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Respiration Supported Nitrogenase Activity of Isolated *Rhizobium meliloti* Bacteroids

R.W. Miller, D.G. McRae, A. Al-Jobore, and W.B. Berndt

Plant Research Centre, Agriculture Canada, Ottawa, Ontario, K1A 0C6, Canada

Bacteroids having a high level of respiration-supported nitrogenase activity were isolated from nitrogen-fixing alfalfa root nodules. Gentle maceration under anaerobic conditions in the presence of sodium succinate and a fatty acid scavenging agent were employed in this method.

A large proportion of isolated bacteroids retained a triple membrane structure as shown by transmission electron microscopy. Dicarboxylic acids of the TCA cycle (malate, fumarate, succinate), but not glutamate or aspartate, supported sufficient respiratory activity to supply the nitrogenase system with ATP and reducing equivalents and to protect the nitrogenase system from inactivation by 4% oxygen over a period of 20–30 min. Sugars did not support nitrogenase activity in intact bacteroids. The properties of the isolated bacteroids were ascribed to minimal damage to the cytoplasmic membrane and peribacteroidal membrane during isolation.

With succinate as substrate and oxygen as terminal electron acceptor, initial nitrogenase activity was determined at 4% oxygen in the gas phase of the assay system employed. At this oxygen concentration, the sustained rate of acetylene reduction by respiring bacteroids was linear up to 30 min. Bacteroid activity declined rapidly with time of exposure to oxygen above 4% in the gas phase. The optimum temperature range for this activity was 10–20°C. Nitrogenase activity was measurable at incubation tempertures below 10°C under 4% oxygen. Functionally intact bacteroids had little nitrogenase activity under anaerobic conditions in the presence of an external source of ATP and reductant. Treatment of the bacteroids with chlorpromazine eliminated respirtation-supported activity and rendered the bacteroid cell membrane permeable to external ATP. Bacteroids treated with chlorpromazine had high acetylene reducing activity with external ATP and dithionite in the absence of oxygen.

Key words: alfalfa, dicarboxylic acid, energy source, chlopromazine, bacteroid, nitrogenase, respiration, rhizobium meliloti

The establishment of effective nitrogen-fixing symbioses between species of *Leguminosae* and species of *Rhizobium* requires the complementary differentiation of both the host and microsymbiont cells. Rhizobium bacteroids differentiate within a membrane structure contributed by the host. This process involves alterations to lipid and respiratory

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proteins of the bacteroid cytoplasmic membrane [1,2] as well as alterations to bacteroid cell wall and the host derived peribacteroidal membrane [3].

In the alfalfa *Rhizobium meliloti* symbiosis, bacteroids differentiate within cortical cells derived from the meristem of root nodules that form in response to infection by free living *R. meliloti*. Undifferentiated, vegetative bacterial cells are released from infection threads within invaginated sacs of the host cell cytoplasmic membrane and undergo an extensive process of differentiation, which leads to increased physical size, increased protein content, and aqueous volume [2]. The bacteroids are osmotically sensitive and readily damaged by harsh physical treatment, osmotic shock, or surfactants such as lysophosphatides and free fatty acids [4]. The expression of the nitrogenase proteins within the bacteroid cytoplasm is accompanied by the development of a specific respiration-driven support system to provide the quantities of ATP and low potential reductant necessary for reduction of dinitrogen [2,4].

Previous attempts [5] to isolate bacteroids from mature nodules of alfalfa by mechanical means have generally resulted in very low or no respiration-supported nitrogenase activity, even though the osmotically sensitive bacteroids were maintained physically intact through the use of a suitable osmoticum. Bacteroids derived from mature nodules of *Pisum sativum* and *Glycine max* have retained significant respiration-supported activity when isolated in the presence of protective substances such as bovine serum albumin, polyvinylpyrrolidone, and diethyldithiocarbamate [4,6,7,8].

Reports of studies with *R. japonicum* and *R. phaseoli* bacteroids have indicated that sugars as well as dicarboxylic acids and pyruvate may be utilised as subtrates by isolated bacteroids to support nitrogenase activity and to provide protection for the nitrogenase activity through respiration by lowering oxygen concentration within the bacteroids. In two reports [9,10], sugars were most effective in supporting nitrogenase activity at low ambient oxygen concentrations. The optimum O_2 level in these reports was $<1\%$ of the assay vessel headspace.

In the case of *R. meliloti* bacteroids isolated from alfalfa nodules, mixtures of lipid-scavenging and reducing agents have not adequately protected the bacteroid cytoplasmic membrane to maintain coupling between nitrogenase and the respiratory chain. The criteria used in this work for successful isolation of bacteroids were a) the maintenance of coupling between the respiratory chain and intracellular nitrogenase activity and b) the retention of the peribacteroidal membrane. Bacteroids isolated with this method have been used to determine the limitations on respiration-supported nitrogenase activity with regard to carbon substrate and oxygen supplies. The question of whether both sugars and TCA-cycle dicarboxylic acids are utilized by *R. meliloti* bacteroids to provide respiratory protection, low potential reductant, and sufficient ATP for the nitrogen enzyme system in vivo is addressed. A membrane-perturbing agent that can uncouple respiration from nitrogenase activity in physically intact cells has been sought, and the results obtained with such an agent are reported herein. The functionally intact bacteroids have allowed the investigation of the presence of a specialized respiratory pathway dedicated to the support of nitrogenase in the Alfalfa *R. meliloti* system. The expression in *R. meliloti* bacteroids of a terminal oxidase system other than the cytochrome a system has been previously suggested [1].

