Plant Physiol. (1985) 77, 335-338
0032-0889/85/77/0335/04/$01.00/0

Effect of Temperature on H_2 Evolution and Acetylene Reduction in Pea Nodules and in Isolated Bacteroids

Received for publication August 16, 1984

HANS BERTELSEN*
Department of Molecular Biology and Plant Physiology, University of Aarhus, DK-8000 Aarhus C, Denmark

ABSTRACT

Nitrogenase (EC 1.7.99.2) activity in pea (*Pisum savitum*) nodules formed after infection with *Rhizobium leguminosarum* (lacking uptake hydrogenase) was measured as acetylene reduction, H_2 evolution in air and H_2 evolution in Ar:O_2. With detached roots the relative efficiency, calculated from acetylene reduction, showed a decrease (from 55 to below 0%) with increasing temperature. With excised nodules and isolated bacteroids similar results were obtained. However, the relative efficiency calculated from H_2 evolution in Ar:O_2 was unaffected by temperature. Measurements on both excised nodules and isolated bacteroids showed a marked difference between acetylene reduction and H_2 evolution in Ar:O_2 with increased temperature, indicating that either acetylene reduction or H_2 evolution in Ar:O_2 are inadequate measures of nitrogenase activity at higher temperature.

Concomitant with N_2 fixation in legume nodules, the enzyme nitrogenase reduces protons to form H_2 (12). The ratio of H_2 produced to N_2 fixed has been suggested from studies with isolated enzyme to occur with a minimum value of 1:1 (13, 15), and it has been proposed that as much as 60% of the electron flow through nitrogenase *in vivo* may lead to proton reduction (16). Under an atmosphere of 90% air and 10% acetylene, essentially all electrons are transferred to acetylene (4). Similarly, assuming that no uptake hydrogenase is present, in an atmosphere of Ar:O_2 the rate of H_2 evolution should equal total electron flow (4, 8, 16). Relative efficiency of N_2 fixation was defined (16) as a parameter for evaluating the significance of H_2 production by nitrogenase:

$$RE^1_{Ac} = 1 - (H_2 \text{ evolved in air})/(C_2H_2 \text{ reduced})$$

or

$$RE_{Ar} = 1 - (H_2 \text{ evolved in air})/(H_2 \text{ evolved in Ar:}O_2)$$

Studies with isolated and purified nitrogenase proteins have shown that a number of factors, *e.g.* pH, ATP concentration, ADP/ATP ratio, flux of electrons through the Mo-Fe protein, can affect the relative distribution of electrons to proton and N_2 reduction (6, 10). Several factors affect RE *in vivo, i.e.* light intensity (2, 9) and plant age (3). However, interpretation of these results (2, 3) is difficult because of the use of a hydrogenase uptake positive (Hup^+) strain. Studies using Hup^- strains offer an opportunity to examine proton and N_2 reduction by the *Rhizobium* nitrogenase enzyme complex *in vivo*. Using Hup^- bacteria, Edie (8) found an effect of plant age on RE_{Ac} and similarly Rainbird *et al.* (14) showed an effect of temperature on RE_{Ar}.

This paper examines the effect of temperature on H_2 evolution, acetylene reduction and relative efficiency, using detached roots, excised nodules and isolated bacteroids, from pea plants infected by a Hup^- bacterium.

MATERIALS AND METHODS

Plant Material. Seeds of pea (*Pisum sativum* var Bodil) from Korn og Foderstof Kompagniet, Aarhus, Denmark, were surface sterilized in 5% NaOCl for 20 min and washed several times in distilled H_2O. After 12 h of inbibition, the seeds were sown in baked porous clay granules, Leca (Lecavaerket, Hinge pr. Randers, Denmark) in plastic pots. After 6 d of germination in the dark, plants were inoculated with *Rhizobium leguminosarum* RCR 1045 (Rothamsted Experimental Station, Harpenden, Herts, England) grown in TY medium (1). The conditions in environmental cabinets were a 16/8 h light/dark, 18/12°C or 12/8°C day/night, light intensity of 170 $\mu E\ m^{-2}\ s^{-1}$ and a RH of 70%. Plants were watered regularly with N-free medium (18).

