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# Hydrogen Reactions of Nodulated Leguminous Plants

## I. EFFECT OF RHIZOBIAL STRAIN AND PLANT AGE[1]

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### ABSTRACT

**The ATP-dependent evolution of $H_2$ catalyzed by nitrogenase and the hydrogenase-catalyzed oxidation of $H_2$ have been implicated as factors influencing the efficiency of energy utilization in the $N_2$ fixation process. The effects of rhizobial strain and plant age on the $H_2$-evolving and $H_2$-utilizing activity of leguminous root nodules are described in this manuscript. Two classes of legume-*Rhizobium* combinations were observed in studies with soybeans (*Glycine max* L. Merr.) and cowpeas (*Vigna unguiculata* L. Walp.). One group evolved $H_2$ in air; the other group did not exhibit net evolution of $H_2$. The latter group metabolized $H_2$ formed within the nodule through the action of a hydrogenase. The capacity to oxidize $H_2$ was strongly linked to the strain of *Rhizobium* used to inoculate cowpeas and soybeans. Although the magnitude of $H_2$ evolution in air changed during vegetative growth of a given symbiont, the ratio of $H_2$ evolved in air to total nitrogenase activity was not appreciably altered during this period. No consistent difference in nitrogenase activity as measured by the $C_2H_2$ reduction assay was observed between symbionts with an active hydrogenase and those that apparently lack the enzyme and evolve $H_2$. The effects of the two reactions of $H_2$ on total $N_2$ fixation and yield must now be established.**

Nitrogen fixation is an energy intensive process. Fossil fuels (primarily natural gas) equivalent to approximately $300 \times 10^6$ barrels of oil were consumed worldwide in 1972 for the industrial synthesis of anhydrous ammonia for fertilizer production (5). The widespread use of fertilizer nitrogen has accounted for a major part of the increased productivity of cereal grain, the world's primary source of food (5, 18). Limitations on use of fossil fuels and the high cost of production of fertilizer nitrogen have raised questions concerning the practicality of increasing industrial nitrogen fertilizer production sufficiently to maintain worldwide agricultural productivity.

For these reasons we must maximize the use of biological nitrogen fixation which now is estimated to account for about two-thirds of the total nitrogen fixed annually (4, 13). Biological fixation utilizes solar energy captured via photosynthesis. In order to maximize the productivity of biological nitrogen fixation we must understand the factors which may limit the biological process. The ATP-dependent evolution of hydrogen from the nitrogenase reaction and $H_2$ uptake via a hydrogenase have been implicated as possible factors involved in the efficiency of the nitrogen-fixing process (18). The hydrogen reactions consist of the ATP-dependent evolution of $H_2$ catalyzed by nitrogenase and the hydrogenase-catalyzed oxidation of $H_2$. One or both of these reactions occur in many bacteria, blue-green algae, and leguminous and nonleguminous root nodules (18). This paper describes investigations to define parameters involved in the expression of $H_2$ evolution and $H_2$ utilization in leguminous root nodules.

### MATERIALS AND METHODS

After surface disinfection, seeds were inoculated by submersion in the desired culture of *Rhizobium* (20). Seeds were germinated on sterile $H_2O$ agar plates and the young seedlings were grown in 20-cm plastic pots containing Perlite. The plants were maintained on nitrogen-free nutrient solution (12) in a controlled environment chamber (day/night temperature, 29/24 C; light intensity, 22,000 lux; photoperiod, 16 hr). Nodules were excised with a small segment (2-3 cm) of root and precautions were taken to keep nodules moist without excessive wetting. Gases were obtained from National Cylinder Gas Company (Portland, Oregon) or from Matheson Gas Products (Newark, California).

Rates of $H_2$ evolution or uptake were measured amperometrically (17). A Lucite chamber designed and constructed to hold two Clark-type probes (YSI 4004, Yellow Springs, Ohio) was used for these experiments. One electrode was used to monitor $O_2$ and the other $H_2$ evolution or uptake. Procedures described previously (17) were used for measurements of acetylene-($C_2H_2$) reducing capacity of excised nodules, nodulated root systems, or intact nodulated plants. Rates of $C_2H_2$ reduction were determined on excised nodules after rates of $H_2$ evolution and $O_2$ utilization were measured. In some cases rates of $H_2$ evolution and $O_2$ consumption were not linear. The values for apparent relative efficiency as used throughout this manuscript are based on the ratio of net $H_2$ evolved in air to total nitrogenase activity (17, 18). These calculations are subject to certain limitations (18) as described under "Discussion." Rates of $H_2$ evolution in air and $H_2$ evolution in $Ar/O_2/CO_2$ used in the calculation of apparent relative efficiency values (17, 18), therefore, were determined during time courses when the rates of respiration were about the same. When rates of respiration in air and in $Ar/O_2/CO_2$ were not comparable, the rate of $H_2$ evolution was calculated by dividing the net $H_2$ evolved by the time of the reaction. Specific details of experiments are listed in legends.