MATERIAL AND METHODS

Growth of Nodules

Alfalfa plants were grown at 25°C in 12.5-cm pots with an 18-hr light 6-hr dark cycle for 7 weeks. Alfalfa seeds (cv. Saranac) were imbibed with a suspension of inoculum (*R. meliloti*, strain Balsac); then 10–20 seeds were planted per pot and reinoculated on days 5 and 10 after germination with 5 ml of a water-washed suspension of the inoculum strain. Inoculum cultures (1 L) were grown on yeast extract-mannitol medium [11] for 40 hr at 30°C before centrifugation and resuspension in 100 ml of sterile distilled water.

Bacteroid Preparation

Nodules were harvested manually at 25°C from water-washed roots of about 200 plants. The yield of fresh nodular mass from 16 pots of 7-week-old plants was approximately 8 g. The nodules were placed in a 30-ml sealed serum bottle, and the gas phase of the bottle was exchanged for ultrapure nitrogen on a gas manifold. The nodules were transferred to an anaerobic glove chamber containing 99% nitrogen and 1% hydrogen gas and placed in a polyethylene bag (11 × 11 cm) containing a piece of starched cheese cloth (Terochem Ltd., Toronto, Canada) and macerated in an anaerobic extraction medium by passing a hand operated, ball bearing grinder (Tecan, AG, Hambrechtikon, Switzerland) over the outside of the bag for 1 min. The medium contained bovine serum albumin, 0.1% (w/v); sodium succinate, 50 mM; sodium N-tris(hydroxymethyl) methyl-2-amino-ethane sulfonate buffer (TES), 50 mM, pH 7.0, and 125 mM KCl. Following maceration of the nodule tissue, the nodule brei was collected from a corner of the bag with a Pasteur pipette. The maceration treatment was repeated a second time after adding 5 ml more of the extraction medium. The resulting brei was pooled with the first extract. The suspension was centrifuged at 100g for 10 min to remove cell debris. The supernatant fluid was withdrawn and recentrifuged at 1,500g for 5 min at 25°C. The resulting dark pellet containing most of the bacteroids was resuspended in 3–5 ml of extraction medium and resedimented at 1,500g for 5 min. The supernatant fluid that contained leghemoglobin was frozen for subsequent purification of this protein.

Assay of Bacteroid Nitrogenase

The bacteroid pellet was suspended in extraction medium, removed from the glove chamber in a sealed vial and divided into aliquots (0.5 ml) in sealed anaerobic, argon-filled 10-ml serum bottles containing other specified reagents in a final volume in 1.0 ml. An ATP generating system and 10 mM dithionite were added where indicated [12], and the bottles were flushed with argon. Aliquots of argon were removed with a syringe, and oxygen was added as indicated to give the required O_2 levels. Acetylene normally was added in a similar manner as 10% (v/v) of the reaction vial headspace. Measured bacteroid nitrogenase activity showed a typical hyperbolic dependence on acetylene concentration, with half saturation occurring at 1–2% of the reaction vial headspace. The reaction mixtures were incubated in reciprocal shaking baths (120 oscillations per at various temperatures. Nitrogenase activity was estimated from ethylene formed as a function of time or at fixed intervals of 15 or 30 min, as previously described [5].

Respiration Rates

Extraction medium (1.4ml) lacking any carbon substrate was equilibrated with a mixture of 4% O_2, 96% A in a thermostated (20°C) Clarke electrode chamber. Bacteroids (1–4 mg protein) that had been washed free of exogenous substrate were added, and a baseline rate of oxygen consumption was established, usually after 5 min. Substrates (50 mM) or an equivalent amount of KCl were then added, and a new rate of oxygen consumption was established. Respiration rates reported are the mean values obtained from three determinations.

Adenylate Concentrations

Adenylate energy charge (AEC)[1] was determined from luminometric analyses of ATP, ADP, and AMP after a 15-min incubation. The bacteroids were extracted with 2.5% (w/v) TCA, and nucleotides were assayed in the supernatant fluid after centrifugation of TCA extracts at 5°C [13]. Protein content of isolated bacteroids was determined by the method of Peterson [14]. The nominal protein concentration (0.1% serum albumin) of the extraction medium was determined by the same method and subtracted from the total protein in the bacteroid preparations before calculation of nitrogenase-specific activities.

Electron Microscopy

Isolated bacteroids and bacteria were fixed with 2% glutaraldehyde and osmic acid (4%) and then dehydrated through an ethanol series. Following infiltration with propylene oxide and Epon 812 resin, the cured pellets were sectioned and mounted on copper grids. The sections were stained with lead citrate-uranyl acetate and examined with a Philips model 300 electron microscope. Whole nodules were fixed, sectioned, and stained in a similar manner.

RESULTS

Structure of Isolated Bacteroids

Figure 1 shows the ultrastructure of intact bacteroids having high levels of respiration-supported nitrogenase activity. The majority of the isolated bacteroids retained the peribacteroidal membrane in close proximity to the outer bacterial and cytoplasmic membranes. The appearance of the peribacteroidal, outer, and innter cytoplasmic membranes were similar to those of the same membranes in intact nodule cells (not shown). Bacteroid membranes sustained less damage than preparations previously described [5] during the isolation procedure and retained functions essential to maintenance of coupling between respiration and nitrogenase activity.

