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Title: Properties of the Hydrogenase System in Bacteroids of Soybean Root Nodules

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The hydrogenase system which catalyzes the oxyhydrogen reaction in soybean nodules produced by strains of Rhizobium japonicum is located in the bacteroids. The hydrogenase complex in intact bacteroids has an apparent K_M for H_2 of 2.8 μM and an apparent K_M for O_2 of 1.3 μM. A linear relationship exists between the rate of H_2 uptake and the concentration of bacteroids in the reaction mixture at all bacteroid concentrations below 900 μg of bacteroid protein in each reaction mixture. The addition of H_2 to bacteroids increases O_2 uptake but decreases CO_2 production, indicating a conservation of endogenous substrates. After correction for the effect of H_2 on endogenous respiration, a ratio of 1.9 ± 0.1 for H_2 to O_2 uptake was determined. Bacteroids from greenhouse or field-grown soybeans that evolved H_2 showed no measurable oxyhydrogen reaction activity

whereas consistent activity was demonstrated by bacteroids from soybean nodules that evolved little or no H_2. In addition, some results of preliminary attempts to demonstrate hydrogenase activity in free-living R. japonicum strains are briefly discussed.

Properties of the Hydrogenase System in Bacteroids of Soybean Root Nodules

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PROPERTIES OF THE HYDROGENASE SYSTEM IN BACTEROIDS OF SOYBEAN ROOT NODULES

INTRODUCTION

ATP-dependent H_2 evolution by nitrogenase occurs *in vivo* in many N_2-fixing nodulated symbionts including soybeans (34,35). Until recently the possible importance of H_2 evolution and uptake in the efficiency of N_2 fixation by nodulated N_2-fixing plants was not fully appreciated (14,34). H_2 evolution via the nitrogenase system not only consumes ATP but also reducing power that is needed for the operation of an efficient N_2-fixing process. Some soybean nodules have been shown to have a hydrogenase system which may increase the efficiency of their N_2-fixing process (7,35). Symbionts with an ability to oxidize H_2 via the hydrogenase complex may recapture at least part of the energy that is lost through H_2 evolution and may therefore have a more efficient N_2-fixing process than symbionts lacking a hydrogenase system. The purpose of this thesis is to characterize the hydrogenase system of soybean root nodules in regard to its location and some of its properties.

LITERATURE REVIEW

A. Introduction

A variety of enzymes have been termed hydrogenases in the literature. Clarification of terminology, therefore, provides a logical starting point. Aside from the hydrogenases obviously present in aerobic hydrogen bacteria (reviewed by Schneider and Schlegel (33) in 1977), three types of enzymes catalyzing either the formation of H_2 from $2H^+ + 2e$ or H_2 uptake have been referred to in the literature, at one time or another, as hydrogenases and are important to the consideration of H_2 metabolism in the N_2-fixing process.

One of these types of enzymes is the "classical" reversible hydrogenase common in strict and facultative anaerobic bacteria such as Clostridium species. Gray and Gest (21) have reviewed the information available on H_2 evolution via reversible hydrogenases and concluded that in anaerobes H_2 evolution generally serves the purpose of disposing of excess electrons released in metabolic oxidations.

Nitrogenase is a second type of enzyme referred to as displaying hydrogenase activity through its catalysis of a nonreversible, ATP-dependent H_2 evolution reaction.

Some of the investigations which provided evidence leading to the conclusion that it was the nitrogenase system catalyzing the evolution of H_2 by N_2-fixing organisms are discussed here. In 1957 Hoch, Little, and Burris (23) showed by mass spectrometric methods that excised soybean root nodules evolved H_2 and that this process required O_2. In 1960 Hoch, Schneider, and Burris (24) showed that the evolution of H_2 by excised soybean root nodules was inhibited by N_2 and N_2O. Their evidence supported the view that H_2 was produced via the N_2-fixing system. Investigations by Bergersen (2) and by Dart and Day (8,9) both confirmed that excised soybean root nodules evolve H_2 and that H_2 evolution and N_2 fixation appeared to be catalyzed by closely related enzyme systems. The capacity of cell-free nitrogenase from soybean nodules to catalyze ATP-dependent H_2 evolution was first demonstrated in 1967 by Koch, Evans, and Russell (28).

The third type of hydrogenase is a unidirectional, H_2-oxidizing enzyme associated with Azotobacter (26,37), nodules of Pisum sativum (10) and some other nodulated symbionts (7,34,35), and perhaps N_2-fixing blue-green algae (3,5,30). The hydrogenase system of soybean root nodule bacteroids referred to in this thesis is of this

third type.

B. H_2 Metabolism in N_2-fixing Organisms

Our current understanding of H_2 metabolism in N_2-fixing organisms is the result of many investigations over a long period of time. In 1938 Wilson, Umbreit, and Lee (41) reported that molecular H_2 was a specific inhibitor of N_2 fixation in red clover plants (Trifolium pratense) inoculated with Rhizobium trifolii. Nitrogen fixation in free-living Azotobacter also was reported to be specifically inhibited by H_2 in 1941 by Wyss and Wilson (44). An effort was made by Phelps and Wilson (31) in 1941 to determine the mechanism of this inhibition by testing Rhizobium and Azotobacter cultures for hydrogenase. They found hydrogenase activity in cultures of both free-living Azotobacter vinelandii and Rhizobium leguminosarum strain 311 bacteroids taken directly from nodules of garden peas (Pisum sativum). Cultures of R. leguminosarum strain 311 grown on the usual laboratory media, however, did not show hydrogenase activity. A review of the early evidence for a relationship between H_2 metabolism and N_2 fixation was presented by Wilson and Burris in 1947 (43). Of particular interest during

