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Glutamine as a Feedback Inhibitor of the *Rhodopseudomonas sphaeroides* Nitrogenase System

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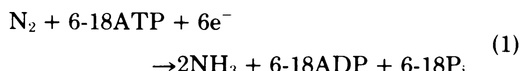
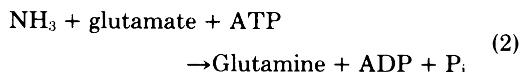
In whole cells of *Rhodopseudomonas sphaeroides*, nitrogen fixation, as measured by hydrogen production and acetylene reduction, was totally inhibited by micromolar concentrations of ammonia. This inhibition could not be duplicated by glutamate or glutamine alone. The inhibition by ammonia was abolished by methionine sulfoximine, a glutamine synthetase inhibitor. Inhibition by glutamine was complete in the presence of methionine sulfone, a preferential inhibitor of glutamate synthase, presumably by permitting a rise in the glutamine pool. The results indicated that the level of the glutamine pool controlled the activity of nitrogenase. None of these effects could be duplicated with cell-free nitrogenase, indicating there is probably a mediator which responds to the glutamine pool and inhibits nitrogenase, rather than glutamine itself being a direct inhibitor.

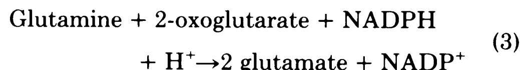
Among nitrogen-fixing bacteria, such as *Klebsiella pneumoniae* and *Anabaena cylindrica*, fixation occurs by the following set of reactions, catalyzed, respectively, by nitrogenase, glutamine synthetase, and glutamate synthase (15, 28, 37):



$$N_2 + 6\text{-}18ATP + 6e^- \rightarrow 2NH_3 + 6\text{-}18ADP + 6\text{-}18P_i \quad (1)$$



$$NH_3 + \text{glutamate} + ATP \rightarrow \text{Glutamine} + ADP + P_i \quad (2)$$



$$\text{Glutamine} + 2\text{-oxoglutarate} + NADPH + H^+ \rightarrow 2\ \text{glutamate} + NADP^+ \quad (3)$$

The sum of reactions 2 and 3 represents a net gain of one fixed ammonia in the form of glutamate.

On the genetic level, the synthesis of nitrogenase and glutamine synthetase, as well as other enzymes which produce glutamine or ammonia, appears to be regulated through glutamine synthetase, probably by changes in its adenylylation state (2, 5, 13, 22, 23, 31, 32). The adenylylation state of glutamine synthetase also controls its own activity (9, 13).

One would expect that, since nitrogenase is an enzyme of high-energy demand, like those of amino acid synthesis (34) and sulfur reduction (25, 27), it would also exhibit some type of regulation at the enzymatic level, such as feedback inhibition by ammonia, glutamate, or glutamine. However, to date, evidence for such regulation has been very limited. For a recent review, see Mortenson (16).

In whole-cell experiments with a variety of organisms, ammonia in the concentration range of 1 to 20 mM had no immediate effect on nitrogenase activity, only a delayed effect consistent with the repression of enzyme synthesis. The organisms tested were *Clostridium pasteurianum* (5, 38), *Azotobacter vinelandii* (30), *Rhodospirillum rubrum* (21), *A. cylindrica* (19), and *K. pneumoniae* (33). In cell-free preparations of nitrogenase from *A. vinelandii* (7, 30) and *K. pneumoniae* (14) 3 to 20 mM ammonia had no effect. A few workers, however, have reported the inhibition of nitrogenase in whole cells of *R. rubrum* (6, 17, 29), *Rhodopseudomonas sphaeroides* (K. Seeland, M.S. thesis, University of Tennessee, Knoxville, 1976), *Rhodopseudomonas capsulata* (11), and *A. cylindrica* (4) by ammonia in the concentration range of 0.8 to 25 mM. There is one report of inhibition of nitrogenase in whole cells of *Rhodopseudomonas palustris* by 50 μM ammonia (39).

In this study, we submit data demonstrating a rapid and total inhibition of the nitrogenase activity in whole cells of *R. sphaeroides* by the addition of ammonia in the concentration range of 20 to 60 μM. We find the inhibition to be nearly instantaneous in its onset and the time required for its relaxation to be proportional to the concentration of ammonia added. We believe this inhibition of nitrogenase at micromolar concentrations of ammonia to be important in the regulation of the metabolically expensive N_2-fixing pathway. A preliminary report including

these data has been presented (B. L. Jones, C.-L. Tsai, J. London, and K. J. Monty, Fed. Proc. **35**:1429, 1978.)

