological and Medical Sciences of The National Science Foundation, Washington, D.C.).
5. Burns, R. C. & Hardy, R. W. F. (1975) *Nitrogen Fixation in Bacteria and Higher Plants* (Springer-Verlag, New York).
6. Bergersen, F. J. (1966) *Biochim. Biophys. Acta* **130**, 304–312.
7. Koch, B., Evans, H. J. & Russell, S. A. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 1343–1350.
8. Hwang, J. C. & Burris, R. H. (1972) *Biochim. Biophys. Acta* **283**, 339–350.
9. Bulen, W. A., Burns, R. C. & LeComte, J. R. (1965) *Proc. Nat. Acad. Sci. USA* **53**, 532–539.
10. Mortenson, L. E. (1966) *Biochim. Biophys. Acta* **127**, 18–25.
11. Burns, R. C. & Bulen, W. A. (1965) *Biochim. Biophys. Acta* **105**, 437–445.
12. Rivera-Ortiz, J. M. & Burris, R. H. (1975) *J. Bacteriol.* **123**, 537–545.
13. Hadfield, L. K. & Bulen, W. A. (1969) *Biochemistry* **8**, 5103–5108.
14. Hwang, J. C., Chen, C. H. & Burris, R. H. (1973) *Biochim. Biophys. Acta* **292**, 256–270.
15. Ljones, T. & Burris, R. H. (1972) *Biochim. Biophys. Acta* **275**, 93–101.
16. Hoch, G. E., Schneider, K. C. & Burris, R. H. (1960) *Biochim. Biophys. Acta* **37**, 273–279.
17. Bergersen, F. J. (1963) *Aust. J. Biol. Sci.* **16**, 669–680.
18. Dixon, R. O. D. (1968) *Arch. Mikrobiol.* **62**, 272–283.
19. Dixon, R. O. D. (1972) *Arch. Mikrobiol.* **85**, 193–201.
20. Dart, P. J. & Day, J. M. (1971) *Plant and Soil* Special Volume 1971, pp. 167–184.
21. Hardy, R. W. F. & Havelka, U. D. (1973) *Plant Physiol.* **51** (Supplement), 190.
22. Klucas, R. V., Koch, B., Russell, S. A. & Evans, H. J. (1968) *Plant Physiol.* **43**, 1906–1912.
23. Schwinghamer, E. A., Evans, H. J. & Dawson, M. D. (1970) *Plant and Soil* **33**, 192–212.
24. Wang, R., Healey, F. P. & Myers, J. (1971) *Plant Physiol.* **48**, 108–110.
25. Burris, R. H. (1975) in *The Biology of Nitrogen Fixation*, ed. Quispel, A. (North-Holland Publ. Co., Amsterdam), pp. 9–33.
26. Ljones, T. & Burris, R. H. (1972) *Anal. Biochem.* **45**, 448–452.
27. Peck, H. D., Jr. & Gest, H. (1956) *J. Bacteriol.* **71**, 70–80.
28. Hyndman, L. A., Burris, R. H. & Wilson, P. W. (1953) *J. Bacteriol.* **65**, 522–531.
29. Davis, L. C., Shah, V. K. & Brill, W. J. (1975) *Biochim. Biophys. Acta* **403**, 67–78.
30. Ching, T. M., Hedtke, S., Russell, S. A. & Evans, H. J. (1975) *Plant Physiol.* **55**, 796–798.
31. Hardy, R. W. F., Burns, R. C. & Holsten, R. D. (1973) *Soil Biochem. Biol.* **5**, 47–81.
32. Bergersen, F. J. (1970) *Aust. J. Biol. Sci.* **23**, 1015–1025.
33. Zelitch, J. (1975) *Science* **188**, 626–633.
34. Minchin, F. R. & Pate, J. S. (1973) *J. Exp. Bot.* **24**, 259–271.
35. National Science Foundation Report (1973) *Proceedings of the Workshop on Bio-Solar Conversion* (Bethesda, Md.).
36. Lien, S. & San Pietro, A. (1975) *An Inquiry into Biophotolysis of Water to Produce Hydrogen* (National Science Foundation report, Washington, D.C.).
37. Bergersen, F. J., Turner, G. L. & Appleby, C. A. (1973) *Biochim. Biophys. Acta* **292**, 271–282.