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Nickel Accumulation and Storage in *Bradyrhizobium japonicum*†

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Hydrogenase-derepressed (chemolithotrophic growth conditions) and heterotrophically grown cultures of *Bradyrhizobium japonicum* accumulated nickel about equally over a 3-h period. Both types of cultures accumulated nickel primarily in a form that was not exchangeable with $NiCl_2$, and they accumulated much more Ni than would be needed for the Ni-containing hydrogenase. The nickel accumulated by heterotrophically incubated cultures could later be mobilized to allow active hydrogenase synthesis during derepression in the absence of nickel, while cells both grown and derepressed without nickel had low hydrogenase activities. The level of activity in cells grown with Ni and then derepressed without nickel was about the same as that in cultures derepressed in the presence of nickel. The Ni accumulated by heterotrophically grown cultures was associated principally with soluble proteins rather than particulate material, and this Ni was not lost upon dialyzing an extract containing the soluble proteins against either Ni-containing or EDTA-containing buffer. However, this Ni was lost upon pronase or low pH treatments. The soluble Ni-binding proteins were partially purified by gel filtration and DEAE chromatography. They were not antigenically related to hydrogenase peptides. Much of the ^{63}Ni eluted as a single peak of 48 kilodaltons. Experiments involving immunoprecipitation of ^{63}Ni-containing hydrogenase suggested that the stored source of Ni in heterotrophic cultures that could later be mobilized into hydrogenase resided in the nonexchangeable Ni-containing fraction rather than in loosely bound or ionic forms.

One of the bacterial enzymes produced during the legume root nodule symbiosis between soybeans and some *Bradyrhizobium japonicum* strains is a hydrogen uptake hydrogenase. In association with a cytochrome-dependent electron transport chain (29), hydrogenase catalyzes the oxidation of hydrogen to water with the production of ATP (10, 13). Hydrogenase is produced both in the bacteroids in root nodules and in free-living cells. In free-living conditions, hydrogenase enzyme synthesis is derepressed by incubation with H_2 and CO_2 in microaerophilic conditions (12, 22).

Bartha and Ordal (3) provided the first unequivocal evidence for nickel-dependent growth of a bacterium; growth of Knallgas bacteria on H_2 and CO_2 required the addition of nickel to the medium. The *B. japonicum* hydrogenase, like many other hydrogenases, is a nickel-containing enzyme (1, 34). In addition to its playing a catalytic role, presumably at the active site of Ni-containing hydrogenases (19), nickel appears to play a role in regulating the expression of hydrogenase synthesis in free-living *B. japonicum* (35).

In addition to hydrogenase, other bacterial nickel-containing enzymes are urease, carbon monoxide dehydrogenase, and methylcoenzyme M reductase of methanogenic bacteria (for a review of sources and properties of nickel-containing enzymes, see references 14 and 19). It would not be surprising that bacteria containing one or more of these enzymes can sequester Ni. Indeed, some of them have been shown to express efficient or high-affinity Ni transport systems (6–8, 16, 20, 21, 37, 38, 40), and progress in determining the mechanisms of bacterial Ni transport, including the genes involved, is evident (9, 31). Almost nothing is known about how a bacterium handles nickel from the time it is taken up to when it is incorporated into an enzyme. However, from the initial observations, it appears that little of the Ni that is rapidly accumulated by bacteria accumulates as nickel ions; most that is not exchangeable with externally added Ni ions (7, 16, 38, 40) is observed in (presumably) protein-containing fractions. In *B. japonicum*, nickel-starved cells accumulate Ni rapidly, and most other metals have little effect on the uptake rate (32). Also, the bulk of the nickel uptake has been shown to be energy independent, and after a brief exposure to Ni, cell-associated nickel is not exchangeable with exogenous Ni over a 15-min period.

In this study, we addressed the sinks for nickel accumulation in *B. japonicum*. Nickel accumulation rates were not greater when cells were synthesizing hydrogenase but were similar in cells incubated under chemolithotrophic and heterotrophic growth conditions. We found that *B. japonicum* can accumulate nickel into cytoplasmic components, that most of the nickel appears to be bound to soluble proteins, and that some nickel-binding proteins can be partially purified. These physiological studies suggest that this nickel can be mobilized into hydrogenase during hydrogenase derepression conditions.

