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Investigation of the H_2 Oxidation System in *Rhizobium japonicum* 122 DES Nodule Bacteroids[1]

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ABSTRACT

The H_2-oxidizing complex in *Rhizobium japonicum* 122 DES bacteroids failed to catalyze, at a measurable rate, $^2H^1H$ exchange from a mixture of $^2H_2$ and $^1H_2$ in presence of ^2H_2O and ^1H_2O, providing no evidence for reversibility of the hydrogenase reaction *in vivo*. In the H_2 oxidation reaction, there was no significant discrimination between $^2H_2$ and $^1H_2$, indicating that the initial H_2-activation step in the over-all H_2 oxidation reaction is not rate-limiting. By use of improved methods, an apparent K_m for H_2 of 0.05 micromolar was determined. The H_2 oxidation reaction in bacteroids was strongly inhibited by cyanide (88% at 0.05 millimolar), theonyltrifluoroacetone, and other metal-complexing agents. Carbonyl cyanide *m*-chlorophenylhydrazone at 0.005 millimolar and 2,4-dinitrophenol at 0.5 millimolar inhibited H_2 oxidation and stimulated O_2 uptake. This and other evidence suggest the involvement of cytochromes and nonheme iron proteins in the pathway of electron transport from H_2 to O_2. Partial pressures of H_2 at 0.03 atmosphere and below had a pronounced inhibitory effect on endogenous respiration by bacteroid suspensions. The inhibition of CO_2 evolution by low partial pressures of H_2 suggests that H_2 utilization may result in conservation of oxidizable substrates and benefits the symbiosis under physiological conditions. Succinate, acetate, and formate at concentrations of 50 millimolar inhibited rates of H_2 uptake by 8, 29, and 25%, respectively. The inhibition by succinate was noncompetitive and that by acetate and formate was uncompetitive. A concentration of 11.6 millimolar CO_2 (initial concentration) in solution inhibited H_2 uptake by bacteroid suspensions by 18%. Further research is necessary to establish the significance of the inhibition of H_2 uptake by succinate, acetate, formate, and CO_2 in the metabolism of the H_2-uptake-positive strains of *Rhizobium*.

During the reduction of N_2 to NH_4^+, a considerable fraction of the electron flow through the nitrogenase complex is utilized in the reduction of protons, resulting in the evolution of H_2 (32). On the basis of electron equivalents transferred, the energy requirement for nitrogenase-dependent H_2 evolution is approximately the same as that for N_2 reduction in legumes. The results of several surveys show H_2 evolution representing a mean loss of 29% of the total electron flow to the nitrogenase reaction (16). The discovery (11-13, 25) of a H_2-oxidizing system in legume nodules and other N_2-fixing organisms (7, 8, 19) has created considerable interest in the H_2-recycling process. Recent evidence supports two of the benefits that Dixon (13) postulated might be derived from possession of the H_2-oxidizing system. Walker and Yates (35) have shown that the H_2-oxidizing system of *Azotobacter chroococcum* provided ATP and electrons for support of nitrogenase activity and demonstrated respiratory protection for the nitrogenase in cells grown under carbon limited conditions. Peterson and Burris (24) and Bothe *et al.* (6-8) have reported that H_2 oxidation supported ATP formation and provided respiratory protection for nitrogenase in blue-green algae. The oxidation of H_2, via the hydrogenase system in *Rhizobium japonicum* 122 DES bacteroids, greatly stimulated nitrogenase activity, increased the steady-state level of cellular ATP, and provided respiratory protection for nitrogenase (14, 29). Schubert *et al.* (31, 33) and Albrecht *et al.* (1) have reported that plants inoculated with H_2-uptake-positive strains of *Rhizobium* produced greater yields of dry matter and accumulated more N in shoots in greenhouse experiments than did plants inoculated with H_2-uptake-negative strains.

Some of the properties of the hydrogenase complexes in the bacteroids of *Rhizobium leguminosarum* and *R. japonicum* (122 DES) have been described by Dixon (12, 13) and Ruiz-Argüeso *et al.* (28), respectively. The membrane-bound hydrogenase from *R. japonicum* (USDA 110) bacteroids was purified by Arp and Burris (3) who reported a mol wt of 65,300 and a K_m for H_2 of 1.4 μM using methylene blue as the acceptor. To understand better the physiological role of the hydrogenase, it must be known whether the K_m for H_2 is sufficiently low to utilize efficiently the H_2 that is produced within the nodule and whether the H_2-activation step in the oxyhydrogen reaction is rate-limiting. Lim (22) and Bethlenfalvay and Phillips (4) claim that the hydrogenase complex catalyzes an exchange reaction, but Dixon (11) reported little or no exchange between $^2H_2$ and $^1H_2$ by the hydrogenase from *R. leguminosarum* bacteroids. Both Dixon (11) and McCrae *et al.* (23) observed that 0.1 atm $^2H_2$ or $^1H_2$ strikingly inhibited respiratory CO_2 evolution; however, nothing is known about the effect of much lower physiological concentrations of H_2 on the conservation of carbon substrates in bacteroids. It is the purpose here to attempt to clarify these and some related questions that seem to be relevant to a better understanding of the $^1H_2$ cycling process in nodules.

