Plant Physiol. (1979) 63, 816–820
0032-0889/79/63/0816/05/$00.50/0

Variation in Nitrogenase and Hydrogenase Activity of Alaska Pea Root Nodules[1]

Received for publication June 13, 1978 and in revised form December 11, 1978

GABOR J. BETHLENFALVAY[2] AND DONALD A. PHILLIPS
Department of Agronomy and Range Science, University of California, Davis, California 95616

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_2 and measuring tissue HTO. Hydrogenase activity saturated at 0.50 millimolar H_2 and was not inhibited by the presence of 0.10 atmosphere C_2H_2, which prevented H_2 evolution from nitrogenase. Total H_2 production from nitrogenase was estimated as net H_2 evolution in air plus H_2 exchange in 0.10 atmosphere C_2H_2. Although such an estimate of nitrogenase function may not be quantitatively exact, due to uncertain relationships between H_2 exchange and H_2 uptake activity of hydrogenase, differences observed in H_2 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_2 production by nitrogenase. Total H_2 production and hydrogenase activity were maximum 28 days after planting. Thereafter, hydrogenase activity and H_2 production declined, but the potential proportion of nitrogenase-produced H_2 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_2 had significantly higher rates of N_2 reduction than those which did not exhibit hydrogenase activity.

Reduction of C_2H_2 to C_2H_4 by nitrogenase is measured easily by gas chromatography (12). As a consequence, this assay has become a major research technique for estimating biological N_2 fixation. In the process of interacting with nitrogenase, however, C_2H_2 inhibits ATP-dependent H_2 evolution by this enzyme complex (6). The first reproducible demonstration of H_2 evolution from soybean root nodules (13) was later confirmed and extended to other legumes (7). Recent work (18) emphasized that H^+ reduction by nitrogenase can result in 40 to 60% of the electron flow through this enzyme complex being lost as H_2 and proposed an equation for the relative efficiency of N_2 fixation, which related the total flow of electrons through nitrogenase (C_2H_2 reduction) to electron flow resulting in the reduction of H^+ to H_2. This equation incorporates data from net H_2 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 apparent that a better understanding of uptake hydrogenase activity and H_2 production by nitrogenase is needed to comprehend factors which affect the efficiency of N_2 fixation.

Whole-plant studies revealed that plant ontogeny (5) and irradiance (4) during 4 weeks of growth markedly altered the relative efficiency of N_2 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_2 evolution resulted from changes in uptake hydrogenase activity or from differences in H_2 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_2 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 $\mu E/m^2 \cdot s$ at emergence level, except in the experiment where growth irradiance was varied. In that case irradiance was 100, 400, 700 or 1,000 $\mu E/m^2 \cdot s$. 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 $CaSO_4$, 1 mM KH_2PO_4, 2 mM K_2SO_4, 0.5 mM $MgSO_4$, and 1 mM NH_4NO_3. Micronutrients were according to Johnson *et al.* (14). In addition, 4.2 nM $CoCl_2$ 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_2 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_2 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

[1] This material is based on research supported by National Science Foundation Grants No. PRF 77-07301 A01 and PCM 78-01146. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the National Science Foundation.

[2] Present address: Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, La. 70803.

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_2 was determined to be 2.27 mCi/ml by radiochromatography (11). When used at a low concentration (16 μM), tritiated H_2 was not diluted with additional H_2, otherwise it was diluted 20-fold with H_2. The reaction vessels were 25-ml bottles stoppered with hard rubber. Gas additives (tritium, CO, C_2H_2) were injected through the rubber stopper. To equalize moisture conditions on the nodule surfaces in all treatments and to remove Vermiculite which adsorbed H_2, 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_2 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_2H_2, except in the experiment involving exposure time. There hydrogenase activity was determined after 15, 30, and 60 min incubation with 16 μM tritiated H_2 after 5-min exposure to 0.02 atm CO or 0.10 atm C_2H_2. 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_2 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_2 Evolution. Measurements of H_2 evolution and C_2H_2-dependent C_2H_4 production were made on nodulated lateral roots from the same plants which were used in the H_2 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_2H_2-dependent C_2H_4 evolution rates were determined separately in 25-ml reaction vessels by GC (5). Total H_2 production by nitrogenase was computed as the sum of net H_2 evolution in air and hydrogenase activity in the presence of 0.10 atm C_2H_2, which prevented H_2 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_2 concentration (16 μM) and shortest incubation time (5 min). Appropriate correction factors therefore were applied in subsequent measurements. When tritiated H_2 was supplied at a low concentration (16 μM), *i.e.* an amount comparable to net H_2 evolution during 1 h in air, hydrogenase activity rates were higher in the presence of 0.10 atm C_2H_2 and lower in the presence of 0.02 atm CO than in the absence of either gas (Fig. 1). As H_2 production was inhibited in the presence of C_2H_2 (no net H_2 evolution was detected with C_2H_2 present in *Rhizobium* strains which failed to exchange tritiated H_2), hydrogenase activity in this system depended entirely on externally supplied H_2. Thus, tritiated H_2 exchange in the presence of C_2H_2 took place without dilution of tritiated H_2 by H_2 generated by nitrogenase. CO, which inhibited C_2H_2 reduction, but permitted H_2 evolution by nitrogenase, showed an inhibition of H_2 exchange compared with ambient conditions.