MATERIALS AND METHODS

Chemicals and glassware. Glassware used for nickel-free growth and derepression of hydrogenase was routinely soaked in 2 M nitric acid and extensively rinsed with deionized H_2O. Radioactive $^{63}NiCl_2$ was purchased from Dupont, NEN Research Products, Boston, Mass., and was of the highest specific activity available (7.4 mCi/mg). Gases were purchased from Potomac Airgas, Baltimore, Md. Ultrapure trace elements (Puratronic grade 1) were purchased from Morton Thiokol, Alfa Products, Danvers, Mass., and were certified to be ≥99.99% pure. CPG-8-hydroxyquinoline (particle size 124 μm; pore diameter, 50.0 nm) was purchased from Pierce Chemical Co., Rockford, Ill. All other chemicals were purchased either from Sigma Chemical Co., St. Louis, Mo., or J. T. Baker Chemical Co., Phillipsburg,

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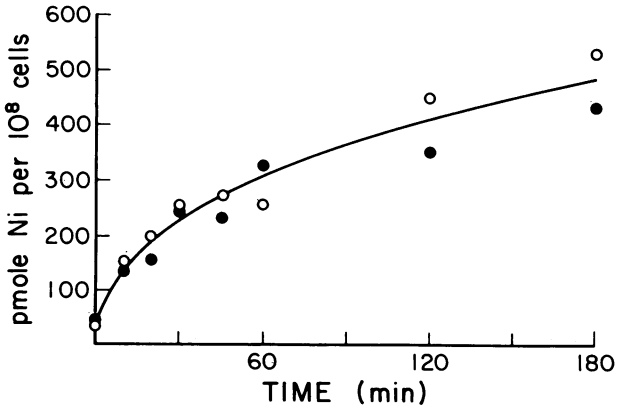

FIG. 1. Nickel accumulation by *B. japonicum*. Ni-starved cells were incubated in heterotrophic medium (○) or derepression medium with H_2 and CO_2 (●) and 5.0 μM $NiCl_2$ containing 0.02 μCi of ^{63}Ni/ml, and cell-bound Ni was determined as described in the text.

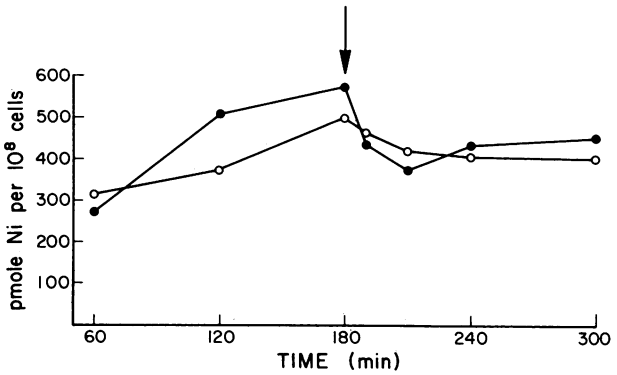

FIG. 2. Exchangeability of accumulated Ni. Cells that had accumulated Ni for 180 min (see the legend to Fig. 1) then received 100 μM nonradioactive $NiCl_2$ (↓), and the remaining cell-associated ^{63}Ni was determined at 10, 30, 60, and 120 min after the shift to excess unlabeled Ni. Cells were grown heterotrophically (○) or in derepression medium (●).

N.J., and were of reagent grade or better. Antiserum against the *Alcaligenes latus* hydrogenase holoenzyme was kindly provided by Daniel Arp.

Strains and growth conditions. *B. japonicum* SR has been described previously (24). Modified Bergersen medium (MB) has been described previously (4). Medium was passed over controlled pore glass 8-hydroxyquinoline (CPG-8-hydroxyquinoline) to remove trace elements by the method of Eskew et al. (11). Glycerol, sodium glutamate, $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$ and all trace elements were added to the medium after the CPG-8-hydroxyquinoline treatment. All cultures were grown at 29°C as described previously (32). For some experiments (dialysis of soluble Ni-containing extract and purification of Ni-binding proteins), cells were grown in MB containing 0.2 g of NH_4Cl per liter instead of sodium glutamate as the N source.