this time was the finding by Lee and Wilson (29) in 1943 that combined nitrogen not only inhibited N_2 fixation by Azotobacter but also markedly decreased the hydrogenase activity. By 1953 Hyndman, Burris, and Wilson (26) had studied the hydrogenase from Azotobacter vinelandii in cell-free extracts and had reported that these crude cell-free extracts carried out the Knallgas reaction ($H_2 + \frac{1}{2}O_2 \longrightarrow H_2O$) with esterification of inorganic phosphate. While the presence of hydrogenase in Azotobacter was being well-established; Shug, Hamilton, and Wilson (36) stated in 1956 that the experiments showing hydrogenase activity in pea nodules (31) were not reproducible. In these early studies however it was not possible to distinguish between H_2 evolution catalyzed by nitrogenase and hydrogenase activity separate from the nitrogenase system, since they were done prior to the demonstration of cell-free nitrogenase preparations.

The presence of hydrogenase activity in pea root nodules as found by Phelps and Wilson (31) remained unconfirmed until 1967 when Dixon (10) demonstrated H_2 uptake by excised pea root nodules. He had inoculated peas (Pisum sativum cv. Meteor) with R. leguminosarum strain 311, excised the nodules produced, and

by mass spectrometric methods determined that the nodules did not evolve H_2 but took up H_2 when this gas was provided in the gas mixture over them. H_2 was evolved, when deuterium was supplied in the gas mixture over the nodules, at a rate which increased with increasing partial pressures of deuterium. Deuterium uptake occurred at the same time that H_2 was evolved. These and other results led Dixon to postulate the involvement of two separate hydrogenase systems, one which evolved H_2 via the nitrogenase system and one which consumed H_2. The following year Dixon (11) showed that the hydrogenase system involved in H_2 uptake in pea nodules was located in the bacteroids and that H_2 uptake was dependent on O_2 uptake. He concluded that the ratio of H_2 uptake to O_2 uptake in washed bacteroid preparations was two to one. Addition of exogenous carbon substrates inhibited H_2 uptake by the washed bacteroid preparations; and H_2, in turn, inhibited utilization of exogenous carbon substrates. From his observations Dixon suggested that H_2 and carbon substrates may compete for electron carriers in the electron transport pathway and that ATP was synthesized by the passage of electrons resulting from H_2 oxidation. Since succinate inhibited H_2 uptake

more than pyruvate or malate, Dixon concluded that the competition was not for the reduction of pyridine nucleotide but at some later point in the electron transport pathway. In 1972 Dixon (12) compared the hydrogenase from pea root nodule bacteroids with the hydrogenase from Azotobacter and concluded that they were similar in all respects tested. ATP was formed as a result of H_2 oxidation in both cases. Dixon (12) also inoculated three different species of host legumes with R. leguminosarum strain 311 and found that the different legumes produced nodules with different amounts of hydrogenase activity. This indicated that the legume host played a role in the expression of hydrogenase activity. He suggested the following three possible functions for the hydrogenase complex in the N_2 fixation process: (a) protection of the O_2 sensitive nitrogenase by utilization of O_2 within the bacteroids; (b) prevention of H_2 inhibition of nitrogenase through the H_2 uptake mechanism; and (c) recovery of part of the energy lost by H_2 evolution through ATP formation associated with H_2 oxidation. The third possible function, which seemed the most likely to Dixon, provided a mechanism for energy conservation by H_2 recycling which could increase the

efficiency of the N_2-fixing process. Later (13) Dixon noted that the oxidation of the H_2 evolved via nitrogenase in pea root nodule bacteroids might have a sparing effect on carbon substrates metabolized since both processes produced the ATP needed by the bacteroids. If nitrogenase activity were limited by carbon substrate supply, then this sparing effect of H_2 oxidation could be significant in the overall efficiency of N_2 fixation.

C. Losses of H_2 *in vivo*

In 1975 it was generally considered that H_2 evolution by nitrogenase under *in vivo* conditions was negligible (4). The significance of Dixon's work with the hydrogenase system in pea root nodule bacteroids therefore was unclear. Research was required to determine the generality of *in vivo* ATP-dependent H_2 evolution by the nitrogenase system in nodulated N_2-fixing plants and the influence of this H_2 evolution on the overall efficiency of the N_2-fixing process *in vivo*.

Results of an initial survey of a large number of nodulated legumes and non-legumes for energy losses from nodules due to H_2 evolution were reported by Schubert and Evans in 1976 (34). Their methods for

estimating energy loss from nodules by H_2 evolution and relative efficiency of energy utilization during N_2 fixation are summarized in these equations:

$$\text{Relative efficiency} = \frac{\text{rate of acetylene reduction} - \text{rate of } H_2 \text{ evolution in air}}{\text{rate of acetylene reduction}}$$

or,

$$\text{Relative efficiency} = 1 - \frac{\text{rate of } H_2 \text{ evolution in air}}{\text{rate of acetylene reduction.}}$$

