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# Selenium Increases Hydrogenase Expression in Autotrophically Cultured *Bradyrhizobium japonicum* and Is a Constituent of the Purified Enzyme†

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**We have investigated the effect of added selenite on autotrophic growth and the time course of hydrogen oxidation derepression in *Bradyrhizobium japonicum* 122DES cultured in a medium purified to remove selenium compounds. In addition, hydrogenase was purified to near homogeneity and examined for the specific incorporation of Se into the enzyme. The addition of Se at 0.1 μM significantly increased total cell protein and hydrogenase specific activity of harvested cells. Also, the addition of $SeO_3^{2-}$ enhanced the time course of hydrogenase derepression by 133%, whereas $VO_3$, $AsO_2^{2-}$, $SO_2^{2-}$, and $TeO_3^{2-}$ failed to substantially affect hydrogenase derepression. During the final chromatographic purification of hydrogenase, a striking coincidence in peaks of protein content, Se radioactivity, and hydrogenase activity of fractions was obtained. The total Se content expressed per milligram of protein increased manyfold during the purification procedure. The mean Se content of the purified hydrogenase was 0.56 ± 0.13 mol of Se per mol of enzyme. These results indicate that Se is an important element in the $H_2$ metabolism of *B. japonicum* and that hydrogenase from *B. japonicum* is a seleno protein.**

The initial evidence demonstrating an important role of Se for microorganisms was provided by Pinsent (15), who showed a requirement for expression of formate dehydrogenase in *Escherichia coli*. Since this early work, there has been a marked increase in our knowledge of the requirements and role of Se, especially in certain anaerobic and facultatively anaerobic bacteria (25, 26). In addition to formate dehydrogenase from several microorganisms, clostridial glycine reductase (4, 25), nicotinic acid hydroxylase (5), xanthine dehydrogenase (29), and hydrogenase from *Methanococcus vannielii* (31) and *Desulfovibrio baculatus* (12) all contain Se as an essential constituent. To our knowledge, there are no reports of the occurrence of Se-containing enzymes in strictly aerobic microorganisms. However, Se has been shown to be essential for the marine diatom *Thalassiosira pseudonana* (16) and the freshwater alga *Chrysochromulina breviturriti* (30). The biochemical role of the element in these algae remains to be established.

The essentiality of Se for animals is well known (14, 23), and a role of this element as a constituent of glutathione peroxidase from animal sources (20) has been established. No conclusive evidence has been presented for a Se requirement for growth of higher plants in general or the presence of a Se-containing glutathione peroxidase (1). The early work of Trelease and Trelease (28) indicated that the addition of Se to a culture medium containing fixed nitrogen improved growth of the Se accumulator *Astragalus racemosus*. These results were questioned by Broyer et al. (2), who observed no beneficial effect of Se on the growth of *Medicago sativa* or *Trifolium subterraneum*. To our knowledge, no one has investigated the possibility of a Se requirement for legumes grown in symbiosis with appropriate rhizobia or a requirement for the culture of rhizobial endosymbionts. The goal of this research, therefore, was to determine whether a representative rhizobial inoculant exhibits a requirement for Se and to identify any site in which this element may play a metabolic role. For most of these experiments we utilized autotrophically cultured *Bradyrhizobium japonicum* because this bacterium under these conditions is dependent upon a functional hydrogenase for the utilization of $H_2$, the only source of energy for growth.

## MATERIALS AND METHODS

**Physiological experiments with purified media. (i) General precautions.** Purified water was prepared from distilled water, redistilled in glass, and then passed through a column (5 by 30 cm) of Amberlite MB-1 mixed bed resin (Mallinkrodt Chemical Co., St. Louis, Mo.). All containers were polypropylene or polycarbonate and were immersed for 2 h in 2% detergent (Count Off; Dupont, NEN Research Products, Boston, Mass.) and then overnight in 0.1 N HCl and rinsed five times in purified water. When sterilization was required, autoclaved containers were rinsed in sterile purified water before use. All liquids requiring sterilization were passed through washed filters because steam sterilization resulted in contamination. Plasticware was new initially, and all equipment used with Se was kept separate from that used with the culture treatments lacking added Se. In all instances cultures without Se addition were incubated in sealed containers separate from those receiving Se.

**(ii) Sources of chemicals.** The chemicals used for the preparation of the basal purified autotrophic medium are listed in Table 1. The macronutrient salts (Puratronic grade), 99.995% KOH, and iron wire were purchased from Johnson Matthey Chemicals Ltd. (Royston, England). Fe-EDTA was obtained by combining $FeCl_3$ (prepared by dissolving iron wire in redistilled 1 N HCl) with 99.5% EDTA (Fluka Chemical Corp., Ronkonkoma, N.Y.) dissolved in purified KOH. All other micronutrient salts (Table 1) were prepared

