

# Variation in Nitrogenase and Hydrogenase Activity of Alaska Pea Root Nodules<sup>1</sup>

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## ABSTRACT

Hydrogenase activity of root nodules in the symbiotic association between *Pisum sativum* L. and *Rhizobium leguminosarum* was determined by incubating unexcised nodules with tritiated H<sub>2</sub> and measuring tissue HTO. Hydrogenase activity saturated at 0.50 millimolar H<sub>2</sub> and was not inhibited by the presence of 0.10 atmosphere C<sub>2</sub>H<sub>2</sub>, which prevented H<sub>2</sub> evolution from nitrogenase. Total H<sub>2</sub> production from nitrogenase was estimated as net H<sub>2</sub> evolution in air plus H<sub>2</sub> exchange in 0.10 atmosphere C<sub>2</sub>H<sub>2</sub>. Although such an estimate of nitrogenase function may not be quantitatively exact, due to uncertain relationships between H<sub>2</sub> exchange and H<sub>2</sub> uptake activity of hydrogenase, differences observed in H<sub>2</sub> exchange under various conditions represent an indication of changes in hydrogenase activity. Hydrogenase activity was lower in associations grown under higher photosynthetic photon flux densities and decreased relative to total H<sub>2</sub> production by nitrogenase. Total H<sub>2</sub> production and hydrogenase activity were maximum 28 days after planting. Thereafter, hydrogenase activity and H<sub>2</sub> production declined, but the potential proportion of nitrogenase-produced H<sub>2</sub> recovered by the uptake hydrogenase system increased. Of five *R. leguminosarum* strains tested two possessed hydrogenase activity. Strains which had the potential to reassimilate H<sub>2</sub> had significantly higher rates of N<sub>2</sub> reduction than those which did not exhibit hydrogenase activity.

Reduction of C<sub>2</sub>H<sub>2</sub> to C<sub>2</sub>H<sub>4</sub> by nitrogenase is measured easily by gas chromatography (12). As a consequence, this assay has become a major research technique for estimating biological N<sub>2</sub> fixation. In the process of interacting with nitrogenase, however, C<sub>2</sub>H<sub>2</sub> inhibits ATP-dependent H<sub>2</sub> evolution by this enzyme complex (6). The first reproducible demonstration of H<sub>2</sub> evolution from soybean root nodules (13) was later confirmed and extended to other legumes (7). Recent work (18) emphasized that H<sup>+</sup> reduction by nitrogenase can result in 40 to 60% of the electron flow through this enzyme complex being lost as H<sub>2</sub> and proposed an equation for the relative efficiency of N<sub>2</sub> fixation, which related the total flow of electrons through nitrogenase (C<sub>2</sub>H<sub>2</sub> reduction) to electron flow resulting in the reduction of H<sup>+</sup> to H<sub>2</sub>. This equation incorporates data from net H<sub>2</sub> metabolism by nitrogenase and any hydrogenase present. Recent findings (16, 17, 19) have confirmed and extended previous reports (8, 9) that uptake hydrogenase activity is present in certain strains of *Rhizobium*. It is

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apparent that a better understanding of uptake hydrogenase activity and H<sub>2</sub> production by nitrogenase is needed to comprehend factors which affect the efficiency of N<sub>2</sub> fixation.

Whole-plant studies revealed that plant ontogeny (5) and irradiance (4) during 4 weeks of growth markedly altered the relative efficiency of N<sub>2</sub> fixation calculated according to Schubert and Evans (18). Although those experiments demonstrated that the host legume could affect the efficiency of bacteroid functioning, there were no data to indicate whether the altered levels of H<sub>2</sub> evolution resulted from changes in uptake hydrogenase activity or from differences in H<sub>2</sub> production by nitrogenase. The present experiments were performed to separate the effects of ontogeny and growth irradiance on nitrogenase and hydrogenase activity in *Rhizobium leguminosarum* 128C53. In addition, other strains of this bacterial species were examined for hydrogenase and nitrogenase activity *in vivo* to determine the mechanisms underlying different N<sub>2</sub> reduction rates which alter photosynthetic efficiency (3).

