The Relationship between \( \text{H}_2 \) Evolution and Acetylene Reduction in \( \text{Pisum sativum-Rhizobium leguminosarum} \) Symbioses Differing in Uptake Hydrogenase Activity

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JOHN D. MAHON* AND LOUISE M. NELSON

Plant Biotechnology Institute, National Research Council of Canada,
Saskatoon, Saskatchewan, S7N0W9 Canada

ABSTRACT

Peas (\textit{Pisum sativum} L.) were inoculated with strains of \textit{Rhizobium leguminosarum} having different levels of uptake hydrogenase (Hup) activity and were grown in sterile Leonard jars under controlled conditions. Rates of \( \text{H}_2 \) evolution and acetylene reduction were determined for intact nodulated roots at intervals after the onset of darkness or after removal of the shoots. Hup activity was estimated using treatment plants or equivalent plants from the growth chamber, by measuring the uptake of \( \text{H}_2 \) or \( \text{H}^2 \text{H}_2 \) in the presence of acetylene. In all cases, the rate of \( \text{H}_2 \) evolution was a continuous function of the rate of acetylene reduction. In symbioses with no demonstrable Hup activity, \( \text{H}_2 \) evolution increased in direct proportion to acetylene reduction and the slopes were similar with the Hup" strains NA502 and 128C79. Hup activity was similar in strains 128C30 and 128C52 but significantly lower in strain 128C54. With these strains, the slopes of the \( \text{H}_2 \) evolution versus acetylene reduction curves initially increased with acetylene reduction, but became constant and similar to those for the Hup" strains at high rates of acetylene reduction. On these parallel portions of the curves, the decreases in \( \text{H}_2 \) evolution by Hup" strains were similar in magnitude to their \( \text{H}_2 \)-saturated rates of Hup activity. The curvilinear relationship between \( \text{H}_2 \) evolution and acetylene reduction for a representative Hup" strain (128C52) was the same, regardless of the experimental conditions used to vary the nitrogenase activity.

In nitrogen-fixing legume—\textit{Rhizobium} symbioses, \( \text{H}_2 \) gas is produced as a consequence of the reduction of protons by the same nitrogenase complex which catalyzes the reduction of \( \text{N}_2 \) to \( \text{NH}_3 \). The relative efficiency of nitrogen fixation, defined as 1—(rate of \( \text{H}_2 \) evolution into air/acetylene-saturated rate of acetylene reduction), has been reported to vary greatly in different symbiotic systems (20). Differences in RE\(^2\) have been attributed to the ability of some \textit{Rhizobium} strains to produce an uptake hydrogenase enzyme which can recycle some or all of the \( \text{H}_2 \) produced by nitrogenase (6, 11). Using single pea genotypes, Nelson and Child (17) and Ruiz-Aguiru et al. (19) were able to demonstrate a wide range of both RE values and Hup activity in symbioses formed with different strains of \textit{Rhizobium leguminosarum}, although none was able to recycle all of the \( \text{H}_2 \) produced, as was the case in some soybean symbioses. Other reports indicate that the host plant genotype can alter the expression of the Hup gene in \textit{R. leguminosarum} strains (1, 3). In some studies with peas, relatively high RE values have been reported even in symbioses having no measurable Hup activity (2, 8–10, 17), and these efficiencies are considered to reflect a more efficient allocation of electrons to the reduction of nitrogen by nitrogenase. The RE of pea-\textit{Rhizobium} symbioses can also be altered by environment and plant age. Generally, environmental conditions which increase the rate of acetylene reduction decrease the RE in both Hup" (4, 5) and Hup" (8–10) symbioses. However, in studies with purified nitrogenase, increasing the flux of electrons through nitrogenase, by a number of different methods, has been shown to increase the rate of \( \text{N}_2 \) reduction relative to that of \( \text{H}_2 \) production (12).