First, samples of excised nodules were examined amperometrically to determine their rates of H_2 evolution in air. Then acetylene reduction rates were measured by gas chromatography using the same nodule samples. Under saturating amounts of acetylene, the total electron flux through the nitrogenase system may be estimated by the rate of acetylene reduction. (The rate of H_2 evolution under argon and O_2 may also be used to estimate the total electron flux through the nitrogenase system, if the nodules being tested are known to lack a hydrogenase.) The estimate of relative efficiency is thus the decimal fraction of the total electron flux through nitrogenase that is actually used in N_2 reduction. This estimate is based on several assumptions (16) and is considered as a convenient index of relative efficiency. Results of the initial

survey showed that the relative efficiencies of nodules from most legumes ranged between 0.52 and 0.70 (16,18). Nodules from cowpeas inoculated with Rhizobium strain 32H1 were a notable exception among the first legumes examined in that they consistently showed a relative efficiency near unity. Also approaching unity were the relative efficiencies of three of the four species of non-legumes collected from their native Oregon habitats (viz., Alnus rubra, Purshia tridentata, and Myrica californica). These results indicated that non-agricultural non-leguminous species, because of their failure to evolve H_2, may be more efficient in their use of energy for N_2 fixation than most of the cultivated leguminous species which have been subjected to common agricultural practices, such as nitrogen fertilization.

Further experiments by Schubert and Evans (34,35) have shown that nodules from either legumes or non-legumes which fail to evolve H_2 in air exhibit a capacity for O_2-dependent H_2 uptake. A preliminary study of the influence of soybean cultivars and Rhizobium japonicum strains on the relative efficiencies of energy utilization during N_2 fixation was presented at the Salamanca Conference (35) by Schubert and Evans in 1976.

The study involved two soybean cultivars, Anoka and Amsoy, inoculated with four different R. japonicum strains, USDA 31, USDA 110, USDA 117, and USDA 138. The relative efficiencies averaged 0.7, except for R. japonicum strain USDA 110 which showed high relative efficiencies on both cultivars. The values were 0.91 and one for cultivars Amsoy and Anoka, respectively (16).

A more thorough study has now been conducted with a series of soybean cultivars and R. japonicum strains to determine the most efficient H_2-recycling combinations (7). From these experiments, Carter et al. concluded that the capacity to recycle H_2 evolved by the nitrogenase system in soybean nodules appears to be determined by the R. japonicum strain. Nodules on soybeans formed by strains USDA 110, USDA 122, USDA 136, 3I1b 6, 3I1b 142, and 3I1b 143 lost little or no H_2 in air and showed a capacity to take up this gas when it was placed over them. In the same experiments soybeans inoculated with strains USDA 16, USDA 31, USDA 117, USDA 120, USDA 135 and 23 other strains produced nodules that lost H_2 in air at rates that averaged approximately 36 percent of the electron flow through the nitrogenase system.

Both intact nodules and bacteroids from Austrian

winter peas inoculated with various strains of R. leguminosarum have been tested for hydrogenase activity by Ruiz-Argüeso, Hanus, and Evans in 1977 (32). Some of the strains showed hydrogenase activity in intact nodules and in bacteroids, but at levels of activity that were too low to recycle all of the H_2 evolved by the nitrogenase system.

D. Importance of the Hydrogenase System

The significance of these findings in agriculturally-important N_2-fixing plants is that it may be possible to select N_2-fixing symbionts that most efficiently utilize the energy provided by photosynthesis for N_2 fixation (34). If the supply of photosynthate is limiting, as indicated by several observations (17), then conservation of the energy lost through H_2 evolution could increase the N_2 fixed (14,34). If photosynthate supply is not limiting, then the decreased energy consumption resulting from hydrogenase system recycling of the H_2 evolved via nitrogenase could cause an increase in dry matter yield (14,34). It is calculated that H_2 evolution via nitrogenase requires about 25 percent of the 28 ATP equivalents needed for N_2 reduction (19). Based on several assumptions, H_2

recycling of this evolved H_2 via the hydrogenase system of nodules might yield a 33 percent recovery of this lost energy (16).

Fixed nitrogen is the major nutrient limiting plant growth (15). Because of the enormous nonrenewable energy input required for commercial synthesis of nitrogen fertilizers, such as its yearly use of 2.5 percent of the United States' annual natural gas consumption (17), biological N_2 fixation represents an increasingly important source of fixed nitrogen since it is not dependent on nonrenewable energy resources (15). The occurrence of a hydrogenase system in nodulated symbionts may provide a means of increasing the efficiency of N_2 fixation in living plants. Soybeans account for almost one-half of the world production of approximately 115×10^6 tons of grain legumes (22) and should therefore receive major attention with regard to increasing their N_2-fixing capabilities. Some soybean nodules have been shown to have a hydrogenase system which may increase the efficiency of their N_2-fixing process (7,35). This hydrogenase system requires characterization. Where is the hydrogenase system of soybean nodules located? What are some of its properties? These questions are the subject of this thesis.

MATERIALS AND METHODS

A. Free-living Rhizobium japonicum

Two methods of assaying for hydrogenase activity in free-living, non-N_2-fixing cultures of Rhizobium japonicum strains were employed. These included: (a) spectrophotometric measurement of the H_2-dependent rate of reduction of methylene blue based on the Modified Thunberg Method developed by Wilson, Burris, and Coffee (42); and (b) the amperometric measurement of H_2 uptake rate using the method of Wang, Healey, and Myers (39).