## MATERIALS AND METHODS

**Growth Conditions.** Pea (*Pisum sativum* L. cv. Alaska) plants were grown under a 16/8 h light/dark cycle at 21/15 °C air temperature and 60/80% RH. Plants were exposed to an irradiance of 800 μE/m<sup>2</sup>·s at emergence level, except in the experiment where growth irradiance was varied. In that case irradiance was 100, 400, 700 or 1,000 μE/m<sup>2</sup>·s. Differences in irradiance were achieved by shielding with wire screen(s). Plants were harvested 28 days after planting except as noted otherwise. Leaf and root temperatures were recorded at all levels of irradiance. Evenly spaced, alternating Sylvania MM400/BU-HOR metal halide and Norelco 160E23/SB/W mercury lamps were used as light sources. Plants were grown in 600-ml plastic pots in Vermiculite which was covered with a layer of white Perlite. Plants were watered daily with excess nutrient solution adjusted to pH 6.0. The solution contained 2 mM CaSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 2 mM K<sub>2</sub>SO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, and 1 mM NH<sub>4</sub>NO<sub>3</sub>. Micronutrients were according to Johnson *et al.* (14). In addition, 4.2 nM CoCl<sub>2</sub> was used. Plants were inoculated with *R. leguminosarum* 128C53, except in the experiment involving different rhizobial strains. There strains TA101 (obtained originally from Dr. J. J. Child, N.R.C. Saskatoon, Sask. Canada), 128C53, 175G10, 92A2, and 92F1 (obtained originally from Dr. J. C. Burton, Nitragin Co., Milwaukee, Wis.) were used, with uninoculated plants as controls. Materials used in this experiment were sterile or surface-sterilized. The Perlite surface was protected by plastic covering after planting.

**Hydrogenase Activity.** Tritiated H<sub>2</sub> was stored in a stainless steel cylinder under pressure. The cylinder was equipped with a pressure regulator and syringe adapter which permitted withdrawal of measured amounts of tritiated H<sub>2</sub> near atmospheric pressure. Precision Sampling Inc. Pressure-Lok syringes were used to control diffusion of gas through the needle. Before injection of tritium into the incubation vessels the syringe contents, which

were at higher than atmospheric pressure following withdrawal from the cylinder, were permitted to equilibrate momentarily with the atmosphere. Specific activity of the tritiated H<sub>2</sub> was determined to be 2.27 mCi/ml by radiochromatography (11). When used at a low concentration (16 μM), tritiated H<sub>2</sub> was not diluted with additional H<sub>2</sub>, otherwise it was diluted 20-fold with H<sub>2</sub>. The reaction vessels were 25-ml bottles stoppered with hard rubber. Gas additives (tritium, CO, C<sub>2</sub>H<sub>2</sub>) were injected through the rubber stopper. To equalize moisture conditions on the nodule surfaces in all treatments and to remove Vermiculite which adsorbed H<sub>2</sub>, roots were rinsed in tap water at 24 C prior to assay. Roots were divided orthostichously. Lateral roots with attached nodules from the same plant were used for the different treatments in the experiments involving hydrogenase activity dependence on tritiated H<sub>2</sub> concentration or exposure time. In the experiments on plant ontogeny, variation of growth irradiance, and rhizobial strains three separate tritium exposed lateral root samples were taken from each plant. All tritium exposures were made at approximately saturating concentrations (0.50 mM) for 20 min and were preceded by a 5-min incubation in 0.10 atm C<sub>2</sub>H<sub>2</sub>, except in the experiment involving exposure time. There hydrogenase activity was determined after 15, 30, and 60 min incubation with 16 μM tritiated H<sub>2</sub> after 5-min exposure to 0.02 atm CO or 0.10 atm C<sub>2</sub>H<sub>2</sub>. The hydrogenase reaction was terminated by freezing nodules on dry ice. Adsorption of hydrogen to dead nodule tissue was determined by exposing prefrozen samples to tritiated H<sub>2</sub> at various concentrations and exposure times. Experimental results were corrected to reflect the adsorption of hydrogen to tissue. Samples were combusted in a Packard sample oxidizer model B306, and radioactivity was determined by scintillation counting. Rates of hydrogenase activity were based on nodule fresh weight.