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TABLE 1. Composition and selenium contamination of basal medium used for autotrophic culture of *B. japonicum*[a]

| Medium components | Concn in final medium | Se content (pg-atoms liter$^{-1}$) of final medium constituents[b] |
|---|---|---|
| Macronutrients | | |
| $KH_2PO_4$ | 1.0 mM | <4.3 |
| $MgSO_4 \cdot 7H_2O$ | 1.5 mM | <6.3 |
| $CaCl_2 \cdot 2H_2O$ | 0.5 mM | <6.3 |
| $NH_4Cl$ | 5.0 mM | <1.6 |
| Micronutrients | | |
| Fe-EDTA | 2.5 μM | <6.3 |
| $NiCl_2 \cdot 6H_2O$ | 0.5 μM | |
| $CuCl_2 \cdot 2H_2O$ | 0.4 μM | |
| $CoCl_2 \cdot 6H_2O$ | 0.2 μM | <6.3 |
| $ZnSO_4 \cdot 7H_2O$ | 0.1 μM | |
| $MnCl_2 \cdot 4H_2O$ | 2.0 μM | |
| $K_2MoO_4 \cdot 2H_2O$ | 0.2 μM | |
| Vitamins | | |
| Thiamine hydrochloride | 250.0 μg liter$^{-1}$ | |
| Nicotinic acid | 250.0 μg liter$^{-1}$ | |
| Pyridoxal hydrochloride | 250.0 μg liter$^{-1}$ | <6.3 |
| Biotin | 4.0 μg liter$^{-1}$ | |
| Inositol | 120.0 μg liter$^{-1}$ | |
| pH adjustment | | |
| KOH (purified solution) | <2.0 mM | <2.4 |

[a] Concentrated stock solutions were prepared of each macronutrient salt, Fe-EDTA, a combination of all micronutrient salts except Fe-EDTA, and a combination of all vitamins. From analyses of these solutions and the purified KOH, the maximum Se content of constituents was calculated.

[b] It should be noted that Se was not detected in any component of the medium. The actual Se content of the medium is unknown and below the minimum detectable levels listed by the methods employed. The Se content of the final medium was less than 39.8 pg-atoms liter$^{-1}$.

by twice recrystallizing reagent-grade salts from purified water, except $K_2MoO_4$, which was made by dissolving reagent-grade $MoO_3$ in purified KOH. Selenous acid was purchased from Johnson Matthey Chemicals, and all other chemicals were the highest grade available from commercial sources. $^{75}$Se-labeled sodium selenite was purchased from ICN Radiochemicals, Inc., Irvine, Calif.

A 60% solution of reagent-grade glycerol was purified by passing it through a column (1.5 by 10 cm) of Amberlite MB-1 resin. The vitamins (Table 1) were purchased from Sigma Chemical Co., St. Louis, Mo.

A gas mixture consisting of 4% $H_2$ and 96% $N_2$ was obtained from commercial sources. In all experiments involving the effect of added Se on hydrogenase derepression or autotrophic growth, the gas mixture and air, delivered with a metering pump, was scrubbed to remove volatile Se compounds by passing sequentially through 250 ml each of 0.1 N NaOH, water-washed activated charcoal (EM Science, Cherry Hill, N.J.), 0.2 M $CuCl_2$, and purified water. $CO_2$ provided to cultures was generated by the addition of redistilled HCl (1 N) to a solution of $NaHCO_3$ (Johnson Matthey Chemicals). The $CO_2$, after being passed through a solution of 0.1 M purified $NaHCO_3$, was delivered directly to the gas supply to cultures by use of a peristaltic pump.

**(iii) Media.** The composition of the basal autotrophic medium and the maximum Se content that could have arisen from contamination is shown in Table 1. The basal heterotrophic medium contained the constituents of the autotrophic medium and also 50 mM purified glycerol. The amount of contaminating Se provided in the quantity of purified glycerol is unknown but is less than 48 pg-atoms of the element per liter of medium. The pH of media was adjusted to 6.8 by using an acid-washed electrode employed solely for these investigations. Purified KOH and redistilled HCl were used for adjustment. The concentrations of Se or other additions to the basal medium are indicated in the tables and figures.

**(iv) Autotrophic growth and hydrogenase derepression.** The procedure for autotrophic growth of *B. japonicum* 122DES (21) is a modification of that of Lepo et al. (10). Cultures were derived from single-colony isolates from a stock culture. Before the initiation of experiments in which *B. japonicum* 122DES was grown autotrophically or derepressed for hydrogenase expression, cultures were grown with shaking at 28°C in 50-ml polypropylene Erlenmeyer flasks, each containing 10 ml of a purified heterotrophic medium (the autotrophic medium in Table 1 to which was added 50 mM purified glycerol). After at least three transfers in this medium, cells were harvested aseptically and washed in purified autotrophic medium, and an inoculum (0.12 mg of protein) was transferred to each of a series of 50-ml polypropylene Erlenmeyer flasks, each containing 5 ml of the purified autotrophic medium. For the autotrophic culture experiment (Fig. 1, Table 2), six replicate cultures in two plastic containers were prepared for each time point when cells were removed in the experiment. The sealed polypropylene containers (12 by 10 cm) each fitted with a gas entrance and exit were gassed continually with a purified mixture of 0.17% $O_2$, 1% $CO_2$, 4% $H_2$, and the balance $N_2$. The $CO_2$ generated from purified $NaHCO_3$ was not passed through the gas purification train (see above). The gas flow rate was approximately 18 ml min$^{-1}$ per plastic container. The autotrophic cultures were incubated on a rotary shaker (150 rpm) at 28°C.

