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Sodium Stimulation of Uptake Hydrogenase Activity In Symbiotic *Rhizobium*[1]

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YORAM KAPULNIK* AND DONALD A. PHILLIPS
Department of Agronomy & Range Science, University of California, Davis, California 95616

ABSTRACT

Initial observations showed a 100% increase in H_2-uptake (Hup) activity of *Rhizobium leguminosarum* strain 3855 in pea root nodules (*Pisum sativum* L. cv Alaska) on plants growing in a baked clay substrate relative to those growing in vermiculite, and an investigation of nutrient factors responsible for the phenomenon was initiated. Significantly greater Hup activity was first measured in the clay-grown plants 24 days after germination, and higher activity was maintained relative to the vermiculite treatment until experiments were terminated at day 32. The increase in Hup activity was associated with a decrease in H_2 evolution for plants with comparable rates of acetylene reduction. Analyses of the clay showed that it contained more Na^+ (29 *versus* 9 milligrams per kilogram) and less K^+ (6 *versus* 74 milligrams per kilogram) than the vermiculite. Analyses of plants, however, showed a large increase in Na^+ concentration of clay-grown plants with a much smaller reduction in K^+ concentration. In tests with the same organisms in a hydroponic system with controlled pH, 40 millimolar NaCl increased Hup activity more than 100% over plants grown in solutions lacking NaCl. Plants with increased Hup activity, however, did not have greater net carbon or total nitrogen assimilation. KCl treatments from 5 to 80 millimolar produced slight increased in Hup activity at 10 millimolar KCl, and tests with other salts in the hydroponic system indicated that only Na^+ strongly promoted Hup activity. Treating vermiculite with 50 millimolar NaCl increased Na^+ concentration in pea plant tissue and greatly promoted Hup activity of root nodules in a manner analogous to the original observation with the clay rooting medium. A wider generality of the phenomenon was suggested by demonstrating that exogenous Na^+ increased Hup activity of other *R. leguminosarum* strains and promoted Hup activity of *R. meliloti* strain B300 in alfalfa (*Medicago sativa* L.).

The nitrogenase enzyme complex of procaryotes catalyzes a concurrent reduction of N_2 and protons to produce NH_3 and H_2. Even under 5 MPa of N_2, the system continues to allocate at least 25% of its reductant to H_2 formation (25). Many N_2-reducing bacteria, and specifically some *Rhizobium* organisms, have evolved a separate Hup[2] system that oxidizes the H_2 to water (12). Potential benefits of the Hup^+ phenotype discussed by Dixon (11) include (a) increased utilization of O_2 which might protect the O_2-sensitive nitrogenase system, (b) removal of H_2 which inhibits nitrogenase, and (c) coupling of H_2 oxidation to ATP synthesis. In addition, recent data suggest that under some conditions reductant produced from the Hup system can be used for N_2 fixation (22). Despite such theoretical advantages of the Hup system, actual beneficial effects on whole plant growth and N_2 fixation have been difficult to prove in a rigorous manner (8, 12). Such difficulties demonstrate the need for increasing our basic understanding of the system.

Various groups have shown that different factors can influence Hup activity in *Rhizobium* cells. In free-living *Rhizobium japonicum* a source of H_2 is required in some strains for derepression of the Hup system, while O_2 and carbon compounds inhibit development but not functioning of Hup activity (13, 19). Cyclic nucleotides affect Hup activity in free-living *R. japonicum* cells (17, 18), possibly through interactions with carbon metabolism, O_2 levels, and nitrogenase synthesis. Measurements of *Rhizobium* Hup activity in pea root nodules showed that $^3H_2$ incorporation/mg nodule mass declined with increasing growth irradiance (6), and partitioning studies with ^{14}C-assimilates in comparable peas indicated that a greater fraction of total photosynthate was translocated to root nodules at higher irradiances (24). Thus, the carbon repression of Hup activity observed in free-living cells may also be a factor in symbiotic systems. Nickel is required for synthesis of an active Hup system in *Rhizobium* (16), but specific effects of other elements on Hup activity remain to be demonstrated.

The present study was initiated as an unexpected by-product of experiments that required a low pH in the rooting medium of pea plants. Because vermiculite is unstable at low pH, a more suitable baked clay commercial product was tested in preliminary experiments. Immediately it became apparent that Hup activity was stimulated significantly in peas growing in the clay, and an investigation of some of the nutrient factors responsible for the phenomenon was initiated.