Nodules incubated with 0.10 atm C_2H_2 to inhibit H_2 evolution by nitrogenase exhibited saturation of hydrogenase activity at about 0.5 mM H_2 (Fig. 2). Hydrogenase activity and production of H_2 by nitrogenase in pea root nodules increased to a maximum at the onset of flowering 28 days after planting and then declined

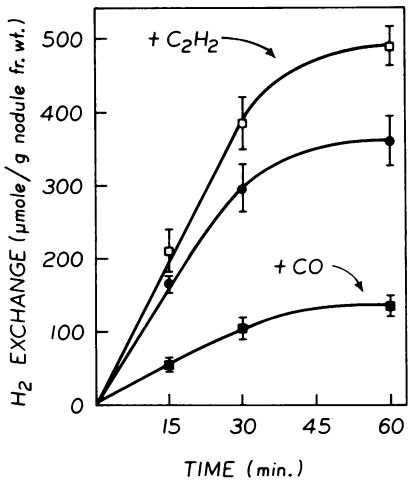

FIG. 1. Tritium exchange from hydrogen gas by *R. leguminosarum* 128C53-pea root nodules in the presence or absence of CO or C_2H_2. Determination of tritium exchange was based on addition of 0.41 μmol (10 μl) of a tritiated H_2 mixture of specific radioactivity 2.27 mCi/ml to 25 ml incubation vessels. Means ± SE were computed from six replicates. (●——●): Air; (□——□): 0.90 atm air + 0.10 atm C_2H_2; (■——■): 0.98 atm air + 0.02 atm CO.

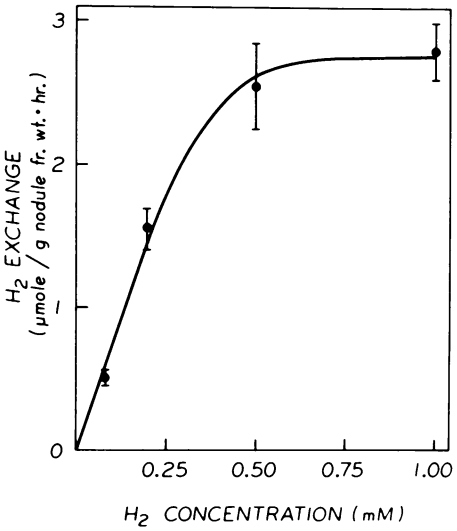

FIG. 2. Saturation characteristics of hydrogenase activity in *R. leguminosarum* 128C53-Alaska pea root nodules. Unexcised nodules were exposed to different concentrations of a mixture of tritiated H_2 of specific radioactivity 0.1135 mCi/ml. Means ± SE were computed from six replicates.

(Fig. 3). Nitrogenase efficiency (NE[3]), a measure of electron allocation by nitrogenase to H^+ or N_2, was computed according to Schubert and Evans (18) but modified in an effort to estimate total H_2 production (net H_2 evolution + tritiated H_2 exchange) by the equation:

$$NE = 1 - \frac{\text{Net } H_2 \text{ evolution} + \text{tritiated } H_2 \text{ exchange}}{C_2H_2 \text{ reduction}} \quad (1)$$

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_2 exchange to total H_2 production by nitrogenase, which may reflect the bacteroid's maximum capacity to recover H_2, increased with time throughout the experiment (Fig. 4).