The procedure for culture of *B. japonicum* on purified heterotrophic medium before the initiation of derepression experiments was the same as the preliminary procedures described for the autotrophic experiments, except that an inoculum equivalent to 3 mg of cell protein was transferred to each of a series of 20-ml culture tubes, each containing 5 ml of the purified autotrophic medium (Table 1). The culture tubes in sealed plastic containers equipped with gas entrance and exit were gassed with the mixture described above for autotrophic cultures or in some cases with a gas mixture the same as used for autotrophic cultures except $CO_2$ was not added (for example, the experiment in Fig. 1). Some experiments were incubated at 28°C with shaking, and others were incubated at the same temperature under stationary conditions. For further details see legends and footnotes of figures and tables.

**(v) Selenium determinations.** Se was determined by a modification of the fluorometric method of Olson (13). This assay could measure less than 0.5 ng of Se per assay of a digested sample of 1 g of dry material or a 10-ml sample of liquid. Increased sensitivity of the method was obtained by use of an excitation wavelength of 375 nm and an emission maximum of 522 nm for fluorometric measurement of piazselenol formed in the assay reaction. Analyses were corrected for any Se detected in the reagents used for sample digestion and the development of the piazselenol for Se determination.

**(vi) Other assays.** $H_2$ uptake rates of cell suspensions were determined by an amperometric method (6). The protein content of cells was measured as described by Herbert et al. (8). $H_2$ and $O_2$ concentrations in gas mixtures provided to

cultures were monitored by use of a gas chromatograph equipped with a thermal conductivity detector with $N_2$ as the carrier gas.

**Hydrogenase purification. (i) Large-scale culture of *B. japonicum*.** For the preparation of sufficient quantities of autotrophically grown *B. japonicum* 122DES for hydrogenase purification, an inoculum was grown initially on an $H_2$-uptake medium (HUM) under conditions that repressed the formation of hydrogenase (11). Six 50.0-ml Erlenmeyer flasks, each containing 20.0 ml of HUM, were placed on a shaking table (160 rpm) in a temperature-controlled (28°C) dark room for 3 days. The cell suspensions were subsequently transferred from the small flasks to 2.8-liter Fernbach flasks, each containing 1.0 liter of HUM for an additional 3 days of growth. Approximately 40.0 ml of cell suspension was transferred to each 2.8-liter flask (4.0% inoculum). Flasks were maintained on the shaking table in the dark at 28°C throughout this growth phase.

The cell suspension was harvested by centrifugation at 9,000 × *g* for 30 min at 4°C, the resulting pellet was suspended in sterile Repaske medium (18, 19) and centrifuged for 1 h at 9,000 × *g* (4°C). This step was taken to remove any remaining HUM from the cells. The remaining pellet was suspended in Repaske medium and transferred to a sterile Pyrex glass carboy containing approximately 36.0 liters of Repaske medium. Cells were cultured autotrophically by a modification of the technique of Purohit et al. (17). Se as $H_2SeO_3$ (0.05 μM) was added to the medium. The final S/Se ratio was approximately 20,000:1. The temperature of the growth medium was maintained at approximately 28 ± 0.5°C.

After 28 days of autotrophic growth on Repaske medium, the cells were harvested by centrifugation by using a continuous-flow centrifuge apparatus (model KSB-R; Ivan Sorvall, Inc., Norwalk, Conn.) at 27,000 × *g* (4°C) and a flow rate of approximately 100 ml $min^{-1}$. $N_2$ gas was continuously spared into the medium in the carboy during harvesting. Pellets were suspended in 20 mM Tris hydrochloride (pH 7.4) and then centrifuged at 27,000 × *g* for 25 min. The resulting pellets of cells were subsequently frozen at −76°C until used in further isolation steps. Approximately 39 g of cells was obtained. Use of a sample taken from the 36.0-liter large-scale culture at the time of harvest for inoculation of plates of yeast extract or nutrient agar medium provided no evidence of contamination after 4 days of growth.

Conditions for the growth of *B. japonicum* in the presence of $^{75}Se$ were essentially identical to those described above. Radioactively labeled Se was added to 0.05 μM total Se, as $Na_2{}^{75}SeO_3$ (50 μCi $liter^{-1}$), and cells were allowed to grow for 39 days. The total volume of the $^{75}Se$-labeled culture was 7.0 liters. Approximately 8 g of cells was harvested.

**(ii) Isolation of membranes.** The pellet was thawed at room temperature under a stream of $N_2$ gas passed over a heated copper-based catalyst (R3-11; Chemical Dynamics Corp.) to remove $O_2$ and then suspended in 20 mM Tris hydrochloride containing 1 mM EDTA (pH 7.4). All buffer solutions used in this portion of the isolation procedure had been deoxygenated before use by sparging the solution with purified $N_2$ gas for 1 h. In addition, purified $N_2$ gas was continuously passed over the cell suspension during all phases of cell breakage.