MATERIALS AND METHODS

Growth of Plants. Pea seeds (*Pisum sativum* L. cv Alaska) were selected for uniformity by weight (0.190–0.220 g), surface sterilized with concentrated H_2SO_4 for 3 min, germinated in sterile paper towels, and planted 48 h after imbibition into one of the two growth systems described below. Unless specified otherwise, the germinated seedlings were inoculated with *Rhizobium leguminosarum* strain 3855 (128C53 Str^r [15]) at the time of planting. In two experiments, other strains of *R. leguminosarum* were used: 128C13, 128C30, 175R1 (all courtesy of L. M. Nelson and J. D. Mahon), or 518 (3855[pRL6JI]::Tn*5-mob* [8]). Both growth systems were maintained under a 16/8 h light/dark cycle at 21/15°C, 50% RH, and a photosynthetic photon flux density (400–700 nm) of 650 μmol m^{-2} s^{-1}.

One growth system employed sterile modified Leonard jar assemblies (10) which were filled either with vermiculite (Terra-Lite, course mix, W. R. Grace and Co., Cambridge, MA) or with baked clay (Saf-T-Sorb, Sierra Chemical Co., West Sacramento, CA). The clay product was ground to a mixture of particle sizes that facilitated movement of nutrient solution from the lower reservoir of the Leonard jar to the plant root system. All Leonard

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[2] Abbreviation: Hup, H_2-uptake.

jars were supplied throughout the experiment with N-free nutrient solution containing 2 mM $CaSO_4$, 1 mM K_2SO_4, 0.1 mM K_2HPO_4, 2 mM $MgSO_4$, 4 μM $CoCl_2$, 1 ml micronutrient solution/L (14), and 18.7 mg/L sequestrene 138 Fe iron chelate (courtesy of Ciba-Geigy). The solution was adjusted to pH 6.8 after sterilizing by autoclaving.

A second growth system, hereafter referred to as the hydroponic system, used plants that were grown for 8 d in vermiculite-containing pots under microbiologically controlled conditions after the initial germination and inoculation procedure. During that period plants received N-free nutrient solution. Then the 10-d-old seedlings were transplanted into a hydroponic system which consisted of a series of 3.8-L plastic containers (12 cm wide × 29 cm long × 21 cm high). Three plants were placed in each container by inserting the root systems through holes in the plastic lid and supporting the cotyledons above the hole with sterile cotton. Containers were connected by 1.5-cm (i.d.) plastic pipe attached to inlet and outlet ports near the top. Each nutrient solution treatment consisted of three containers in series, and the solution bathing the roots of the nine plants in that treatment was circulated from a 6-L reservoir through the three containers and back to the reservoir at a rate of 0.5 ml s^{-1}. Pumps were activated 4 h each day during the experiment, and all solutions were adjusted to pH 6.6 to 6.8 every other day. Plants were acclimated to the hydroponic system by growing them with the standard N-free nutrient solution for 6 d. Then the solution was replaced with fresh, sterile N-free nutrient solution supplemented to contain various salt treatments for an additional 12 d before physiological measurements were made. Solutions prepared by adding 20 mmol of $CaSO_4$ or $CaCl_2$ per liter of nutrient solution formed some precipitates after autoclaving. It is doubtful, therefore, that those treatments contained 20 mM concentrations of Ca^{2+}.

Alfalfa plants (*Medicago sativa* L. cv moapa 69) were sterilized and grown in the Leonard-jar system with vermiculite or baked clay, like the peas, except that plants were maintained under glasshouse conditions during the months of March through May 1986, with minimum and maximum temperatures of 15 and 25°C, respectively. Plants were inoculated with *R. meliloti* strain B300, which was constructed by transferring the recombinant plasmid pIJ1008 from *R. leguminosarum* into a field isolate of *R. meliloti* (2). Plasmid pIJ1008 contains the genetic determinants for Hup activity on pRL6JI (9), the plasmid responsible for Hup activity in *R. leguminosarum* strains 3855 and 518.

