Nickel: A micronutrient element for hydrogen-dependent growth of *Rhizobium japonicum* and for expression of urease activity in soybean leaves (hydrogenase)

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**ABSTRACT** Soybean plants and *Rhizobium japonicum* 122 DES, a hydrogen uptake-positive strain, were cultured in media purified to remove Ni. Supplemental Ni had no significant effect on the dry matter or total N content of plants. However, the addition of Ni to both nitrate-grown and symbiotically grown plants resulted in a 7- to 10-fold increase in urease activity (urea amidohydrolase, EC 3.5.1.5) in leaves and significantly increased the hydrogenase activity (EC 1.18.3.1) in isolated nodule bacteroids. When cultured under chemolithothrophic conditions, free-living *R. japonicum* required Ni for growth and for the expression of hydrogenase activity. Hydrogenase activity was minimal or not detectable in cells incubated either without Ni or with Ni and chloramphenicol. Ni is required for derepression of hydrogenase activity and apparently protein synthesis is necessary for the participation of Ni in hydrogenase expression. The addition of Cr, V, Sn, and Pb in place of Ni failed to stimulate the activity of hydrogenase in *R. japonicum* and urease in soybean leaves. The evidence indicates that Ni is an important micronutrient element in the biology of the soybean plant and *R. japonicum*.

Nickel is an essential element in several biological processes, including H₂O oxidation in some bacteria and urea hydrolysis by plants (1). Although its function is not completely understood, Ni is a constituent of urease (urea amidohydrolase, EC 3.5.1.5) from jackbean (2) and soybean (3) seeds, CO dehydrogenase from *Clostridium thermoaceticum* (4), and factor F₉₀₀ from certain species of Methanobacterium (5, 6). Additionally, hydrogenases (EC 1.18.3.1) from *Methanobacterium thermoautotrophicum* (7), *Alcaligenes eutrophus* (8, 9), *Desulfovibrio gigas* (10), and *Vibrio succinogenes* (11) contain Ni, which appears to participate in an oxidation-reduction reaction during catalysis (10, 12). Ni is a required micronutrient for the synthesis of functional hydrogenase in several other microorganisms (13, 14) and many, but not all, of the H₂-oxidizing bacteria require Ni for growth. The biological significance of Ni recently has been discussed by Welch (15). Growth of soybean tissue cultures (16) and intact *Lemna paucicostata* (17) in media containing urea as the sole source of nitrogen is greatly stimulated by Ni. Recently, Eskew and Welch (18) reported a marked decrease in urease activity in seeds and appearance of a necrosis of leaf tips of soybean plants grown in nutrient solutions containing nitrates but lacking added Ni.

The potential importance of energy loss via H₂ evolution from nitrogenase in nodules has been discussed in a review (19). H₂ uptake via the hydrogenase system is important not only in bacteroids but also for chemolithothrophic growth of free-living rhizobia (20, 21). This study was conducted to determine whether the addition of Ni affected (i) the growth of soybean plants cultured either symbiotically or with nitrate, (ii) the expression of urease activity in soybean leaves, and (iii) the expression of hydrogenase activity in nodule bacteroids. In addition, we have examined the effect of adding Ni on the growth of and derepression of hydrogenase in *R. japonicum* under chemolithothrophic conditions.

**MATERIALS AND METHODS**

**Source of Chemicals.** All chemicals, except otherwise indicated, were reagent grade and, as indicated below, some were purified to remove Ni. Fe, Co, Zn (as metals), MnCl₂, CuCl₂, PbF₂, V₂O₅, SnO₂, and Cr₂O₅ were Speccure grade and purchased directly from Johnson, Matthey (London) or through a United States supplier (Alfa–Venton, Danvers, MA). [¹⁴C]Urea (55 mCi/mmol, 1 Ci = 3.7 × 10¹⁰ Bq) was purchased from ICN and diluted with ultra-pure urea (Schwarz/Mann). Triton X-100 was Sintrex reagent grade (Baker).

**Purification Procedure.** The procedures described by Thiers (22) with modification by Viellon and Vallee (23) and by Ahmed and Evans (24) were generally followed to minimize contamination by Ni. Purified water was prepared by double distillation in a glass still followed by passage through a mixed-bed ion-exchange column (24). This water contained no Ni detectable by diphénylthiocarbazone (dithizone) extraction and was used in preparing all solutions as well as in washing procedures. CHCl₃ and HCl were redistilled in a glass still. Polyehtylene containers that were washed with 20% (vol/vol) HNO₃ and 2 M distilled HCl and rinsed with water were used routinely. Glassware was acid-washed with a solution of 9 M H₂SO₄ plus 7.5 M HNO₃. Acid-washed plastic gloves were worn whenever contact with plants—direct or indirect—was made.