MATERIALS AND METHODS

Selection of colony derivatives (*R. japonicum* USDA 122 DES) and preparation of bacteroids have been described elsewhere (28). Bacteroid suspensions were prepared daily from freshly harvested nodules. H_2 and O_2 were determined amperometrically as previously described (14). The specific activity of hydrogenase in the fresh bacteroid preparations ranged between 1.5 and 2.0 μmol/h·

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mg dry weight. In most of the experiments, HMP buffer[4] was used as the buffer. In some experiments, 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing 2.5 mM $MgCl_2$ was utilized. For the chromatographic determination of H_2, a Hewlett-Packard model 5830A gas chromatograph with a thermal conductivity detector was used. The chromatograph was equipped with a 6.4-mm × 2-m column of Molecular Sieve 5-A and operated at a temperature of 120 C. The carrier gas was N_2 used at a flow rate of 40 ml/min. For the measurement of CO_2 evolution, 0.5-ml samples were withdrawn from 22-ml vials, each containing the bacteroid suspension in 50 mM HMP buffer (pH 7.5). The samples were assayed by use of a Carle gas chromatograph equipped with a thermal conductivity detector and a 7.5-m × 6.4-mm column of Porapak Q at a temperature of 75 C. The flow rate was 15 ml He/min. Carbon substrates used in assays were dissolved in buffer and the pH was adjusted prior to use. CO_2 was added as a CO_2-saturated buffer solution to assays in amperometric chambers. Experimentally determined solubilities of H_2, O_2, and CO_2 were approximately the same as the values listed by Umbreit, Burris, and Stauffer (34). From analyses by J. Hanus of this laboratory, the solubility of $^2H_2$ at 22 C and 760 mm Hg is 20.2 ml/H_2O. Formation of $^2H^1H$ was determined by a Varian mass spectrometer MAT model CH7. $^2H^1H$ exchange assays were measured in 10-ml vials containing a bacteroid suspension or *Clostridium pasteurianum* hydrogenase in HMP buffer.

The kinetic data were calculated from amperometric progress curves from which tangents were drawn to determine the rates of H_2 uptake at the indicated concentrations of dissolved H_2. Kinetic experiments were performed in the range where the concentrations of added H_2 in solution was 30 to 120 times the K_m value for H_2 (0.05 μM). Calculations of the K_m from data obtained with H_2 concentrations in the range of 7- to 30-fold the K_m for H_2 produced values that agreed closely with those in the range of 30- to 120-fold the K_m.

H_2, O_2, CO_2, N_2, and argon of the highest purities available were obtained from Airco Industrial Gases, Vancouver, WA. Hepes, potassium phosphates, succinic acid, sodium acetate, and sodium formate and the inhibitors listed in Table II were obtained from Sigma or Aldrich Chemical Co., San Leandro, CA. $^2H_2$ was purchased from Matheson Gas Products, East Rutherford, NJ.

RESULTS AND DISCUSSION

K_m for H_2. The apparent K_m values reported for H_2 for the H_2-oxidizing hydrogenase from *R. japonicum* (28) and *R. leguminosarum* bacteroids (30) have ranged between 2.2 and 4.2 μM. As discussed by Ruiz-Argüeso *et al.* (28), considerable experimental difficulties are encountered in measuring rates of H_2 uptake at low H_2 concentrations; K_m values reported, therefore, are estimations. The sensitivity of the H_2 electrode is not constant over a wide range in H_2 concentrations. Calibration by addition of sufficient dissolved H_2 in buffer to obtain final concentrations from 2 to 40 μM H_2 produced a nonlinear standard curve that was used to measure more accurately the rate of H_2 uptake at desired H_2 concentrations. The change in sensitivity at different H_2 concentrations appears to be a property of the electrode and not the result of electronic aberrations. The sensitivity of the electrode varied from day to day and also varied during the course of daily runs. Calibration at a series of different concentrations was performed before each assay to ensure more accurate measurements.