The suspension of cells in buffer was passed twice through a French press at 40,000 kPa. The resulting crude extract was placed into the chamber of a prechilled (−76°C) Eaton press and allowed to freeze completely before breakage (40,000 kPa). The remaining unbroken cells, cell walls, and cellular debris were removed by centrifugation for 20 min at 12,000 × *g* (15°C). The supernatant, containing membranes and soluble protein, was centrifuged at 100,000 × *g* for 1 h (15°C). The pellet was suspended in 20 mM Tris hydrochloride–1 mM EDTA–500 mM NaCl (pH 7.4) and centrifuged at 100,000 × *g* for 1 h. The resulting pellet consisted primarily of cellular membranes. For the isolation of $^{75}Se$-labeled hydrogenase, labeled membranes were mixed with unlabeled membranes in a ratio of 1:6.

**(iii) Hydrogenase isolation and characterization.** Hydrogenase was isolated by using a technique developed by Seefeldt and Arp (personal communication); this is a modification of an earlier method of Seefeldt and Arp (24). The pellet was rinsed by suspending it in 20 mM Tris hydrochloride containing 1 mM EDTA (pH 7.4), centrifuged for 1 h at 100,000 × *g*, and then suspended in 20 mM Tris hydrochloride–1 mM EDTA–0.4% detergent (Emulgen 913; Karlan Chemical Corp., Torrance, Calif.)–2 mM dithionite and incubated in a shaking water bath (200 rpm) at 30°C for 1 h. The suspension was centrifuged at 100,000 × *g* for 1 h, and the supernatant was dialyzed against 10 mM succinate hydrochloride (pH 5.0) containing 2 mM dithionite at 4°C for approximately 18 h.

The dialysate was loaded onto a carboxymethyl-Sepharose CL6B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1.0 by 8.0 cm) pre-equilibrated with 10 mM succinate hydrochloride–2 mM dithionite (pH 5.0). Fractions were eluted with a solution of 10 mM succinate hydrochloride and 150 mM NaCl (pH 5.0) and loaded onto an octyl-Sepharose CL4B (Pharmacia) column (0.8 by 7.0 cm) equilibrated with 20 mM Tris hydrochloride–2 mM dithionite (pH 7.4). Samples were eluted with 20 mM Tris hydrochloride–40% ethanol–2 mM dithionite (pH 7.4). Fractions were collected anaerobically and assayed for hydrogenase activity by the methylene blue reduction assay monitored spectrophotometrically at 600 nm by using an empirically determined extinction coefficient of 33.2 $mM^{-1}$ $cm^{-1}$ (24). Individual fractions were frozen in liquid nitrogen.

The protein contents of extracts were determined by utilizing the Bio-Rad protein assay. Se concentration was measured fluorimetrically as described above. The molecular weight of hydrogenase subunits and homogeneity of samples were determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the technique of Laemmli (9). The fraction and standard proteins were diluted with sample buffer (2:1) containing 30% (vol/vol) glycerol–500 mM Tris–6% (wt/vol) sodium dodecyl sulfate–15% (vol/vol) 2-mercaptoethanol, and the samples were loaded onto a 10% (wt/vol) acrylamide gel (90 by 75 by 0.8 mm). Proteins were visualized by staining with Coomassie blue. The radioactivity of $^{75}Se$-labeled fractions was measured with a Beckman gamma counter (model 8000; Beckman Instruments, Inc., Fullerton, Calif.).

## RESULTS

**Effects of Se on autotrophic growth and hydrogenase derepression.** The purified culture medium (Table 1), which contained less than 39.8 pg-atoms of Se per liter, was used in a series of experiments to determine whether the addition of Se influenced growth and the time course of hydrogenase derepression in *B. japonicum*. The addition of $SeO_3{}^{2-}$ at 0.1 μM to replicate cultures significantly increased the total protein content of harvested cells by averages of 39, 46, and 50% at growth periods of 11, 20, and 27 days, respectively. Furthermore, the hydrogenase specific activities of cells grown in media containing 0.1 μM $SeO_3{}^{2-}$ at 7, 11, 20, and

TABLE 2. Effect of added selenite on $H_2$ uptake activity in autotrophically cultured *B. japonicum*[a]

| $H_2SeO_3$ added | Rate of $H_2$ uptake (nmol $min^{-1}$ mg of protein) on day: | | | | |
|---|---|---|---|---|---|
| | 0 | 7 | 11 | 20 | 27 |
| 0.1 μM | <10 | 159 ± 13 | 129 ± 9 | 135 ± 18 | 213 ± 28 |
| None | <10 | 140 ± 16 | 88 ± 11 | 73 ± 12 | 85 ± 16 |

[a] The procedure for autotrophic culture of *B. japonicum* is described in Materials and Methods. At 18 days after inoculation, the $O_2$ concentration in the gas phase over cultures was increased to 0.43%. Data presented are means ± standard errors of the means of determinations on six replicate cultures. At the time points indicated, cultures were harvested by centrifugation and washed in autotrophic medium, and the protein contents of cells and $H_2$ uptake rates were determined. The percent increase after 7, 11, 20, and 27 days was 13.6, 46.6, 84.9, and 150.6%.