Anaerobic Preparation of Bacteroids. Nodules from 27- to 31-d-old plants were homogenized in a glove box under a stream of nitrogen, using a mortar and pestle. The homogenizing buffer (1 ml/200 mg nodules) consisted of 50 mM K-phosphate, 0.3 M sucrose, 1 mM $MgCl_2$, 100 mM ascorbate, 1% w/v PEG grade 6000, adjusted to pH 7.1. The homogenate was filtered through two layers of Miracloth (Calbiochem-Bering Corp.) into a 50-ml screwcap centrifuge tube made gastight with a rubber packing. Bacteroids were obtained by differential centrifugation: After centrifugation at 300*g* for 10 min the supernatant was decanted into a new centrifuge tube and centrifuged at 10,000*g* for 10 min. The pellet was resuspended in fresh buffer and centrifuged at 300*g* for 10 min. The supernatant containing bacteroids was stored under nitrogen on ice until use.

Assay Procedures. *Detached Roots.* Acetylene reduction and H_2 evolution in air were conducted in 375-ml marmalade jars closed with a screwcap mounted with a serum cap for gas injections and sampling. Samples were taken after 0, 20, and 40 min for H_2 evolution and 2, 20, 40 min for acetylene reduction. H_2 evolution in air and acetylene reduction were measured successively on the same root system. After measurements, nodule weight was determined. Enzyme activity was computed from appropriate standards and expressed as $\mu mol/h \cdot g$ nodule fresh weight.

Excised Nodules. Evolution of hydrogen from excised nodules were recorded continuously by use of a standard Clark electrode (Rank Brothers, Cambridge, England). After 10 min of evolution in air, the atmosphere was changed to 80% Ar/20% O_2 by flushing for 4 min. H_2 standards were recorded before and after each two measurements. Linear rates were computed from the last 5 min of evolution. Acetylene reduction assays of excised

[1] Abbreviation: RE, relative efficiency.

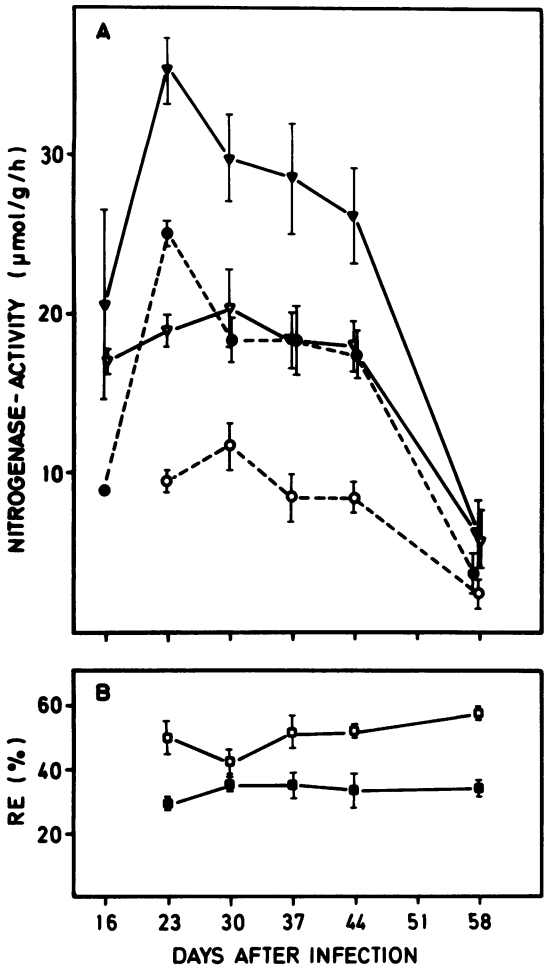

FIG. 1. Effect of temperature on acetylene reduction, H_2 evolution in air, and RE_{Ac}, seasonal variation. Measurements were performed on detached roots. Mean ± SE was computed from six replicates. Plants grown at 12/8°C day/night. A, (▼, ▽) acetylene reduction; (●, ○), H_2 evolution in air. B, (■, □), RE_{Ac}. Open symbols indicate assays performed at 12°C and closed symbols indicate assays performed at 22°C.