Physiological Analyses. Whole-plant H_2 evolution and acetylene dependent ethylene production (acetylene reduction) were measured sequentially on detached root systems within 23 min after removing shoots at the cotyledonary node (3). Relative efficiency of N_2 fixation was calculated as 1 − (H_2 evolved/acetylene reduced) (23). Uptake hydrogenase (Hup) activity was measured on separate plants using $^3H_2$-incorporation techniques described previously (8).

Chemical Analyses. Plants were dried to constant weight at 60°C before measuring reduced N by the Kjeldahl method. Total N_2 fixation was calculated by subtracting original seed N content from total Kjeldahl N values at the end of the experiment. Ground plant samples were digested (5 ml concentrated HNO_3 + 2 ml 70% $HClO_4$ for each 150 mg of tissue) and analyzed for Na^+ and K^+ by atomic absorption spectrophotometry with a Perkin Elmer model 5000 instrument. Cations in the vermiculite and baked clay were extracted with 0.1 N HCl for atomic absorption analyses.

All experiments were conducted at least twice. Hydroponic studies contained a minimum of three plants in each of three replicates; Leonard jar studies were run with six replicate jars containing one plant each.

RESULTS

Hup activity of *R. leguminosarum* 3855 expressed on a root nodule mass basis was promoted as much as 100% in Alaska pea plants growing in the clay substrate relative to comparable plants growing in vermiculite (Fig. 1). A potential promotive effect of the clay material was evident at the first sampling date, and clearly significant increases in Hup activity were measured in 24- to 32-d old pea plants. The inhibitory effect of the clay medium on plant growth was evident in whole-plant acetylene-reduction values, but as the relative efficiency measurement indicated, the increased Hup activity clearly produced a relative decrease in total H_2 evolution from the root nodules (Table I). Baked clay had no statistically significant effect on the total number or mass of nodules per plant relative to the vermiculite treatment. Values averaged across the two treatments for 32-d old Alaska pea plants were 111 nodules/plant and 33 mg nodule dry weight/plant, respectively.

Atomic absorption measurements of extractable ions removed from clay and vermiculite showed notable differences in values for Na^+ and K^+. Clay contained more Na^+ (29 *versus* 9 mg Na/kg) and less K^+ (6 *versus* 74 mg K^+/kg) than the vermiculite. Similar measurements on the plants grown in the two rooting media showed that Na^+ concentration and content were significantly higher in clay-grown plants (Fig. 2, a and c) but that there was no significant decrease in K^+ concentration of the same plants on d 24 when the first stimulation of Hup activity was measured (Fig. 2b). Total K^+ content of plants grown in vermiculite was generally greater than that of plants in clay (Fig. 2d) because of the general inhibition of growth in clay (*e.g.* the 32-

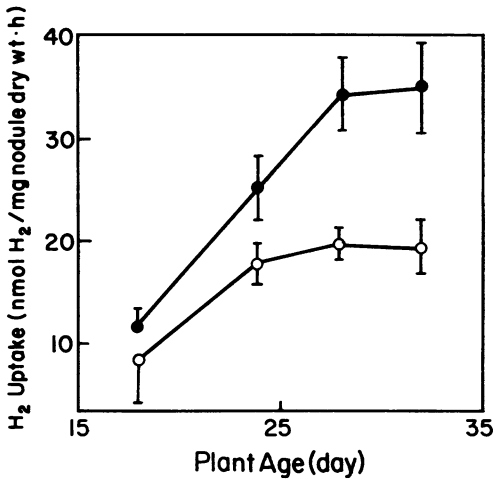

FIG. 1. Hydrogen uptake activity in root nodules formed by *R. leguminosarum* strain 3855 on Alaska peas growing in vermiculite (O) or baked clay (●) as a rooting substrate. Each value represents the mean ± SE of six plants.

Table I. *Root Nodule Activities in 28-Day-Old Alaska Peas Inoculated with R. leguminosarum Strain 3855 and Grown in Two Rooting Substrates*

Relative efficiency was calculated as 1 − (H_2 evolved/C_2H_2 reduced). All values represent the mean ± SE of six plants.