The experiments reported here were undertaken to compare the relationship between \( \text{H}_2 \) evolution and acetylene reduction in the nodules of intact pea plants inoculated with single strains of \textit{R. leguminosarum} reported to differ in RE and Hup activity. Symbiotic combinations with two \textit{Pisum sativum} genotypes were examined as nitrogenase activity decreased in response to substrate deprivation, to see if RE responded similarly in Hup" and Hup" symbioses. The relationship was also examined in a single Hup" symbiotic combination, assayed under a number of treatment conditions which have been reported to affect the rate of acetylene reduction.

MATERIALS AND METHODS

Bacterial Strains. \textit{Rhizobium leguminosarum} strains 128C52, 128C30, 128C54, and 128C79 were obtained from the Nitragin Co. (Milwaukee, WI) and strain NA502 was obtained from the Australian Inoculation Control Service, Narara, Australia.

Plant Culture. Pea (\textit{Pisum sativum} L.) seed cv. Homesteader was obtained from a commercial supplier (Early Seed and Feed, Saskatoon, Canada), and cv. Trapper from Dr. A. E. Slinkard (Crop Development Centre, Univ. Saskatchewan, Saskatoon, Canada). For most experiments, seeds were surface sterilized for 10 min in a 0.6% solution of commercial NaOCl, washed, sown 2 cm deep into Turface (International Minerals and Chemical Corp., Mundelein, IL) in sterile Leonard jar assemblies, and inoculated with single strain suspensions of \textit{R. leguminosarum} (17). In one series of experiments with strain 128C52, open plastic pots were used for plant culture rather than Leonard jars. For these experiments, the pots were washed in 0.6% NaOCl, and the seeds were surface sterilized before inoculation. During growth, an N-free nutrient solution (14) was supplied three times per week and water on the other days. The same nutrient solution was added to the reservoirs of the Leonard jars before they were

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\(^2\) Abbreviations: RE, relative efficiency; Hup, uptake hydrogenase; FI, fluorescent and incandescent lamps; HID, high pressure sodium and metal halide lamps.
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autoclaved. Plants were grown in controlled environment chambers with 16 h photoperiods and day/night temperatures of 20/15°C. Light was supplied by either mixed Fl or equal numbers of HID.

Gas Exchange Measurements. Rates of H2 evolution and acetylene reduction, as well as root zone temperatures, were determined using a computer-controlled automated gas exchange system (14). Four intact plants in their containers were sealed into the four root chambers, the lamps (1000 W metal halide) were turned on, and air was passed through each root chamber at 90 L h\(^{-1}\). A secondary air circulation in each chamber, part of which passed directly through the rooting medium, also flowed through the temperature controlled chamber jacket to provide rapid mixing of the gases and temperature control. Each secondary circulation loop could be directed to the gas chromatographs for determination of either acetylene or H2 concentration and back to the chamber so that with the inlet and outlet valves closed, each chamber operated as a closed system and only the volume of the sample loops was lost during each injection. This represented less than 0.2% of the gas in the closed system.

To determine rates of H2 evolution, the chambers were closed at 2 min intervals, and the internal gas was sampled for determination of H2 concentration, 10, 20 and, in some experiments, 30 min after chamber closure. Because the steady state concentration of H2 in the chambers at the time of chamber closure can be calculated to be 18% of that accumulating in the closed system during a 10 min period, the rates of H2 evolution were calculated from the accumulation of H2 between 10 and 20 min or from the mean accumulation in the 10 to 20 min and 20 to 30 min intervals. After the final H2 determination, the inlet and outlet valves were opened and the chambers were flushed with air for 10 to 20 min. Acetylene reduction was determined by closing the inlet and outlet valves and injecting acetylene into each chamber to a final concentration of 10% (v/v). Ethylene was determined 10, 20, and, in some cases, 30 min later. Because the background ethylene concentration was not detectable, rates of ethylene production during the initial 10 min period were included.

Measurements on plants grown in open pots were carried out using a similar procedure, except that H2 and ethylene were determined by manual injection of samples into gas chromatographs with disposable syringes. Ethylene was determined 10 and 20 min after injection of acetylene to a final volume of 5% (v/v) as previously described (13). H2 was determined 15 and 30 min after chamber closure by injecting 2 ml samples of gas onto the column of a gas chromatograph, similar to that used in the automated system but including a linearizing circuit which gave peak areas proportional to the concentration of H2 over the range of 2 to 150 nmol ml\(^{-1}\).