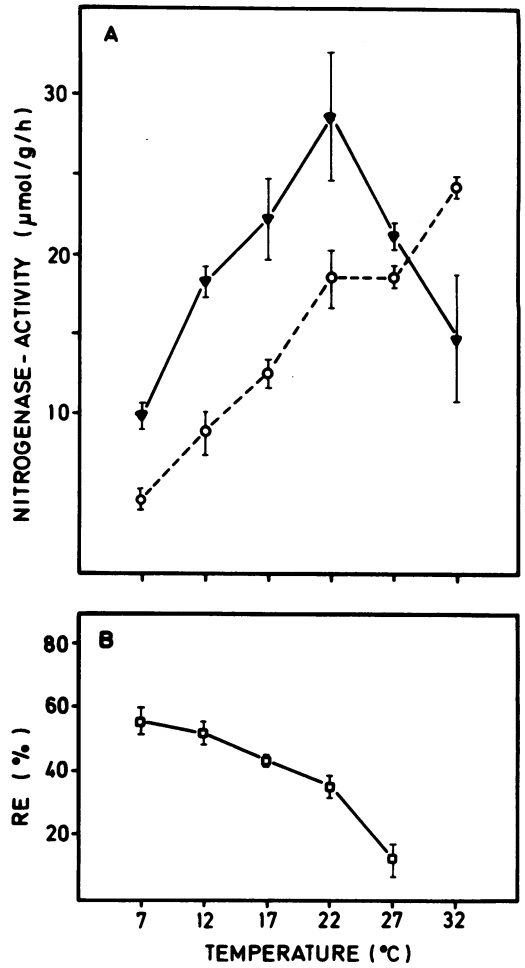

FIG. 2. Effect of temperature on acetylene reduction, H_2 evolution in air, and RE_{Ac}. Measurements were made on detached roots from 37-d-old plants grown at 12/8°C day/night. Mean ± SE was computed from three replicates. A, (▼), acetylene reduction; (○), H_2 evolution in air. B, (□), RE_{Ac}.

nodules were conducted in 120-ml serum flasks, 10% acetylene added and samples taken after 5, 10, and 20 min. Enzyme activity was computed from appropriate standards and expressed as μmol/h·g nodule fresh weight.

Isolated Bacteroids. Assays were carried out in 60-ml serum flasks capped with serum stoppers. The final volume of reaction mixture was 2.5 ml consisting of 1 ml bacteroid preparation and 1.5 ml homogenizing buffer. Malate was found to be the best carbon source and was used at 10 mM concentration. The optimal O_2 concentration in the gas phase with malate as carbon source was around 1.3%. The bacteroids could sustain linear rates of acetylene reduction for at least 50 min. Assays were stopped after 40 min by injection of 0.5 ml 1 N NaOH. Rates of H_2 evolution in N_2/1.3% O_2, H_2 evolution in Argon/1.3% O_2, and acetylene reduction in Argon/1.3% O_2/10% acetylene were computed and activities expressed in nmol/h·assay.

Variation of Temperature. Temperatures were varied by placing marmalade jars or serum flasks in a waterbath. The nodules or detached roots were allowed to equilibrate for 10 min. With bacteroids, assays were performed in parallel in five shaking waterbaths set at different temperatures. In experiments with the electrode, the electrode chamber temperature was controlled by a thermostated cap around the chamber. The temperature in the chamber was checked with a digital thermometer with the sensor placed in the suba seal lid.

Gas Chromatographic Determinations. Ethylene was quantified using a Beckman GC-65 gas chromatograph with a FID detector, mounted with a poropak T column and hydrogen was determined using a Gow Mac series 572 gas chromatograph with TCD detector, mounted with a molecular sieve 5A column and argon as carrier gas.

Calculation of RE. The RE of electron transfer to nitrogen via nitrogenase was defined by Schubert and Evans (16):

RE_{Ac} = (1 - [rate of H_2 evolution in air]/[rate of acetylene reduction]) × 100%

RE_{Ar} = (1 - [rate of H_2 evolution in air]/[rate of H_2 evolution Ar:O_2]) × 100%

For whole root systems and excised nodules, RE was calculated for each plant, while for bacteroids RE was calculated from mean values of H_2 evolutions and acetylene reduction rates.