**H<sub>2</sub> Evolution.** Measurements of H<sub>2</sub> evolution and C<sub>2</sub>H<sub>2</sub>-dependent C<sub>2</sub>H<sub>4</sub> production were made on nodulated lateral roots from the same plants which were used in the H<sub>2</sub> uptake assays. To achieve comparability with uptake data, gas evolution rates also were based on nodule fresh weight. Specific nodule activity (μmol product/g fresh weight · h) was averaged from six replicates. Hydrogen and C<sub>2</sub>H<sub>2</sub>-dependent C<sub>2</sub>H<sub>4</sub> evolution rates were determined separately in 25-ml reaction vessels by GC (5). Total H<sub>2</sub> production by nitrogenase was computed as the sum of net H<sub>2</sub> evolution in air and hydrogenase activity in the presence of 0.10 atm C<sub>2</sub>H<sub>2</sub>, which prevented H<sub>2</sub> evolution from nitrogenase but did not inhibit hydrogenase activity.

## RESULTS

Adsorption of hydrogen to dead nodule tissue was proportional to tissue fresh weight except at the lowest H<sub>2</sub> concentration (16 μM) and shortest incubation time (5 min). Appropriate correction factors therefore were applied in subsequent measurements. When tritiated H<sub>2</sub> was supplied at a low concentration (16 μM), *i.e.* an amount comparable to net H<sub>2</sub> evolution during 1 h in air, hydrogenase activity rates were higher in the presence of 0.10 atm C<sub>2</sub>H<sub>2</sub> and lower in the presence of 0.02 atm CO than in the absence of either gas (Fig. 1). As H<sub>2</sub> production was inhibited in the presence of C<sub>2</sub>H<sub>2</sub> (no net H<sub>2</sub> evolution was detected with C<sub>2</sub>H<sub>2</sub> present in *Rhizobium* strains which failed to exchange tritiated H<sub>2</sub>), hydrogenase activity in this system depended entirely on externally supplied H<sub>2</sub>. Thus, tritiated H<sub>2</sub> exchange in the presence of C<sub>2</sub>H<sub>2</sub> took place without dilution of tritiated H<sub>2</sub> by H<sub>2</sub> generated by nitrogenase. CO, which inhibited C<sub>2</sub>H<sub>2</sub> reduction, but permitted H<sub>2</sub> evolution by nitrogenase, showed an inhibition of H<sub>2</sub> exchange compared with ambient conditions.

Nodules incubated with 0.10 atm C<sub>2</sub>H<sub>2</sub> to inhibit H<sub>2</sub> evolution by nitrogenase exhibited saturation of hydrogenase activity at about 0.5 mM H<sub>2</sub> (Fig. 2). Hydrogenase activity and production of H<sub>2</sub> by nitrogenase in pea root nodules increased to a maximum at the onset of flowering 28 days after planting and then declined

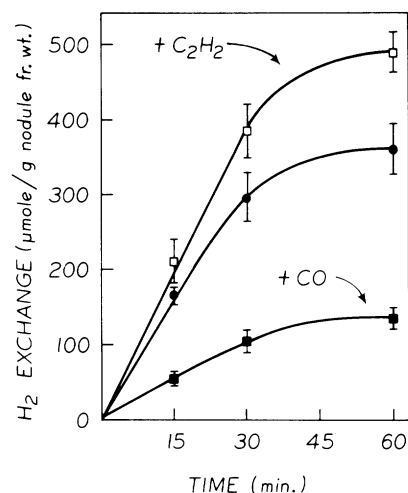


FIG. 1. Tritium exchange from hydrogen gas by *R. leguminosarum* 128C53-pea root nodules in the presence or absence of CO or C<sub>2</sub>H<sub>2</sub>. Determination of tritium exchange was based on addition of 0.41 μmol (10 μl) of a tritiated H<sub>2</sub> mixture of specific radioactivity 2.27 mCi/ml to 25 ml incubation vessels. Means ± SE were computed from six replicates. (●—●): Air; (□—□): 0.90 atm air + 0.10 atm C<sub>2</sub>H<sub>2</sub>; (■—■): 0.98 atm air + 0.02 atm CO.