MATERIALS AND METHODS

R. sphaeroides 1.2 was the gift of W. Sistrom. DL-Malic acid, L-glutamic acid (monosodium salt), L-glutamine (grade III), and DL-methionine-DL-sulfoximine were purchased from Sigma Chemical Co. (St. Louis, Mo.). DL-Methionine sulfone was purchased from the California Foundation for Biochemical Research (Los Angeles). Other chemicals were purchased from Fisher Scientific Co. (Fair Lawn, N.J.) or J. T. Baker Chemical Co. (Phillipsburg, N.J.) and were ACS grade. Hydrogen and ultra-high-purity argon were purchased from Selox, Inc. (Chattanooga, Tenn.).

Acetylene was generated from CaC_2 purchased from Sargent-Welch Scientific (Skokie, Ill.).

The argon was scrubbed of oxygen by bubbling through a solution of 5% pyrogallol in 12 M NaOH.

Anaerobic basal salts phosphate buffer (pH 6.8) (21) and anaerobic water were prepared by boiling and subsequent cooling under argon. The anaerobic water was used to prepare solutions. Glutamate and glutamine solutions were stored over Permutit to remove free ammonia.

Cells were grown anaerobically in the light on a minimal medium with glutamate (8 mM) as the sole added nitrogen source.

Small anaerobic cultures (10 ml) were grown in Kimax test tubes (15 by 125 mm) stoppered with Vacutainer tops (Becton, Dickinson & Co., Rutherford, N.J.). Cultures were incubated on a tube rotator (60 rpm) at 33°C with an applied tungsten lamp intensity of 33 J/m^2 per s, measured at the edge of the rotator.

Cultures were harvested by centrifugation of these culture tubes at top speed in an International Clinical Centrifuge for 5 min, washed once with 10 ml of anaerobic basal salts-phosphate buffer (pH 6.8), and centrifuged as before. The washed cells were suspended in 2.5 ml of anaerobic buffer.

Batch cultures were grown anaerobically at 33°C in 465-ml polycarbonate centrifuge bottles (Beckman catalog no. 33994) that had been modified to accept a septum in the cap. Agitation was provided by a New Brunswick gyratory water bath, and average light intensity was 100 J/m^2 per s. Cells were harvested by centrifugation (7,000 × *g*, 25 min) in a Beckman J-21 centrifuge at 25°C, washed in basal salt-phosphate buffer (pH 6.8), and finally suspended in 120 ml of buffer.

All cells were held at 33°C in a water bath until used.

Assays. The production of H_2 was assayed by a polarographic technique using a Clark electrode (Yellow Springs Instruments, Yellow Springs, Ohio) (10, 35) polarized at +0.6 V and monitored on a chart recorder driven at 3 inches (ca. 76.2 mm) per min. Full-scale deflection of the recorder was obtained with 15 to 40 nmol of H_2 in the 1.9-ml reaction mixture (8 to 21 μM). Deflection was calibrated with each use of the electrode. When the deflection reached full scale during an assay, an offset control was used. The Gilson water-jacketed electrode chamber was maintained at 33°C. Illumination of 860 J/m^2 per s was provided by an Aminco side illumination accessory (J4-9610) (American Instrument Co., Silver Spring, Md.). The chamber was capped by a chimney with a capillary (2 mm) opening, through which additions to the reaction mixture were made using a hypodermic syringe and fine needles (25 gauge). The chimney, when kept full of reaction medium, provided adequate limitation for the entrance of O_2 by diffusion. Cells (ca. 1.5 to 2.5 mg of protein) were suspended in 1.9 ml of basal salts-phosphate buffer containing malate at 15 mM. H_2 production was dependent upon light, the presence of malate, and the absence of O_2.