RESULTS

Effect of Temperature on Nitrogenase Activity of Detached Roots. The seasonal variation of nitrogenase activity, measured as acetylene reduction and H_2 evolution in air, was markedly affected by the assay temperature (Fig. 1A). As expected, nitro-

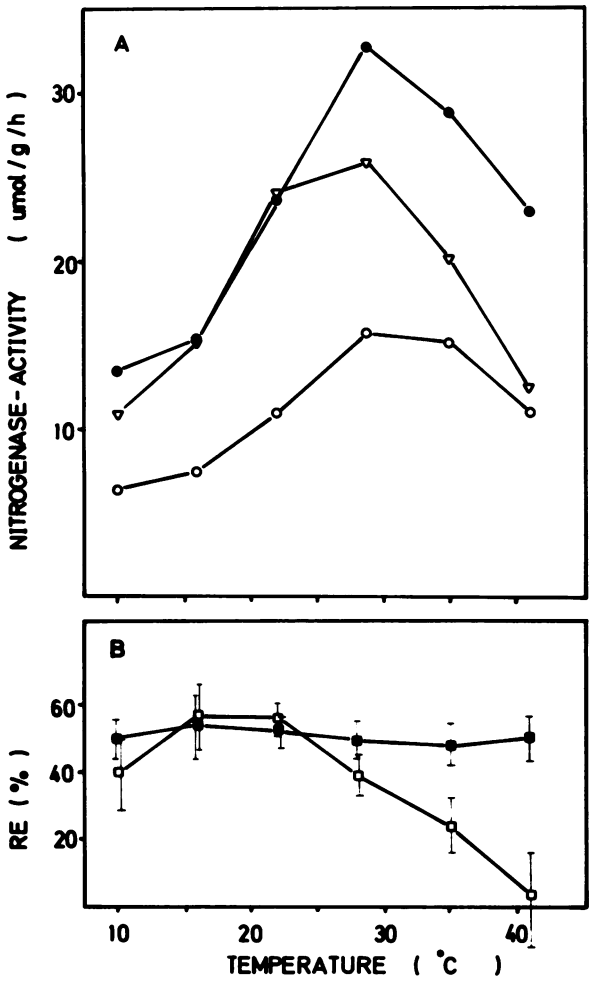

FIG. 3. Effect of temperature on acetylene reduction, H_2 evolution, and RE. Measurements were made on exised nodules from 25- to 28-d-old plants grown at 18/12°C day/night. Mean ± SE was computed from six replicates. A, (●), H_2 evolution in Ar:20% O_2; (○), H_2 evolution in air; (▽), acetylene reduction. B, (■), RE_{Ar}; (□), RE_{Ac}.