Respiration-Supported Nitrogenase Activity

Bacteroids isolated by the prescribed method had much higher respiration-supported nitrogenase activity per milligram of total protein than previously found for alfalfa bacteroids [5]. The isolation of bacteroids in an isotonic medium under anaerobic con-

[1]Adenylate energy charge (AEC) is the ratio of the sum of intracellular (ATP) and 0.5 (ADP) concentrations to total adenylate concentration:

$$AEC = \frac{(ATP) + 0.5\,(ADP)}{(ATP + ADP + AMP)}$$

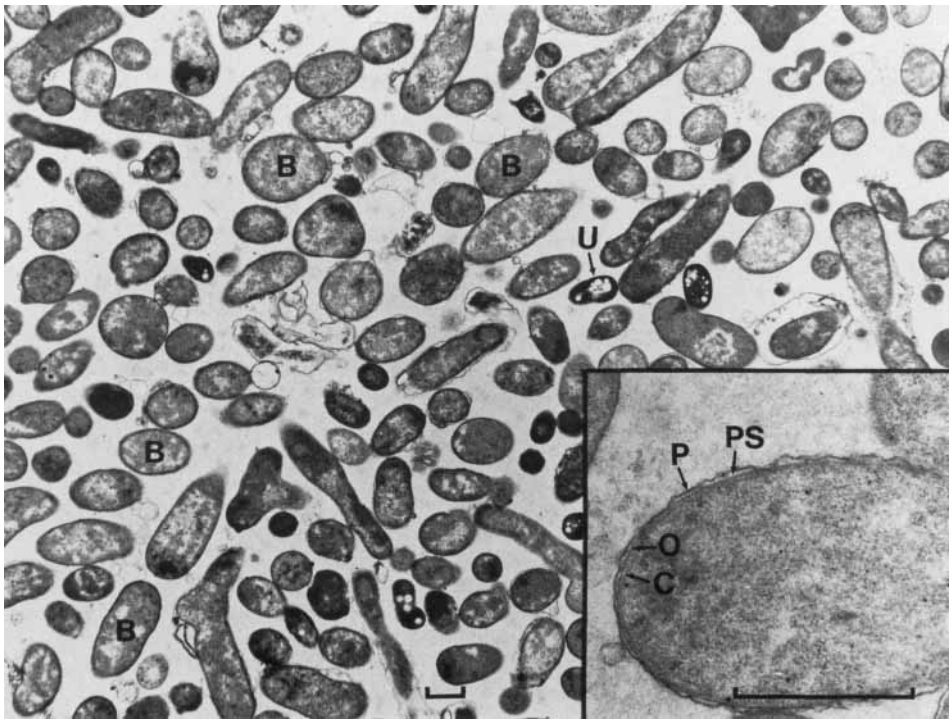

Fig. 1. Elecron micrographs of glutaraldehyde and osmium fixed bacteroids at magnification X10,000 and X80,000 (inset). Abbreviations: C, inner cytoplasmic membrane of bacteroid; O, bacteroid outer membrane; P, peribacteroidal membrane; PS, periplasmic space; B, bacteroids; U, undifferentiated bacteria.

ditions and the inclusion of serum albumin to bind surfactant lipids were beneficial in maintaining coupling between the respiratory chain and the nitrogenase enzyme system and in preventing swelling and membrane damage. Gentle maceration of the nodule tissue was essential, as was low-oxygen tension, to maintain respiration-supported nitrogenase activity intact until assayed. Measured activity declined by 40–60% after storage of bacteroids for 2 hr (data not shown). Anaerobic storage of bacteroids at O°C reduced the rate of decline in activity but did not eliminate it.

The addition of oxygen to reaction mixtures containing the isolated bacteroids allowed respiration-coupled nitrogenase activity to be estimated. Ethylene production is shown as a function of time in Fig. 2. The rate of ethylene formation was linear up to 30 min at 4% oxygen. At oxygen levels greater than 4% of the head space volumn, however, initial activity declined radiply with time under the specified conditions of assay. Data were therefore obtained at 4% oxygen unless otherwise stated.

Buffers other than TES[2] were successfully used, including sodium HEPES, MES, MOPS, and Tricine buffers. However, tris-hydrochloride buffers were not compatible with maximum respiration-supported nitrogenase activity, and the use of tris resulted in a 60–70% reduction in acetylene-reducing activity. Rapid recentrifugation and pelleting

[2]Abbreviations: TES, N-tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid; MES, 2[N-morpholino]ethane sulfonic acid; MOPS, morpholinopropane sulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-tris(hydroxymethyl)methyl glycine.