Three variations of the Modified Thunberg Method were used. A summary of the methodology of these variations is given in Table 1. All three of these included the following procedures. Firstly, each tube in which the progress of the reaction was followed spectrophotometrically contained one ml of sterile 0.00005 M methylene blue, three ml of sterile M/15 phosphate buffer (pH 7.5), five ml of distilled water, and one ml of bacterial suspension (approximate optical densities, as measured at 660 nm, of suspensions used in the experiments reported were: variation A = 1.1; variation B = 0.41; and variation C prior to concentration

Table 1. Summary of Methods Used in Attempts to Demonstrate Hydrogenase Activity in Free-living Rhizobia by the Modified Thunberg Method (42)

Method referred to as:	Type of tube used	O_2 removed by:	Method of H_2-adaptation
Variation A	Sterile test tube sealed with rubber serum stopper	Flushing ten times with O_2-free N_2	Five days under an initial one percent H_2 by volume
Variation B	Sterile "H-tube"	Addition to one arm of two ml of alkaline pyrogallol (after flushing with N_2 to remove O_2)[a]	24 hr under an initial ten percent H_2 by volume
Variation C	Sterile "H-tube"	Addition to one arm of six ml of 33.3 mM $Na_2S_2O_4$ and 0.167 mM benzyl viologen (after flushing with N_2 to remove O_2)[b]	USDA 31: two hr under an initial ten percent H_2 by volume USDA 110: five hr under an initial ten percent H_2 by volume

Table 1. Footnotes

[a]Two cm^3 of gas were removed from an "H-tube" (1) which had been flushed with O_2-free N_2 for at least five minutes. One ml of saturated pyrogallol solution and one ml of alkaline solution (15 percent K_2CO_3 and ten percent NaOH) (6), both of which were under N_2, were then added separately to one arm of the "H-tube".

[b]One µmole of benzyl viologen in five ml of 0.1 M Tes buffer (pH 7.5) and a filter paper "wick" to increase surface area were placed in one arm of an "H-tube" prior to flushing with O_2-free N_2 for ten minutes. After flushing, one ml of freshly-prepared, thoroughly N_2-sparged 0.2 M $Na_2S_2O_4$ in 0.1 M Tes buffer (pH 7.5) was added to the same arm of the "H-tube".

of the bacterial cultures = 0.26 for strain USDA 31 and 0.39 for strain USDA 110). Secondly, the optical density readings of the tubes, which were held near 35^{0}C in a constant-temperature water bath, were measured with a Bausch and Lomb 340 colorimeter at a wavelength of 660 nm. Thirdly, an effort was made to remove all traces of O_2 from the tube. The three variations mentioned involved different methods for O_2 removal. In the simplest method (variation A), Thunberg tubes were replaced by sterile test tubes which were filled with all components except the bacterial suspension; and then each tube was sealed with a sterile rubber serum stopper. At least ten cycles of alternate evacuation and flushing with O_2-free N_2 were used to free each tube of O_2 prior to the addition of the bacterial suspension and the initiation of the assay. By use of a manifold, several tubes were evacuated and flushed at the same time. Variations B and C employed sterile "H-tubes" each of which consists of two approximately 30 ml test tubes joined together by male and female ground glass fittings which are held together by use of a clamp (1). The assay mixture was placed in one arm of each assembly and the other arm was utilized for the addition of an O_2-removing system.

In variation B, O_2 was removed, after flushing each "H-tube" with O_2-free N_2 for five minutes, by the addition of two ml of alkaline pyrogallol (6). In variation C, O_2 was removed, after flushing with O_2-free N_2 for ten minutes, by the addition of six ml of a solution containing 33.3 mM $Na_2S_2O_4$ and 0.167 mM benzyl viologen. Assays were performed under either one atm H_2 or one atm N_2 in variations A and B. In variation C, either 0.1 atm H_2 and 0.9 atm N_2 or one atm N_2 was added for the assay.

R. japonicum strains were provided by Dr. George Ham of the University of Minnesota. Bacterial suspensions used in assays involving the Modified Thunberg Method were 5 to 12 day-old R. japonicum cultures grown in yeast extract mannitol broth (38) on a shaker at 30^0C. Optical densities of cultures ranged from 0.26 to 1.1. All cultures were H_2-adapted as specified in Table 1. In variation A, a yeast extract mannitol broth culture of R. japonicum strain USDA 110 was adapted to H_2 by addition of an initial one percent H_2 by volume to the culture for a period of five days. In variation B, ten percent H_2 by volume was added to the broth culture and allowed to remain for 24 hours. In variation C of the method, cells were collected aseptically from

broth cultures by centrifugation and resuspended in 10 to 15 percent of the original volume of broth medium. Then the resuspended cells were incubated under an initial ten percent H_2 by volume for the periods of time specified in Table 1 (i.e., two or five hours).

Bacterial suspensions used in the amperometric hydrogenase assay were made from 12 to 16 day-old R. japonicum cultures grown on 25 ml of yeast extract mannitol agar which was allowed to solidify on one side of a 250 ml prescription bottle. Incubation was at 24^0C. The suspensions of cells were made by adding to each culture bottle about 15 sterile glass beads and 12 to 15 ml of sterile yeast extract mannitol broth. Each bottle was then sealed by use of a sterile rubber serum stopper. The procedure followed was an adaptation of a method used by Wilson (40). The bottles were then made micro-aerophilic by flushing them with a one percent H_2:99 percent argon gas mixture, followed by the addition of approximately 0.1 percent O_2 by volume to each bottle. Cultures were then allowed to adapt to H_2 for periods of 4 or 22 hours. Just prior to assay by the amperometric method, the beads in each bottle were agitated gently in order to loosen the

bacteria from the agar surface. The very dense suspension of cells from each bottle was removed for assay by use of a hypodermic syringe. This prevented exposure of the cells to O_2 during the transfer.