27 days were 13.6, 46.6, 84.9, and 150.6% higher, respectively, than comparable data for cells harvested from a medium lacking added $SeO_3^{2-}$ (Table 2). From the magnitude of the responses during culture incubation and the relatively small variation among replicate measurements, it seems obvious that the addition of $SeO_3^{2-}$ had a definite positive effect on hydrogenase specific activities.

Another experiment was conducted that is analogous to that described in Fig. 1, except that the medium contained 50 mM purified glycerol in addition to the components of the autotrophic medium (Table 1). The addition of $SeO_3^{2-}$ at 0.03 or 0.1 μM had no significant effect on the protein contents or optical densities of replicate cultures of *B. japonicum* 122DES during a 15-day growth period (data not shown). $SeO_3^{2-}$ concentrations of 1.0 or 3 μM in the medium resulted in decreases in culture absorbance of 14 and 25%, respectively, after a 13-day culture period. From the results of autotrophic (Fig. 1, Table 2) and heterotrophic experiments containing added glycerol with less than 48 pg-atoms of Se per liter, we conclude that a Se requirement for heterotrophic growth, if it exists, must be more than 2 orders of magnitude lower in concentration than that needed for autotrophically cultured cells.

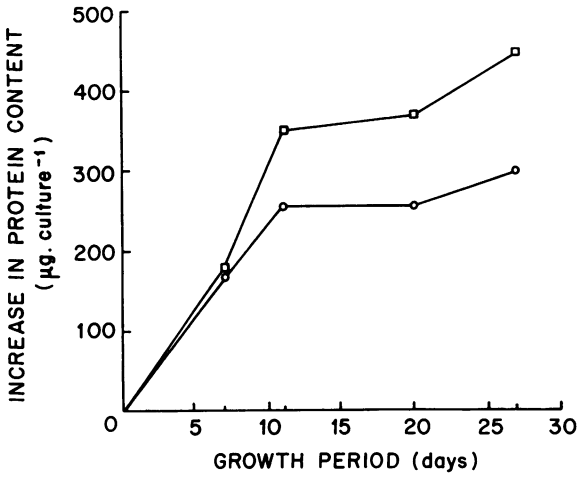


FIG. 1. Effect of selenite on the protein content of *B. japonicum* 122DES cultured under autotrophic conditions. The protein content of the inoculum at the beginning of the experiment was 118 μg. The details of this experiment are presented in Materials and Methods. The standard error of the mean of protein determinations on six replicate cultures collected at 7, 11, 20, and 27 days for the treatment with no additions (○) were 8, 10, 0, and 20 μg per culture and for the 0.1 μM $SeO_3^{2-}$ treatment (□) were 20, 15, 30, and 5 μg per culture, respectively.

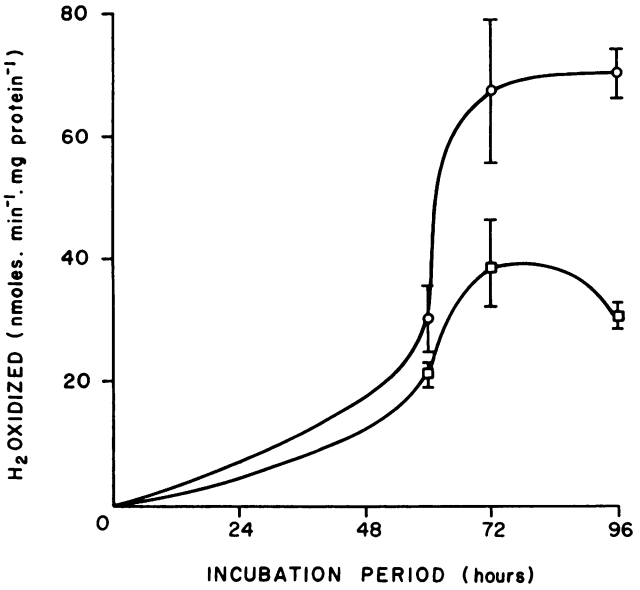


FIG. 2. Effect of the addition of selenite on the derepression of uptake hydrogenase in stationary cultures of *B. japonicum* without added $CO_2$ (see Materials and Methods). Eight replicate cultures in two plastic containers were prepared for each time point shown in the figure above. One series of cultures (○) received 0.1 μM $SeO_3^{2-}$; a second series (□) lacked added $SeO_3^{2-}$. The sealed containers were continually gassed with a mixture of 95.7% $N_2$, 4% $H_2$, and 0.3% $O_2$ and were incubated at 28°C. The result presented at each time point is the mean ± standard error of the mean of determinations on eight replicate cultures.