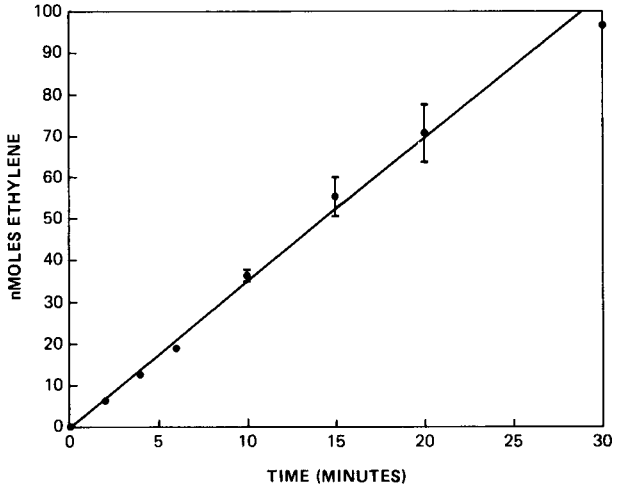

Fig. 2. Time course of ethylene production by isolated bacteroids under 4% oxygen. The incubation medium contained succinate (50 mM) and other components of the extraction medium as described under Materials and Methods. Triplicate analyses were carried out at the indicated times, and ethylene was determined by flame ionization detection following gas chromatography on a 2-m Porapak-N column at 75°C.

of the bacteroids did not result in loss of activity. Phospholipase inhibitors such as 10μM dibucaine had little effect on bacteroid activity or stability. The addition of polyethylene glycol, polyvinylpyrollidone, or diethyldithiolcarbamate did not increase the nitrogenase activity or stability of bacteroids isolated under these conditions.

Effect of Osmotic Strength

The osmotic strength of the isolation medium was an important factor in determining nitrogenase activity in isolated bacteroids. Figure 3 shows that 300 mM KCl gave maximum activity under the experimental conditions, whereas with sorbitol as osmoticum, optimal activity was obtained at 400mM (data not shown). The data of Fig. 3 were obtained with 10 mM TES buffer and 50 mM succinate contributing to total ionic strength.

pH and Temperature Optima

Plots of respiration-supported nitrogenase activity against medium pH peaked sharply at pH 7, with no activity observable at pH 6.0. Half of the peak activity was observed at pH 8.

The effect of temperature on nitrogenase activity was surprising, in that the highest activities were observed under 4% oxygen in the gas phase at 15–20°C (Fig. 4). This effect suggests an oxygen limitation on activity because the solubility of oxygen is increased by 30% in this range, compared with 30°C, the temperature generally used for anaerobic assay of isolated nitrogenase [12]. Significant respiration-supported activity was observed with bacteroids at 5°C, well below the temperature at which isolated nitrogenase ceases to catalyze acetylene reduction in vitro [15].

Effect of Oxygen Concentration on Succinate-Supported Activity

The initial rate of acetylene reduction by isolated bacteroids was dependent on oxygen concentration, as shown in Fig. 5. Activity in the 15-min assay at 20°C increased

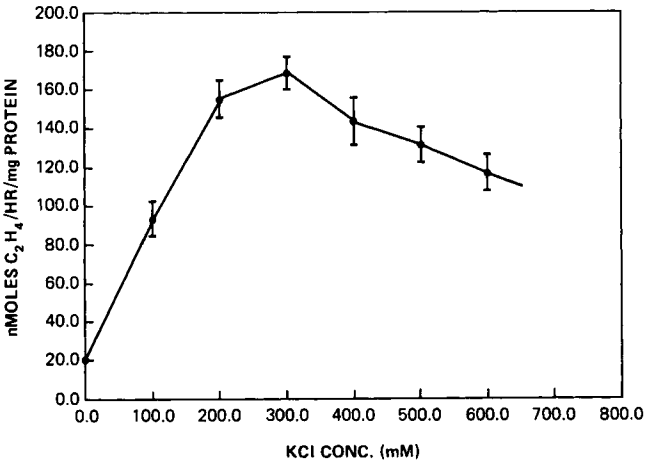

Fig. 3. Effect of KCl concentration on bacteroid nitrogenase activity. Acetylene reduction was determined after 15 min incubation at 20°C. Substitution of sorbitol for KCl resulted in a similar curve, with maximum activity occurring 400 mM.

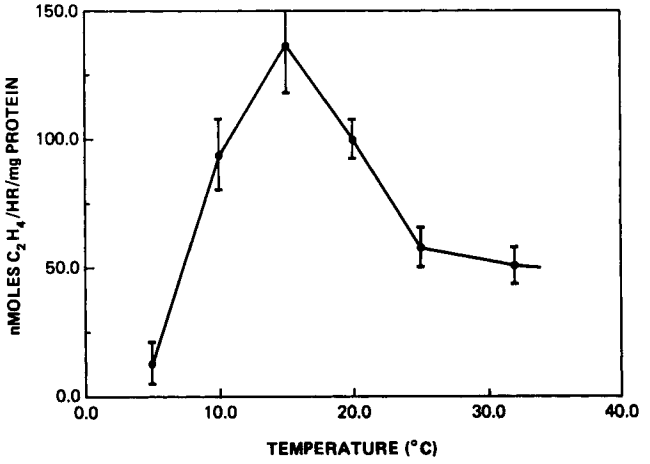

Fig. 4. Effect of temperature on bacteroid nitrogenase activity. Respiration-supported nitrogenase activity was measured after 15 min incubation with a gas phase containing 4% O_2, as detailed under Materials and Methods. Temperature and oscillation rates (120 per minute) were kept constant within ±0.2°C and ±5 oscillations per minute.

as oxygen was raised to 10% (v/v) of the assay vessel head space; activity then declined at higher oxygen levels. If the bacteroids were incubated with acetylene for 30 min, highest activity was obtained at 4% oxygen. At 30°C, activity increased up to 20% O_2 before declining in 15-min assays; therefore, oxygen effects were highly temperature dependent because of increased solubility at lower temperatures. Only at 4% oxygen (or lower) in the optimal temperature range was activity maintained in a linear fashion for up to 30 min incubation. Thus, these conditions were selected for determinations of the effects of substrate concentration and other parameters on nitrogenase activity.