Hydrogenase derepression. Cells grown in MB were harvested at mid-log phase, washed, and suspended to an optical density at 540 nm (OD_{540}) of 0.5 in CPG-8-hydroxyquinoline-treated mineral medium consisting of (grams per liter) K_2HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.1), $MgCl_2 \cdot 6H_2O$ (0.51), and NH_4Cl (0.1) (derepression medium). Suspended cells (10 ml) were placed in 250-ml prescription bottles, nickel and EDTA were added as noted in the text, and the bottles were sealed with a rubber serum stopper. The bottles were then sparged through the serum stopper with 10% H_2–5% CO_2–1% O_2–84% N_2 and derepressed for 24 h at 29°C, shaking at 120 rpm (23). These cells were then used as the derepressed samples (Fig. 1 and 2). After growth with or without Ni, cells were washed three times in Ni-free derepression medium prior to the start of derepression (Table 1).

Hydrogen uptake assay. Hydrogen uptake in whole cells was measured amperometrically by using H_2-saturated H_2O as a standard (34, 39). Cell numbers were calculated from a standard curve of OD_{540} versus viable cell number.

Nickel accumulation assays. Nickel uptake by whole cells and the exchangeability of the incorporated Ni were assayed similarly to previously described procedures (32). Cells grown in Ni-free MB (5.5×10^8 cells per ml) or derepression medium (3.6×10^8 cells per ml) with H_2 and CO_2 (see text above) were suspended in the same medium and conditions but with added Ni (5.0 μM $^{63}NiCl_2$ at 0.02 μCi per ml of cells). Samples (0.5 ml) were removed at the times indicated and were rapidly filtered (32), and the filters were washed with two 10-ml amounts of 50 mM MES [2-(*N*-morpholino) ethanesulfonic acid], pH 6.0 (32). The membrane filters (25-mm diameter, HA type; Millipore Corp.) were placed into 5-ml scintillation vials into which 4 ml of Optifluor (Dupont, NEN) was then added. The vials were vortexed, and radioactivity was counted in a Rackbeta scintillation counter (LKB Instruments, Inc., Rockville, Md.). Three replicate 0.5-ml filtered samples were used for each datum point, and counts per minute from control experiments (without cells) were subtracted from the sample values. Background levels from reaction mixtures containing ^{63}Ni but without cells were about 1,100 cpm, while the experimental values ranged from 12,700 to 440,000 cpm. For the exchangeability experiment, after 180 min of Ni accumulation from 5.0 μM $^{63}NiCl_2$, 100 μM nonradioactive nickel ($NiCl_2$) was added and samples were removed for assay as described above and in the legend to Fig. 2. Similar procedures were used for determining Ni retention after dialysis, membrane fractionation, or purification steps, except that 0.1-ml samples were assayed for ^{63}Ni. Cell-free background counts (protein-containing extracts without ^{63}Ni) for these latter experiments ranged from 0 to 55 cpm.

Dialysis conditions. Cells used in dialysis experiments were grown on the mineral medium described above in the presence of $^{63}NiCl_2$ (15 μCi/liter) to an OD_{540} of 0.5. The cells were harvested and washed two times with 0.05 M potassium phosphate, pH 6.9, containing 0.1 M NaCl. The sus-

TABLE 1. Effect of derepression conditions and previous Ni status on whole-cell hydrogenase activity

Derepression condition	Hydrogenase activity (nmol of H_2 uptake/h per 10^8 cells)[a]		
	Grown without Ni	Grown with Ni and washed with Ni-free medium	Grown with Ni and washed with EDTA medium
No nickel	14	227	194
Ni (5 μM)	170	264	230
EDTA (6 μM)	22	115	125
Ni (5 μM) plus EDTA (6 μM)	112	197	139
10% O_2	<2	<2	<2

[a] Each value is the average of two independent amperometric H_2 uptake determinations.

pended pellet was then broken in a French pressure cell (27). Phenylmethylsulfonyl fluoride was added to 1 mM prior to breaking. Membranes were removed by centrifuging the crude extract at 100,000 × *g* for 2 h (27). The resulting supernatant was dialyzed by using cellulose membrane dialysis tubing (Sigma Chemical Co.) at 4°C for 72 h against three changes of 0.05 M potassium phosphate, pH 6.9, containing 0.1 M NaCl. Other aspects of these experiments are noted in the text.