### RESULTS

Since results of an initial survey indicated that cowpeas (*Vigna unguiculata* L. Walp. cv. Whippoorwill) inoculated with *Rhizobium* strain 32H1 exhibited extremely low rates of $H_2$ evolution, this species was selected to examine the effect of rhizobial strain on the net evolution of $H_2$. In this experiment cowpeas (cv. Whippoorwill) were inoculated with each of a series of 13

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different strains of "cowpea" *Rhizobium*. Rates of $H_2$ evolution from excised nodules in an atomsphere of either air or a mixture of $Ar/O_2/CO_2$ and rates of $C_2H_2$ reduction were used in the calculation of the relative efficiency values for the 13 strains (Fig. 1). The relative efficiency of nodules from cowpeas varied with the specific strain of cowpea *Rhizobium* used to inoculate the seed. Nodules from plants inoculated with either strain 41Z2 or 176A27 exhibited relative efficiency values less than 0.70. In contrast, nodules from plants inoculated with any of the other 11 strains had relative efficiency values approaching unity. For these symbionts rates of $H_2$ evolution were extremely low and in some cases net $H_2$ uptake was observed. High relative efficiencies were correlated with the ability to recycle $H_2$ via hydrogenase. When two of the strains, 176A27 and 176A28, were used to inoculate an alternate host *Cajanus cajan* (pigeon peas) similar results were obtained. Nodules from pigeon peas inoculated with 176A27 evolved $H_2$ (relative efficiency value = 0.69) whereas nodules from plants inoculated with 176A28 were capable of metabolizing externally supplied $H_2$. These results support the conclusion that the *Rhizobium* strain is the important factor controlling the relative efficiency value.

To better assess the role of both the host and the endophyte in the expression of hydrogenase activity and higher relative efficiencies, an experiment analogous to that described in Figure 1 was conducted. Each of four series of replicated cultures of Anoka and Amsoy 71 cultivars of soybeans (*Glycine max* L. Merr.) was inoculated with one of four different strains of *Rhizobium japonicum*. For both cultivars tested, relative efficiency varied as a function of the rhizobial strain (Fig. 2). Although plants inoculated with USDA strain 110 exhibited the highest relative efficiencies, significant differences between the two cultivars were observed. Soybean cultivar Anoka inoculated with USDA 110 exhibited net $H_2$ uptake whereas Amsoy 71 inoculated with USDA 110 evolved $H_2$ at a low rate, because of the limited scope of this study, insufficient data were accumulated to assess thoroughly the role of the host legume in the expression of hydrogenase activity.

The magnitudes of $H_2$ evolution varied during the growth cycle and these changes seemed to be correlated with changes in nitrogenase activity as measured by rates of $C_2H_2$ reduction. Relative efficiencies, however, were fairly constant during the growth period. Also, as shown in Figure 3, relative efficiencies of nodules from cowpeas inoculated with strain 176A27 were fairly constant at each of four harvests during a 49-day growth period and were consistently lower than relative efficiencies of nodules from plants inoculated with strain 176A28. Excised nodules from cowpeas inoculated with strain 176A28 showed a capability to utilize an exogenous supply of $H_2$ while nodules from plants inoculated with 176A27 evolved $H_2$ in air at a rate equivalent to about 30 or 40% of the total nitrogenase activity as measured by $C_2H_2$ reduction. The trends of these results were similar to those obtained in experiments with Anoka and Amsoy 71 soybeans (Fig. 2).