Rooting Substrate	Acetylene Reduced	H_2 Evolved	Relative Efficiency	H_2 Uptake
	μmol/plant·h			*nmol/mg nodule dry wt·h*
Vermiculite	15.0 ± 2.0	9.1 ± 1.3	0.38 ± 0.09	18.4 ± 1.3
Baked clay	5.5 ± 2.0	0.7 ± 0.6	0.87 ± 0.06	35.0 ± 2.4

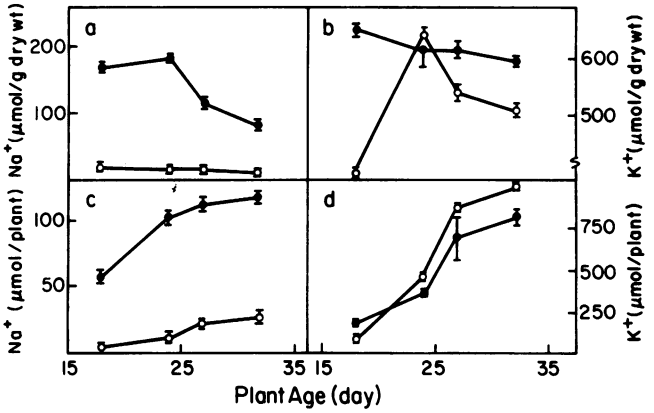

FIG. 2. The effect of rooting substrate (vermiculite, ○; baked clay, ●) on Na^+ and K^+ concentration (a, b) and total accumulation (c, d) in Alaska pea shoots. Each value represents the mean ± SE of the six plants reported in Figure 1.

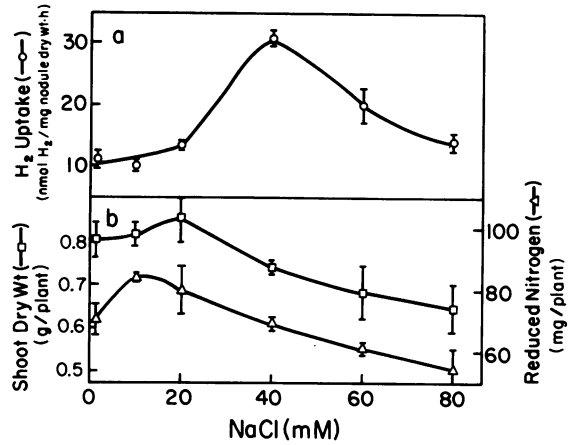

FIG. 3. Hydrogen uptake activity of root nodules formed by *R. leguminosarum* strain 3855 and dry matter and reduced nitrogen content of Alaska pea shoots from plants growing in a hydroponic system with different external concentrations of NaCl. Each value represents the mean ± SE of nine plants.

d-old plants in vermiculite and clay had dry weights of 1000 ± 40 mg and 732 ± 70 mg, respectively).

Tests with NaCl in the hydroponic growth system produced an obvious, highly significant, and very reproducible increase in Hup activity (Fig. 3a). In various experiments Hup activities in treatments containing 40 to 60 mM NaCl were as much as 300% higher than Hup activity of plants that were not supplemented with NaCl. In no case, however, was the increase in Hup activity associated with a significant increase in N_2 fixation or shoot growth (*e.g.* Fig. 3b). No significant increase or decrease in root dry weight was measured with different NaCl treatments in the hydroponic system (data not shown). Atomic absorption measurements of the shoots in those studies showed that increases in external NaCl were associated with increased uptake of Na^+ (Fig. 4, a and c) and decreases in K^+ uptake (Fig. 4, b and d).

Tests with Na^+ and Cl^- in combination with other ions showed that the promotive effect of 40 mM NaCl on Hup activity resulted from the Na^+ and not the Cl^- (Table II). Although the 40 mM KCl treatment increased Hup activity slightly in some experiments (Table II), repeated attempts to measure a significant promotive effect of KCl were unsuccessful (*e.g.* Table III). Although there were occasional hints that 7.5 to 10 mM KCl treatments increased Hup activity, the effects were not significant at $P \leq 0.05$ either in *t* tests or in Fisher's (protected) LSD tests

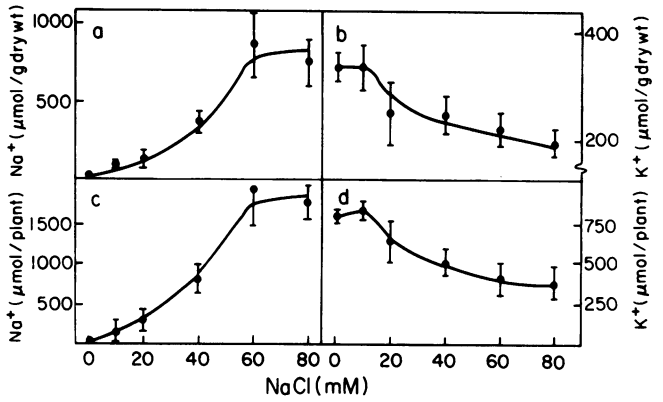

FIG. 4. The effect of external NaCl in a hydroponic system on Na^+ and K^+ concentration (a, b) and total accumulation (c, d) in Alaska pea shoots. Each value represents the mean ± SE of the nine plants reported in Figure 3.