Hydrogenase activity was lower in *Rhizobium*-pea associations grown under higher irradiance, while total H_2 production by

[3] Abbreviation: NE: nitrogenase efficiency.

nitrogenase was lowest at the severely limiting growth irradiance of 100 $\mu E/m^2 \cdot s$ and highest at 700 $\mu E/m^2 \cdot s$ (Fig. 5). At the highest growth irradiance used (1,000 $\mu E/m^2 \cdot s$) 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 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 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 H_2 exchange to 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 H_2 (Table I). This capacity of strains 128C53 and 92F1 was associated with significantly lower ($P \leq 0.05$) net H_2 evolution and higher N_2 reduction and NE than was found in strains TA101, 175G10, and 92A2, which did not exchange H_2. The difference between associations based on hydrogenase activity was not noted in comparisons of total H_2 production. According to this latter criterion only one strain incapable of H_2 exchange (175G10) had significantly greater($P \leq 0.05$) total H_2 production than those which exchange H_2. Evaluation of 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).

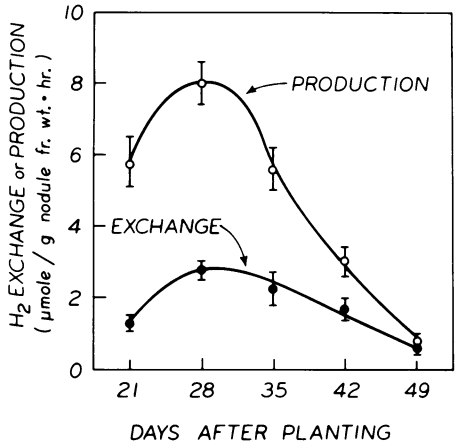

FIG. 3. H_2 production by nitrogenase and hydrogenase activity in pea root nodules during host plant ontogeny. Plants were inoculated with *R. leguminosarum* 128C53. H_2 production by nitrogenase was estimated as the sum of net H_2 evolution in air and H_2 exchange in the presence of 0.10 atm C_2H_2. Means ± SE were computed from six replicates.

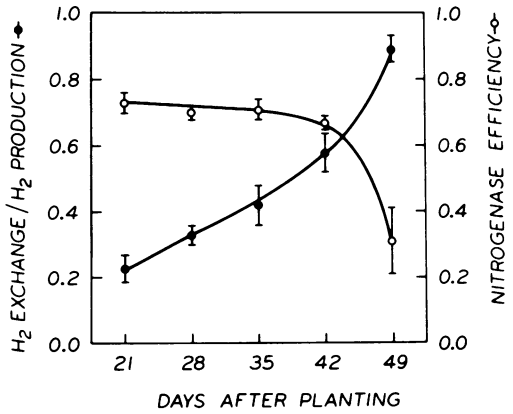

FIG. 4. Changes in efficiency of symbiotic N_2 fixation during host plant ontogeny. NE was computed as shown in equation 1. Tritiated H_2 exchange/H_2 production represents a relative measure of the capacity of the entire nodule to recover H_2 produced by the nitrogenase enzyme complex. Calculations were based on data in Figure 3.

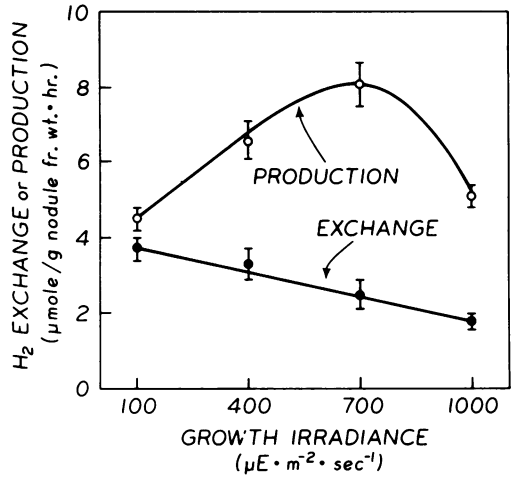

FIG. 5. H_2 production by nitrogenase and hydrogenase activity in root nodules of pea plants grown at different irradiances. Plants were inoculated with *R. leguminosarum* strain 128C53. H_2 production by nitrogenase was estimated as the sum of net H_2 evolution in air and H_2 exchange in the presence of 0.10 atm C_2H_2. Means ± SE were computed from six replicates.