B. Bacteroids Prepared from Soybean Root Nodules

R. japonicum strains and soybean seeds were obtained from Drs. George Ham and Deane Weber. In greenhouse experiments where different strains were used, seeds of Glycine max (cultivar Anoka) were surface disinfected (38) and germinated on water agar plates. Young seedlings were inoculated with seven to ten day-old yeast extract mannitol broth cultures of R. japonicum such that each seedling received at least 10^3 bacteria (38). The inoculated seedlings were then planted in a sand-vermiculite mixture in modified Leonard jars (38). A nitrogen-free nutrient solution (27) was provided to the plants, which were kept in a greenhouse near 27^0C during the day and 21^0C at night. Supplemental light equivalent to 5380 Lux was provided during a 16-hour light period. Uninoculated soybean controls in Leonard jar assemblies did not form nodules.

Nodules were collected from a field experiment which was conducted to determine the relationship

between activity of the hydrogenase system in nodules and yield of soybeans. In this experiment surface disinfected soybean seeds (cultivar Portage) were treated with a series of inocula prepared from sterile peat and pure cultures of R. japonicum strains. Of the eight strains, four were hydrogenase-positive and four were hydrogenase-negative (7,35). Sufficient inoculum was added to insure that each seed received at least 10^3 bacteria (38). Uninoculated control plots were included, and these showed only sparse nodulation.

Bacteroids were prepared from 27 to 36 day-old greenhouse-grown plants as described by Dixon (11), except that anaerobic conditions were not maintained during the preparation procedure. No difference was found in hydrogenase activity of aerobically and anaerobically prepared bacteroids; however, the high respiratory rate of the concentrated bacteroids maintained O_2 at a very low level. Bacteroids prepared in this way exhibited no nitrogenase activity. Heat-treatment of bacteroids at 80^0C for 15 minutes under anaerobic conditions destroyed hydrogenase activity. Protein contents of the washed bacteroid suspensions were determined as described by Goa (20).

H_2 uptake rates by bacteroid suspensions were

measured amperometrically using the method of Wang, Healey, and Myers (39). O_2 uptake was measured by a Clark type O_2 probe purchased from Yellow Springs Instrument Company, Yellow Springs, Ohio. CO_2 evolution by bacteroid suspensions was determined with a Carle 8500 gas chromatograph using a thermal conductivity detector. The 3.2 mm x 45.7 cm column was packed with Porapak Q (Waters Associates, Inc., Framington, Massachusetts). Helium was used as the carrier gas at a flow rate of 17 ml per minute. The column temperature was $72^{0}C$.

RESULTS AND DISCUSSION

A. Free-living Rhizobium japonicum

Variations of the Modified Thunberg Method

As indicated in Table 2, only the simplest variation, variation A, showed any really significant difference in the rates of reduction of methylene blue under N_2 and H_2 by free-living, non-N_2-fixing R. japonicum strain USDA 110. When an O_2-removing system such as alkaline pyrogallol (variation B) or $Na_2S_2O_4$ with benzyl viologen (variation C) was included in one arm of an "H-tube" with the assay mixture to prevent O_2 interference with the measurement of the rate of reduction of methylene blue, the difference between the rates of methylene blue reduction under N_2 and H_2 decreased markedly. This effect may have been due to the diffusion of gaseous products of the O_2-removing reactions into the atmosphere above the assay mixture, with resultant inhibition of the activity of any hydrogenase present.

Amperometric Measurement of H_2 Uptake Rates

Using the amperometric method for measuring H_2

Table 2. Relative Rates of Methylene Blue Reduction by Free-living Strains of *Rhizobium japonicum* under N_2 or H_2 as Measured by Variations of the Modified Thunberg Method

Variation[a]	A		B		C			
Strain	USDA 110		USDA 110		USDA 31		USDA 110	
Gas present	N_2	H_2	N_2	H_2	N_2	H_2	N_2	H_2
Time elapsed, hr	O.D.[b]	O.D.	O.D.	O.D.	O.D.	O.D.	O.D.	O.D.
0	0.63	0.66	0.70	0.67	1.8	1.8	1.8	1.8
0.5	0.62	0.63	0.69	0.66	1.5	1.5	1.6	1.5
1.0	0.62	0.55	0.68	0.64	1.4	1.4	1.2	1.2
1.5	0.62	0.49	0.68	0.64	1.1	1.1	1.0	1.0
Rate of methylene blue reduction, O.D./hr[c]	0.01	0.11	0.01	0.02	0.47	0.47	0.53	0.53

[a] See Table 1 for these variations.

[b] O.D. means optical density of the assay mixture.

[c] Rate of reduction determined during a 1.5 hr incubation.

uptake rates, no H_2 uptake was observed for R. japonicum strain USDA 31. R. japonicum strain USDA 110 also generally exhibited no H_2 uptake unless cells of this strain were diluted with argon-saturated yeast extract mannitol broth just prior to the amperometric assay. When one to one or one to two dilutions, by volume, of USDA 110 bacterial suspension to fresh argon-saturated yeast extract mannitol broth were prepared just before an assay, these USDA 110 dilutions exhibited H_2 uptake rates ranging from 0.18 to 0.50 nmoles of H_2 consumed per minute. These low rates were measured in the presence of very low initial concentrations of H_2 ranging from two to 3.7 μM. Some data indicated that increasing amounts of O_2 tended to decrease the H_2 uptake rate. This observation might account for the apparent requirement of fresh yeast extract mannitol broth for H_2 uptake activity by strain USDA 110 if it is assumed that this fresh medium supplied readily available oxidizable substrates which allowed the organism to rapidly utilize O_2 which otherwise might have destroyed hydrogenase activity.