Table II. *Effect of Various Salt Treatments on H_2-Uptake Activity in Alaska Pea Root Nodules Formed by R. leguminosarum Strain 3855 on Plants Grown in a Hydroponic System*

Each value represents the mean ± SE of nine, 28-d-old plants.

Treatment	H_2 Uptake
	nmol/mg nodule dry wt·h
Control	8.3 ± 0.3
40 mM NaCl	19.2 ± 0.7
40 mM KCl	11.8 ± 1.1
20 mM Na_2SO_4	18.0 ± 0.6
20 mM $CaSO_4$	8.3 ± 0.4
20 mM $CaCl_2$	6.1 ± 0.4

between each K^+ level and the control. The normal N-free nutrient solution used in all experiments contained 2.2 mM K^+, and no attempt was made to alter that background value at any time.

Conclusive evidence that part of the original difference in Hup activity between clay-grown and vermiculite-grown plants (Fig. 1) was associated with Na^+ was obtained in separate experiments in which the N-free nutrient solution of vermiculite-containing Leonard jars was supplemented to contain 50 mM NaCl (Table IV). In those cases Hup activity was promoted significantly by the NaCl treatment, and Na^+ content of the plants also was increased. A similar promotive effect on Hup activity was measured with 75 mM NaCl in four other strains of *R. leguminosarum* grown with vermiculite in the Leonard-jar system (Table V).

In root nodules of 51-d-old, flowering alfalfa seedlings, Hup activity of *R. meliloti* strain B300 was significantly greater for plants grown in baked clay than for those rooted in vermiculite. Representative values were 6.51 and 2.55 nmol H_2 uptake/mg nodule dry weight·h for baked clay and vermiculite, respectively.

DISCUSSION

Data from this study show clearly that the sodium ion stimulates Hup activity in pea root nodules containing *R. leguminosarum* 3855 bacteria. The increase in Hup activity observed in a particular clay rooting medium relative to a vermiculite substrate (Fig. 1) was associated with increases in the availability and accumulation of Na^+ (Fig. 2); tests in a hydroponic system demonstrated a maximum Hup activity in the presence of 40 mM Na^+ (Fig. 3; Table II) which could not be produced with equivalent ionic strengths of K^+, Cl^-, or $SO_4^=$ (Table II); and supplementing vermiculite with 50 mM NaCl increased Hup activity significantly in this symbiotic association (Table IV).

Table III. *Hydrogen-Uptake Activity of Alaska Pea Root Nodules Formed by R. leguminosarum Strain 3855 on Plants Grown in a Hydroponic System with Different Supplemental Concentrations of KCl*

Each value represents the mean ± SE of nine, 28-d-old plants.

	KCl Concentration (mM)								
	0.0	5.0	7.5	10	15	20	40	60	80
	nmol H_2/mg nodule dry wt·h								
H_2 uptake	10.5 ± 0.5	10.9 ± 0.6	12.7 ± 1.5	14.0 ± 2.1	11.5 ± 2.0	11.8 ± 1.1	11.8 ± 0.6	11.2 ± 0.6	2.3 ± 0.7

Table IV. *Effect of Exogenous NaCl on H_2-Uptake Activity of Alaska Pea Root Nodules Formed by R. leguminosarum Strain 3855 on Plants Grown in Vermiculite*

All values represent the mean ± SE of six plants.