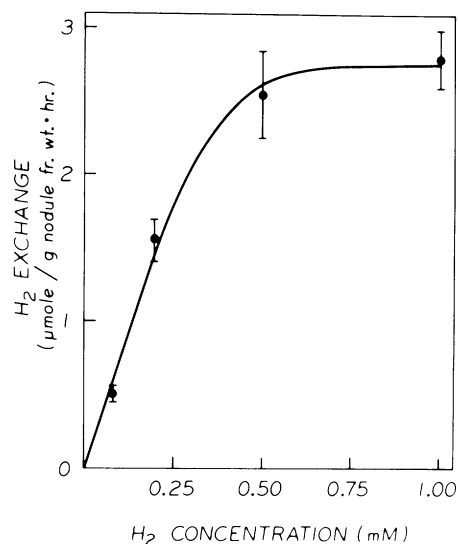


FIG. 2. Saturation characteristics of hydrogenase activity in *R. leguminosarum* 128C53-Alaska pea root nodules. Unexcised nodules were exposed to different concentrations of a mixture of tritiated H<sub>2</sub> of specific radioactivity 0.1135 mCi/ml. Means ± SE were computed from six replicates.

(Fig. 3). Nitrogenase efficiency (NE<sup>3</sup>), a measure of electron allocation by nitrogenase to H<sup>+</sup> or N<sub>2</sub>, was computed according to Schubert and Evans (18) but modified in an effort to estimate total H<sub>2</sub> production (net H<sub>2</sub> evolution + tritiated H<sub>2</sub> exchange) by the equation:

$$NE = 1 - \frac{\text{Net H}_2 \text{ evolution} + \text{tritiated H}_2 \text{ exchange}}{\text{C}_2\text{H}_2 \text{ reduction}} \quad (1)$$

NE was constant until the end of the 6th week after planting (Fig. 4). Its decline thereafter coincided with early pod filling. The ratio of tritiated H<sub>2</sub> exchange to total H<sub>2</sub> production by nitrogenase, which may reflect the bacteroid's maximum capacity to recover H<sub>2</sub>, increased with time throughout the experiment (Fig. 4).

Hydrogenase activity was lower in *Rhizobium*-pea associations grown under higher irradiance, while total H<sub>2</sub> production by

<sup>3</sup> Abbreviation: NE: nitrogenase efficiency.

nitrogenase was lowest at the severely limiting growth irradiance of  $100 \mu\text{E}/\text{m}^2 \cdot \text{s}$  and highest at  $700 \mu\text{E}/\text{m}^2 \cdot \text{s}$  (Fig. 5). At the highest growth irradiance used ( $1,000 \mu\text{E}/\text{m}^2 \cdot \text{s}$ ) the shoot system was markedly sclerophyllous with small, hard, yellowish leaves, shortened internodes, and profuse branching. Nodules in these plants were larger and less numerous than in plants grown at lower irradiance. The decline in  $\text{H}_2$  production by nitrogenase at this growth irradiance may have been influenced by suboptimal growth conditions. Leaf and root temperatures prevailing under experimental conditions at a growth irradiance of 100, 400, 700, 800, or 1,000 were: 20.5, 21.0, 22.4, 23.1, 25.6 or 21.5, 22.0, 23.5, 24.2, 25.0 C, respectively. In the dark, leaf and root temperatures were 15 C. The fraction of total  $\text{H}_2$  produced by nitrogenase, which could have been reassimilated by the nodules according to tritium exchange measurements, was greatest at the lowest growth irradiance. NE values paralleled the trend shown by the ratio of  $\text{H}_2$  exchange to  $\text{H}_2$  production (Fig. 6).

Associations of Alaska pea with different strains of *R. leguminosarum* could be divided into two groups based on the capacity of the microsymbionts to exchange  $\text{H}_2$  (Table I). This capacity of strains 128C53 and 92F1 was associated with significantly lower ( $P \leq 0.05$ ) net  $\text{H}_2$  evolution and higher  $\text{N}_2$  reduction and NE than was found in strains TA101, 175G10, and 92A2, which did not exchange  $\text{H}_2$ . The difference between associations based on hy-