Ni was removed from concentrated solutions of major nutrient salts, organic substrates, vitamins, H₂BO₃, and Na₂MoO₄ by three to five extractions at pH 6.5–7.5 with 0.02% dithizone in redistilled CHCl₃. Extractions were done in Teflon-stoppered glass separator funnels containing 50 ml of the dithizone solution and 500 ml of the nutrient solution. Completeness of extraction was tested on each nutrient solution by a final extraction with 0.0002% dithizone in redistilled CHCl₃. The dithizone reagent was removed by five to seven extractions with 50–100 ml of distilled CHCl₃ per extraction. Residual CHCl₃ was removed by bubbling filtered air through the solution. KHCO₃ was purified by the same procedure as described for the major nutrient salts except 1% 8-hydroxyquinoline replaced dithizone and the pH was 5.3. Purified CaCO₃ was prepared by combining purified solutions of KHCO₃ and CaCl₂, collection

**Abbreviations:** Mes, 2-(N-morpholino)ethanesulfonic acid; Hup⁺, hydrogen uptake-positive.

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of the precipitated CaCO₃ by filtration, and extensive washing of the precipitate with purified water. CaCO₃ was used as a suspension. Ni was extracted from sodium citrate as described for the major nutrient salts except equal volumes of 0.02% dithizone in 6 M HCl were used to chelate Ni. Ferric citrate was prepared by dissolving Specpure Fe sponge in redistilled 6 M HCl and converting the FeCl₃ to ferric citrate by boiling with an equivalent amount of purified sodium citrate (24). V, Cr, Sn, and Pb were added as V₂Os, Cr₂O₃, SnO₂, and PbF₂ after solution in redistilled 6 M HCl. Solutions of NiCl₂, ZnCl₂, and CoCl₂ were prepared by dissolving Specpure NiO, Zn rod, and Co sponge, respectively, in redistilled HCl.

Plants. Soybean seeds [Cystine coker (Linnaeus) Merr] cv. Wilken were surface sterilized by ethanol and sodium hypochlorite, rinsed, inoculated with R. japonicum 125 DES (20) that had been grown on a purified defined medium (25), and germinated in cylinders of acid-washed cheesecloth. During germination, the seedlings were kept moist by placing one end of the cylinders into a purified nutrient solution (26) at 0.5 concentration and supplemented with purified CaCl₂ (2 mM) and 2 mM 2-(N-morpholino)ethanesulfonic acid (Mes) at pH 6.7. After 5 days, four seedlings were reincubated and suspended through a cover into a 15-liter acid-washed polyethylene container lined with two acid-washed polyethylene bags and containing 13.6 liters of purified nutrient solution. Plants were grown in solution culture in a modified purified nutrient solution (25) at one-half concentration for 29 days and the concentration of the nutrient solution was increased to the full concentration for the final 34 days. Final concentrations of nutrients in the basic solution were 73.5 μM K₂HPO₄, 76.5 μM KH₂PO₄, 2.0 mM CaCl₂, 2.0 mM MgCl₂, 1.25 mM K₂SO₄, 7.75 μM H₂BO₃, 0.15 mM CuCl₂, 1.5 μM MnCl₂, 0.6 μM ZnCl₂, 0.05 μM Na₂MoO₄, 15 μM ferric citrate, 15 mM CoCl₂, and 2 mM Mes. Solutions into which the roots were suspended were continuously aerated with filtered air and maintained between pH 6 and 7 by the addition of CaCO₃ or redistilled HCl. The experiment was designed as randomized blocks with four replicates of each treatment. The six treatment variables utilized are presented in the legend of Table 1. Purified water was added routinely to each container to compensate for losses.

Plants were grown in a growth chamber with a 14-hr, 29°C day and 10-hr, 24°C night. The light intensity was 350 μE·m⁻²·hr⁻¹ (E = einstein, 1 mol of photons). Cotyledons were removed from plants 14 days after germination. The initial shoots were harvested 30 days after seed germination by cutting stems immediately above the first node above the cotyledons. After growth of the axillary shoots, they were harvested by cutting between the second and third node above the cotyledons 49 days after seed germination. These procedures were employed to lower the endogenous level of Ni in the plants. The final harvest of shoots, roots, and nodules was completed 69 days after seed germination.