Substrates for Respiration-Supported Nitrogenase Activity

Bacteroids prepared in the presence of 50mM sodium succinate and then washed with buffer, in which succinate was replaced by 50 mM KCl (control) or a 50-mM

alternative substrate, were used to test the effectiveness of various carbon sources in supporting nitrogenase activity. Figure 6 shows activities obtained with some Krebs cycle dicarboxylic acids, sugars, amino acids, and other alternative carbon substrates. Only succinate, malate, and fumarate and oxaloacetate supported high nitrogenase activity in *R. meliloti* bacteroids isolated under the present conditions. Pyruvate supported significant nitrogenase activity, as did alanine (data not shown). This activity was 10% of activity observed with the four carbon dicarboxylates.

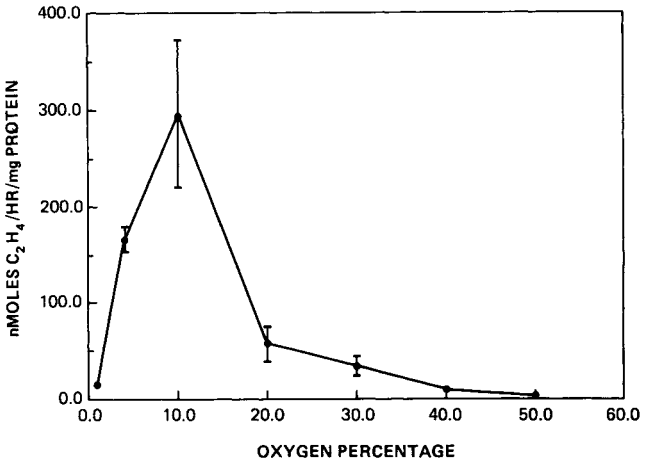

Fig. 5. Effect of oxygen partial pressure on nitrogenase activity of bacteroids. Oxygen was mixed with argon in sealed serum bottles and incubated 15 min with bacteroids at 20°C. Nitrogenase activity was measured, as is detailed under Materials and Methods.

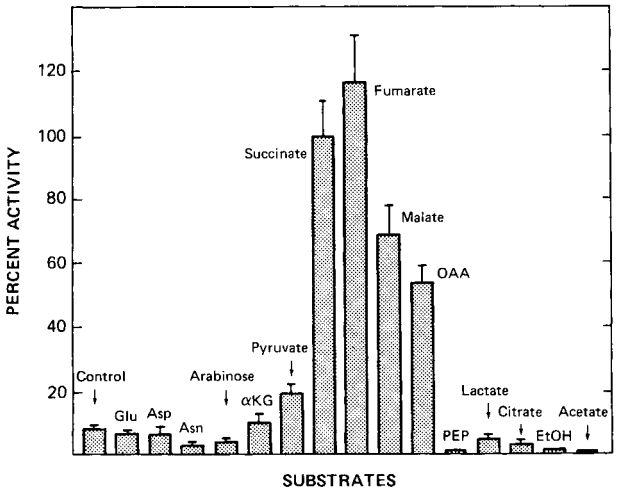

Fig. 6. Substrates used by bacteroids to support nitrogenase activity. Bacteroids were isolated in 50 mM succinate and washed free of external substrate with 175 mM KCl buffer extraction medium under anaerobic conditions. Nitrogenase activity was measured at 20°C, as described under Materials and Methods. Abbreviations: Glu, glucose; Asp, sodium aspartate; Asn, asparagine; αKG, sodium α-keto-glutarate; OAA, oxaloacetate; ETOH, ethanol.

Figure 7 compares activity with succinate and added sugars as a function of oxygen concentration in the gas phase of assay bottles. Sugars, including glucose and sucrose, gave no more activity than controls lacking any added substrate at any oxygen concentration tested.

Effect of Succinate Concentration

When bacteroids were isolated in the absence of any substrate under carefully controlled anaerobic conditions ($O_2 \leq 5$ ppm), it was possible to observe respiration-supported nitrogenase activity at levels similar to those reported above for succinate-isolated bacteroids. The effect of succinate concentration on nitrogenase activity was determined with bacteroids isolated in this way or in 50 mM succinate and then washed free of succinate by centrifugation, suspension in succinate-free medium, and recentrifugation. Half-saturation of activity was obtained at between 2 and 3 mM succinate with either method. However, when bacteroids were isolated in 50 mM succinate and nitrogenase activity then was determined without washing in succinate-free medium, much higher levels (~80 mM) of succinate were required for full saturation of the respiration-supported nitrogenase activity. Replacement of succinate with 50mM pyruvate in the extraction medium also gave bacteroids with high succinate-supported nitrogenase activity. This treatment did not result in increased nitrogenase activity with pyruvate as carbon source, however.