Consistent and clear-cut results could not be readily obtained by use of free-living R. japonicum strain USDA 110. It was decided therefore to test

R. japonicum bacteroids from soybean root nodules for the presence of the hydrogenase system.

B. Bacteroids Prepared from Soybean Root Nodules

Characterization of the Hydrogenase System

Initial evidence of activity of the hydrogenase system in nodules of Anoka soybeans inoculated with R. japonicum strain USDA 110 was shown by O_2-dependent H_2 uptake by intact nodules (35). Experiments performed by Dr. Dave Emerich and other workers in the laboratory failed to detect hydrogenase activity in the cytosol of nodules formed by R. japonicum strains whose intact nodules exhibited activity of the hydrogenase system.[1] Bacteroids prepared from nodules formed by USDA 110 consistently exhibited activity of the hydrogenase system. The effect of H_2 concentration on the rate of H_2 uptake at an initial O_2 concentration of 22.1 μM is presented in Figure 1. H_2 saturation of the bacteroid hydrogenase system from nodules formed by strain USDA 110 occurred near 30 μM. The apparent K_M for H_2 as determined from a linear regression analysis of an

[1] Personal communication

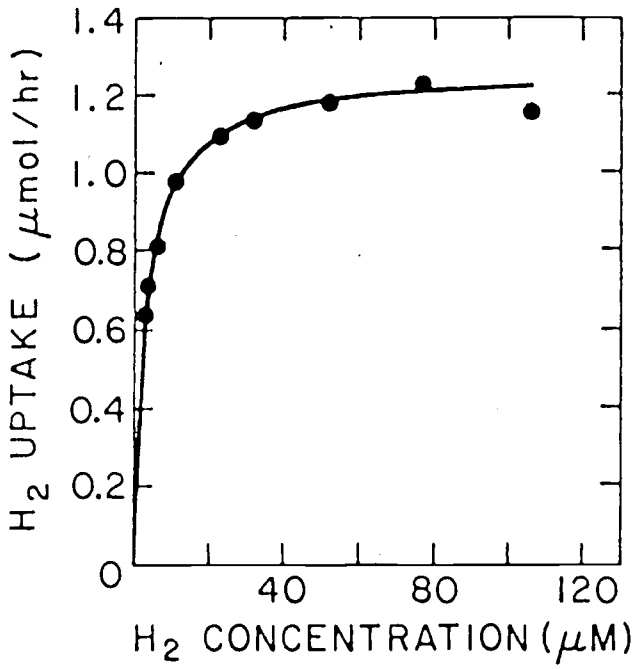

Figure 1. H_2 uptake by bacteroids of soybean nodules as a function of H_2 concentration. The bacteroid suspension, prepared from nodules formed by R. japonicum strain USDA 110, contained 111 µg protein per ml in a reaction volume of 2.8 ml at 23°C. The initial concentration of O_2 in each assay was 22.1 µM.

Eadie-Hofstee plot (25) of the data in Figure 1 was approximately 2.8 μM. At saturating concentrations of H_2, 10 μM O_2 or greater concentrations resulted in maximum rates of H_2 uptake (Figure 2). The apparent K_M for O_2 was approximately 1.3 μM. Methylene blue also functioned as an acceptor; however, under the same conditions 5.4 mM methylene blue produced a rate of H_2 uptake that was only 5.6 percent of the rate with 44 μM O_2. The ineffectiveness of methylene blue and some other acceptors with freshly prepared bacteroids may be associated with problems of the permeability of cells to these compounds and further work is in progress by other workers in the laboratory to clarify these matters. Nodules formed by R. japonicum strain USDA 31 lost H_2 at rapid rates and showed no capacity for H_2 uptake (35). Bacteroid preparations from nodules formed by USDA 31 also exhibited no activity of the hydrogenase system. Mixtures of bacteroids from nodules formed by strain USDA 31 with bacteroids formed by USDA 110 caused no inhibition of the activity of the USDA 110 hydrogenase system. This observation provided no evidence for the presence of an inhibitor of the hydrogenase system in the bacteroids formed by strain USDA 31.