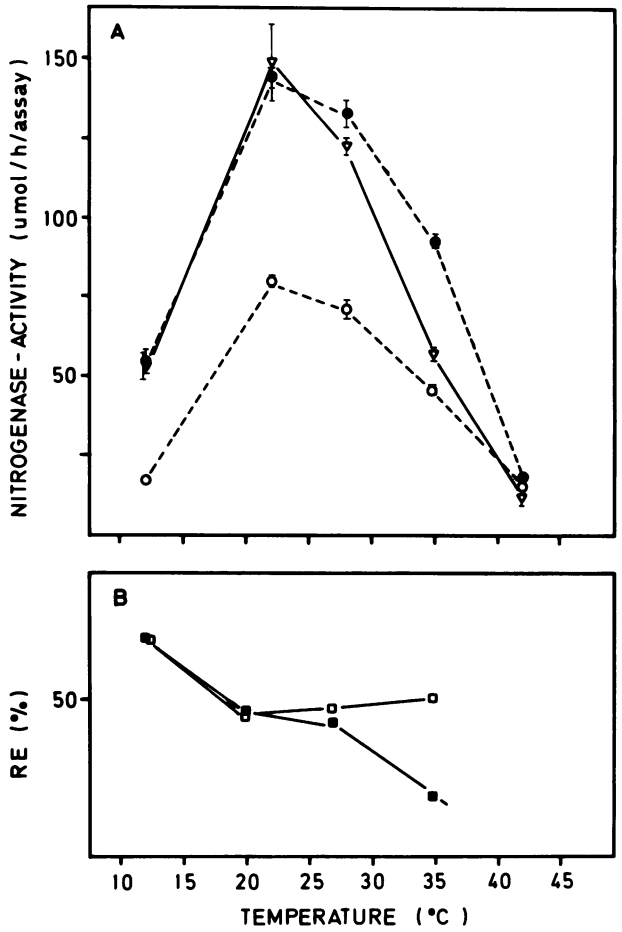

FIG. 4. Effect of temperature on acetylene reduction and H_2 evolution. Measurements performed with anaerobically isolated bacteroids. Mean ± SE was computed from three replicates. A, (▽), acetylene reduction; (○), H_2 evolution in N_2; (●), H_2 evolution in argon. B, (■), RE_{Ar}; (□), RE_{Ac}.

genase activity was lower at 12°C as compared to 22°C. However, plants assayed at 12°C showed a RE_{Ac} of 50%, while plants assayed at 22°C had a RE_{Ac} of 35% (Fig. 1B) which was not affected by plant age. A significant decrease in RE_{Ac} was observed, when temperature was increased from 7 to 32°C (Fig. 2B). Acetylene reduction showed a temperature optimum around 22°C, while H_2 evolution in air increased with temperature in the range examined (Fig. 2A). RE_{Ac} decreased from 55% at 7°C to even negative value at 32°C (H_2 evolution in air even higher than acetylene reduction).

Effect of Temperature on Nitrogenase Activity of Excised Nodules. To establish the significance of plant metabolites, nodules were excised from the roots, nitrogenase activity was measured as H_2 evolution in air, H_2 evolution in Ar:O_2 and acetylene reduction at a range of temperatures (10–41°C). At temperatures above 22°C, a difference between H_2 evolution in Ar:O_2 and acetylene reduction is observed (Fig. 3A). As with detached roots, RE_{Ac} decreased with increasing temperature (55–5%) while RE_{Ar} appeared constant over the temperature range tested (Fig. 3B).

Effect of Temperature on Nitrogenase Activity of Isolated Bacteroids. To test whether the observed differences are caused by changes in plant metabolism or are caused by properties of the nitrogenase enzyme, the above experiments were performed with anaerobically isolated bacteroids (Fig. 4). Acetylene reduction and H_2 evolution were maximal at 22°C and a difference between acetylene reduction and H_2 evolution in Ar:O_2 at higher temperatures was observed (Fig. 4A), resulting in a similar decrease in RE_{Ac} (Fig. 4B) as for intact nodules. To test that the observed phenomenon is not simply due to the lower solubility of gas with increasing temperatures, the oxygen concentration in the atmosphere over the bacteroids was varied and the nitrogenase activity at 28°C was measured as above (Fig. 5). The results indicate that RE decrease by lowering the oxygen tension, but give no indication of a difference between H_2 evolution in Ar:O_2 and acetylene reduction caused simply by lowering oxygen tension.

For all three types of experiments the possibility exists that lower acetylene reduction at higher temperature was caused by the lowering of acetylene solubility with increasing temperature. To check this, H_2 evolution in acetylene was measured and was found to be low and did not influence the results (data not shown). Similarly, hydrogen uptake was measured for every batch of nodules and no hydrogen uptake was ever found.

DISCUSSION

In experiments performed with detached roots, assay temperature markedly affected acetylene reduction and H_2 evolution in air (Fig. 1A). In addition, RE_{Ac} was higher at 12°C than at 22°C (Fig. 1B). This tendency was confirmed by assaying over a wider temperature interval (Fig. 2A); RE_{Ac} is decreasing with increasing temperature (Fig. 2B). Similar results were obtained with excised