By use of the improved calibration method, K_m values of approximately 0.05 μM (Fig. 1) were obtained. When measurements based upon a calibration with a single high concentration of H_2 (26 μM) were made, K_m values near 2.0 μM were obtained.

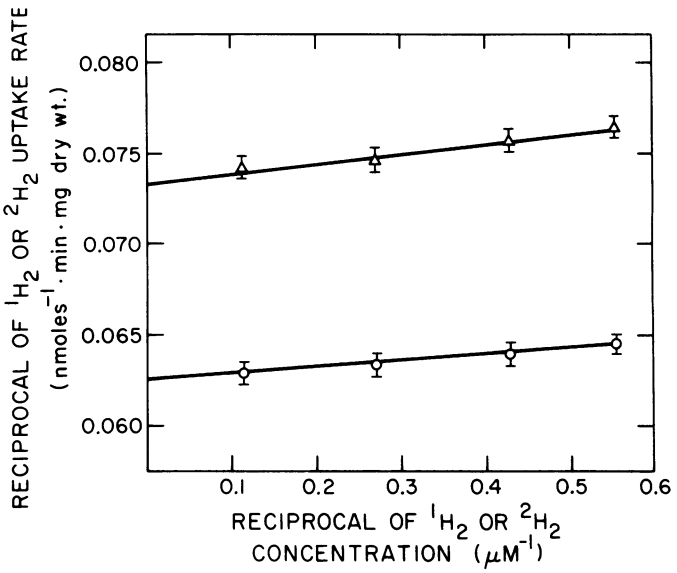

FIG. 1. Isotope discrimination between $^2H_2$ and $^1H_2$ by *R. japonicum* 122 DES bacteroids. Bacteroids were prepared from fresh nodules as described previously (14). The equivalent of 0.67 mg dry weight of bacteroids was added to the amperometric assay chamber (2.8 ml) containing HMP buffer to initiate the reaction. The initial concentration of dissolved gas was typically: O_2, 260 μM; $^1H_2$, 26 μM; and $^2H_2$, 28 μM. Calibration of the electrode and determination of the rates of $^1H_2$ or $^2H_2$ uptake were performed as described. The lines were drawn by linear regression analyses and the values reported are means of five determinations (± SE). (○——○), $^1H_2$; (△——△), $^2H_2$.

Even higher K_m values were observed when H_2 was determined by the relatively insensitive gas chromatographic method. As discussed by Cleland (10), the K_m determinations should be carried out at substrate concentrations ranging from 0.2 to 5 times the K_m. Due to the insensitivity of the method, it was not possible here to adhere to Cleland's recommendation. The most reliable values obtained here, as judged from the least variation in the replicate measurements, were obtained using H_2 concentrations which ranged from 30 to 120 times the K_m. It is believed that the value of 0.05 μM represents a more accurate estimate of the apparent K_m for H_2 under *in vivo* conditions.

Brocklehurst and Cornish-Bowden (9) have argued that, to maximize the rate of product formation for an apparently irreversible enzyme reaction, the substrate concentration should be about 10% of the K_m. Since the concentration of dissolved H_2 within the nodule is not known and the estimated value of the K_m is limited by the method of measurement, the results cannot be evaluated by the approach of Brocklehurst and Cornish-Bowden (9). The system can be considered to be physiologically efficient because little or no H_2 ordinarily is evolved from nodules containing bacteroids possessing the H_2-oxidizing system.

Isotope Exchange and Discrimination. The membrane-bound H_2 oxidation system in *R. japonicum* 122 DES bacteroids, which contains cytochromes as part of the electron transport chain (unpublished data), provides the cells with a mechanism for H_2-supported ATP synthesis (14). The increase in H_2 uptake activity resulting from the addition of certain high-potential electron acceptors to the H_2 oxidation complex suggests, but does not prove, that the H_2 activation step *per se* is not rate-limiting (28). As illustrated in the reciprocal plots in Figure 1, the rate of $^2H_2$ uptake was 87% the rate of $^1H_2$ uptake (13% inhibition). The isotope effects on V_{max} and V/K are 1.17 and 1.51, respectively. These results indicate that the initial H_2 activation step is not rate-limiting. If it were rate-limiting, the substitution of $^2H_2$ for $^1H_2$ would normally be expected to result in a 50% or greater inhibition

[4] Abbreviation: HMP buffer, 50 mM Hepes, 2.5 mM $MgCl_2$, 1.0 mM K_2PO_4 (pH 7.5).