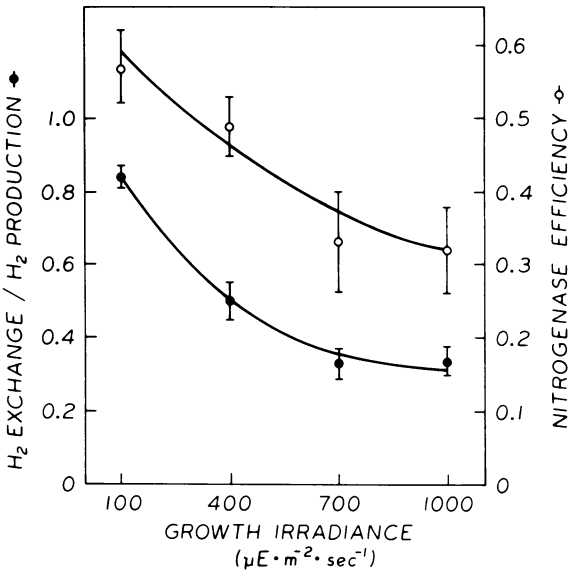

FIG. 6. Changes in efficiency of symbiotic N_2 fixation due to growth of host plants at different irradiances. NE was computed as shown in equation 1. Tritiated H_2 exchange/H_2 production represents a relative measure of the capacity of the entire nodule to recover H_2 produced by the nitrogenase enzyme complex. Calculations were based on data in Figure 5.

DISCUSSION

Unlike in free living, N_2-fixing bacteria (20), 0.10 atm C_2H_2 did not inhibit hydrogenase activity in *R. leguminosarum* strain 128C53 while preventing H_2 evolution from nitrogenase (Fig. 1). The opposite effects of 0.02 atm CO and 0.10 atm C_2H_2 on 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_2 production by nitrogenase was estimated as the sum of net H_2 evolution in air and H_2 exchange in the presence of 0.10 atm C_2H_2. Reduction of N_2 was computed as (C_2H_2 reduced-total H_2 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 ($p \leq 0.05$) for the indicated parameter.

N_2 fixation parameters		*Rhizobium* Strain				
		TA101	175G10	92A2	128C53	92F1
Tritium exchange	(μmole/g fresh weight·hr)	--	--	--	2.3 a	1.0 b
Net H_2 evolution	(" " ")	7.7 c	8.9 c	6.7 c	2.5 d	4.0 d
Total H_2 production	(" " ")	7.7 ef	8.9 e	6.7 ef	4.8 f	5.0 f
C_2H_2 reduction	(" " ")	12.9 g	16.5 gh	14.5 g	17.2 h	19.8 h
N_2 reduction	(" " ")	1.8 i	2.5 i	2.6 i	4.4 j	5.0 j
NE [1-(Total H_2 production/C_2H_2 reduction)]		0.32 k	0.46 kl	0.53 l	0.74 m	0.76 m
Tritium exchange/total H_2 production (%)		--	--	--	47	22

H_2 by H_2 produced in the nodules. This interpretation was supported by the lack of any measurable H_2 evolution in the presence of 0.10 atm C_2H_2, whether or not the *Rhizobium* strain exhibited hydrogenase activity. An evaluation of hydrogenase activity independent of H_2 evolution from nitrogenase therefore was possible. An expression of NE, which estimates the efficiency of nitrogenase in allocating electrons to H^+ or N_2 was determined according to equation 1.

Although tritiated H_2 exchange cannot be accepted as a measure of H_2 uptake in the absence of a measured decline in external H_2 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_2 produced by nitrogenase which could be recovered by hydrogenase in this system will require H_2 uptake measurements in the presence of 0.10 atm C_2H_2. 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_2 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_2. The ratio of H_2 exchange to H_2 production by nitrogenase and the calculated NE values (Figs. 4 and 6) likewise represent relative relationships between enzymes important for energetically efficient N_2 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_2 evolution with increasing growth irradiance previously had been ascribed either to a shift in electron allocation by nitrogenase from N_2 to H^+ 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_2 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_2 recapture in older plants.