To further examine the role of Se in hydrogenase expression, *B. japonicum* cultures were grown on the purified heterotrophic medium where no hydrogenase activity is expressed. Then cells were removed and incubated under conditions where the enzyme was derepressed (see Materials and Methods) (Fig. 2). The addition of 0.1 μM $SeO_3^{2-}$ to the cultures in an autotrophic medium (Table 1) strikingly increased the time course of hydrogenase derepression (Fig. 2). With the 96-h incubation period, the mean increase from the Se treatment of eight replicate cultures was 133%. Other experiments (data not shown) in which cultures were incubated with shaking, with or without $CO_2$, resulted in similar responses to added $SeO_3^{2-}$.

Additional derepression experiments were carried out to determine the specificity of the Se effect and the optimum concentration of Se for hydrogenase derepression. The addition of vanadate, arsenite, sulfite, or tellurite at 0.02 μM failed to significantly affect hydrogenase derepression, but the addition of $SeO_3^{2-}$ at 0.02 μM resulted in a mean increase in hydrogenase specific activity in six replicate cultures from 44.5 without added $SeO_3^{2-}$ to 81.5 with added $SeO_3^{2-}$ (Table 3). Furthermore, these data show an approximate linear increase in hydrogenase specific activity from additions of 0, 0.02, and 0.1 μM $SeO_3^{2-}$. The addition of 0.5 μM $SeO_3^{2-}$ was excessive, resulting in an approximate 25% decline in activity.

**Hydrogenase isolation and characterization.** Hydrogenase was purified to a specific activity of 28.44 ± 4.83 μmol $min^{-1}$ mg of $protein^{-1}$ which represents a 61-fold purification (Table 4). These numbers are the mean and standard error of eight separate pooled fractions showing hydrogenase activity. Use of the carboxymethyl-Sepharose and octyl-Sepharose columns (Table 4) resulted in an 8.6-fold hydrogenase

TABLE 3. Effect of trace element additions and concentrations of Se on hydrogenase derepression in *B. japonicum* 122DES[a]

| Element added[b] | Concn in medium (μM) | Mean hydrogenase sp act (nmol of $H_2$ $min^{-1}$ mg of $protein^{-1}$ ± SEM) | % Increase[c] |
|---|---|---|---|
| None | | 44.5 ± 9.0 | |
| Se | 0.02 | 81.5 ± 9.6 | 83.1 |
| Se | 0.10 | 134.4 ± 13.7 | 202.0 |
| Se | 0.50 | 101.6 ± 10.9 | 128.3 |
| S | 0.02 | 42.4 ± 4.4 | 3.8 |
| V | 0.02 | 49.4 ± 4.4 | 11.0 |
| Te | 0.02 | 51.8 ± 4.7 | 16.4 |
| As | 0.02 | 44.6 ± 4.3 | 0.2 |

[a] Conditions for growth of *B. japonicum* and depression of hydrogenase on purified media were as described in Materials and Methods, except the purified gas mixture over cultures contained 0.08% $O_2$, 0.1% $CO_2$, 4% $H_2$, and the balance $N_2$. The six replicate cultures for each treatment were grown with shaking at 28°C and were assayed for hydrogenase activity and cell protein after 96 h of incubation.

[b] Trace elements were added as the following compounds: Se as $H_2SeO_3$, As as $NaAsO_2$, S as $Na_2SO_3$, Te as $K_2TeO_3$, and V as $V_2O_5$ (dissolved in purified KOH). The additions had no measureable effect on the pH of the final medium, which was 6.8.

[c] Calculated as the percent increase over the treatment with no addition.

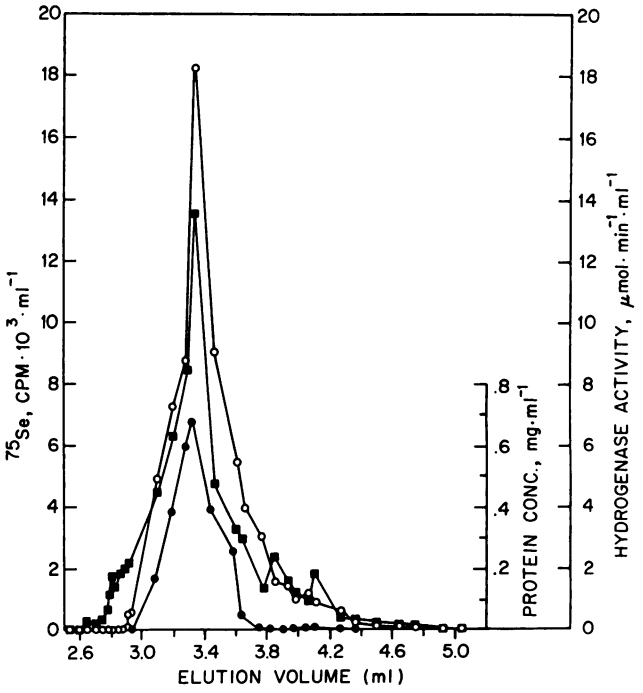


FIG. 3. Chromatography of $^{75}$Se-labeled preparation on octyl-Sepharose. Protein concentration (●), hydrogenase activity (○), and radioactivity (■) were determined for individual fractions eluted from an octyl-Sepharose column (see Materials and Methods for details). The protein content of the initial column eluate is not shown.