(21).

Substitution of $^2H_2$ also increased the K_m approximately 30% (Fig. 1). Kleiner and Burris (20) and Erbes and Burris (15) reported that the substitution of $^2H_2$ for $^1H_2$ in a reaction containing the reversible hydrogenase from *C. pasteurianum* had no significant effect on the maximum velocity at pH 7.0. The K_m for $^2H_2$, however, was only 63% of that for $^1H_2$. The effect of $^2H_2$ on the K_m probably is due to the difference in polarizability of the bonds in ^2H—^2H and ^1H—^1H(21).

An experiment (Table I) was conducted to determine whether $^2H^1H$ was formed in the gas phase during the oxidation of a mixture of $^2H_2$ and $^1H_2$ by the bacteroids. ^2H_1H formation was measured as atom per cent excess ^2H in $^2H^1H$. Positive results from an experiment of this type would demonstrate exchange activity, whereas incorporation of $^2H_2$ or $^3H_2$ from the gas phase into the liquid phase would not distinguish between exchange and $^2H_2$ or $^3H_2$ oxidation. Since the rate of isotope incorporation due to the reaction of $^2H_2$ with $^1/_2O_2$ to yield ^2H_2O usually greatly exceeds the rate of catalytic isotope exchange in the reaction of $^2H_2$ and $^1H_2$ to yield 2 $^2H^1H$(21), care must be exercised to ensure that the appropriate parameter, exchange or incorporation, is being measured.

Repeated experiments with intact *R. japonicum* bacteroids 122 DES has provided no evidence of a significant rate of true exchange (Table I). Formation of $^2H^1H$ could occur only in a unidirectional H_2-oxidizing system if either the $^1H_2$ or the $^2H_2$ bond were broken before the irreversible step in the oxidation pathway, allowing the reaction intermediates to interact with neighboring $^1H_2$ or $^2H_2$ molecules. This would result in a back-reaction with the exchange of atom partners as follows: 2[$^1H^*$]-reacting with $^2H_2$ to yield 2[$^2H^2H$] where H* represents an activated state. In the experiment in Table I, the reversible hydrogenase from *C. pasteurianum* was included as a positive control. In Table I, the H_2-oxidizing system of *R. japonicum* bacteroids was compared with the purified, reversible hydrogenase from *C. pasteurianum*. The purified enzyme from *Clostridium pasteurianum* was used as a control in these experiments because its reactivity has been characterized (15, 20) and because the H_2 metabolism of whole cells of *C. pasteurianum* is complex, involving a reversible hydrogenase and an H_2-oxidizing hydrogenase in addition to nitrogenase-dependent H_2 evolution. The bacteroids were prepared aerobically and, as a consequence, contained only H_2-oxidizing activity.

Table I. *Isotope Exchange by Hydrogenases from R. japonicum 122 DES Bacteroids and C. pasteurianum*

Assays were conducted in 10-ml Vacutainers containing a total liquid volume of 1.0 ml with HMP buffer. Reaction vials contained either a preparation of 122 DES bacteroids (0.77 mg dry weight equivalent; 1.54 μmol H_2 consumed/min·mg dry weight) or a partially purified hydrogenase preparation from *C. pasteurianum* (1.8 mg protein; 1.53 μmol H_2 consumed/min·mg protein). Reaction vials were incubated with shaking for 100 min. The gas phase for the bacteroid assays consisted of either 0.1 atm $^1H_2$, 0.1 atm $^2H_2$, 0.2 atm O_2, and 0.6 atm N_2 or 0.1 atm $^1H_2$, 0.1 atm $^2H_2$, and 0.8 atm N_2 and that for *C. pasteurianum* hydrogenase consisted of 0.1 atm $^1H_2$, 0.1 atm $^2H_2$, and 0.8 atm N_2. Assays were terminated by placing vials in dry ice. Gas samples (100 μl) were withdrawn for mass spectrometric determinations of $^2H^1H$. Each value reported represents the mean of four determinations. Methyl viologen, when present, was at a final concentration of 1 mM.