Fractionation and partial purification: cell growth. Cells used in the partial purification were grown on the medium described above (MB containing 0.2 g of NH_4Cl per liter substituted for glutamate) and 15 μCi of $^{63}NiCl_2$ per liter. Crude supernatant was prepared in the same manner as for the dialysis experiments. For isolation and fractionation of cells into soluble, inner membrane, and outer membrane fractions, cells were grown in MB, and crude extracts were prepared (27). Crude extract (2 ml) containing 8.5 mg of protein per ml was layered onto a 10 to 65% sucrose density step gradient. Conditions were essentially as described previously for separation of inner and outer membrane fractions of *R. japonicum* SR (27), except that the centrifugation time was 20 h at 22,000 rpm and the rotor was an SW25.1. Fractions (5 ml) were removed, starting from the bottom of the gradient tube, and 75-μl amounts were assayed for ^{63}Ni. The fraction labeled IM (inner membrane) contained succinate oxidase activity, and hydrogenase activity was detected in this fraction when isolated from Hup^c strain SR470 (25) grown in MB.

Chromatographic fractionations. Gel filtration chromatography was performed by using Sephacryl S-200 Superfine beads (Pharmacia, Inc., Piscataway, N.J.). Crude supernatant (8 to 10 ml) was loaded onto a 350-ml included volume column and allowed to settle into the gel. This was then overlaid with 0.05 M potassium phosphate (pH 6.9) buffer containing 0.1 M NaCl and run at approximately 6 ml/h. The fractions collected were assayed for the presence of ^{63}Ni, and peak fractions were pooled and transferred into 0.01 M Tris, pH 8.5, containing 0.1 M NaCl by ultrafiltration with a YM10 membrane (Amicon Corp., Danvers, Mass.). The pool was washed and concentrated to about 10 ml. This pool was then loaded onto a DEAE-Sepharose CL-6B column (Sigma), and the bound protein was eluted with a linear 0.1 to 0.5 M NaCl gradient. Again, the collected fractions were assayed for the presence of ^{63}Ni (see above). Protein concentration was determined by the dye binding method of Bradford (5).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (18). Silver staining was performed by the method of Morrissey (26).

Immunoprecipitation of ^{63}Ni-labeled hydrogenase. SR cells were grown in 1 liter of MB containing 50 μCi of ^{63}Ni (final Ni concentration, 0.15 μM) to an OD_{540} of 0.65 (approximately 7×10^8 cells per ml) and then incubated in the same medium containing nonradioactive nickel (200 μM) for 3 h. Then the cells were harvested by centrifugation, and the cell pellet was washed two times with derepression medium lacking nickel. The final suspension was split into two equal 500-ml amounts and centrifuged. One cell pellet was kept on ice for 36 h, and the other pellet was suspended in fresh derepression medium to a cell concentration of 5×10^8 cells per ml and derepressed by incubation in an atmosphere of 10% H_2–5% CO_2–1% O_2–84% N_2 for 36 h (23). The MB-grown and the derepressed cells were broken by passage through a French pressure cell (27) after suspension of cells in 0.05 M Tris, pH 7.4, containing 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride. The crude extract was centrifuged at 100,000 × *g* to harvest membranes, and the membranes were suspended in immunoprecipitation buffer composed of 0.05 M Tris hydrochloride (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100, as described by Suissa and Reid (36). After solubilization of hydrogenase by homogenization and incubation for 15 min at 23°C, the extract was again subjected to ultracentrifugation to remove nonsolubilized material. Immunoprecipitation was performed similarly to that described previously (28), except that antiserum against *B. japonicum* hydrogenase subunits was not used as it reacts poorly with the intact holoenzyme. The supernatant was transferred to a vial, and antiserum against *A. latus* hydrogenase (2) was added at 1:200 dilution. The solution was incubated overnight at 4°C with the antibody, *Staphylococcus aureus* with protein A (Pansorbin; Calbiochem-Boehring) was added and incubated for 60 min (28), and the mixture was washed five times with immunoprecipitation buffer (6,000 × *g*, 23°C). The final washed pellet was suspended in immunoprecipitation buffer, and samples were counted for ^{63}Ni. The solubilized membrane extracts contained 2.9 and 3.3 mg of protein per ml for the MB-grown and derepressed cells, respectively. Because these extracts contained detergent, the BCA protein assay system (Pierce) was used for these samples.