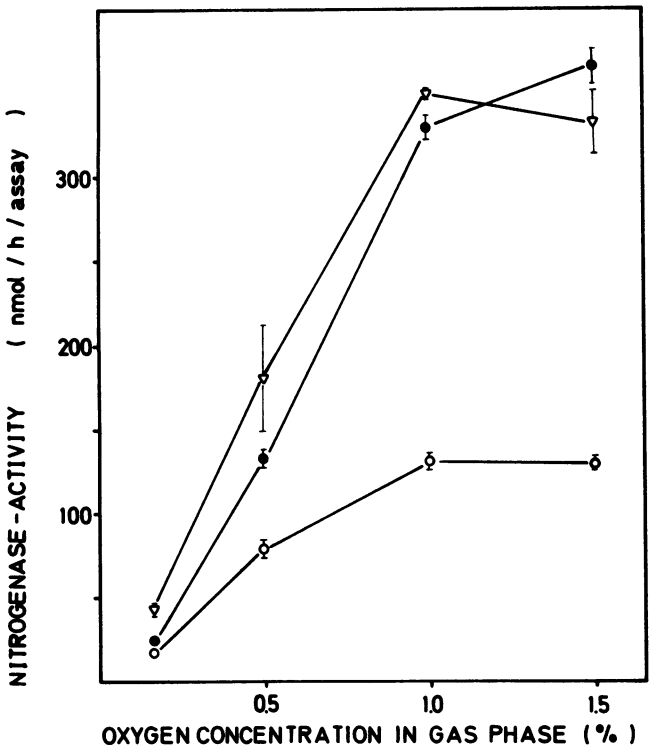

FIG. 5. Effect of oxygen concentration on acetylene reduction and H_2 evolution. Measurements were performed with anaerobically isolated bacteroids at 28°C. Mean ± SE was computed from three replicates. (▽), acetylene reduction; (○) H_2 evolution in N_2; (●), H_2 evolution in argon.

nodules (Fig. 3B) and isolated bacteroids (Fig. 4B). Dart and Day (5) also observed an effect of assay temperature on H_2 evolution and acetylene reduction. Acetylene reduction in *Vicia sativa* showed an optimum around 20 to 25°C and, interestingly, temperature seemed to affect the ratio of H_2 evolved in air to acetylene reduction, but little significance was attached to the observation as the two measurements were made on different sets of plant material. By measuring the same parameters on pea nodules at two different temperatures, Dixon and Blunden (7) found that RE_{Ac} was higher at 6°C as compared to RE_{Ac} at 25°C. Rainbird *et al.* (14) found with cowpea nodules infected with a Hup^- strain that both H_2 evolution in air and H_2 evolution in $Ar:O_2$ had an optimum temperature at 35°C. N_2 reduction was calculated from these data and they found a higher proportion of electrons to proton reduction than to N_2 reduction by increasing temperature. Results presented here with pea demonstrate a similar effect of temperature on H_2 evolution compared to acetylene reduction, while RE_{Ar} seems to be constant for pea.

At temperatures above 22°C a difference between H_2 evolution in $Ar:O_2$ and acetylene reduction is observed on both excised nodules (Fig. 3A) and isolated bacteroids (Fig. 4A), while at lower temperatures they are nearly equal. Earlier findings with intact nodules (8, 16) show that H_2 in $Ar:O_2$ and acetylene reduction were equal at room temperature. However, using purified nitrogenase components from *Klebsiella pneumonia*, Thorneley and Eady (17) found that H_2 evolution in argon was 25% higher than acetylene reduction at 30°C. The possibility that the lower acetylene reduction at higher temperatures was caused by lower solubility of acetylene with increasing temperature was checked by measuring H_2 evolution in acetylene. It was found to be negligible. Likewise solubility of other gases (*i.e.* oxygen and nitrogen) is lowered by increasing temperature. The effect of lowered oxygen solubility in intact nodules is difficult to evaluate due to the presence of leghemoglobin. With intact nodules, Dixon and Blunden (7) found that RE_{Ac} increased with decreasing oxygen tension. Assays with bacteroids at different oxygen concentrations (Fig. 5) indicate that RE decreases with decreasing oxygen tension. Work with *Azotobacter* (19) similarly showed that lowering the oxygen tension decreases the RE. The effect of lowering N_2 concentration has been demonstrated with isolated enzyme (15). H_2 evolution is increased by lowering the partial pressure of N_2, but the effect is small at partial pressures around 0.8 atm. H_2 is a competitive inhibitor of N_2 reduction (11); thus, with increasing H_2 evolution as temperature increases, N_2 reduction may be progressively inhibited.