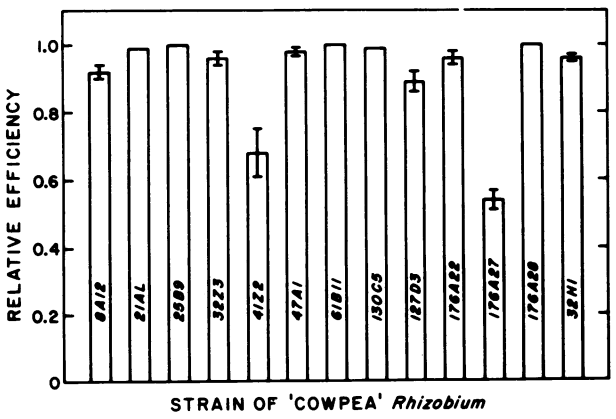


FIG. 1. Relative efficiency of cowpea plants inoculated with various strains of cowpea *Rhizobium*. Cowpea seeds (cv. *Whippoorwill*) were surface-disinfected and inoculated with the indicated rhizobial strains which were provided by J. Burton of The Nitragin Company. Plants were cultured in a growth chamber and results are expressed as means (± standard error of the mean, SEM) of replicated determinations during growth of plants. If no error bar is shown, the SEM was less than 0.005 of the value presented. Relative efficiencies calculated on the basis of $C_2H_2$ reduction or $H_2$ evolution in argon were about the same in this particular experiment.

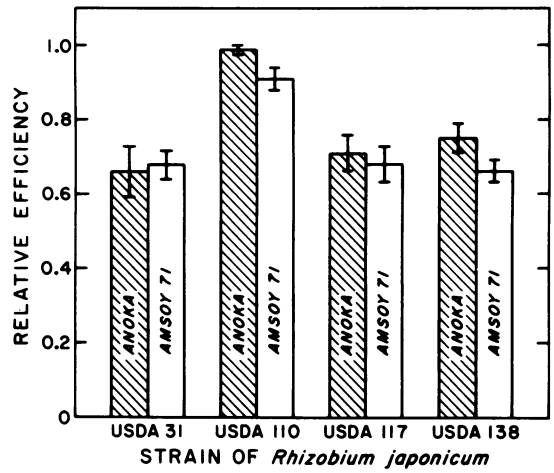


FIG. 2. Relative efficiency as a function of the combination of soybean host cultivar and strain of *R. japonicum*. Seeds of *G. max* (cv. Anoka or Amsoy 71) were inoculated with one of four strains of *R. japonicum*. Both the seeds and strains were a gift from G. Ham. Uninoculated control plants were not nodulated. Measurements of relative efficiency were conducted on excised nodules from plants from five replicated treatments when plants were 21, 28, and 35 days of age. Values given are the means (± SEM) of determinations at all three times of sampling.

Rates of $C_2H_2$ reduction as a function of time were measured for $H_2$-evolving and non-$H_2$-evolving nodules. Replicated samples of nodulated soybean root systems were excised from Anoka soybean plants inoculated with rhizobial strains USDA 31 and 110, and exposed to 0.1 atm of $C_2H_2$. Gas samples were taken at intervals and results are shown in Figure 4A. Rates of $C_2H_2$ reduction decreased after the first 10 to 30 min of incubation. After 30 min, rates decreased more slowly or remained approximately linear up to 2 hr. Initially there were no measurable differences in the rates of $C_2H_2$ reduction between soybean plants inoculated with USDA 31 and those inoculated with USDA 110. After 30 min, however, the rate of $C_2H_2$ reduction by root systems detached from plants that were inoculated with strain 110 was lower than the corresponding rate for nodules from plants inoculated with strain 31. Experiments of this general design were repeated using cowpea plants inoculated with either strain 176A27 or 176A28. The trends of results of this experiment (Fig. 4B) were similar to those obtained for soybeans. Although initial rates were about the same, after 15 to 30 min root systems from plants inoculated with the strain exhibiting hydrogenase activity (176A28) reduced $C_2H_2$ at lower rates than nodules from plants which actively evolved $H_2$ (176A27). The reason for the faster decline in rates of $C_2H_2$ reduction for symbionts with hydrogenase activity is not known, but may be related to the recent report of $C_2H_2$ inhibition of hydrogenase (2, 19).