RESULTS

Nickel accumulation. In initial studies of nickel accumulation in *B. japonicum* (32), we presumed that hydrogenase was a major sink for Ni and therefore that Ni accumulation rates would coincide with the level of hydrogenase expression. However, despite many attempts, we could not observe any correlation between Ni uptake rates and hydrogenase expression or the onset of derepression (data not shown). In fact, when Ni^{2+} uptake was measured in 5.0 μM Ni^{2+} over a 3-h period in previously Ni-starved cells, both hydrogenase derepressed (chemolithotrophic) and heterotrophic growth-cultured cells accumulated nickel about equally (Fig. 1). The hydrogenase derepressed culture (Fig. 1) had low (10 nmol/h per 10^8 cells) hydrogenase activity, probably due to Ni limitation. Even initial Ni uptake rates were not significantly greater in Ni-starved, hydrogenase-derepressed cultures than in heterotrophically incubated cultures (data not shown). Therefore, the ability to steadily accumulate Ni was not dependent on or correlated with the derepression of hydrogenase.

Nickel exchangeability. Previously, it was shown that most (about 85%) of the ^{63}Ni taken up in 1 min by *B. japonicum* SR473 was not exchangeable with an excess of nonradioactive Ni (34). It was possible that the Ni which accumulated in the heterotrophic cells (Fig. 1) was either loosely or nonspecifically bound to the cell surface or remained in ionic forms as nickel salts whereas the Ni in the chemolithotrophic incubated cells was bound to intracellular components, such as hydrogenase. Therefore, we wished to determine the exchangeability of the Ni accumulated over the 3-h period in chemolithotrophic versus heterotrophic cells. Figure 2 shows an experiment in which the exchangeability of Ni (from 5.0 μM Ni) accumulated in 3 h was compared for the two types of cells. Some accumulated Ni was lost upon addition of 100 μM nonradioactive Ni, but in both cases most of the cell-associated Ni that was lost was eliminated in the first 10 min and both types of cells eventually lost about 20% of the nickel that had accumulated in the first 180 min.

Therefore, for both types of cells, most of the nickel taken up appears to be tightly associated with cellular components and does not merely accumulate in cells as $NiCl_2$ or other ionic forms. In contrast, similar experiments with *Alcaligenes eutrophus* (37) showed that a large portion of the accumulated Ni was exchangeable with nonradioactive Ni in 20 min.

Derepression in the presence or absence of Ni. Previous studies showed that hydrogenase of *B. japonicum* is a nickel-containing enzyme (1, 34) and that *B. japonicum* requires nickel in order to synthesize hydrogenase (34). We set out to determine if the nickel accumulated by heterotrophically grown *B. japonicum* could later be mobilized to allow hydrogenase activity during derepression in the absence of nickel. Cells were grown either in the presence or absence of 5 μM $NiCl_2$ and then harvested and thoroughly washed (three consecutive suspensions and centrifugations in Ni-free derepression medium [see Materials and Methods]) in order to remove as much nickel as possible that might remain associated with the cell. Cells were also repeatedly washed with Ni-free, EDTA-containing medium to ensure removal of nonspecifically bound Ni. The cells were then derepressed and assayed for hydrogenase activity (Table 1). Cells that had previously been grown with nickel were able to express hydrogenase in the absence of nickel, while cells that were grown in the absence of nickel expressed only low levels of hydrogenase activity. The level of activity in cells grown with Ni and then derepressed without Ni was about the same as that in those derepressed with Ni. This suggested that internally accumulated nickel could be utilized later during hydrogenase formation and was of sufficient quantity to supply the entire Ni requirement of hydrogenase.