Source of Hydrogenase	^2H_2O in Liquid Phase	O_2 in Gas Phase	Excess ^2H in $^2H^1H$
	%	*atm*	*atom %*
122 DES bacteroids	0	0.2	0.003
122 DES bacteroids	0	0.0	0.005
122 DES bacteroids	50	0.2	−0.005
122 DES bacteroids	50	0.0	−0.002
122 DES bacteroids (boiled)	0	0.2	0.000
122 DES bacteroids (boiled)	0	0.0	0.000
122 DES bacteroids (boiled)	50	0.2	0.000
122 DES bacteroids (boiled)	50	0.0	0.000
C. pasteurianum extract and methyl viologen	0	0.0	0.046
C. pasteurianum extract and methyl viologen	50	0.0	0.880

In the absence of ^2H_2O, the *C. pasteurianum* hydrogenase generated 0.046 atom % excess ^2H in $^2H^1H$, whereas the 122 DES bacteroids produced an insignificant amount of $^2H^1H$. Conducting the assays in a medium containing a mixture of ^2H_2O and ^1H_2O provided an opportunity for generation of $^2H^1H$ during the evolution of gas by the reversible hydrogenase. The addition of ^2H_2O increased the rate of formation of $^2H^1H$ by 19-fold in a reaction containing *C. pasteurianum* hydrogenase, but the addition of ^2H_2O had no effect on $^2H^1H$ formation in reactions containing bacteroids. It was concluded that the catalytic mechanism of the bacteroid hydrogenase does not permit measureable isotope exchange during a 100-min incubation period and that the bacteroid hydrogenase *in vivo* catalyzes an oxidation reaction that appears to be unidirectional. These results are in general agreement with those of Dixon (13) who reported that *R. leguminosarum* bacteroids catalyzed no exchange between $^2H_2$ and $^1H_2$ during a 4-h period. Hyndman *et al.* (19) reported that the exchange between $^2H_2$ and $^1H_2$ in cell-free preparations of *Azotobacter vinelandii* were insignificant in assays incubated up to 20 h. Lim (22) claimed that *R. japonicum* catalyzed an exchange reaction between $^3H_2$ and H_2O; however, O_2 was not eliminated in his experiments and no measurements of $^3H^1H$ in the gas phase were reported. It seems highly probable that he observed $^3H_2$ oxidation rather than exchange. Reports (4) of $^3H_2$ exchange by nodules under aerobic conditions undoubtedly are due to $^3H_2$ oxidation via the oxyhydrogen reaction rather than exchange.

Inhibition of Electron Transport. A series of inhibitors that affect the uptake of H_2 and O_2 by *R. japonicum* 122 DES bacteroids are listed in Table II. The compounds that are classified as Cyt *c* oxidase inhibitors and used at concentrations ranging up to 10 mM strongly inhibited H_2 uptake and the uptake of O_2 in the presence and absence of H_2. Although the electron transport chain in *R. japonicum* bacteroids is complex and may not involve a classical Cyt *c* oxidase (2), these results suggest that H_2 oxidation is occurring through a pathway that involves Cyt or other metalloenzymes. The data here are consistent with those of Bothe *et al.* (7) and Peterson and Burris (24), both of whom reported that cyanide inhibited the oxyhydrogen reaction in blue-green algae.

Most of the compounds that were added as possible uncouplers of oxidative phosphorylation inhibited H_2 uptake at least 50% at the highest concentrations utilized (Table II). Concentrations of carbonyl cyanide *m*-chlorophenylhydrazone of 0.005 and 0.05 mM inhibited H_2 oxidation 62 and 73%, respectively. These results are consistent with previous reports (14) and also with the finding of Bothe *et al.* (7) that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone inhibited H_2 uptake in blue-green algae. In contrast with the observations here, 2,4-dinitrophenol failed to inhibit appreciably H_2 uptake in either blue-green algae (7) or *Hydrogenomonas* H20 (5). Carbonyl cyanide *m*-chlorophenylhydrazone, pentachlorophenol, 2,4-dinitrophenol, and 2,6-dibromophenol at some of the concentrations utilized stimulated O_2 uptake in the absence of added H_2 and thus were the only compounds showing clear evidence of uncoupling capability. Among the other compounds tested, inhibition by *o*-phenanthroline and thenoyltrifluoroacetone suggest the involvement of nonheme iron proteins in the oxidative pathway (27), whereas inhibition of O_2 uptake by 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide in *Hydrogenomonas* H20

Table II. *Effect of Inhibitors on Hydrogen and Oxygen Uptake by Bacteroids*

Inhibitors were dissolved in 50 mM K-phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$ and incubated with 122 DES bacteroids (0.38 mg dry weight/ml) for 30 min in a shaker at 23 C. The suspension was sparged with argon and 2.8 ml (1.06 mg dry weight bacteroids with a hydrogenase specific activity of 1.5 μmoles/min·mg dry weight) were injected in the amperometric electrode chamber. H_2 and O_2 at final concentrations of 26 and 22 μM, respectively, were provided as H_2- and O_2-saturated solutions of buffer. The rates of uptake of H_2 and O_2 were determined amperometrically. Results are expressed as the percentage of control reactions without added inhibitors.