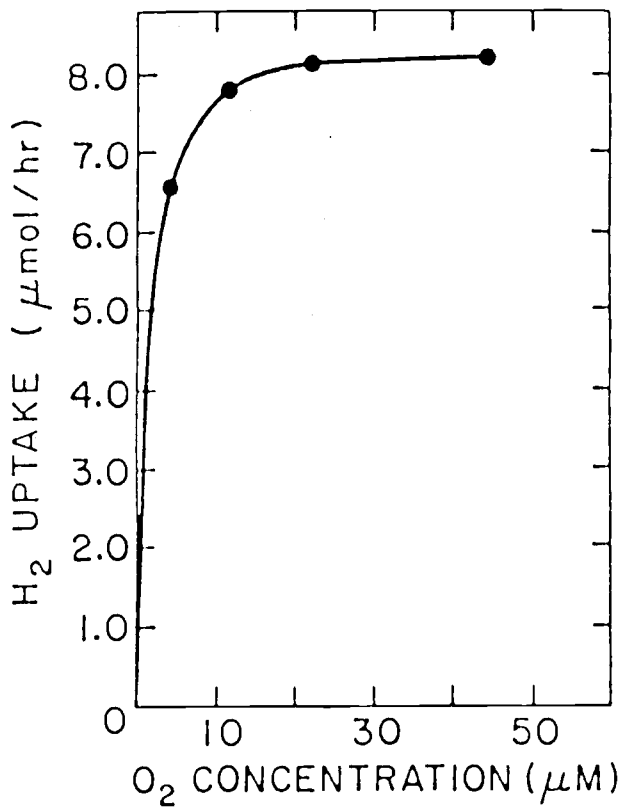

Figure 2. H_2 uptake by bacteroids of soybean nodules as a function of O_2 concentration. The bacteroid suspension, prepared from nodules formed by R. japonicum strain USDA 110, contained 555 µg protein per ml in a reaction volume of 2.8 ml at 23°C. The initial concentration of H_2 in each assay was 52.1 µM.

In reactions containing less than 2.2 ml of bacteroid suspensions and saturating amounts of H_2 and O_2, a linear relationship exists between the rate of H_2 uptake and the concentration of bacteroids in the reaction mixture (Figure 3). The nonlinearity at bacteroid concentrations greater than 2.2 ml may be due to incomplete mixing of the more viscous bacteroid suspensions.

By use of bacteroids formed by USDA 110, measurements were made of the concomitant H_2 and O_2 uptake rates in an attempt to define the stoichiometry of the nodule bacteroid hydrogenase system. Table 3 shows the results obtained from three replicate determinations. At first the endogenous O_2 uptake rate in the absence of H_2 was subtracted from the O_2 uptake rate with H_2 present in an effort to determine the H_2-dependent O_2 uptake rate. This calculation amounted to a correction of the total O_2 uptake rate in the presence of H_2 for that part of the rate that presumably was due to a constant rate of endogenous respiration. This type of calculation, however, did not yield the expected two to one ratio of H_2 to O_2 uptake. The possibility that H_2 might inhibit endogenous respiration was tested by gas chromatographic measurement of CO_2 evolution rates

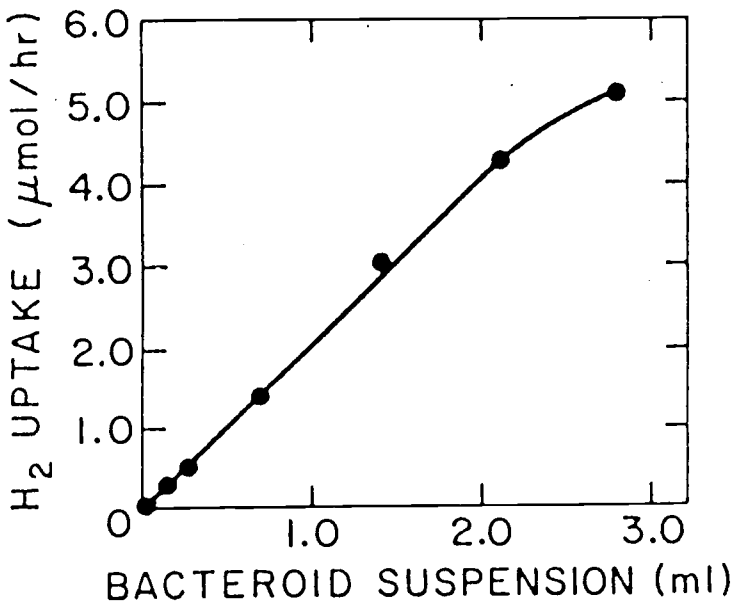

Figure 3. Proportionality between H_2 uptake and volume of bacteroid suspension (450 µg protein per ml) added to a reaction volume of 2.8 ml at 23°C. Bacteroids were obtained from nodules formed by R. japonicum strain USDA 110. The initial H_2 and O_2 concentrations in each assay were 39.1 µM and 22.1 µM, respectively.

Table 3. The Stoichiometry of the Hydrogenase Reaction in Bacteroids from Nodules of Soybeans Inoculated with Strain USDA 110 of *R. japonicum*[a]

Expt.	H_2 uptake	O_2 uptake with H_2	Endogenous O_2 uptake without H_2	Calculated endogenous O_2 uptake in presence of H_2[b]	H_2 dependent O_2 uptake	$\frac{H_2 \text{ uptake rate}}{H_2 \text{ dependent } O_2 \text{ uptake rate}}$[c]
	(nmoles per minute)					(ratio)
1	19.7	13.4	8.5	3.4	10.0	2.0
2	18.6	12.5	6.9	2.8	9.7	1.9
3	19.5	13.5	6.5	2.6	10.9	1.8

Table 3. Footnotes

[a]The reaction chamber contained 2.8 ml of bacteroid suspension (0.13 mg protein per ml). Initial concentrations of H_2 and O_2 for measurement of H_2 uptake were 52.1 μM and 22.1 μM, respectively. For measurements of O_2 uptake rates in the absence of H_2, 44.2 μM O_2 was initially present. The figures given represent three replicate determinations. The mean ratio of H_2 to O_2 uptake is 1.9 ± 0.1 (standard error of the mean).