Acetylene reduction was assayed in the following manner. From a batch culture, 100 ml of cells was divided evenly into two argon-filled reaction vessels, and the atmosphere was raised to 20% with respect to acetylene. The reaction vessels were incubated at 33°C in a shaking water bath. Illumination was provided by four 100-W light incandescent bulbs, producing an intensity of 130 J/m^2 per s at the surface of the reaction vessels. Gas samples were withdrawn at appropriate intervals with 1-ml tuberculin syringes and analyzed at ambient temperature using a Bendix 2600 gas chromatograph fitted with a column (0.25 inches by 6 feet [ca. 6.35 mm by 182.88 cm]) of Porapak R (3). Concentrations of acetylene and ethylene were determined by peak height analysis (3, 7). Protein was determined by the method of Lowry et al. (12). Crystalline bovine serum albumin was used as a standard.

RESULTS

Presented in Fig. 1 is a typical demonstration of the kinetics of H_2 production. When the light

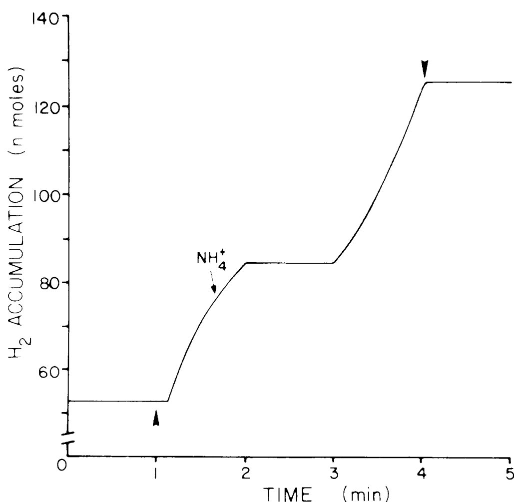


FIG. 1. *Effect of ammonia on hydrogen production. The reaction vessel contained cells (2.26 mg of protein) in basal salts-phosphate buffer. At 1.6 min NH_4^+ was added to a final concentration of 30 μM. Light on (▲) and off (▼).*

was turned on, H_2 production was apparent after a lag of 8 to 10 s. The introduction of ammonium ion produced a complete inhibition of the phenomenon within 20 s. Hydrogen release resumed about 1 min later. The process stopped instantly when the actinic light was removed.

There was a range of NH_4^+ concentrations over which the inhibition of H_2 production was proportional to the amount of inhibitor present. Inhibition was detectable at ammonia concentrations as low as 4 μM, and was complete at about 16 μM (Fig. 2). Higher concentrations of NH_4^+ produced periods of complete inhibition like that shown in Fig. 1, with the duration proportional to the amount of NH_4^+ introduced, as has been seen in *R. rubrum* (29) and *R. palustrus* (39). Recovery was always characterized by resumption of the original uninhibited rate of H_2 production.

That the formation of H_2 observed in these cells was in fact due to the action of nitrogenase rather than to a hydrogenase is argued from several circumstantial points. It was observed only in cells grown in the absence of NH_4^+, i.e., on media where either glutamate or N_2 serve as the sole source of nitrogen. It was not detectable in cells grown in the presence of O_2 and was rapidly lost from active cells upon exposure to O_2. Furthermore, the capacity of cells to form ethylene from acetylene correlated in all of the above points with the capacity to produce H_2, as is shown below.

The measurement of the rate of ethylene formation was not as sensitive as the measurement of the rate of H_2 formation, nor could the response to inhibitors be observed as rapidly as with H_2 production. Nevertheless, the data of Fig. 3 clearly show that ethylene production, like the production of H_2, was inhibited by the introduction of NH_4^+, and that the inhibition was completely relaxed after a few minutes. The longer apparent lag in the onset of NH_4^+ inhibition was probably a result of the relatively large gas space used in these experiments to permit the repeated withdrawal of samples. The slower recovery time by comparison with that

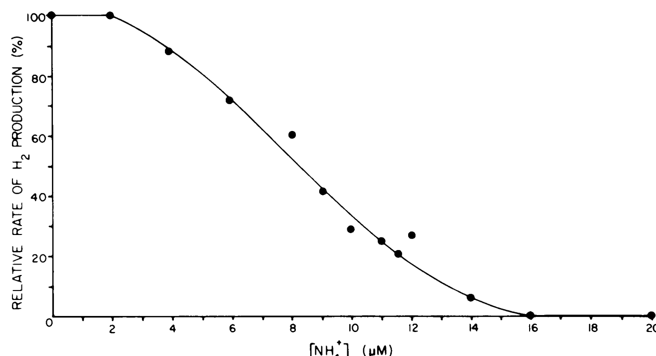


FIG. 2. *Effect of varying the ammonia concentration on the rate of hydrogen production. Rates were determined from slopes of maximum inhibition as shown in Fig. 1. Each point represents a separate sample. Rates were normalized for the amount of cell protein.*