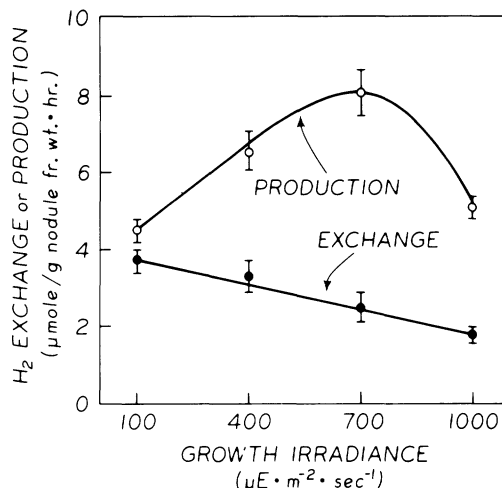


FIG. 5.  $\text{H}_2$  production by nitrogenase and hydrogenase activity in root nodules of pea plants grown at different irradiances. Plants were inoculated with *R. leguminosarum* strain 128C53.  $\text{H}_2$  production by nitrogenase was estimated as the sum of net  $\text{H}_2$  evolution in air and  $\text{H}_2$  exchange in the presence of 0.10 atm  $\text{C}_2\text{H}_2$ . Means  $\pm$  SE were computed from six replicates.

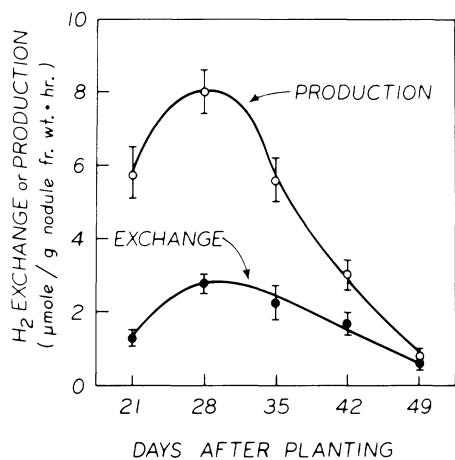


FIG. 3.  $\text{H}_2$  production by nitrogenase and hydrogenase activity in pea root nodules during host plant ontogeny. Plants were inoculated with *R. leguminosarum* 128C53.  $\text{H}_2$  production by nitrogenase was estimated as the sum of net  $\text{H}_2$  evolution in air and  $\text{H}_2$  exchange in the presence of 0.10 atm  $\text{C}_2\text{H}_2$ . Means  $\pm$  SE were computed from six replicates.

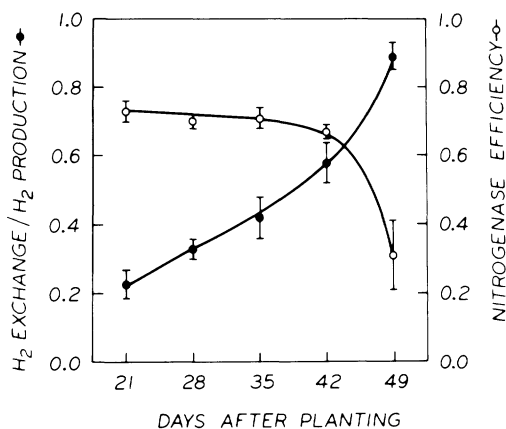


FIG. 4. Changes in efficiency of symbiotic  $\text{N}_2$  fixation during host plant ontogeny. NE was computed as shown in equation 1. Tritiated  $\text{H}_2$  exchange/ $\text{H}_2$  production represents a relative measure of the capacity of the entire nodule to recover  $\text{H}_2$  produced by the nitrogenase enzyme complex. Calculations were based on data in Figure 3.

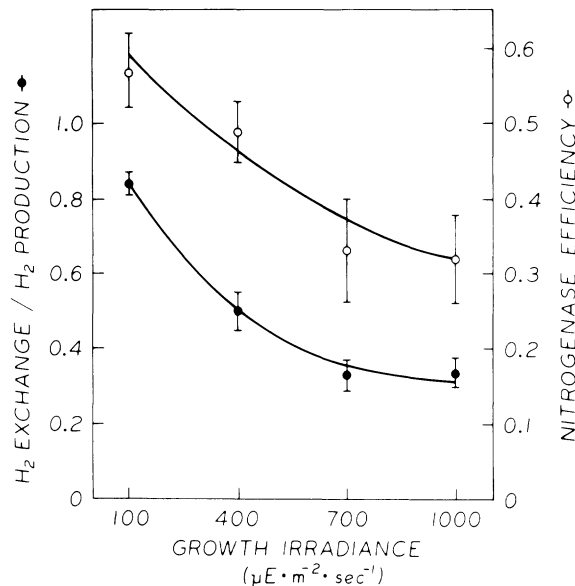


FIG. 6. Changes in efficiency of symbiotic  $\text{N}_2$  fixation due to growth of host plants at different irradiances. NE was computed as shown in equation 1. Tritiated  $\text{H}_2$  exchange/ $\text{H}_2$  production represents a relative measure of the capacity of the entire nodule to recover  $\text{H}_2$  produced by the nitrogenase enzyme complex. Calculations were based on data in Figure 5.