Previous results showed that 6 μM EDTA inhibited hydrogenase derepression of *B. japonicum* in Ni-poor medium (34), presumably by chelating trace amounts of nickel in the derepression media. Like the Ni-free derepression conditions for cells grown without Ni, low hydrogenase activity was observed with cells derepressed in the presence of 6 μM EDTA. However, results from Table 1 show that the addition of 6 μM EDTA to the derepression flasks did not greatly inhibit the derepression ability of cells that had been previously allowed to accumulate nickel. This result indicates that the nickel utilized during derepression originated from the cells and not from possible trace amounts of the metal in the derepression medium; this finding strengthens the conclusion that previously accumulated nickel could be mobilized for use in hydrogenase expression. Adding nickel along with EDTA allowed both sets of cells (Ni starved and Ni sufficient) to derepress hydrogenase, as expected, if the results are truly due to nickel status and not another (damaging) physiological factor.

Immunoprecipitation of ^{63}Ni-containing hydrogenase. The above results suggested that internalized Ni pools, accumulated during heterotrophic growth, could later be mobilized into hydrogenase. Also, experiments (Fig. 2) showed that about 20% of the nickel accumulated as $NiCl_2$, as other ionic forms, or at least as loosely bound nickel, whereas about 80% could not be exchanged with externally added Ni. To determine if the source of Ni that was mobilized into hydrogenase came from the nonexchangeable pool, we grew cells in ^{63}Ni, then exchanged a portion of the accumulated Ni with a 100-fold greater level of nonradioactive Ni, and then derepressed the cells for hydrogenase in the absence of Ni. If Ni in hydrogenase came from the nonexchangeable pool accumulated during heterotrophic growth of the cells, then the finally isolated hydrogenase should contain label. The derepressed culture contained a within-normal range of hydrogenase activity, 148 nmol of H_2 per h per 10^8 cells. Immunoprecipitation of hydrogenase with hydrogenase-specific antibody from Triton X-100-solubilized extracts (see Materials and Methods) of membranes revealed that the solubilized membranes of hydrogenase-derepressed cells contained 466 cpm/mg of solubilized membrane protein compared with 122 cpm for the control (nonderepressed cells). The results suggest that the ^{63}Ni in hydrogenase can originate from the nonexchangeable pool accumulated during heterotrophic growth and strengthens the conclusion that Ni stored during heterotrophic growth can later be mobilized into hydrogenase.

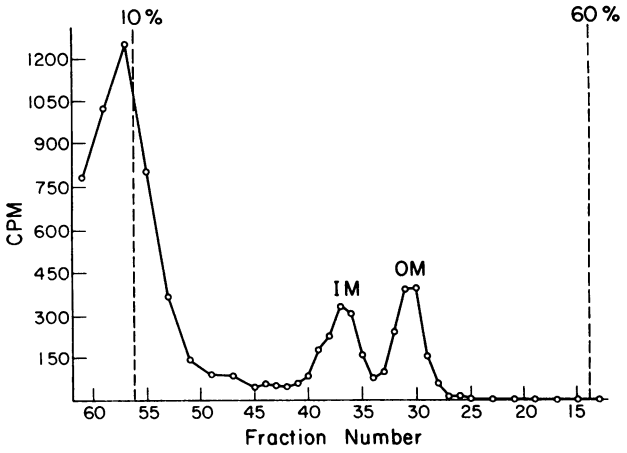

FIG. 3. Sucrose gradient separation of ruptured ^{63}Ni-grown cells into ^{63}Ni-containing soluble, inner membrane (IM), and outer membrane (OM) fractions. The 10% and 60% dashed lines show the centers of the 10% and 60% sucrose layers.

Internalized Ni pools. The results described above suggested that *B. japonicum* was able to use previously accumulated nickel. In order to further study this, we decided to determine the sources of the internalized ^{63}Ni in *B. japonicum*. A crude extract from cells grown in ^{63}Ni was fractionated on a sucrose gradient by centrifugation. The inner membrane, outer membrane, and soluble fractions were distinctly observable (Fig. 3). Of the three fractions, most of the ^{63}Ni was associated with the soluble components and did not migrate with particulate matter. About 70% of the total ^{63}Ni remained in the soluble fraction (Fig. 3), but this fraction contained only about 35% of the total cell protein (data not shown). When this soluble supernatant fraction was dialyzed against phosphate buffer for 72 h (Materials and Methods), 73% of the ^{63}Ni was retained (Table 2). An excess of cold nickel (10 μM) was not able to exchange with the supernatant fraction ^{63}Ni, nor was EDTA able to chelate it away. Reduction of the protein in this fraction by including dithiothreitol in the dialysis buffer (to disrupt sulfhydryls) was unable to remove the bound ^{63}Ni. However, when the soluble portion was dialyzed against pH 2.5 glycine buffer, essentially all of the ^{63}Ni counts were lost in the dialysis. Likewise, incubation of the soluble supernatant with 5 mg of pronase at 37°C for 30 min prior to dialysis resulted in the loss of most of the ^{63}Ni of the dialyzed supernatant fraction. Incubation of the soluble supernatant with DNase had no effect on bound ^{63}Ni (data not shown). The fractionation and dialysis experiments (Fig. 3 and Table 2) suggest that the bulk of nickel internalized by *B. japonicum* is bound to soluble proteins.