A positive association between the efficiency of N_2 fixation and the presence of an H_2 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_2 production probably was minimized in strains 128C53 and 92F1 by hydrogenase activity. These strains also had relatively low rates of total H_2 production and high rates of C_2H_2 reduction (Table I), resulting in significantly higher N_2 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_2 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^+ or N_2 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_2, the present work suggests that even species which may be measurably inefficient at times can evolve less H_2 under other conditions. A more complete understanding of N_2 fixation efficiency and functional relationships between host legumes and rhizobial symbionts may result in identifying symbioses which reduce more N_2 for each unit of photosynthate supplied.

Acknowledgments—The authors are grateful to R. C. Aune, B. E. Gordon, and W. R. Irwin of the Lawrence Berkeley Laboratory, Berkeley, California, for assistance in designing the ^3H-dispensing apparatus and for determination of the specific activity of tritiated H_2.

LITERATURE CITED

1. ANAND SR, AI KRASNA 1965 Catalysis of the H_2-HTO exchange by hydrogenase. A new assay for hydrogenase. Biochemistry 4: 2747–2753
2. BETHLENFALVAY GJ, SS ABU-SHAKRA, DA PHILLIPS 1978 Interdependence of nitrogen nutrition and photosynthesis in *Pisum sativum* L. I. Effect of combined nitrogen on symbiotic nitrogen fixation and photosynthesis. Plant Physiol. 62: 127–130
3. BETHLENFALVAY GJ, SS ABU-SHAKRA, DA PHILLIPS 1978 Interdependence of nitrogen nutrition and photosynthesis in *Pisum sativum* L. II. Host plant response to nitrogen fixation by *Rhizobium* strains. Plant Physiol 62: 131–134
4. BETHLENFALVAY GJ, DA PHILLIPS 1977 Effect of light intensity on efficiency of carbon dioxide and nitrogen reduction in *Pisum sativum* L. Plant Physiol 60: 868–871

5. BETHLENFALVAY GJ, DA PHILLIPS 1977 Ontogenetic interactions between photosynthesis and symbiotic nitrogen fixation in legumes. Plant Physiol 60: 419–421
6. BURNS RC RWF HARDY 1975 Nitrogen Fixation in Bacteria and Higher Plants. Springer-Verlag, New York
7. DART PJ, JM DAY 1971 Effects of incubation temperature and oxygen tension on nitrogenase activity of legume root nodules. *In* TA Lie, EG Mulder, eds, Biological Nitrogen Fixation in Natural and Agricultural Habitats. Plant Soil Special Vol, pp. 167–184
8. DIXON ROD 1968 Hydrogenase in pea root nodule bacteroids. Arch Microbiol 62: 272–283
9. DIXON ROD 1972 Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch Microbiol 85: 193–201
10. GINGRAS G, RA GOLDSBY, M CALVIN 1963 Carbon dioxide metabolism in hydrogen-adapted *Scenedesmus*. Arch Biochem Biophys 100: 178–184
11. GORDON BE, WR IRWIN, M PRESS, RM LEMMON 1978 Non-destructive, high temperature gas radiochromatography. Anal Chem 50: 179–182
12. HARDY RFW, RD HOLSTEN, EK JACKSON, RC BURNS 1968 The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluation. Plant Physiol 43: 1185–1207
13. HOCH GE, HN LITTLE, RH BURRIS 1957 Hydrogen evolution from soybean root nodules. Nature 179: 430–431
14. JOHNSON CM, PR STOUT, TC BROYER, AB CARLTON 1957 Comparative chlorine requirements of different plant species. Plant Soil 8: 337–353
15. LIM ST 1978 Determination of hydrogenase in free-living cultures of *Rhizobium japonicum* and energy efficiency of soybean nodules. Plant Physiol 62: 609–611
16. MCCREA RE, J HANUS, HJ EVANS 1978 Properties of the hydrogenase system in *Rhizobium japonicum* bacteroids. Biochem Biophys Res Commun 80: 384–390
17. RUIZ-ARGÜESO T, J HANUS, HJ EVANS 1978 Hydrogen production and uptake by pea nodules as affected by strains of *Rhizobium leguminosarum*. Arch Microbiol 116: 113–118
18. SCHUBERT KR, HJ EVANS 1976 Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc Nat Acad Sci USA 73: 1207–1211
19. SCHUBERT KR, NT JENNINGS, HJ EVANS 1978 Hydrogen reactions of nodulated leguminous plants. Plant Physiol 61: 398–401
20. SMITH LA, S HILL, MG YATES 1976 Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. Nature 262: 209–210