Inhibitor	Concentration	Inhibition		
		H_2 Uptake	O_2 Uptake	
			+ H_2	− H_2
	mM		%	
Inhibitors of cytochrome *c* oxidase				
Potassium cyanide	0.01	38	43	17
	0.05	88	71	31
	0.1	90	77	37
Sodium azide	0.1	25	39	2
	1	70	55	0
	10	90	98	86
Hydroxylamine	0.05	35	38	55
	1	76	77	71
	5	98	98	91
Sodium sulfide	0.5	28	45	10
	10	91	78	53
Uncouplers				
2-4-Dinitrophenol	0.5	26	−5	−68
	5	81	70	49
CCCP[a]	0.001	2	−119	−358
	0.005	62	32	−62
	0.05	73	54	43
Dicoumarol	0.025	5	26	4
	0.25	15	19	4
Sodium arsenate	0.5	6	−1	−5
	50	25	14	−5
Pentachlorophenol	2	45	20	−40
	6	76	54	5
2-6-Dibromophenol	0.2	29	24	−17
	2	60	64	72
Other inhibitors				
Iodoacetate	10	0	0	32
	50	39	40	77
HQNO[a]	0.05	39	30	−10
	0.1	35	42	0
TTFA[a]	0.2	17	13	17
	2	49	52	40
o-Phenanthroline	4	64	44	−14
	10	80	68	4
p-CMB[a]	0.025	21	20	84

[a] CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TTFA, thenoyltrifluoroacetone; HQNO, 2-*n*-heptyl-8-hydroxyquinoline-*N*-oxide; *p*-CMB, *p*-chloromercuribenzoate.

was interpreted as an interference with Cyt *b* reduction (5).

Iodoacetate and *p*-chloromercuribenzoate were unique among the inhibitors because they strongly inhibited endogenous O_2 uptake in the absence of H_2 and weakly inhibited the oxyhydrogen reaction. This differential inhibition of the two reactions by iodoacetate was used (14) to demonstrate H_2-dependent ATP formation in bacteroids of *R. japonicum* 122 DES. The increased inhibition of O_2 uptake in bacteroids by iodoacetate has been interpreted to mean a greater participation of sulfhydryl-containing dehydrogenases in the oxidation of endogenous substrates than in the oxyhydrogen reaction (14).

As pointed out by Ruiz-Argueso *et al.* (28), the bacteroid membrane may not be permeable to some of the inhibitor compounds and, for this reason, interpretation is complicated. Further work is needed to clarify the pathway of electron transport from H_2 to O_2.

Interaction between H_2 Uptake and Endogenous Respiration. McCrae *et al.* (23) reported that the addition of saturating concentrations of H_2 to a suspension of *R. japonicum* bacteroids decreased the endogenous rate of respiration. The effect of different partial pressures of H_2 on the rate of H_2 uptake and on endogenous respiration (CO_2 evolution) by bacteroids of *R. japonicum* 122 DES is shown in Figure 2. Increasing partial pressures of H_2 increased the rate of H_2 uptake and decreased the rate of CO_2 evolution. At less than saturating partial pressures of H_2, small changes in H_2 partial pressures resulted in large changes in both the rates of H_2 uptake and CO_2 evolution. Definite inhibition of CO_2 evolution was observed at a partial pressure of H_2 of 0.01 atm, which is equivalent to 8 μM H_2 in solution. H_2 oxidation may result in a sparing of endogenous carbohydrate supplies at partial pressures of H_2 sufficiently low to be expected to occur in N_2-fixing organisms. A carbon-sparing effect might be expected to increase the N_2-fixing capacity of the legume symbiotic association because energy, ordinarily derived from photosynthate, is presumed to limit N_2 fixation (18, 26).