Studies with isolated enzyme have shown that a number of factors (pH, ATP concentration, ADP/ATP ratio, ratio of Fe-protein to Mo-Fe protein, and flux of electron through the Mo-Fe protein) can affect the relative distribution of electrons to substrates (10). Any one of these parameters or a combination hereof might be modulated *in vivo* by changing temperature.

Due to the current interest in improving rate, efficiency, and reliability of measurements of nitrogen fixation, it would be of particular relevance to perform experiments using purified nitrogenase protein in order to determine whether the differential response to temperature described here is caused by bacteroid metabolism or whether it is a property of the enzyme. Furthermore, a direct measurement of N_2 fixation is needed to establish whether the same effects can be obtained with the natural substrate nitrogen, as was observed with acetylene.

Acknowledgments—I would like to thank Bent E. Sørensen for operating the growth chambers and Dr. Bjarne U. Jochimsen for carefully reading the manuscript.

LITERATURE CITED

1. BERINGER JE 1974 R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84: 188–198
2. 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
3. BETHLENFALVAY GJ, DA PHILLIPS 1977 Ontogenetic interaction between photosynthesis and symbiotic nitrogen fixation in Legumes. Plant Physiol 60: 419–421
4. BURNS RC, RWF HARDY 1975 Nitrogen fixation in bacteria and higher plants. Springer-Verlag, New York, pp 121–132
5. DART PJ, JM DAY 1971 Effects of incubation temperature and oxygen tension on nitrogenase activity of legume root nodules. Plant Soil (Special Volume): 167–184
6. DAVIS LC, VK SHAH, WJ BRILL 1975 Effect of component ratio, ATP and H_2 on the distribution of electrons to alternative substrates. Biochim Biophys Acta 403: 67–78
7. DIXON ROD, EAG BLUNDEN 1983 The relative efficiency of nitrogen fixation in pea root nodules. Plant Soil 75: 131–138
8. EDIE SA 1983 Acetylene reduction and hydrogen evolution by nitrogenase in a *Rhizobium*-legume symbiosis. Can J Bot 61: 780–785
9. EDIE SA, DA PHILLIPS 1983 Effect of the host legume on acetylene reduction and hydrogen evolution by *Rhizobium* nitrogenase. Plant Physiol 72: 158–160
10. HAGEMAN RV, RH BURRIS 1980 Electron allocation to alternative substrates of *Azotobacter* nitrogenase is controlled by the electron flux through dinitrogenase. Biochim Biophys Acta 591: 63–75
11. HARDY RWF, RC BURNS, JT STASNY, GW PARSHALL 1975 The nitrogenase reaction. *In* WDP Stewart, ed, Nitrogen Fixation by Free-Living Microorganisms. Cambridge University Press, London, pp 351–376
12. HOCH GE, HN LITTLE, RH BURRIS 1957 Hydrogen evolution from soybean root nodules. Nature 179: 430–431
13. MORTENSEN LE 1978 Regulation in nitrogen fixation. Curr Top Cell Regul 13: 179–227
14. RAINBIRD RM, CA ATKINS, JS PATE 1983 Effect of temperature on nitrogenase functioning in cowpea nodules. Plant Physiol 73: 392–394
15. RIVERA-ORTIZ JM, RH BURRIS 1975 Interactions among substrates and inhibitors of nitrogenase. J Bacteriol 123: 537–545
16. SCHUBERT KR, HJ EVANS 1976 Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc Natl Acad Sci USA 73: 1207–1211
17. THORNELEY RANF, RR EADY 1977 Nitrogenase of *Klebsiella pneumonia*. Biochem J 167: 457–461
18. TRUELSEN TA, R WYNDAELE 1984 Recycling efficiency in hydrogenase uptake positive strains of *Rhizobium leguminosarum*. Physiol Plant 62: 45–50
19. WALKER CC, CDP PARTIDGE, MG YATES 1981 The effect of nutrient limitation on hydrogen production in continuous cultures of *Azotobacter chroococum*. J Gen Microbiol 124: 317–327