Culture of R. japonicum. Several precautions were followed to minimize contamination of cultures. Acid-washed polypropylene containers and covers were routinely used. Sterilization of containers and media was accomplished by autoclaving in closed polypropylene bags. For solutions requiring sterilization by filtration, acid-washed plastic syringes with an attached Swinnex filter holder enclosing an acid-washed Duroapore (Millipore) filter were used. R. japonicum 125 DES was cultured through three growth cycles in a purified mineral salts/vitamins medium (27) at pH 6.8 to which was added carbon substrate. The constituents were as follows: 30 mM K₂HPO₄, 1 mM K₂SO₄, 15 mM NH₄Cl, 4 mM MgCl₂, 12 mM KHCO₃, 70 μM CaCl₂, 0.1 μM Na₂MoO₄, 0.4 μM CuCl₂, 0.2 μM CrCl₃, 0.2 μM CoCl₂, 0.1 μM ZnCl₂, 0.2 μM MnCl₂, 1 mg of thiamin·HCl per liter, 1 mg of nicotinic acid per liter, and 15 μg of biotin per liter. Carbon substrates added in g per liter were: 0.5 of sucrose, 0.5 of sodium gluconate, 0.5 of sodium glutamate, and 1.0 of arabinose. After the final growth cycle, cells were harvested by centrifugation of acid-washed polycarbonate tubes and suspended in a purified salts/vitamins medium (27) as described above.

The derepression experiment (Fig. 2), the cell suspension was divided into aliquots for four treatments with duplicates. Treatments included (i) no added NiCl₂, (ii) NiCl₂ (5 μM) added at time, (iii) preinoculation with chloramphenicol (8 μM) for 30 min and then adding NiCl₂ (5 μM) at 0 time, and (ii) NiCl₂ (5 μM) added at 12 hr. Three milliliters of each cell suspension was placed in acid-washed 17 × 100 mm polycarbonate tubes, which were sealed with serum stoppers. The tubes were evacuated, gassed with a mixture containing 4% H₂, 5% CO₂, 1% O₂, and 90% N₂ (vol/vol), and incubated without shaking at 27°C. Duplicate cultures were removed at each sampling, sparged with N₂, and then assayed for hydrogenase activity (25).

In the experiment (Fig. 3) in which the effect of Ni on chemolithotrophic growth of R. japonicum was determined, cells were prepared as described under Culture of R. japonicum, above. Additional details of the experiment are described in the legend of Fig. 3.

Assays. Samples (about 0.5 g) of nodules were removed from plants (treatments 1 through 4) 29, 48, and 68 days after seed germination. Bacteroids were isolated and H₂ uptake was measured amperometrically as described (28). Leghemoglobin was measured in the plant cytosolic fraction of nodules by the method of Appleby and Bergersen (29). Urease activity was measured in leaf disks by the evolution of ¹⁴CO₂ from [¹⁴C]urea, using an in situ method described by Kerr et al. (30) except Triton X-100 was used as the detergent. Duplicates of eight disks from four older trifoliate leaves were assayed for each sample. The specific activities of urease in young leaves (at 30 days) and older leaves (at 69 days) were not greatly different, with values of 10.6 ± 0.9 and 6.4 ± 1.0 mol of CO₂·hr⁻¹ per g dry wt, respectively. After each harvest, plant material was dried at 80°C, weighed, ground, and assayed for total N by the Kjeldahl method. Protein contents of bacteria were determined as described by Eisbrenner and Evans (31).

RESULTS AND DISCUSSION

Effect on Yield and N Content of Plants. Soybean plants were grown symbiotically and with nitrate, using purified nutrients in solution cultures. Added Ni had no significant effect on the total accumulation of dry matter or the N content of shoots and roots of plants receiving most of the treatments (Table 1). Also, there was no effect of Ni on the first and second harvest of shoots (data not presented). The combination of metal ions (Sn, Pb, Cr, and V) also had no effect on symbiotic growth. Significant differences in dry matter and N content were observed between plants grown symbiotically and those supplied with nitrate, but the addition of Ni had no significant effect on these parameters (Table 1). The addition of Ni resulted in a significantly lowered N content of roots of plants growth with nitrate (Table 1), but interpretation of this effect must be delayed until tissues are analyzed for nitrogen constituents. Because Eskew and Welch (18) have described a necrosis of soybean leaves that was prevented by adding Ni, we suggest that Ni from seed or from other sources in our experiments may have been sufficient to meet minimal growth requirements.