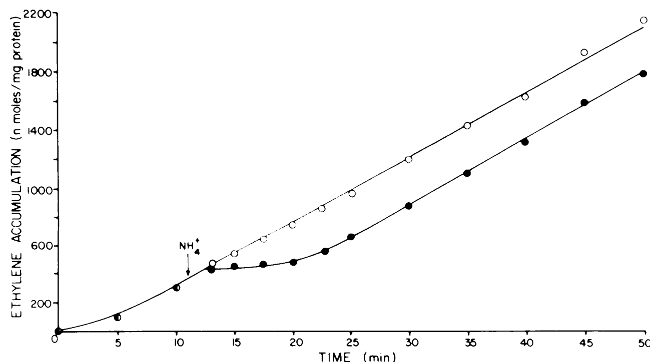


FIG. 3. *Effect of ammonia on acetylene reduction. Two reaction vessels, containing cells from a common culture, were incubated in parallel, and the light was turned on at t = 0. At 11 min, NH_4^+ was added to one reaction vessel at a final concentration of 60 μM.* (○) *Control;* (●) *addition of NH_4^+.*

seen in Fig. 1 was directly the result of the larger amount of ammonia presented to the cells and agreed with the duration of inhibition seen with H_2 production. As with H_2 production, acetylene reduction was light dependent, oxygen sensitive, and observable only in cells grown in the absence of NH_4^+. Also characteristic of nitrogenase system was the fact that H_2 formation by these cells was completely inhibited by the introduction of acetylene (2.8 mM) and inhibited to a maximum of 35% by N_2 (70 μM), both of which have been observed in extracts of *Azotobacter* and *Clostridium* (8).

The introduction of extracellular ammonia caused marked changes in the intracellular pool concentrations of a number of metabolites in *Escherichia coli* (26) *A. cylindrica* (37), *Anabaena variabilis* (15), and *Cylindrospermum licheniforme* (15). The data of Table 1 demonstrate that the two metabolites which were the most direct products of ammonia assimilation, glutamine and glutamate, failed to produce the complete inhibitions seen upon addition of ammonia, even when introduced at 10^3-times-higher concentrations. Unlike ammonia, however, these amino acids probably gained entrance to intracellular spaces by way of rather slow uptake processes as seen in *E. coli* (36). To circumvent any uncertainties about the impact of permease systems, we explored the use of inhibitors which would limit the pathway of ammonia assimilation.

Methionine sulfoximine (MSX) is an irreversible inhibitor of ovine glutamine synthetase (24). The corresponding enzyme from *Klebsiella aerogenes* was inhibited 70% by MSX at a concentration of 10 μM, while glutamate synthase from the same organism was inhibited only 40% by a 100-fold-higher concentration (1). At 1 mM, it prevented synthesis of glutamine from ammonia in *A. cylindrica* (37), *A. variabilis* (15), and *C. licheniforme* (15). In our laboratory, growth of

TABLE 1. *Effect of glutamate and glutamine on acetylene reduction and H_2 production*[a]

Determination	Ethylene formation		H_2 formation	
	nmol/min per mg	%	nmol/min per mg	%
Control	27.3	100	20.6	100
10 mM glutamate	20.8	76	19.8	96
Control	25.0	100	21.4	100
10 mM glutamine	10.6	42	15.6	76

[a] Acetylene reduction was measured as described for Fig. 3, and H_2 production was measured as described for Fig. 1, except that glutamate or glutamine was added as indicated.

R. sphaeroides on N_2 or NH_4^+ as sole nitrogen sources did not occur in the presence of MSX at a concentration above 1 mM, but growth on glutamine was unaffected (J. F. London, unpublished data). Thus, MSX can apparently abolish the synthesis of glutamine from ammonia in vivo.

From the data of Fig. 4, it is apparent that MSX rapidly abolished the ability of ammonia to inhibit the nitrogenase-mediated formation of hydrogen. The partial inhibition by glutamine, however, remained unchanged (data not shown). This result suggested that the inhibition may be produced, not by ammonia itself, but by some product derived from the assimilation of ammonia.