Experiment 1. Trapper and Homesteader peas, inoculated with strains 128C52, 128C54, NA502, or 128C79, were grown for 4 weeks with a PAR of 400 mmol quanta m\(^{-2}\) s\(^{-1}\) (HID). For each genotype, one plant inoculated with each strain was placed into a different chamber on each of 4 consecutive days. The plants were sealed into the chambers at 0830 h with a PAR at the shoot tops of 600 mmol quanta m\(^{-2}\) s\(^{-1}\) and a root temperature of 20°C. The two gas chromatographs were calibrated and sampling began at 1100 h. At 2 h intervals until 0700 the following day rates of H2 evolution and acetylene reduction were determined from measurements of H2 and ethylene made 10 and 20 min after chamber closure or acetylene injection. The lamps were turned off after the first set of measurements (1245 h). After the gas exchange measurements, roots and nodules were washed, dried, and weighed together. Hup activity was determined using equivalent plants on the same sampling day. A portion of the root with attached nodules was removed from each plant and sealed into a small vial. Acetylene was injected (10% v/v) and samples were taken after 10 min for ethylene determination. Immediately thereafter, 3H uptake was determined as previously described (17). The remainder of the root and nodule system was assayed for acetylene reduction activity and then dried and weighed. The combined rates of acetylene reduction from the two samples were used to adjust the rates of Hup to a total root + nodule dry weight basis.

Experiment 2. Two groups of Homesteader peas, inoculated with strains 128C52, 128C54, NA502, or 128C79 were planted 1 week apart and grown in sterile Leonard jars for 28 to 31 d at a PAR (HID) of 400 mmol quanta m\(^{-2}\) s\(^{-1}\). For each experimental run, two plants inoculated with each of two strains were sealed into the chambers at 0830 h with a PAR at the shoot tops of 600 mmol quanta m\(^{-2}\) s\(^{-1}\) and a root temperature of 20°C. H2 evolution and acetylene reduction were determined at 90 min intervals from 1100 h to 0630 h on the following day from determinations of H2 and ethylene, sampled at 10 and 20 min after chamber closure or acetylene injection. The plants were automatically irrigated with N-free nutrient solution for 15 min at 1930 h (14). At 1650 h the shoots of two plants, one inoculated with each strain, were excised just above the chamber seal. The procedure was repeated with other combinations of strains over the next 3 days and with the second group of plants 1 week later. After the gas exchange measurements, plants were removed from the chambers and the shoots of the control plants were excised. Roots and nodules from each plant were sealed into scintillation vials and 2.4 ml of air were removed and replaced with acetylene and H2 to give final concentrations of 10 and 1% (v/v), respectively. Samples of the gas were immediately withdrawn and injected onto a molecular sieve column in a gas chromatograph with a thermal conductivity detector, to determine the initial H2 concentration. After 90 min, a second sample was removed and assayed for H2 and the roots and nodules were removed, dried, and weighed together. Rates of H2 uptake were estimated from the decrease in H2 and were expressed per g of root + nodule dry weight.