Effect on Urease Activity in Leaves. The addition of NiCl₂ to the purified nutrient solutions enhanced by at least 10-fold the urease activity in leaves from symbiotically cultured soybeans (Fig. 1), but addition of Cr, Pb, Sn, and V instead of Ni
had no significant effect. Furthermore, the addition of these metal compounds to cultures with Ni had no measurable effect on urease activity. The provision of Ni to purified nutrient solutions containing nitrate also increased urease activity of leaves about 7-fold. Among plants supplemented with Ni, the activity of leaf urease in symbiotically grown plants was significantly higher than that of nitrate-grown plants. These results are consistent with the well-established observations that soybean plants grown with nitrates translocate considerably lower levels of ureides than do symbiotically cultured soybeans (32). Ureases from soybean leaves and seeds differ in several basic properties and may be different enzymes (33). In respect to Ni requirement, our results with leaf urease and the reports on ureases from seeds of jackbean (2) and soybean (3, 18) indicate that ureases from these different sources behave similarly. Presumably, urease plays a role in the metabolism of urea derived from the catabolism of ureides, the major compounds in which fixed N is transported from nodules in soybean plants (32). Leaves, therefore, would be expected to be major organs where urea is metabolized.

Effect of Ni on Root Nodules and Bacteroids. At the final harvest, nodules had begun to senesce and, as a consequence, nodules were not separated from roots for dry weight measurements. No obvious effects of treatments on nodules or roots were apparent. Mean levels of leghemoglobin in nodules from 48-day-old plants ranged between 93 and 102 nmol per g fresh wt and no statistically significant differences among the four treatments were observed. Hydrogenase activity of bacteroids isolated from nodules just before the first harvest of shoots (29-day-old plants) ranged between 112 and 128 nmol of H$_2$ taken up per min per mg of protein, but the effect of treatments was not significant (Table 2). In the subsequent two harvests, however, hydrogenase activity was significantly higher in nodules from plants that received supplementary Ni. Nodule bacteroids from plants receiving Ni and collected just before the second harvest of shoots (45-day-old plants) showed 33% higher hydrogenase specific activity than comparable bacteroids from plants grown without added Ni or bacteroids from plants provided with Cr, Pb, Sn, and V instead of Ni. No synergistic effect was observed from the combination of metals and Ni. Although nodules from 68-day-old plants had begun to senesce, a significant difference in hydrogenase activities of bacteroids from plants provided with and without Ni was still evident (Table 2). Ni added to isolated bacteroids during the H$_2$ uptake assay did not enhance hydrogenase activity.

Effect of Ni on Hydrogenase Derepression and Chemo-lithotrophic Growth of R. japonicum. Hydrogen uptake-positive (Hup$^+$) wild-type strains of R. japonicum are capable of growing as chemo-lithotrophs (20), utilizing H$_2$ for energy and CO$_2$ fixation via ribulose bisphosphate carboxylase as a source of carbon (34). In many ways, R. japonicum resembles A. eutrophus, the hydrogen bacterium, which requires Ni for chem-
olithotrophic growth and derepression of hydrogenase (9, 15).
It was of interest, therefore, to determine whether Ni had an
effect on derepression of hydrogenase in a Hup+ strain of R.
japonicum.

*R. japonicum* was grown heterotrophically for three genera-
tions in a purified medium to deplete endogenous levels of Ni.
Cells were then incubated under conditions required for hy-
drogenase derepression (see legend of Fig. 2). In the absence
of added Ni, hydrogenase activity was not detected or was
minimally expressed (<0.5 nmol of H2 taken up per min per mg of
protein) during the 24-hr incubation period (Fig. 2). When 0.5
μM NiCl2 was included in the medium, cells expressed hy-
drogenase activity (9.2 nmol of H2 taken up per min per mg of
protein), reaching a maximum level within 12 hr (Fig. 2). This
maximum was considerably lower than hydrogenase activities
in cultures of chemolithotrophically grown *R. japonicum*
previously reported from this laboratory (2). The lower hydrogenase
specific activity reported here may be related to a suboptimal
level of O2 (21) to cultures or to other limitations associated
with the strict conditions demanded in order to assure minimal Ni
contamination.

In the presence of 5 μM NiCl2 and 8 μM chloramphenicol
to inhibit protein synthesis, no appreciable hydrogenase was ex-
pressed during a 24-hr incubation period (Fig. 2). *R. japonicum*
cells that were incubated initially for 12 hr without Ni exhibited
low levels of hydrogenase activity within 6 hr after Ni had been
added, and the activity increased 3-fold during the next 4–5 hr.