[b]This number was obtained by multiplying the endogenous O_2 uptake rate without H_2 present by 0.40, the fraction of the endogenous respiration rate remaining in the presence of hydrogenase-saturating concentrations of H_2 (see Table 4).

[c]The ratio of H_2 to O_2 taken up by the bacteroids was calculated by dividing H_2 uptake by H_2-dependent O_2 uptake.

by soybean root nodule bacteroids in the presence of 70 μM H_2 and in the absence of H_2 (Table 4). From these experiments it was established that the rate of CO_2 evolution in the presence of 70 μM H_2 was only 40 percent of the rate of CO_2 evolution in the absence of H_2. This indicates that not only does H_2 inhibit the oxidation of exogenous substrates as Dixon has shown with pea root nodule bacteroids (11,13), but also that added H_2 acts to conserve endogenous substrates in intact bacteroids. As shown in Table 3, the H_2-dependent O_2 uptake rate, which was obtained by subtraction of the corrected endogenous respiration rate in the presence of H_2, was approximately 50 percent of the rate of H_2 uptake. The ratio of rates of H_2 uptake to H_2-dependent O_2 uptake was 1.9 ± 0.1 (standard error of the mean). It is clear therefore that the bacteroid hydrogenase system catalyzes the oxyhydrogen reaction.

Activities of the Bacteroid Hydrogenase System in Relation to Strains

Nodules were collected from field and greenhouse experiments in which strains that produce nodules showing H_2 loss were compared with strains that form

Table 4. CO_2 Evolution by Bacteroids from Nodules of Soybeans Inoculated with Strain USDA 110 of R. japonicum in the Presence and Absence of H_2[a]

Expt.	Description	Gases added: H_2	Gases added: O_2	CO_2 in incubation mixture[b] (nmoles per reaction ± SEM)
1	Control	-	-	57.8 ± 8.6[c,d]
2	With H_2	+	+	135.7 ± 9.4
3	Without H_2	-	+	254.6 ± 4.1

[a]Inhibition of endogenous respiration by H_2 was determined by gas chromatographic measurement of CO_2 evolution by bacteroids in the presence and absence of H_2. In experiments one, two, and three, each 21 ml serum bottle contained five ml of bacteroid suspension (0.13 mg protein per ml). Initial concentrations of H_2 and O_2 (when added) in the N_2 atmosphere above the bacteroids were 0.10 and 0.05 atm, respectively.

[b]Experiment one, the control, measured CO_2 evolved from the incubation mixture at zero time. Experiments two and three were analyzed for CO_2 after 3.5 hr of incubation at 23°C on a shaker. In all three experiments, 0.5 ml of 0.6 N hydrochloric acid (an amount found experimentally to bring five ml of the bacteroid suspension to a pH of 1.8) was injected into each incubation mixture to release CO_2 from solution at the time of analysis.

Table 4. Footnotes (continued)

[c]All data are means of three replicate determinations plus or minus the standard error of the mean (SEM).

[d]Calculation of the percent inhibition of endogenous respiration by 70 μM H_2 was done as follows:

	With H_2 (nmoles per reaction)	Without H_2 (nmoles per reaction)
Mean of respiratory CO_2 evolved after 3.5 hr	135.7	254.6
Mean of CO_2 present in controls	57.8	57.8
Endogenous respiratory CO_2 evolution	77.9	196.8.

The percentage of the endogenous respiration rate of the bacteroids which remains in the presence of hydrogenase-saturating concentrations of H_2 is 77.9/196.8 x 100 percent = 40 percent of the endogenous respiration rate in the absence of H_2. This represents a 60 percent inhibition of the endogenous respiration rate by 70 μM H_2.

nodules that lose little or no H_2 (7). No measurable rate of H_2 uptake could be detected in bacteroids from nodules that lost H_2, whereas all bacteroid preparations from nodules that evolved little or no H_2 actively took up H_2 (Table 5). No activity was observed when O_2 was omitted from the reaction chamber. These results are consistent with those reported previously for intact nodules (7,35) and provide strong evidence that the hydrogenase complex, in bacteroids of nodules that do not evolve H_2, participates in an H_2 recycling process both in greenhouse-grown and in field-grown plants.

Table 5. Activity of the Hydrogenase System in Bacteroids from Nodules of Soybeans Inoculated with Different Strains of *R. japonicum*[a]

Strains	H_2 loss from nodules[b]	H_2 uptake ($\frac{\mu mol}{hr}$ x mg protein)
Field-grown plants		
USDA 16	+	<0.002
USDA 117	+	<0.002
USDA 120	+	<0.002
USDA 135	+	<0.002
USDA 110	-	8.770
USDA 122 (CB 1809)	-	3.730
3I1b 6	-	9.680
3I1b 143	-	6.134
Greenhouse-grown plants		
USDA 31	+	<0.002
USDA 110	-	4.083

Table 5. Footnotes

[a]The reaction chamber contained 2.8 ml of bacteroid suspension (0.14 to 1.95 mg protein per ml). The initial concentrations of H_2 and O_2 in all cases were greater than 39 μM and 16 μM, respectively. The values for field-grown nodules are the means of determinations on samples from two replicate plots. The values for greenhouse-grown nodules are means of three replicate determinations. Bacteroids were prepared from field-grown soybeans (cultivar Portage) and greenhouse-grown soybeans (cultivar Anoka) as described in Materials and Methods.

[b]Based upon data from Carter *et al.* (7).

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