Methionine sulfone (MSF) is a reversible inhibitor of the glutamate synthase of *K. aerogenes*, producing ca. 55% inhibition at 0.2 mM (1). This compound also inhibited glutamine synthetase to a somewhat lesser extent (1). In our laboratory, MSF at a concentration of 1 mM inhibited the growth of *R. sphaeroides* on N_2, NH_4^+, or glutamine as sole nitrogen sources (unpublished data). It appears that MSF can be used to block the enzyme glutamate synthase in vivo, but the blockage was reversible.

The effect of MSF on hydrogen production is shown in Fig. 5. When present alone, it had no effect, but when glutamine was added a complete inhibition not characteristic of glutamine alone resulted. This inhibition was transitory, and partial recovery at a rate equal to that seen with glutamine alone ensued. Inhibition by glutamate was potentiated in a fashion qualitatively similar to, but quantitatively less than, that displayed with glutamine (data not shown). It appears that MSF, by inhibiting glutamate synthase, permitted the cell to achieve higher pool concentrations of glutamine either from uptake of extracellular glutamine or through synthesis catalyzed by glutamine synthetase. The transitory nature of the inhibition reflected the fact that binding of MSF was reversible and perhaps that MSF was displaced from the cell by glutamine. (The result displayed in Fig. 5 required that MSF be added before glutamine—the reverse order produced no effect of MSF. The inhibitor may enter the cell competitively with glutamine.) When higher concentrations (0.3 mM) of MSF were used, neither NH_4^+ nor glutamate could cause inhibition of H_2 production, but the effect of glutamine remained as shown in Fig. 5.

DISCUSSION

R. sphaeroides manifests the properties of H_2 formation and acetylene reduction after anaerobic, light-supported growth with either N_2 or

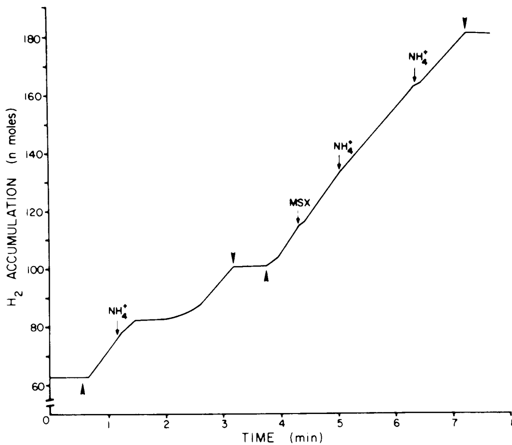


FIG. 4. *Effect of MSX on the ammonia inhibition of hydrogen production. Conditions were as described for Fig. 1 except that 1.65 mg of cell protein was present. Additions were as follows to produce the final concentrations indicated: at 1.15 min, 30 μM NH_4^+; at 4.33 min, 0.1 mM MSX; at 5.05 min, 30 μM NH_4^+; and at 6.53 min, 100 μM NH_4^+. Light on (▲) and off (▼).*

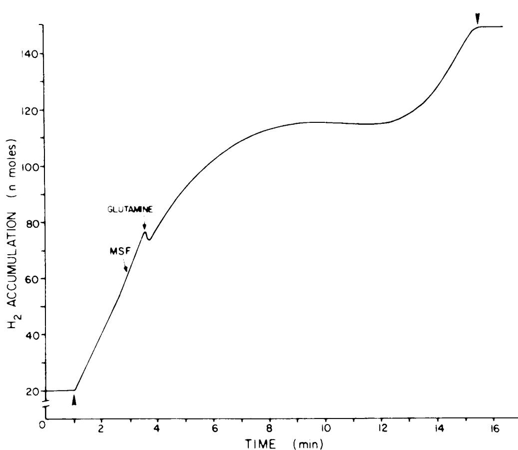


FIG. 5. *Effect of MSF on the glutamine inhibition of hydrogen production. Conditions were as described for Fig. 1 except that 1.27 mg of cell protein was present. Additions were as follows to produce the final concentrations indicated: at 2.9 min, 0.2 mM MSF; at 3.5 min, 10 mM glutamine. Light on (▲) and off (▼).*

glutamate as the sole source of nitrogen. No nitrogenase activity was detectable in either whole-cell or cell-free preparations from ammonia-grown cultures (unpublished data). It appears that *R. sphaeroides* manifests the repressive regulation of nitrogenase seen in so many bacterial systems. The parallelism extends to the observations that glutamine synthetase varies in level and in metal sensitivity in response to growth conditions of *R. sphaeroides* like the corresponding enzyme in *E. coli* (C.-L. Tsai, M.S. thesis, University of Tennessee, Knoxville, 1977) and in so doing changes its degree of adenylylation (H. Karimi and K. J. Monty, unpublished data). It is important to distinguish the slow responses of the repressive regulation system from the very quick responses detailed in this manuscript. The kinetics of repressive regulation in *R. sphaeroides* are measurable in a time frame calibrated in hours. The inhibitions and relaxations described in the present data occur in seconds and minutes.