Effect of Bacteroid Protein Concentration

Respiration supported nitrogenase activity was directly related to bacteroid protein concentration only within a narrow range. Activity increased linearly, and specific activity remained constant up to 1 mg protein per assay vessel; however, above this level activity became independent of protein concentration (1–2 mg/ml), and at concentrations greater than 2 mg/ml, there was a decline in activity. This phenomenon is likely to be related

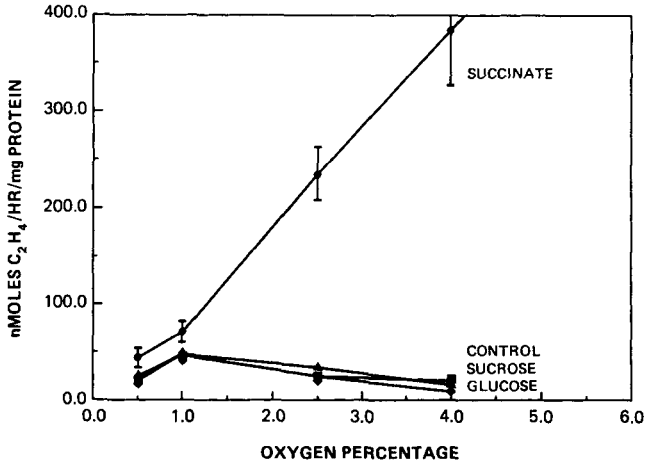

Fig. 7. Oxygen limitation on respiration-supported nitrogenase activity. Nitrogenase activity was determined at 20°C after 15 min incubation at the indicated oxygen tensions. Bacteroids were washed free of succinate before incubation with the indicated substrates.

to oxygen limitation of a respiration pathway specifically supporting the nitrogenase reaction, as discussed below.

Adenylate Energy Charge

At oxygen tensions obtained at less than 4% O_2 in the assay headspace, AEC was found to be in the range 0.5–0.6, and because an adequate supply of ATP is required to participate in the nitrogenase reaction, these levels may be suboptimal. Moreover, ADP is a potent inhibitor of acetylene and dinitrogen reduction by isolated nitrogenase. At 4% oxygen and above, AEC was found to approach a value of 0.9 (Fig. 8), so that under the standard assay conditions employed here, ATP production was not a factor limiting nitrogenase activity. However, even at lower AEC values, nitrogenase activity remained coupled to respiration.

Treatment With Chlorpromazine

The lipophilic, cationic anaesthetic chlorpromazine was found to be a potent inhibitor of respiration-supported nitrogenase activity in isolated bacteroids, as shown in Table I. This potentially useful effect was first noticed when intact nodules were found to lose all nitrogenase activity in the presence of 4% oxygen on incubation with 4 mM chlorpromazine solution. Chlorpromazine undergoes photochemical degradation; all studies with this compound therefore were carried out in the absence of light. Table I shows the effect of varying concentrations of chlorpromazine on bacteroid nitrogenase activity. Chlorpromazine at 0.5 mM eliminated all respiration-supported activity, lowered energy charge, and significantly reduced total bacteroid adenine nucleotides, as determined

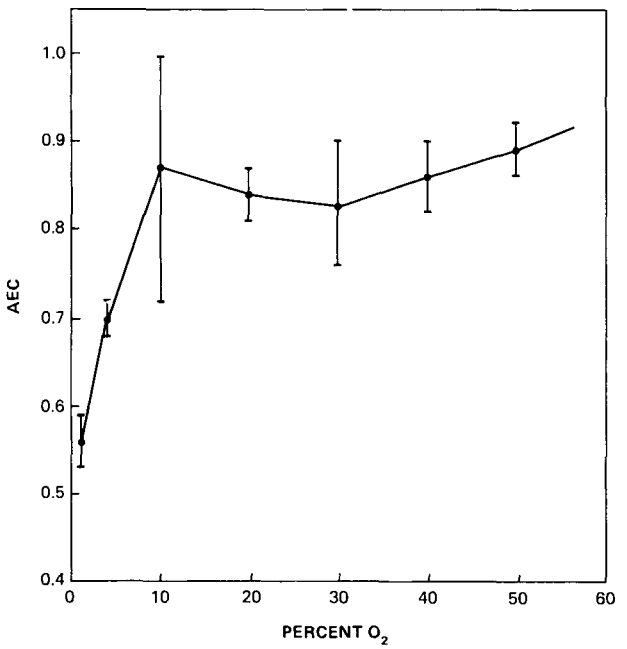

Fig. 8. Effect of oxygen partial pressure on AEC. AEC was determined at 20°C, as indicated under Methods. Adenylates were determined luminometrically [13].

luminometrically [13] At 4.0 mM, this agent caused 80–90% of adenine nucleotides to leak from the cells, although energy charge remained nominally in the 0.5 range.

Bacteroids treated anaerobically with 4 mM chlorpromazine gained the abiltity to admit external ATP and dithionite, presumably because of loss of the permeability barriers associated with the bacteroid cytoplasmic membrane. Nitrogenase present inside the physically intact chlorpromazine-treated bacteroid cells remained active in the acetylene-reduction assay. Three to four times more activity was measured with chlorpromazine and external ATP and reductant, as compared with respiration-supported activity under the experimental conditions of Table II. However, the ratio of respiration-supported activity in untreated bacteroids to activity supported by external ATP and dithionite in chlorpromazine-treated cells was found to vary with bacteroid-protein concentration. At lower bacteroid concentrations (<.2 mg/ml), respiration-supported and externally supported nitrogenase were about equal, although results were variable (data not shown).