Experiment 3. Homesteader peas, inoculated with strains 128C52, 128C54, NA502, or 128C79, were grown for 28 to 32 d with a PAR (HID) of 600 mmol quanta m\(^{-2}\) s\(^{-1}\). For each sampling group, a plant inoculated with each strain was sealed into one of the chambers. PAR at the shoot tops was 600 mmol quanta m\(^{-2}\) s\(^{-1}\) and root temperature was 20°C. The lamps were turned off at 2155 h, plants were irrigated for 30 min at 0500 h, the lamps were turned on at 0555 h, and the gas chromatographs were calibrated at 0900 h. Beginning at 1000 h, rates of H2 evolution and acetylene reduction were determined at 2 h intervals by measuring H2 or ethylene 10, 20, and 30 min after chamber closure or acetylene injection. Dark treatments were begun at 1355 h and control plants remained in continuous light until the end of the experiment at 1400 h on the following day. On the same day as plants were removed from the chambers, one plant inoculated with each strain was removed from the grown chamber and used for estimating H2 uptake of the whole root + nodule system. Excised roots and nodules were sealed into 140 ml glass jars. Acetylene was injected to a final concentration of 10% (v/v) and after 10 min, 0.2 ml samples of the gas were used to determine ethylene concentrations. Immediately thereafter H2 was injected to a final concentration of 10% (v/v) and 120 μl of 3H2 (188 μCi m\(^{-1}\)l\(^{-1}\)) were injected. After 30 min at room temperature, the nodulated roots were removed, sealed into a polyethylene bag, dipped into a dry ice/methanol bath until frozen, and stored at -20°C. To determine 3H incorporation, the frozen roots were put into a 50 ml cylinder and water was added to give a total volume of 25 ml. The roots were homogenized (Polytron, Brinkman, NY) for 1 min. Three 1.5 ml samples were transferred to scintillation vials and 15 ml of scintillation cocktail (Aquasol II, New England Nuclear) were
added. The liquid was sparged for 1 min with H\textsubscript{2} gas to remove dissolved \textsuperscript{3}H\textsubscript{2} from the samples and the samples were counted (17). Parallel assays were carried out on root and nodule material which was autoclaved before the assay and all sample values were adjusted to compensate for any \textsuperscript{3}H recovery which was not related to metabolic activity.

**Experiment 4.** Nitrogenase activity of Trapper peas, inoculated with strain 128C52, was varied by a number of different methods. Plants were sealed into chambers and allowed to equilibrate for 1 h before initial rates of H\textsubscript{2} evolution and acetylene reduction were determined. Initial rates were determined on 4-week-old plants at 8, 16, 24, and 35°C as well as at 20°C beginning at 0900, 1300, and 1700 h. In other trials, the shoots were excised immediately after the initial measurements and H\textsubscript{2} evolution and acetylene reduction were determined at 20°C, beginning 1, 3, and 6 h after the treatment onset. These time courses were repeated with plants grown for 3, 4, and 5 weeks at a PAR (FI) of 300 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) and for 4 weeks at a PAR (HID) of 1000 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\). A similar protocol was used to follow the decline in H\textsubscript{2} evolution and acetylene reduction after the shoots of 4-week-old plants were darkened.

**Statistical Procedures.** Because there were four chambers, experiments were randomized block designs with four replicates of each treatment, one in each chamber. In all but experiment 3, rates of H\textsubscript{2} evolution and acetylene reduction were expressed on a unit root + nodule dry weight basis to adjust for variations in plant size. Data which showed significant heterogeneity of variances among treatments by Bartlett's test, were transformed using the formula \(Y = \ln (X + 1)\). All experimental data were subjected to analysis of variance, with sequential measurements on the same plants being considered as subplots. Means were compared by Duncan's new multiple range test. Because acetylene reduction was usually determined 30 min after H\textsubscript{2} evolution, in excision experiments where rates decreased rapidly, the rates of acetylene reduction were adjusted by linear interpolation to correspond to the time at which H\textsubscript{2} evolution was determined. To estimate rates of H\textsubscript{2} evolution at specific rates of acetylene reduction for experiment 3, linear or second order polynomial regressions were determined. For graphic presentation of the data from other experiments, linear relationships were drawn from regression equations and nonlinear curves were fitted to the data points by eye.

**RESULTS**

In these experiments, the gas phase was sampled for ethylene determination after two or three time intervals, to see if there was an appreciable acetylene induced decrease in the rate of acetylene reduction as has been reported in peas under some conditions in an open flow-through measuring system (15) and detected in other experiments with this closed system (14). In experiments 1, 2, and 4 there was no significant effect of time after acetylene injection on the rates of acetylene reduction. There were also no significant interactions between the time interval and either plant genotype (experiment 1) or *Rhizobium* strain (experiments 1 and 2). In experiment 3, however, the rates of acetylene reduction by strain NA502 decreased significantly after the injection of acetylene, while the rates were constant with the other three strains (Fig. 1). To have comparable values for the examination of the H\textsubscript{2} evolution-acetylene reduction relationships, in the first three experiments only the rates from 0 to 10 min were used but because there was no indication of decling rates in experiment 4, the mean rates between 0 and 20 min were used.