The conclusion that *R. sphaeroides*, unlike all of the nonphotosynthetic nitrogen-fixing bacteria, possesses a highly responsive feedback system for the modulation of the function of nitrogenase in response to changes in pools of metabolic intermediates is inescapable. In fact, this bacterium's modulating system is more sensitive to changes in external concentrations of ammonia than any of the other photosynthetic bacteria (see Introduction).

We interpret the observations in the following manner. The intracellular pool of glutamine is the key to the feedback modulation of nitrogenase activity. Because ammonia gains quick access to intracellular spaces, the addition of even micromolar concentrations of that ion produces a quick rise in glutamine, made possible by the very low K_m (0.25 mM) for ammonia displayed by that enzyme in *R. sphaeroides* (C.-L. Tsai, unpublished data). The feedback effect is transitory because the assimilation processes quickly exhaust the available ammonia and the glutamine pool falls. The inhibition of glutamine synthetase by MSX abolishes any synthesis of glutamine from ammonia and, therefore, any response of the nitrogenase activity to the presence of ammonia.

The response to added glutamine is less pronounced probably because the rate of entry of glutamine into the cell is not rapid enough to raise the intracellular pool in the face of the rate of utilization of glutamine by glutamate synthase and other quantitatively less important reactions. Upon inhibition of glutamate synthase with MSF, the feedback inhibition by glutamine rises to 100%. This inhibition under our experimental conditions is transitory, not because the added glutamine is exhausted, but because of the competitive relationship of glutamine and MSF in their interaction with glutamate synthase and probably with a permease as well.

Response of the nitrogenase system to glutamate is very sluggish because the synthesis of a glutamine pool from this compound requires either the oxidative release of some ammonia followed by the recombination of that ammonia

with other molecules of glutamate, or the oxidative reversal of the glutamate synthase reaction, or a combination of these two processes. Both will be unfavorable processes for the anaerobic metabolism demanded in our experimental conditions. Inhibition of glutamate synthase with MSF makes possible some rise in the pool of glutamine synthesized from glutamate. The inhibitor MSX does obviate any inhibitory effect of glutamate upon the nitrogenase system, presumably by blocking glutamine synthetase.

Although the argument for glutamine as a unique control agent is circumstantial, it is obvious that this bacterium does possess a feedback system for the control of nitrogenase activity.

Indeed, there is evidence that the ADP/ATP ratio may be important in the regulation of nitrogenase activity (16). In *A. cylindrica*, the addition of 50 μM ammonia causes a major increase in the ADP/ATP ratio and that increase can be prevented by inhibition of glutamine synthetase with MSX (20). While changes in the ADP/ATP ratio can be used to explain the ammonia inhibition and relief with MSX that we observe, we can think of no reason why the ADP/ATP ratio would change to give the inhibition seen with glutamine in the presence of MSF. The most probable explanation is that both the glutamine pool and the ADP/ATP ratio are important in the regulation of nitrogenase activity. This would permit a cell when faced with an abundance of fixed nitrogen and energy to turn off nitrogenase via a rise in the glutamine pool or when faced with insufficient energy and fixed nitrogen to turn off nitrogenase via a rise in the ADP/ATP pool.

The failure of early studies with cell-free nitrogenase to reveal any feedback control by nitrogen-containing compounds (7, 14, 30) is probably not due to a uniqueness of *R. sphaeroides*. In preliminary experiments with cell-free hydrogen production (unpublished) using dithionite as an electron source and reaction conditions similar to those employed with extracts of *R. rubrum* (18), we have been unable to demonstrate any inhibitory effects of ammonia, glutamine, or glutamate. It appears possible that the feedback regulation of nitrogenase by glutamine, like the control of synthesis of nitrogenase, is mediated through a macromolecular system which is sensitive to the intracellular glutamine pool.

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