![Fig. 2](image)

**Fig. 2.** Effect of Ni on hydrogenase derepression in *R. japonicum*
122 DES. Cells were grown heterotrophically in a defined purified me-
dium and carried through three growth cycles to late logarithmic
phase in order to dilute initial Ni. Cells were washed aseptically three
times in a purified saline medium and divided into four aliquots. The cell sus-
pensions were treated as follows: o, NiCl2 (5 μM) at 0 hr; ∆, no NiCl2 added;
△, chloramphenicol (8 μM) added before adding NiCl2 (5 μM) at
0 hr; ■, NiCl2 (5 μM) added at 12 hr. Three milliliters of cell suspension
was introduced aseptically into sterile acid-washed 17 × 100 mm poly-
propylene tubes, which were sealed with serum stoppers. At 0 hr, the
tubes were evacuated and refilled with a gas mixture containing 4%
H2, 5% CO2, 1% O2, and 90% N2. Duplicate cultures of each treatment
were removed at each time indicated, briefly sparged with N2 to re-
move H2, and then assayed amperometrically.

In other experiments in which Ni was omitted and 1 μM con-
centrations each of Cr, Sn, V, and Pb were added individually
or in combination, no stimulation or inhibition of the derepres-
sion of hydrogenase was observed. These results indicate con-
siderable specificity of Ni as a micronutrient element for hy-
drogenase expression in *R. japonicum*. Because no synthetic chelating agents were included in the medium and all of the trace
elements known to be required by *Rhizobium* were provided,
the observed Ni requirement for hydrogenase derepression must
have been due to the Ni rather than some nonspecific effect.

Because Ni was required for hydrogenase derepression in *R.
japonicum*, experiments were conducted (Fig. 3) to determine
whether Ni is required for chemolithotrophic growth of this
bacterium. Cultures without added NiCl2 showed no increase in
absorbance during the 27-day incubation period (Fig. 3). In contrast,
cultures with 0.5 μM NiCl2 began to increase in ab-
sorbance after 11 days and reached a maximum of about 0.45
OD after 20 days. It seems probable that the plateau in growth
of the cultures was due to O2 limitation, because it has been
shown that chemolithotrophic cultures of *R. japonicum* will at-
tain optical densities of 1.5 or more when provided with a gradu-
al programmed increase in O2 supply (21). At intervals
throughout the growth period in the experiment described
in Fig. 3, protein contents and O2-dependent H2 uptake rates
of cells were measured. During the first 6 days no hydrogenase
activity of cells was detected, then the specific activity (nmol of
H2 taken up per min per mg of protein) increased rapidly from
8 at 11 days to 19 at 15 days, then 28 at 21 days, where a plateau
was reached. The curve (not presented) of increasing hy-
drogenase specific activities during growth approximately paralleled
the curve showing increase in growth (Fig. 3).

These results indicate that Ni is required for hydrogenase de-
pression and chemolithotrophic growth of a Hup+ strain of *R.
japonicum*. From reports by Friedrich et al. (8, 9) with *A. eu-

![Fig. 3](image)

**Fig. 3.** Effect of Ni on the chemolithotrophic growth of *R. japon-
icum*. Cultures of the Hup+ strain 122 DES were grown in acid-washed
polypropylene screw-cap vials containing 5 ml of purified mineral salts/
vitamins medium. The inoculum was prepared and washed as de-
scribed in the legend of Fig. 2. Two culture vials without Ni (□) and two
with 0.5 μM NiCl2 (○) were placed in each of a series of 1.9-liter glass
jars and sealed. The composition of the gas phase in the jars was ad-
justed to 4% H2, 5% CO2, 0.1% O2, and 90.8% N2. The jars were incu-
bated at 27°C on a shaker at 60 strokes per min. Gases in the jars were
monitored daily by removal of samples through a rubber serum cap and
concentrations were adjusted when necessary. Individual jars were re-
moved at designated intervals and the cell suspensions were assayed
for optical density, protein content, and H2-oxidizing capacity.
trophus, by Partridge and Yates (13) with Azobacter chroo-
coccum, by Takakuwa and Wall (14) with Rhodopseudomonas
sphaeroides, and the information summarized in reviews (1, 15),
it appears that Ni is required for the function of membrane-bound
hydrogenases in most microorganisms. The results of our in-
vestigation extend the biological role of Ni to R. japonicum, a
bacterium of great importance in agriculture.

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