**Experiment 1.** Acetylene reduction was significantly \((P < 0.001)\) affected by the plant genotype (Table I), but neither the *Rhizobium* strain (Table I) nor the genotype \(\times\) strain interaction had a significant effect \((P > 0.05)\). H\textsubscript{2} evolution, relative effici-
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Fig. 2. Mean rates of acetylene reduction (open symbols) and mean relative efficiencies (closed symbols) of intact nodulated roots of *P. sativum-R. leguminosarum* symbioses during dark treatment. Values are means from Hup⁺ strains 128C52 and 128C30 (circles) and Hup⁻ strains NA502 and 129C79 (squares) with Trapper and Homesteader peas. Arrow shows beginning of dark treatment and LSD is for RE values.

Fig. 3. H₂ evolution as a function of acetylene reduction in intact nodulated roots of Trapper or Homesteader peas inoculated with strains 128C52 (○), 128C30 (●), NA502 (□), or 128C79 (■) of *R. leguminosarum*. Plants were grown for 4 weeks and rates were determined at 2 h intervals during darkness.

In neither case was there a significant strain by measurement time effect on acetylene reduction (P > 0.05).

In excised roots, the mean rate of acetylene reduction for all strains decreased to 5% of the initial value and in control plants it increased to 118%. Thus, the rates of both H₂ evolution and acetylene reduction were determined over a wide range of nitrogenase activities and because control plants were included, high rates of acetylene reduction were not confounded with initial measurements, as was the case in experiment 1. The relationships between H₂ evolution and acetylene reduction (Fig. 4) were similar to those seen in experiment 1, in that for Hup⁺ strains (NA502, 128C79) it was approximately proportional and for Hup⁻ strains (128C52, 128C54) it was distinctly curvilinear. However, in experiment 1 the Hup⁻ strains were almost identical, whereas in this experiment, strain 128C54 had greater H₂ evolution than strain 128C52 for all rates of acetylene reduction. The extended data obtained in this experiment indicate that the relationship between H₂ evolution and acetylene reduction approached linearity even in Hup⁻ strains, as nitrogenase activity increased. Although there is some indication that the slopes of these linear portions might be different, a statistical comparison of the regressions from all data with rates of acetylene reduction

Fig. 4. H₂ evolution as a function of acetylene reduction in nodulated roots of Homesteader peas inoculated with strains 128C52 (○), 128C54 (●), NA502 (□), or 128C79 (■) of *R. leguminosarum*. Rates were determined at 14 × 1.5 h intervals in four intact plants and four plants from which the shoots had been excised. Data points are means for measurements grouped according to rates of acetylene reduction.

Table II. Acetylene Reduction and H₂ Uptake Determined by ³H₂ Uptake in Detached Roots and Nodules and the Difference in H₂ Evolution Between Strain 128C52 or 128C54 and the Hup⁻ Strains for an Equivalent Rate of Acetylene Reduction

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acetylene Reduction</th>
<th>³H₂ Uptake</th>
<th>Difference in H₂ Evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>128C52</td>
<td>17.2 a*</td>
<td>1.96</td>
<td>1.52 a NS†</td>
</tr>
<tr>
<td>128C54</td>
<td>14.9 a</td>
<td>0.83 b</td>
<td>0.67 b NS</td>
</tr>
<tr>
<td>NA502</td>
<td>16.9 a</td>
<td>0.01 c</td>
<td></td>
</tr>
<tr>
<td>128C79</td>
<td>17.1 a</td>
<td>0.00 c</td>
<td></td>
</tr>
</tbody>
</table>

* Values in a column followed by a common letter do not differ significantly (P > 0.05). † Values followed by NS do not differ significantly (P > 0.05) from corresponding rates of ³H₂ uptake.

Greater than 40 μmol g⁻¹ h⁻¹ showed no significant differences (P > 0.05).