TABLE 2. Effect of dialysis conditions on the retention of Ni by the soluble extract fraction

Dialysis condition(s) or pretreatment	% Retention[a]
Potassium phosphate[b]	73
Plus 10 μM $NiCl_2$	71
Plus 15 μM EDTA	76
Plus dithiothreitol (0.15 mg/ml)	76
pH 2.5	<1
Pronase pretreatment[c]	12

[a] Each value is the ratio of ^{63}Ni remaining after dialysis to ^{63}Ni loaded in counts per minute. A 0.75-ml sample of soluble proteins, containing approximately 2,000 cpm, was loaded into the dialysis tubing. Dialysis was performed at 4°C for 72 h.

[b] Potassium phosphate buffer (0.05 M, pH 6.9) containing 100 mM NaCl. This is the solution in which the extract was prepared.

[c] Extract was treated with 5 mg of pronase per ml for 30 min prior to the start of dialysis.

Partial purification of nickel-binding proteins. We attempted to purify the soluble proteins that contain nickel. Gel filtration chromatography showed that a large amount of ^{63}Ni eluted as a single peak with a molecular mass of about 48 kilodaltons (Fig. 4). The major peak in Fig. 4 contained about 25% of the total ^{63}Ni loaded. Most of the protein eluted in the void volume (data not shown), but no ^{63}Ni was detected in the void volume fractions. The peak fractions from Fig. 4 (fractions 58 to 62) were then run over a DEAE ion-exchange column. Again, the ^{63}Ni eluted from the column as a single peak. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the pooled DEAE fractions containing most of the nickel showed approximately six bands, but the number of bands varied (up to eight) with different preparations (data not shown). Therefore, we do not know which specific bands are associated with Ni-binding peptides, and in spite of many attempts, further progress was impossible due to a continual loss of ^{63}Ni signal from the partially purified preparation. The Ni-associated peptides are not antigenically related to hydrogenase, as immuno-dot blot assays (33) of the soluble fraction from free-living *B. japonicum* showed no reaction with anti-hydrogenase antibodies (35; data not shown).

DISCUSSION

Nickel is required for the synthesis and function of *B. japonicum* hydrogenase (34, 35). Cells exposed to nickel are able to accumulate it, and the data presented here suggest that the cells are able to sequester the nickel and store it until needed. Ni accumulation was not dependent on the synthesis of hydrogenase, but previous Ni nutritional status affected the ability of cells to synthesize hydrogenase later. The fact that most of the nickel taken up is bound to soluble protein suggests that the cells are able to produce proteins that can bind nickel once it is internalized. It should be pointed out that none of the nickel-binding protein(s) described here is hydrogenase, nor is it structurally related to hydrogenase peptides. Wild-type SR cells do not produce hydrogenase unless derepressed, and no proteins in the nonderepressed cells cross-react with anti-hydrogenase antiserum (35). Cells used in the fractionation, dialysis, and partial purification were not subjected to derepression conditions.

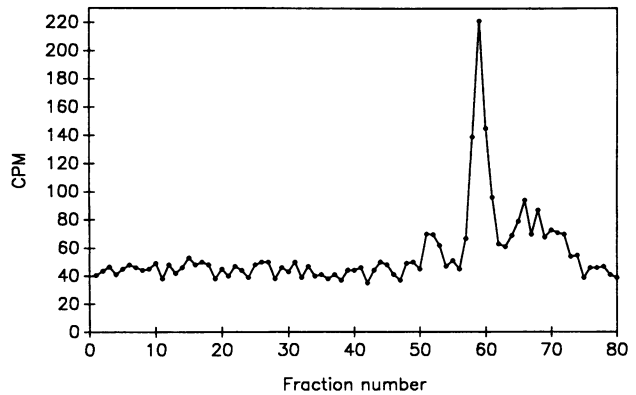

FIG. 4. Fractionation of ^{63}Ni proteins on a gel filtration column. The soluble extract (see text) was loaded, and fractions were removed for ^{63}Ni determination. Most of the protein was eluted in the initial (void volume) fractions (see text).