drogenase activity was not noted in comparisons of total  $\text{H}_2$  production. According to this latter criterion only one strain incapable of  $\text{H}_2$  exchange (175G10) had significantly greater ( $P \leq 0.05$ ) total  $\text{H}_2$  production than those which exchange  $\text{H}_2$ . Evaluation of  $\text{N}_2$  fixation and NE data by a multiple range test showed no significant differences ( $P \leq 0.05$ ) among strains which possessed or did not possess hydrogenase activity; differences between the two groups, however, were significant ( $P \leq 0.05$ ) (Table I).

## DISCUSSION

Unlike in free living,  $\text{N}_2$ -fixing bacteria (20), 0.10 atm  $\text{C}_2\text{H}_2$  did not inhibit hydrogenase activity in *R. leguminosarum* strain 128C53 while preventing  $\text{H}_2$  evolution from nitrogenase (Fig. 1). The opposite effects of 0.02 atm CO and 0.10 atm  $\text{C}_2\text{H}_2$  on  $\text{H}_2$  exchange compared with the ambient standard suggest that ambient conditions and 0.02 atm CO resulted in a dilution of tritiated

Table I. Nitrogenase and hydrogenase activity of Alaska pea root nodules formed by different strains of *Rhizobium leguminosarum*.

Total H<sub>2</sub> production by nitrogenase was estimated as the sum of net H<sub>2</sub> evolution in air and H<sub>2</sub> exchange in the presence of 0.10 atm C<sub>2</sub>H<sub>2</sub>. Reduction of N<sub>2</sub> was computed as (C<sub>2</sub>H<sub>2</sub> reduced-total H<sub>2</sub> produced)/3. Means were calculated from four replicates. Nitrogen fixation data were evaluated statistically by a multiple range test. *Rhizobium* strains not annotated by the same letter were significantly different ( $p < 0.05$ ) for the indicated parameter.

N <sub>2</sub> fixation parameters		<i>Rhizobium</i> Strain				
		TA101	175G10	92A2	128C53	92F1
Tritium exchange	( $\mu\text{mole/g fresh weight}\cdot\text{hr}$ )	--	--	--	2.3 a	1.0 b
Net H <sub>2</sub> evolution	( " " " )	7.7 c	8.9 c	6.7 c	2.5 d	4.0 d
Total H <sub>2</sub> production	( " " " )	7.7 ef	8.9 e	6.7 ef	4.8 f	5.0 f
C <sub>2</sub> H <sub>2</sub> reduction	( " " " )	12.9 g	16.5 gh	14.5 g	17.2 h	19.8 h
N <sub>2</sub> reduction	( " " " )	1.8 i	2.5 i	2.6 i	4.4 j	5.0 j
NE [1-(Total H <sub>2</sub> production/C <sub>2</sub> H <sub>2</sub> reduction)]		0.32 k	0.46 kl	0.53 l	0.74 m	0.76 m
Tritium exchange/total H <sub>2</sub> production (%)		--	--	--	47	22

H<sub>2</sub> by H<sub>2</sub> produced in the nodules. This interpretation was supported by the lack of any measurable H<sub>2</sub> evolution in the presence of 0.10 atm C<sub>2</sub>H<sub>2</sub>, whether or not the *Rhizobium* strain exhibited hydrogenase activity. An evaluation of hydrogenase activity independent of H<sub>2</sub> evolution from nitrogenase therefore was possible. An expression of NE, which estimates the efficiency of nitrogenase in allocating electrons to H<sup>+</sup> or N<sub>2</sub> was determined according to equation 1.