Interaction of H_2 Oxidation and Exogenous Substrate Respiration. Ruiz-Argüeso *et al.* (28) have shown that a whole series of carbon substrates stimulate O_2 uptake by 122 DES bacteroids. Succinate, acetate, and formate are the only substrates found to inhibit the rate of H_2 uptake significantly. The rate of O_2 uptake in the presence or absence of H_2 increased with increasing succinate concentration (Fig. 3). Respiratory O_2 uptake, either in the presence or absence of H_2, was saturated at approximately 5 mM

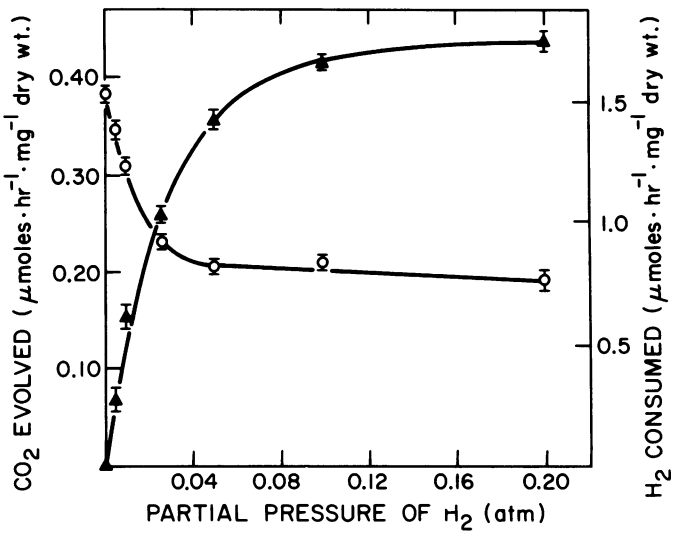

FIG. 2. Effect of different partial pressures of H_2 on H_2 consumption and CO_2 evolution by *R. japonicum* 122 DES bacteroids. Assays were conducted in 22-ml vaccine bottles containing: 2.4 ml HMP buffer and 0.1 ml bacteroids (equivalent of 13.4 mg dry weight). The gas composition in the assay bottles initially consisted of 0.2 atm O_2, partial pressures of H_2 as indicated, and N_2 to 1 atm. Bacteroids were injected into the bottles to initiate the reaction. Gas samples (0.5 ml) were withdrawn from the assay bottles after 10, 20, and 30 min of incubation at 23 C with shaking (150 cycles/min) and analyzed by gas chromatography for H_2 and CO_2 as described. Values are means of three replicate determinations (± SE); (▲——▲), H_2 uptake; (○——○), CO_2 evolution.

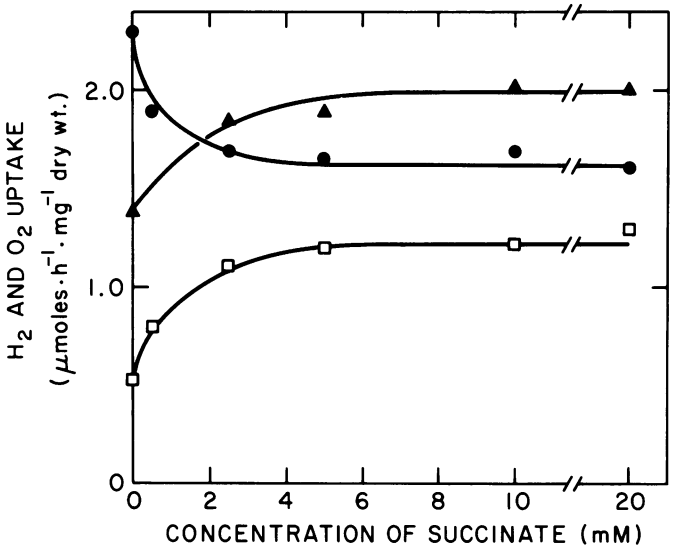

FIG. 3. Effect of succinate on H_2 and O_2 uptake by *R. japonicum* 122 DES bacteroids. Amperometric measurement of H_2 and O_2 consumption were conducted as described previous (14). The amperometric chamber (2.8 ml) contained 50 mM K-phosphate buffer (pH 7.0) with 2.5 mM $MgCl_2$ and succinate at the concentrations indicated. Initial concentrations of H_2 and O_2 were typically 26 and 22 μM, respectively. Bacteroids, equivalent to 0.68 mg dry weight, were injected into the chamber to initiate the assay. (□——□), endogenous O_2 uptake (no H_2 present); (▲——▲), O_2 consumption in presence of H_2; (●——●), H_2 uptake.

succinate. H_2 uptake was inhibited by increasing concentrations of succinate and maximal inhibition was observed at approximately 5 mM. The addition of H_2 resulted in a decrease in succinate-stimulated CO_2 evolution in a manner that was similar to the effect of H_2 on endogenous respiration. These results could be explained by the assumption that the pathways of succinate-stimulated respiration and H_2 oxidation share a common electron transport chain component(s). According to Dixon (12), H_2 uptake and succinate oxidation are competitive processes in *R. leguminosarum* bacteroids.