While we have not yet assigned a function to any of the proteins associated with the Ni-containing fractions, the idea of a trace element-binding protein that plays a storage role is not a new one. Pienkos and Brill (30) found that *Azotobacter vinelandii* was able to take up and store molybdenum. They purified a protein capable of binding at least 15 molybdenum atoms per molecule of protein. Hinton and Mortenson (15) isolated a molybdenum storage protein from a mixture of molybdenum-binding proteins in crude extracts of *Clostridium pasteurianum*. It has also been suggested the *Escherichia coli* and *Salmonella typhimurium* are capable of storing iron (17). It remains to be seen whether the proteins we isolated in the ^{63}Ni-containing fractions serve as a nonspecific sink for nickel or have some other (i.e., storage) function.

Intracellular nickel pools have been studied in other nickel-requiring bacteria, but these pools have not been characterized. Most of the nickel taken up by *Methanobacterium thermoautotrophicum* grown in Ni-limited conditions is used to synthesize the nickel tetrapyrrole factor F_{430} and hydrogenase (12). However, when grown in high-Ni conditions (2 μM Ni^{2+}), 50% of the Ni was associated with unidentified proteins other than hydrogenase (12). Likewise, much of the Ni sequestered by *Methanobacterium bryantii* was precipitable with acetone (16), and Ni-containing extracts of *Acetogenium kivui* (40) and *Clostridium thermoaceticum* (21) were composed of unidentified low-molecular-weight anionic species on the basis of gel electrophoresis. Thus far, there is no evidence that these fractions may act as temporary storage pools for Ni. In most cases, the proportion of Ni as free nickel (nickel ions) among the internal pool is low; after uptake of nickel by *Rhodobacter capsulatus* (38), *M. bryantii* (16), *A. kivui* (40), and *B. japonicum* (this study), little of the internal ^{63}Ni is exchangeable with exogenous Ni. One exception is the report that *A. eutrophus* effluxes 62% of the internalized Ni within 20 min of incubation in 60 μM Ni (37), although the bulk of the Ni accumulated by the low uptake activity, Ni-specific transport system of *A. eutrophus* is thought to be utilized for hydrogenase formation (20).

We observed no correlation between hydrogenase derepression and Ni accumulation ability. This is really not surprising if one considers the small effect of hydrogenase (with one Ni atom per hydrogenase molecule) as an Ni sink in view of the fact that the bacterium is capable of accumulating over 300 pmol per 10^8 cells (32; this study). Even if hydrogenase in this bacterium is a major component of the total protein pool, much less than a few picomoles of Ni should be needed per 10^8 cells. In contrast to the results for *B. japonicum*, *C. pasteurianum* sequestered Ni more rapidly

when incubated with an initial gas phase containing H_2 (6) and *A. eutrophus* Ni uptake rates were 10-fold greater when cells were cultured chemolithotrophically with H_2 and CO_2 versus heterotrophically (37).

B. japonicum is capable of sequestering significant levels of nickel even during heterotrophic growth, and physiological studies suggest the Ni accumulated during such growth can be utilized later for active hydrogenase synthesis. Experiments with EDTA-washed cells indicated that the source of the Ni for hydrogenase came from Ni tightly associated with cell components. Other experiments in which ^{63}Ni-containing hydrogenase was immunoprecipitated after exchanging with nonradioactive Ni indicated that the nonexchangeable ^{63}Ni pool is the stored source of nickel used for hydrogenase. The properties of the soluble proteins capable of sequestering a large part of the accumulated nickel are of obvious interest. Some ^{63}Ni-containing proteins could be identified as possible storage forms of nickel. If ^{63}Ni with a higher specific activity can be obtained, perhaps these Ni-binding components could be further characterized.

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