Rates of H₂ uptake in the presence of acetylene were variable, and analysis showed no significant difference (P > 0.05) difference between the plants from which the shoots had been removed early in the measurement period and those which had remained intact. There was, however, a significant (P < 0.01) effect of the strain used to inoculate the plants. H₂ uptake by strains NA502 and 128C79 was not significantly greater than zero, but with strain 128C52 and 128C54 it averaged 2.8 ± 0.5 and 2.5 ± 0.6 μmol g⁻¹ h⁻¹, respectively.

Experiment 3. Acetylene reduction was not significantly different (P > 0.05) among the plants inoculated with the four strains. The initial rate of acetylene reduction per plant after equilibration overnight in the chambers, averaged 32.6 μmol h⁻¹. This rate did not change significantly in the control plants for 20 h, after which it decreased to 22.5 μmol h⁻¹. However, acetylene reduction decreased significantly (P < 0.05) within 4 h of the onset of darkness and continued to decline to a final value of 0.53 μmol h⁻¹. The mean values for estimates of ³H₂ uptake by the whole root + nodule systems of plants inoculated with the four inoculants (Table II) showed no significant hydrogen uptake ability with strains NA502 and 128C79. The relationship between H₂ evolution and acetylene reduction was proportional and similar with these two strains, as was the case in experiments 1 and 2. Thus, for the purposes of describing the relationship for Hup⁻ strains, the data from the two strains were pooled to give the regression equation for H₂ evolution versus acetylene reduction $Y = 0.31 + 0.384X (r^2 = 0.978; n = 52)$. Plants inoculated
with strains 128C52 and 128C54 exhibited significant but different rates of \(^3\)H\(_2\) uptake on a whole plant basis (Table II). The plots of \(H_2\) evolution versus acetylene reduction were also similar to those in experiment 2 in that both curves upwards and \(H_2\) evolution by plants inoculated with 128C54 was consistently greater than that of 128C52 for the same rate of acetylene reduction. The regressions of \(H_2\) evolution versus acetylene reduction for all values are \(Y = -0.031 + 0.313X + 0.000105X^2\) \((r^2 = 0.993; n = 26)\) for 128C52 and \(Y = 0.292 + 0.339X + 0.00094X^2\) \((r^2 = 0.9987; n = 26)\) for 128C54. Using the appropriate Hup\(^+\) regression, the pooled Hup\(^+\) regression, and the rate of acetylene reduction determined immediately before the \(H_2\) uptake assays, the Hup\(^+\) and Hup\(^-\) rates of \(H_2\) evolution and the difference between them were calculated for each Hup\(^+\) plant assayed for \(H_2\) activity. The mean estimated difference in \(H_2\) evolution for strain 128C52 was significantly greater \((P < 0.05)\) than that for strain 128C54, and neither was significantly different \((P > 0.05)\) from the measured rate of \(H_2\) uptake (Table II).

**Experiment 4.** Rates of acetylene reduction by plants inoculated with strain 128C52 were varied by several experimental manipulations including darkening and shoot excision treatments, used in the previous experiments. The data from all these tests, expressed on a per g root + nodule dry weight basis showed \(H_2\) evolution was similarly related to the rate of acetylene reduction, regardless of the treatment used to vary nitrogenase activity (Fig. 5). As was seen in other experiments, the relationship between \(H_2\) evolution and acetylene reduction was distinctly curvilinear.