The experiments with excised nodules indicate that initial rates of $C_2H_2$ reduction of $H_2$-evolving and non-$H_2$-evolving symbionts were not experimentally different. This conclusion was supported when rates of $C_2H_2$ reduction for intact cowpea

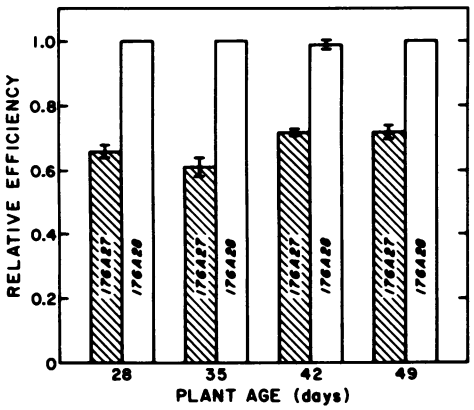


FIG. 3. Relative efficiency of excised nodules of cowpeas as a function of plant age. Cowpea seeds (cv. Whippoorwill) were inoculated with cultures of either cowpea *Rhizobium* strain 176A27 or 176A28. Strains were obtained from The Nitragin Company. Determinations of relative efficiency were made on nodules excised from plants from 10 replicated treatments at four times during the growth of the plants (4, 5, 6, and 7 weeks). Results are expressed as means (± SEM) of the replicated determinations. If no value is shown for the SEM, then the SEM was less than 0.005 of the value presented.

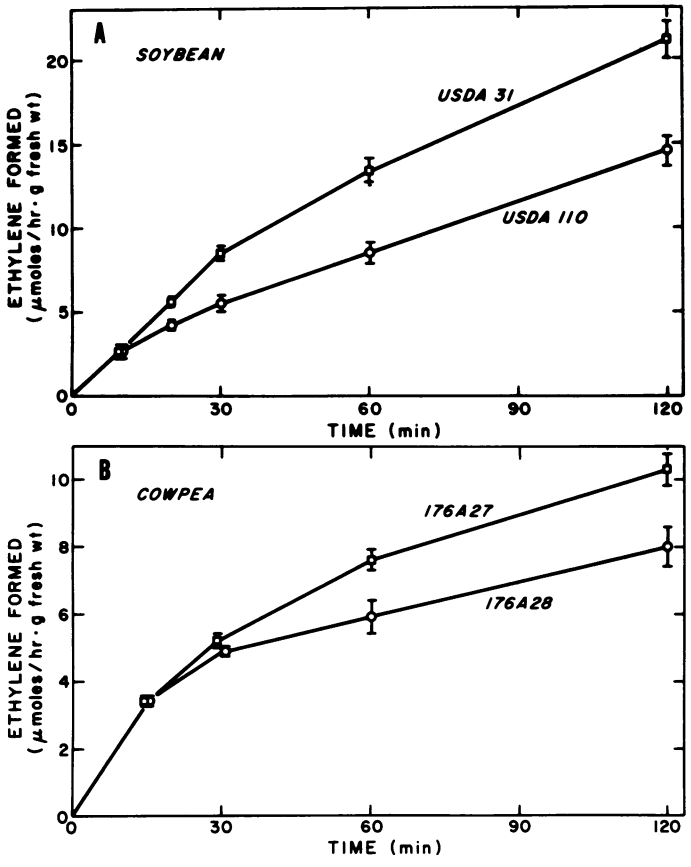


FIG. 4. Measurements of rates of $C_2H_2$ reduction of nodulated root systems from soybean or cowpea plants inoculated with different strains of *Rhizobium*. Assays were conducted in 250-ml Erlenmeyer flasks sealed with rubber stoppers containing a sampling port with serum stopper. Twenty-five ml of gas was removed from the flask and 25 ml of $C_2H_2$ was added to initiate the assay. Values given are means ± the standard error of the mean for 10 replicated samples. Nodulated root systems from soybean plants cv. Anoka, inoculated with either *R. japonicum* strain USDA 31 (□) or USDA 110 (○) were used for results in A; roots from cowpea plants cv. Whippoorwill inoculated with either strain 176A27 (□) or 176A28 (○) were used in B.