Experiment	Age of Plants	External Na^+	H_2 Uptake	Shoot Dry Weight	Shoot Na^+
	d	*mM*	*nmol/mg nodule dry wt·h*	*g/plant*	*μmol/g dry wt*
I	27	0	8.8 ± 0.3	0.56 ± 0.03	26 ± 0.9
I	27	50	16.0 ± 0.2	0.67 ± 0.07	240 ± 13
II	32	0	36.4 ± 9.2	1.48 ± 0.11	9 ± 2
II	32	50	72.0 ± 13.0	1.44 ± 0.15	250 ± 74

Table V. *Effect of Exogenous NaCl on H_2-Uptake Activity of Alaska Pea Root Nodules Formed by Various Strains of R. leguminosarum*

All values represent the mean ± SE of six, 35-d-old plants.

R. leguminosarum Strain	External NaCl	H_2 Uptake Activity
	mM	*nmol/mg nodule dry wt·h*
128C13	0	11.1 ± 1.6
128C13	75	22.4 ± 3.4
128C30	0	13.8 ± 1.2
128C30	75	25.5 ± 3.4
175R1	0	3.9 ± 0.3
175R1	75	12.2 ± 3.1
518	0	9.6 ± 0.8
518	75	31.3 ± 3.6

The promotive effect of Na^+ on Hup activity apparently is not restricted to *R. leguminosarum* strain 3855. Results in Table V indicate that Hup activity in four very different strains of *R. leguminosarum* was increased significantly by NaCl, and alfalfa plants inoculated with *R. meliloti* strain B300 had much higher Hup activity when they were grown in baked clay than in vermiculite. Presumably Na^+ was the active element in each experiment, as was demonstrated for strain 3855. Although these results certainly do not suggest that Hup activity in all types of rhizobia would be increased in the presence of NaCl, the strains selected for these tests represent a diverse sample from *R. leguminosarum*. Nelson *et al.* (20), for example, used *hup*-specific DNA hybridization probes from various sources to demonstrate different hybridization characteristics in strains 128C13, 128C30, 128C53, and 175R1. The Hup system in *R. meliloti* strain B300 is determined by the same plasmid, pRL6JI, present in *R. leguminosarum* 128C53, 3855, and 518 (8, 15), but the genetic background of *R. meliloti* is quite different from the *R. leguminosarum* strains. These results suggest, therefore, that the promotive effect of NaCl on Hup activity occurs in many strains other than just *R. leguminosarum* 3855.

The significance at the whole-plant level for the phenomenon demonstrated in this study remains to be determined. Other workers have reported that 120 to 144 mM NaCl inhibited growth and N_2 fixation in various *Rhizobium*-legume symbioses (26, 27). Likewise, in the present hydroponic experiments the higher concentrations of NaCl tended to decrease carbon and nitrogen assimilation in pea shoots during the 12 d plants were exposed to the salt treatment (Fig. 3). Whether the increased Hup activity observed with 40 mM NaCl counteracted the general inhibitory effect of salt and benefited plant growth cannot be determined from the present study. A more critical comparison would examine the effect of Hup^+ *versus* Hup^- rhizobia on plant growth in the presence of 40 mM NaCl. Such tests with the Hup^+ strain 3855 and isogenic Hup^- mutants produced by Tn5-*mob* mutagenesis (15) are in progress. Previous work from this laboratory has shown that different pea cultivars produce varying levels of Hup activity in *R. leguminosarum* (3, 5, 8) and that a factor responsible for that effect can be transmitted across grafts between the cultivars (4). The present data on Na^+ probably do not explain those observations because no Na^+ was supplied in the nutrient solution used in the work cited. However, the possibility that the small K^+ effects on Hup activity (Table III) may be important is being investigated.

The Na^+-induced increases in Hup activity might result from several different biochemical mechanisms. At one level, the amount of some protein component in the Hup system might be increased. It also is possible that the functional properties of Hup, rather than the total amount of this membrane-bound system in *Rhizobium* (1), could be affected directly through the disruptive displacement of Ca^{2+} by Na^+ (7). Alternatively, such injuries might affect the concentration of potentially critical factors such as phosphate (21) internal or external to the *Rhizobium* cells in the plant cell cytoplasm. Other potential explanations for the increase in Hup activity include the possibility that the Hup system is an important source of ATP during extreme stress or that the Hup system functions to control O_2 concentration when normal O_2 levels are altered by changes in membrane properties. It is also possible that exogenous Na^+ acts indirectly by altering internal K^+ levels (Fig. 4) and membrane potential, which could affect Hup activity by various biochemical mechanisms. Attempts to distinguish among these possible explanations obviously will require studies with isolated *Rhizobium* bacteroids.

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