purification. The specific activity of the individual fractions obtained from the octyl-Sepharose column were relatively constant. Values obtained in this experiment for the specific activity of hydrogenase were somewhat less than those previously reported for peak fractions of the enzyme from autotrophically cultured *B. japonicum* (7). The calculated Se content per milligram of protein increased over 1,100-fold during the purification procedure (Table 4). Due to the inaccuracy of the Se analyses at very low concentrations, considerable error may be involved in this calculation. However, repeated protein and Se determinations on fractions obtained from hydrogenase purification on a separate batch of cells produced data in general agreement with those reported here. This increase strongly suggests a specific association between Se and hydrogenase. The correlation coefficient between enzyme specific activity and Se content per milligram of protein during purification was greater than 0.99. The isolation of hydrogenase requires strict anaerobic conditions. Thus, losses in enzyme activity during purification may be due, in part, to inactivation by $O_2$ during purification. Loss in enzyme activity may account for the relatively greater increase in the content of Se per unit of protein than increase in the specific activity of hydrogenase.

Hydrogenase eluted from the octyl-Sepharose column in a single peak (Fig. 3). During the purification of hydrogenase obtained from the $^{75}$Se-labeled culture, radioactively labeled membranes were mixed with unlabeled membranes (1:6) to increase the accuracy of assays for protein content and hydrogenase activity. The radioactivity values reported here, therefore, are approximately 17% of those to be expected from use of $^{75}$Se-labeled cells without dilution with unlabeled cells. The peaks of Se radioactivity, hydrogenase activity, and protein content all occurred in the same fraction eluted from the column (Fig. 3). These results indicate that Se is strongly associated with hydrogenase activity in individual fractions. The values presented above are typical of those obtained in five separate hydrogenase isolations.

Hydrogenase appears to be essentially homogeneous as shown by sodium dodecyl sulfate-polyacrylamide gel elec-

TABLE 4. Purification of hydrogenase from *B. japonicum* and Se content at various purification stages from the unlabeled culture

| Purification stage | Total protein (mg) | Total activity (U)[a] | Sp act (U/mg) | Purification (fold) | Selenium content[b] (ng mg of $protein^{-1}$) |
|---|---|---|---|---|---|
| Crude membranes[c] | 423.6 | 197.4 | 0.47 | 1.0 | 0.20 |
| Tris-EDTA-NaCl-washed membranes | 126.2 | 62.0 | 0.49 | 1.1 | 0.35 |
| Emulgen extract[d] | 41.0 | 105.8 | 2.58 | 5.5 | |
| Succinate dialysate | 37.0 | 121.9 | 3.30 | 7.1 | 2.12 |
| Octyl-Sepharose eluate[e] | 0.4 | 11.6 | 28.44 | 61.0 | 235.90 |

[a] Micromoles of $H_2$ oxidized per minute.

[b] Concentration of Se and protein content were determined for various preparation stages during hydrogenase purification (see Materials and Methods for details).

[c] The crude membranes consisted of soluble proteins and broken membrane fragments.

[d] Emulgen 913, a detergent used to solubilize membranes.

[e] Octyl-Sepharose chromatography of fractions collected from a carboxymethyl-Sepharose column (see Materials and Methods). Numbers represent average pooled values for eight individual fractions showing hydrogenase activity.

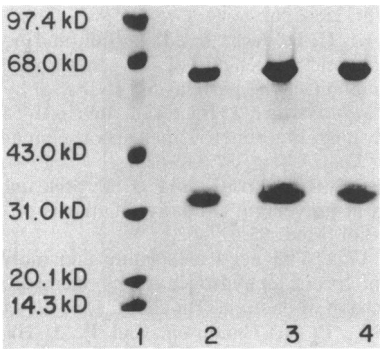


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified hydrogenase from *B. japonicum*. Lanes: 1, standard proteins (top to bottom) phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme; 2, 3, and 4, three individual peak fractions obtained between 3.28 and 3.46 ml of elution volume from the column of octyl-Sepharose (Fig. 3).

trophoresis (Fig. 4). The large and small subunits of the enzyme in the dried gel were excised, and the radioactivity in each portion was determined. The radioactivities in the gel fractions containing the two subunits were essentially identical (small subunit, 1,640 cpm $mg^{-1}$; large subunit, 1,465 cpm $mg^{-1}$), indicating the presence of Se in both subunits of hydrogenase. Gel fractions on either side of the bands containing the hydrogenase subunit showed background levels of radioactivity of about 10 cpm $mg^{-1}$. These results were further confirmed by autoradiography (data not shown). Treatment of purified hydrogenase with 5% trichloroacetic acid failed to release a significant amount of Se from the enzyme. Approximately 91% of the radioactivity originally present in the enzyme was retained in trichloroacetic acid-treated samples. The average Se content per mol of hydrogenase was 0.56 ± 0.13 g-atom (mean and standard error of five determinations) in three enzyme isolations.