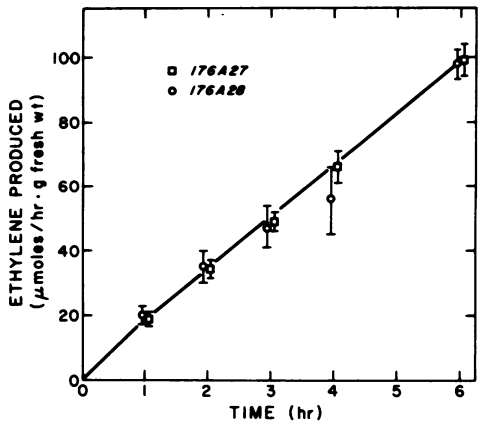


FIG. 5. Measurement of rates of $C_2H_2$ reduction for intact cowpea plants. Plants were inoculated with either strain 176A27 (□) or 176A28 (○) and cultured in the greenhouse with supplemental lighting. Two days before the experiment, plants were placed in the growth chamber. Pots were placed in Saran bags and bags were sealed around sampling tubes and stems of plants with Plasticene as previously described (17) so that shoots of plants were maintained under lights (temperature at root level 31 C, light intensity 22,000 lux). Approximately 0.1 atmosphere of $C_2H_2$ was added to each bag. Samples were removed and analyzed by gas chromatography. Results are expressed as μmol $C_2H_4$ formed/hr·g fresh wt of nodules. Values are means ± standard error of the mean for five replicated samples.

plants inoculated with either 176A27 or 176A28 were examined. As shown in Figure 5, the differences in the rates of $C_2H_2$ reduction between strains were within the limits of experimental error.

## DISCUSSION

Nitrogen-fixing organisms apparently catalyze one or both of the following reactions of $H_2$. The first is the ATP-dependent production of $H_2$ which is catalyzed by nitrogenase. This reaction occurs concomitantly with the reduction of $N_2$ in all organisms examined (2-4, 9, 14, 17-19). The second reaction is the hydrogenase-dependent oxidation of $H_2$ in some nitrogen-fixing organisms such as free living bacteria (6, 15, 19), blue-green algae (2), and root nodule bacteriods (7-9, 17, 18). The first reaction may decrease the energy available for the reduction of $N_2$ (1, 2, 9-11, 17-19) and for this reason has been used in the calculation of a relative efficiency value of energy utilization for $N_2$ reduction (17, 18). This value has been defined as the energy used to reduce $N_2$ divided by the total energy consumed for nitrogenase-catalyzed substrate reduction (17, 18). Relative efficiency values vary among organisms. The nodulated nitrogen-fixing symbionts examined that had relative efficiencies approaching unity apparently were capable of recycling $H_2$ via a hydrogenase. In cases where part or all of the $H_2$ evolved from nitrogenase is metabolized via hydrogenase it is not possible to use the rate of $H_2$ evolution under argon for the calculation of the relative efficiency values as originally defined. The calculation of relative efficiency should be based on an accurate rate of $N_2$ reduction and net $H_2$ evolution. If this is not feasible then the inherent errors should be considered and appropriate corrections made if possible.

In these investigations cowpeas and soybeans were inoculated with selected *Rhizobium* strains and grown under controlled conditions. The loss of $H_2$ from nodules from one group of strains amounted to 25 to 35% of the total electron flux through the nitrogenase system. This loss is in the same range as values reported for cell-free preparations of nitrogenase under $N_2$ pressures of an atmosphere or higher (3, 14, 16, 17). A second group of cowpeas and soybeans inoculated with other *Rhizobium* strains were capable of utilizing $H_2$ via a hydrogenase and

consequently evolved insignificant quantities of $H_2$ in air. Nodules from the first group of legumes may contain a hydrogenase, but the level of activity may be too low for detection. Alternatively, the genetic information for the expression of hydrogenase may be cryptic. This study and those of Dixon (7) support the conclusion that the strain of *Rhizobium* plays an important role in the synthesis of active hydrogenase in nodules. The evidence presented here does not exclude the possibility, however, that the host legume may influence the induction of hydrogenase within nodule bacteria.

A major unanswered question concerns the role of the two $H_2$ reactions in nitrogen-fixing capacity and plant yield. The use of the $C_2H_2$ reduction assay to measure total nitrogenase activity provides some evidence that the nitrogenase activities in symbionts with $H_2$-evolving and non-$H_2$-evolving nodules were similar. Since $C_2H_2$ is a known inhibitor of the $H_2$-evolving reaction and also inhibits some hydrogenases, care must be taken in interpreting results (2, 4, 19). Acetylene may effectively reduce total nitrogenase activity by eliminating the recycling of $H_2$ and its use as a source of energy to support $N_2$ fixation.

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