Bacteroid Respiration

Freshly prepared bacteroids washed free of external succinate and resuspended in extraction medium containing 125 mM KCl had high endogenous respiration rates, as shown in Table II. However, this endogenous respiration was not able to provide ATP

TABLE I. Effect of Chlorpromazine on Nitrogenase and Nucleotide Levels of Bacteriods

V	Oxygen in gas phase (% v/v)	External ATP-dithionite	Chlorpromazine (mM)	Nitrogenase activity (nmoles/h/mg protein)[a]	Total nucleotides (nmoles/mg protein)[b]	AEC
1	4	–	0	86.5	6.5	0.53
2	4	–	0.2	94.7	6.6	0.48
3	4	–	0.5	0	4.2	0.37
4	4	–	4.0	1.3	0.74	0.52
5	0	+	0	31.2	—	—
6	0	+	0.2	42.5	—	—
7	0	+	0.5	78.0	—	—
8	0	+	4.0	368	[83][c]	[1.0][c]

[a]SEM = ± 10% of given values.
[b]SEM = ± 0.35 nmoles/mg bacteroid protein.
[c]Calculated value from concentration of ATP in generating systems. Bacteroid protein concentration was 3 mg/ml; external ATP was 10 mM; dithionite concentrations was 10 mM where indicated.

TABLE II. Respiration Rates of Isolated Bacteroids*

Substrate	Concentration (mM)	Rate O_2 uptake (nmol-hr^{-1}-mg^{-1})	Stimulation over control (%)
None	—	1,442	(0)
Malate	10	1,508	4.3
Succinate	10	2,320	61
Fumarate	10	1,528	6.0
Sucrose	10	830	—
Succinate + 0.5 mM chlorpromazine	10	1,173	—

*Reaction vessels contained 1.5 mg protein and 83 μM O_2. Oxygen consumption rates were determined with a thermostated Clark electrode at 20°C.

or low potential reductant to the nitrogenase system, because in the absence of added four carbon dicarboxylates, bacteroids had very low activity (Fig. 6). In the presence of added succinate, the respiration rate was stimulated by up to 60% over the endogenous rate, whereas the addition of fumarate and malate stimulated overall respiration rates only by 5% and 4%, respectively. In contrast to the four carbon dicarboxylates, added sucrose generally inhibited respiration by 10%.

DISCUSSION

The presence of succinate in the bacteroid isolation medium stimulated rates of respiration over the endogenous rate in the bacteroids. The combination of endogenous and succinate-supported respiration provided an efficient means for removal of traces of oxygen from bacteroid suspensions in the anaerobic glove chamber used for the isolation procedure. Neither succinate nor any other exogenous substrate was absolutely required for this purpose, however; active bacteroids were also isolated readily in the absence of exogenous substrate, provided that extra precautions were taken to minimize oxygen in extraction media. Damage that occurred during isolation under anaerobic conditions primarily affected the permeability of the bacteroids to adenine nucleotides. This property is associated with the bacteroid membranes because cells damaged during isolation or by 4mM chlorpromazine were depleted in nucleotides but still contained active nitrogenase proteins capable of acetylene reduction when supplied with *external* ATP and dithionite. Thus, the intact peribacteroidal and cytoplasmic membranes demonstrated by electron microscopy may be essential to maintenance of respiration-supported nitrogenase in *R. meliloti* bacteroids.

Retention of respiration-supported nitrogenase activity in isolated bacteroids clearly requires maintenance of coupling between substrate oxidation, oxidative phosphorylation, and production of low potential reductants capable of passing electrons to the nitrogenase enzyme system. The data reported in Fig. 6 indicate that relative to the effect of succinate (100%), fumarate and malate stimulated nitrogenase activity by 120% and 60%, respectively. All these substrates apparently have ready access to the portion of the bacteroid respiratory chain that supports nitrogenase activity in vivo. Whereas respiration of all three dicarboxylates was clearly coupled to nitrogenase activity, fumarate and malate stimulated overall respiration rates by only 6% and 4%, respectively, over endogenous rates. Hence, there was no direct relationship between the overall rate of respiration and observed nitrogenase activity. It appears that only a certain portion of the respiratory system, i.e., that portion responsible for respiration of externally added 4 carbon TCA cycle intermediates, is effectively coupled to nitrogenase activity in isolated bacteroids.

The respiration-supported nitrogenase activity of isolated *R. meliloti* bacteroids with 4 carbon Krebs cycle dicarboxylic acids was equivalent to about 3 nmoles of acetylene reduced per minute per milligram protein. This is comparable to activities of bacteriods of *R. japonicum* isolated from soybean nodules but lower than specific activities of nonsymbiotic diazotrophs grown on N-free media [8,16,17]. Based on the assays reported here, isolated bacteroids had specific activities around twice that of whole alfalfa nodules [15].

Because only Krebs cycle 4 carbon dicarboxylates supported nitrogenase activity in bacteroids, it is probable that the metabolism of glutamate, aspartate, arabinose, glucose, or sucrose is not linked directly to the support of nitrogenase activity in situ. The substrate saturation behaviour of bacteroids isolated in the absence of succinate indicated that under physiological conditions, succinate is actively taken up and half-

saturates the nitrogenase activity supported by the bacteroid cytoplasmic membrane oxidative system at 2–3 mM. This value exceeds by many times the apparent Km reported for the succinate transport system in free living in *R. meliloti* cells [18]. When bacteroids were routinely isolated in 50 mM succinate, 80 mM succinate was required to approach saturation of the same system with regard to the overall maximum nitrogenase specific activity. The physiological dicarboxylate transport system may have been inhibited by the presence of a high concentration (50 mM) of succinate during isolation, and therefore succinate levels greatly exceeding physiological concentrations were required for maximum nitrogenase activity under these conditions.