Although tritiated H<sub>2</sub> exchange cannot be accepted as a measure of H<sub>2</sub> uptake in the absence of a measured decline in external H<sub>2</sub> concentration, the technique is a valid test for hydrogenase activity (10) which has been used in pure cultures of *Rhizobium japonicum* (15). The advantage of the exchange assay is that it measures activation of molecular hydrogen directly rather than the transfer of the activated hydrogen to an electron acceptor, a process which is altered by changes in oxidation-reduction potentials of the cell (1). The exchange assay, therefore, is ideally suited for measuring effects of plant ontogeny or growth irradiance on relative hydrogenase activity. Quantitative estimates of H<sub>2</sub> produced by nitrogenase which could be recovered by hydrogenase in this system will require H<sub>2</sub> uptake measurements in the presence of 0.10 atm C<sub>2</sub>H<sub>2</sub>. The latter technique, however, will not allow any conclusion about hydrogenase activity as a separate enzyme system because it will measure net activation of H<sub>2</sub> by hydrogenase and transfer to appropriate electron carriers. Results from the present study may be interpreted as showing an effect of host plant ontogeny and long term growth irradiance on *Rhizobium* hydrogenase activity (Figs. 3 and 5) required for activation of H<sub>2</sub>. The ratio of H<sub>2</sub> exchange to H<sub>2</sub> production by nitrogenase and the calculated NE values (Figs. 4 and 6) likewise represent relative relationships between enzymes important for energetically efficient N<sub>2</sub> reduction. Correction for isotope effects could yield information on the maximum possible efficiencies which could be attained if all oxidation-reduction potentials involved were favorable, but such a correction probably would not change the general nature of the relationships reported.

An increase in net H<sub>2</sub> evolution with increasing growth irradiance previously had been ascribed either to a shift in electron allocation by nitrogenase from N<sub>2</sub> to H<sup>+</sup> reduction, or to higher levels of uptake hydrogenase activity at lower growth irradiance (4). Present results reveal that both mechanisms probably operate (Fig. 5). The proportionate increase in hydrogenase activity relative to total H<sub>2</sub> production with plant age (Fig. 4) indicates that these processes responded differently to decreasing photosynthate availability with increasing plant age (5). While the absolute magnitudes of both nitrogenase and hydrogenase specific activity decreased in the aging nodules, the decrease occurred at markedly different rates (Fig. 3). This may have resulted in increasing rates of H<sub>2</sub> recapture in older plants.

A positive association between the efficiency of N<sub>2</sub> fixation and

the presence of an H<sub>2</sub> uptake system in leguminous root nodules has been demonstrated recently (17, 19). The present data, based on five strains of *R. leguminosarum*, support these findings. Energy lost to H<sub>2</sub> production probably was minimized in strains 128C53 and 92F1 by hydrogenase activity. These strains also had relatively low rates of total H<sub>2</sub> production and high rates of C<sub>2</sub>H<sub>2</sub> reduction (Table I), resulting in significantly higher N<sub>2</sub> reduction and NE than observed in the other three strains. This observation suggests a causal relationship between the increased energy potentially available from hydrogenase activity and increased nitrogenase activity. The difficulty of proving such a causal relationship is compounded by the fact that peas which receive combined N or are nodulated by strains which fix more N<sub>2</sub> are more photosynthetically efficient at the developmental stage studied (2, 3). Thus, plants infected with strains 92F1 or 128C53 could be expected to provide more photosynthate to the nodules than plants infected with TA101, for example. The significantly greater NE values calculated for strains 92F1 and 128C53 may reflect these facts or represent another mechanism which affects allocation of reductant to H<sup>+</sup> or N<sub>2</sub> independent of any uptake hydrogenase. In either case, the end result is an increase in Kjeldahl N values at this stage of growth as reported previously (3).

Dixon (9) demonstrated that different species of host plants induced various levels of uptake hydrogenase activity in *Rhizobium* bacteroids. Data reported in this study provide the first clear evidence that hydrogenase activity within a single host plant can vary with plant age and growth irradiance, an environmental parameter which interacts with the symbiosis through the host plant. Although Dixon's report (9) provided hope for identifying leguminous species which could develop efficient symbioses evolving less H<sub>2</sub>, the present work suggests that even species which may be measurably inefficient at times can evolve less H<sub>2</sub> under other conditions. A more complete understanding of N<sub>2</sub> fixation efficiency and functional relationships between host legumes and rhizobial symbionts may result in identifying symbioses which reduce more N<sub>2</sub> for each unit of photosynthate supplied.

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