### DISCUSSION

Strains NA502 and 128C79 have previously been classified as Hup\(^+\) (18) and in these experiments, they had no significant ability to take up hydrogen as estimated by \(3\)H\(_2\) uptake in experiments 1 and 3 or by net \(H_2\) uptake in the presence of acetylene in experiment 2. Plants inoculated with these strains had rates of \(H_2\) evolution which were directly proportional to the rates of acetylene reduction and thus RE values which were constant (Fig. 2). In no case was there a significant difference in the regressions of \(H_2\) evolution versus acetylene reduction between plants inoculated with these two strains, nor did host plant genotype change the relationship (Fig. 3). Thus, these results do not demonstrate the significant changes in RE of Hup\(^-\) Pisum/\(R.\) leguminosarum symbioses which have been reported by other workers (2, 8-10). For these experiments, the mean regression coefficients were 0.43 \pm 0.02 as for NA502 and 0.44 \pm 0.02 for 128C79. These slopes and the lack of any significant intercept values indicate a constant allocation of 56 to 57% of the electrons to nitrogen fixation. At the present time it is not known whether the very stable efficiencies in these experiments are due to the use of intact plant systems or the particular plant and bacterial combinations used. However, they do show that the inverse relationship between RE and nitrogenase activity which has been reported in Hup\(^+\) pea symbioses (8, 9) is not a universal phenomenon.

The \(H_2\) uptake capacities of bacteroid preparations from the three Hup\(^+\) strains used in these experiments have been ranked in descending order as 128C30, 128C52, 128C54 (18). In experiment 1, however, there was no apparent difference in the results with strains 128C30 and 128C52 (Table I; Fig. 3). For this reason, strain 128C54 was substituted for strain 128C30 in experiments 2 and 3. In both cases strain 128C54 evolved \(H_2\) at consistently greater rates than 128C52 for equivalent rates of acetylene reduction as indicated by Figure 4 and the regression equations from experiment 3. Moreover, \(H_2\) activity estimated by the \(H_2\) evolution rate in the experiment to whole root, and the proportion into whole root and bacteroid systems was significantly greater in strain 128C52 than in 128C54 (Table II).

Plants inoculated with these Hup\(^+\) strains did not evolve \(H_2\) at rates proportional to the rate of acetylene reduction. Nevertheless, except for some points corresponding to the initial measurements in experiment 1, \(H_2\) evolution appeared to be a continuously increasing, but curvilinear function of the rate of acetylene reduction. It is believed that these few deviant points were related to the adjustment of plants to the measuring conditions (14), because in experiment 2 when plants were equilibrated overnight in the system and measurements were made on intact plants at high PAR as well as on plants from which the shoots had been removed, there was no indication that \(H_2\) evolution reached a plateau or decreased at high rates of acetylene reduction (Fig. 4). In fact, the data show that the relationship between \(H_2\) evolution and acetylene reduction is curvilinear only at low rates of acetylene reduction, becoming linear in the higher ranges. This is also indicated by the results in experiment 1 for Trapper peas which achieved acetylene reduction rates greater than 60 \(\mu\)mol g\(^{-1}\) dry weight h\(^{-1}\) (Fig. 3).

This general relationship between \(H_2\) evolution and acetylene reduction predicts a change in RE values with differences in nitrogenase activity. The concurrence of the data from a number of different methods of varying the rate of acetylene reduction (Fig. 5) suggests that anything which restricts the flux of electrons through nitrogenase in intact nodules will cause similar changes in the RE of Hup\(^+\) symbioses. In other studies with Hup\(^+\) strains of \(R.\) leguminosarum, RE has also been shown to increase as acetylene reduction decreased during pod development (4) or in response to lower levels of irradiance (5). The results of the experiments reported here suggest that RE may not be the most appropriate parameter to describe the \(H_2\) metabolism of Hup\(^+\) symbioses. For example, Table I shows a significant difference in RE between Trapper and Homestead peas, and a comparison of the results for these genotypes combined only with the Hup\(^+\) strains 128C52 and 128C30 indicates a difference in genotypic RE which is significant at a probability level of 0.01. Nevertheless, the plots of \(H_2\) evolution versus acetylene reduction (Fig. 3) shows virtually identical relationships, and the difference in RE is due solely to the decreased nitrogenase activity of Homestead peas. Similarly, the curves from experiments 2 and 4 indicate mean RE values for strain 128C52 which range from 0.72 to 0.96 (Fig. 4) and from 0.65 to 0.95 (Fig. 5).