Physiological oxygen concentrations of the order of 10–100 nM are normally found in nitrogen-fixing nodule cells, and in the presence of the oxygen-transporting protein leghemoglobin, there is sufficient flux of oxygen to the bacteroid cytoplasmic membrane to support bacteroid respiration [19]. It has been shown previously that nodules account for 20% of total respiration rates in axenically grown alfalfa seedling roots [20]. Nitrogenase activity in bacteroid suspensions described here is clearly limited by oxygen supply, even when shaken at 120 oscillations per minute during assay. This activity of the isolated bacteroids showed an abnormally low temperature optimum (oxygen solubility effect), as compared with isolated nitrogenase proteins.

Adenylate energy-charge values of whole-nodule cells [15] and isolated bacteroids in the presence of 1% O_2 have been shown to be near 0.5 to 0.6, indicating the presence of a large proportion of ADP (~⅓) of total adenylates in the cytoplasm. These values are typical of diazotrophic bacteria [17] and are a reflection of the prodigious rate of ATP utilization by the nitrogenase system expressed under diazotrophic conditions. MgADP is a potent inhibitor of nitrogenase activity in vitro. Therefore, the ratio of ATP to ADP may be a limiting factor over nitrogenase activity expressed in bacteroids and nodules under physiological conditions. The results reported previously [15] show that although nodule AEC is limited by oxygen supply below 4% O_2 in the gas phase, there appears to be an additional effect on nitrogenase activity of increasing oxygen above 4% O_2 that is independent of AEC. This additional limitation may be related to the supply of low potential reductant, which is also produced by the respiratory chain and coupled to nitrogenase electron carriers [4]. In the present work, nitrogenase activity of functionally intact isolated bacteroids peaked at 10% O_2 and was inhibited at higher oxygen tensions. The observed dependence of enzyme activity on oxygen concentration was also influenced by overall respiratory activity, and this factor may explain differing reports of optimal O_2 levels for bacteroids [9,10].

Chlorpromazine has been identified as a membrane-perturbing agent that eliminated respiration-supported nitrogenase activity (but not respiration) at low concentrations ($\leq$.5 mM) and caused the bacteroid membranes to become freely permeable to ATP and dithionite at a higher concentration (4 mM). Although the overall respiration rate was inhibited only 50% at low chlorpromazine concentrations, coupling between respiration and nitrogenase was completely inhibited specifically. Because high nitrogenase activities were still observed with external ATP and dithionite, however, this treatment did not lead to direct damage to the nitrogenase proteins. Chlorpromazine did not inhibit isolated nitrogenase proteins when assayed for acetylene reduction or proton reduction in vitro (R.W. Miller, unpublished data). Chlorpromazine therefore allowed separation of the two essential functions of bacteroid respiratory substrates, i.e., the prevention of oxygen damage to the nitrogenase proteins and the provision of ATP and low potential reductant to the nitrogenase system. Respiration was maintained at 0.5 mM chlorpromazine,

although the supply of reductant and ATP to the nitrogenase proteins was not. In the *R. meliloti* bacteroid system, Kreb's cycle 4 carbon dicarboxylate intermediates fulfil both these roles. Under anaerobic conditions, activity with 4 mM chlorpromazine and external ATP and dithionite exceeded respiration-supported activity. The externally supported nitrogenase activity may, in these circumstance, be regarded as the total potential nitrogenase activity of the bacteroids, because ATP and dithionite were supplied at optimal levels, based on studies with the isolated proteins. [12].

It has been proposed previously that oxygen limitation of nodular nitrogenase activity in alfalfa provides one mechanism whereby nitrogenase activity may be maintained at lower temperatures than is observed with the isolated nitrogenase proteins [15]. The observed low-temperature optimum of isolated bacteroids may be due to the fact that increasing oxygen solubility at temperatures between 5°C and 15°C tends to partially compensate for adverse effects of lower temperatures on reaction rates. Increased energy charge, ATP to ADP ratios as previously found in nodules [15], and reductant supply to nitrogenase (via respiration) may cause activity to be maintained below 10°C [15]. In addition, the relative efficiency of energy utilization by the nodular nitrogenase system has been reported to increase at the subambient incubation temperatures [21]. Ratios of hydrogen produced to total electron flux through the nitrogenase system decline when nodules are assayed at subambient temperatures. Thus, there appear to be several possible mechanisms by which nitrogen-fixing root nodules may compensate for the expected thermodyamic reaction rate effects of lowered temperatures on nitrogenase activity in vivo. The results reported here and previously imply that the catalytic activity of the nitrogenase proteins contained within the bacteroids is not maximal under physiological conditions at temperatures above 15°C. This excess catalytic capacity allows nodules to maintain nitrogenase activity to suboptimal temperatures by manipulation of the energy supply to the system. Lower temperatures are not unusual in soils of the north temperate zone; therefore, the ability to fix nitrogen effectively at these physiological temperatures represents a distinct ecological advantage of alfalfa and other *Leguminosae*.

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