Fig. 4a shows that succinate is a noncompetitive inhibitor of H_2 oxidation in *R. japonicum* 122 DES bacteroids. Since the H_2-oxidation system undoubtedly contains several electron transport components and the rate-limiting step in the H_2 oxidation system remains to be identified, an interpretation by steady-state inhibitor kinetics cannot be made. Although the noncompetitive inhibition results (Fig. 4) indicate a reversible interaction between some of the components in the H_2 and succinate oxidation pathways, no precise definition of the interaction is possible from the data available.

The inhibition of H_2 oxidation by acetate was uncompetitive (Fig. 4b). A similar reciprocal plot for formate also shows uncompetitive inhibition (Fig. 4c). These results provide no evidence for a reversible connection between H_2 oxidation and utilization of either acetate or formate. Appleby (2) showed that the electron transport chain in *R. japonicum* bacteroids is branched and complex. Further work is necessary to determine the significance of the inhibition of H_2 oxidation by succinate, acetate, and formate.

Inhibition of H_2 Uptake by CO_2. In contrast to free-living *R. japonicum*, which is capable of utilizing CO_2 and H_2 for autotrophic growth (17), the addition of CO_2 to bacteroid suspensions inhibited H_2 uptake. Figure 4d shows that CO_2 is an uncompetitive inhibitor of the H_2 uptake reaction. Care was taken to ensure that inhibition was due to CO_2 rather than to an effect of CO_2 on the pH of the suspension. Experiments also were conducted which demonstrated that CO_2, and not HCO_3^-, was the inhibitory species. H_2 inhibited CO_2 evolution by nodule bacteroids and added

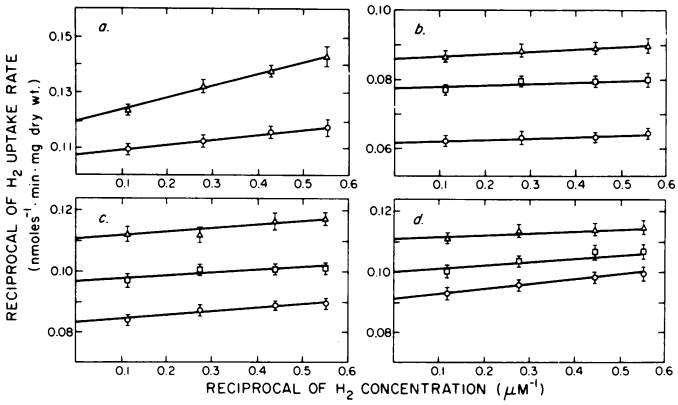

FIG. 4. Inhibition by various carbon compounds of H_2 uptake by *R. japonicum* 122 DES bacteroids. Assays were performed in the amperometric chamber (2.8 ml) containing HMP buffer. Succinate, acetate, and formate, when present, in the experiments described below were incorporated into the buffer at the indicated final concentrations. CO_2 was added as a saturated solution in buffer. Initial concentrations of H_2 and O_2 were typically 26 and 260 μM, respectively. The reactions were initiated by injecting the bacteroids into the chamber. Rates of H_2 uptake were calculated at the indicated concentrations of H_2 from progress curves. Lines were drawn by linear regression analyses. Experiment a contained the equivalent of 0.53 mg dry weight of bacteroids; values are means of five determinations (± SE); (○——○), no succinate; (△——△), 50 mM succinate. Experiment b contained the equivalent of 0.69 mg dry weight of bacteroids; values are means of three determinations (± SE) for assays containing acetate and means of seven determinations (± SE) for assays without added acetate; (○——○), no acetate; (□——□), 20 mM acetate; (△——△), 50 mM acetate. Experiment c contained the equivalent of 0.70 mg dry weight of bacteroids; values are means of three determinations (± SE); (○——○), no formate; (□——□), 20 mM formate; (△——△), 50 mM formate. Experiment d contained the equivalent of 0.64 mg dry weight of bacteroids; values are means of four determinations (± SE); (○——○), no CO_2; (□——□), 2.9 mM CO_2; (△——△), 11.6 mM CO_2 (initial concentrations). The specific activities of the hydrogenase preparations ranged between 1.5 and 2.0.

CO_2 inhibited H_2 uptake. The addition of CO_2, however, did not inhibit O_2 uptake by bacteroid suspensions that were not supplied with H_2. The reciprocal effects of CO_2 and H_2 on the pathways involving these molecules possibly might exert metabolic regulation between these two pathways.

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