A comparison of the \(H_2\) evolution versus acetylene reduction relationships shows an almost constant difference in the rate of \(H_2\) evolution between Hup\(^+\) and Hup\(^-\) strains when rates of

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**Fig. 5.** \(H_2\) evolution as a function of acetylene reduction in nodulated roots of Trapper peas inoculated with \(R.\) leguminosarum strain 128C52. Plants were grown for different numbers of weeks (AGE) at a PAR of 300 or 1000 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) (LIGHT). \(H_2\) evolution and acetylene reduction are from initial measurements at different times of day (TIME) or at different temperatures (TEMP) or from measurements at intervals after shoot excision (EXCISE) or darkening (DARK).
acetylene reduction exceeded 40 μmol g⁻¹ dry weight h⁻¹. The magnitude of this difference in H₂ evolution was estimated for the rates of acetylene reduction determined in the control plants which were assayed for Hup activity. It was not significantly different from the H₂-saturated rate of ³H₂ uptake by the same tissues from either of the Hup⁺ strains (Table II). Thus, it appears that at high rates of electron flux, the only difference between Hup⁺ and Hup⁻ symbioses was the ability of the former to recycle H₂ through the Hup. If this is so, then the slopes of the H₂ evolution versus acetylene reduction relationships on this linear portion of the curves should represent the proportion of electrons allocated to H₂ production in any symbiotic combination. While some of the curves presented in Figure 3 appear to have different slopes, a comparison of the linear regression coefficients showed no significant differences (P > 0.05). Moreover, as was the case with the Hup⁺ symbioses, there is no indication of an increasing slope as nitrogenase activity increased, as would be expected if higher rates of H₂ production could produce concentrations of H₂ within the nodules, of sufficient magnitude to shift the allocation of electrons from N₂ fixation to H₂ production (7, 8).

At lower rates of nitrogenase activity, the H₂ evolution versus acetylene reduction curves for Hup⁺ and Hup⁻ symbioses converged. The curvature of the Hup⁺ relationships could have been caused by increased H₂ production in response to an elevated H₂ concentration in the nodules, if the diffusive resistance of the nodules increased in response to carbohydrate starvation (16). However, unless the resistance change was specific to the Hup⁺ symbioses, a similar change in electron allocation would be expected in the Hup⁻ combinations. Because no such changes were seen in these experiments, it seems more likely that the decreasing difference in H₂ evolution between the Hup⁺ and Hup⁻ symbioses was related to a changing rate of H₂ recycling. Such a change in H₂ uptake activity can be most easily interpreted as a response of the uptake hydrogenase system to H₂ concentration within the nodules. As nitrogenase activity increases from zero, the internal concentration of H₂ would also rise because of its increased rate of production by nitrogenase. The rate of H₂ recycling would increase in response to the increasing substrate concentration until a maximum rate was achieved when the uptake hydrogenase enzyme was saturated with respect to H₂. Beyond this point, further increases in H₂ concentration would have no effect on H₂ recycling and the difference between the curves for Hup⁺ and Hup⁻ symbioses would be constant and equal to the rates estimated by ³H₂ uptake at a saturated level of H₂.

In the experiments reported here, RE values for Hup⁺ P. sativum L.-Rhizobium leguminosarum symbioses were inversely related to the nitrogenase activity, regardless of the treatments used to vary this activity. The plots of H₂ evolution versus acetylene reduction for these Hup⁺ symbioses were curvilinear at low nitrogenase activities, but became linear as nitrogenase activity increased, and it is proposed that this pattern reflects a linear increase in H₂ production combined with a variable rate of Hup activity, dependent on the internal H₂ concentration. Because of this nonproportional relationship, estimates of RE will decrease continuously as nitrogenase activity increases, even on the linear portions of the curves. Thus, comparisons of RE values in different symbiotic combinations must be interpreted with caution, as they may be affected by the growth or assay conditions. In Hup⁺ symbioses, no similar changes in RE occurred as nitrogenase activity varied. H₂ evolution was in all cases proportional to acetylene reduction and the regression coefficients were similar to those of the linear portions of the Hup⁺ curves. If the proposed explanation of RE dependence on nitrogenase activity in Hup⁺ symbioses is correct, then the similar changes in RE with nitrogenase activity have been reported in other Hup⁺ systems, must be mechanistically different.

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