## DISCUSSION

We have determined that Se is involved in hydrogenase expression and autotrophic growth of *B. japonicum* under conditions where $H_2$ is the only energy source. The addition of $SeO_3^{2-}$ at 0.1 μM increased hydrogenase derepression as indicated by the rate of $H_2$ oxidation in the range of 133 to over 200% (Fig. 2, Table 4). Among the responses to several anions tested the response to $SeO_3^{2-}$ was relatively specific. Furthermore in the experiment with autotrophically cultured cells, the addition of $SeO_3^{2-}$ resulted in a marked increase in the rate of $H_2$ oxidation (Table 2) and also in a 50% increase in cell protein at the end of the experiment (Fig. 1). These results have not shown an essential role of Se for hydrogenase expression and autotrophic growth of *B. japonicum*, but they have established an important role of the element in $H_2$ metabolism in this bacterium. The failure to demonstrate an absolute requirement for Se in autotrophic growth and hydrogenase derepression of *B. japonicum* is complicated by the difficulty in preparing a growth medium with no contaminating Se present and the apparently low concentration of Se required by *B. japonicum* (<39.7 pg of Se per liter of autotrophic medium, Table 1). The necessity for utilization of purified chemicals, pure water, clean culture equipment, and filter sterilization to consistently demonstrate responses to added $SeO_3^{2-}$ in *B. japonicum* was emphasized in this research. Although the effect of $SeO_3^{2-}$ on expression of hydrogenase in *B. japonicum* was substantial and reproducible, the order of response to the optimum level of $SeO_3^{2-}$ was considerably less than the 20-fold increase in formic dehydrogenase activity resulting from the addition of 0.03 μM $SeO_3^{2-}$ to cultures of *E. coli* (15). A concentration of 0.1 μM $SeO_3^{2-}$, which produced the maximum hydrogenase expression in *B. japonicum*, is of the same order of magnitude as the concentration needed for growth of *C. breviturriti* (29). In contrast, *T. pseudonana* (16) responded to 1 nM $SeO_3^{2-}$, a much lower concentration than the level needed for *B. japonicum*.

The positive responses to the addition of $SeO_3^{2-}$ to *B. japonicum* discussed above raise questions regarding a possible role of Se in symbiotically cultured legumes inoculated with rhizobia capable of recycling $H_2$ evolved as a by-product of the $N_2$ fixation reaction. Some preliminary experiments have been conducted in which the effects of the addition of $SeO_3^{2-}$ to nutrient solutions on bacteroid $H_2$ uptake rates, nodule nitrogenase activities, and dry weights of *Glycine max*, *Vicia faba*, and *Lupinus alba* inoculated with $Hup^+$ inoculants were measured (data not shown). The addition of $SeO_3^{2-}$ to the nutrient medium of these legumes resulted in a statistically significant increase (68%) in the $H_2$ uptake rate of nodule bacteroids from only *V. faba* plants; the effect of the $SeO_3^{2-}$ treatment on $H_2$ uptake rates of bacteroids from *G. max* and *L. alba* were not statistically significant at the $P = 0.05$ level. In these experiments plants were provided with reagent-grade rather than purified chemicals. Dry plant shoots from cultures not receiving $SeO_3^{2-}$ contained from 0.28 to 0.54 ng of Se per g (dry weight), and the seed from which plants were grown contained 10.2 ng of Se per g (dry weight). Obviously, conclusions regarding the importance of Se in $H_2$ metabolism of legume nodules cannot be made until experiments can be designed that will minimize Se contamination during plant growth trials.

The purification of hydrogenase from *B. japonicum* resulted in a striking coincidence of peaks in protein content, hydrogenase activity, and $^{75}Se$ radioactivity (Fig. 3) of individual fractions during the final chromatographic purification step. In addition, Se tended to copurify with the enzyme, resulting in a more than 1,100-fold concentration of Se in the most highly purified hydrogenase fractions. Since the increase in Se content per unit of protein was much greater than that of hydrogenase specific activity, it seems possible that an inactive Se-containing protein (likely inactive hydrogenase) copurified with the active enzyme. These results strongly indicate a specific incorporation of Se in hydrogenase. Determination of the stoichiometry of Se incorporation into *B. japonicum* hydrogenase has revealed a mean value of 0.56 ± 0.13 g-atoms of Se per mol of protein. This may be compared with values ranging from 0.1 to 0.5 g-atom $mol^{-1}$ for nicotinic acid hydroxylase (5), 3.8 g-atom $mol^{-1}$ for hydrogenase from *M. vannieli* (31), and 1.08 g-atom $mol^{-1}$ for the hydrogenase from *Desulfovibrio salexigens* (27). Less than 1 g-atom of Se per mol of hydrogenase in *B. japonicum* might be explained by a loss of the element during purification. However, an explanation of the observed occurrence of $^{75}Se$ in both subunits of the *B. japonicum* hydrogenase is not obvious at present. Many of the chemical properties of Se and S are similar; as a consequence, a nonspecific replacement of S with Se in metabolites may be occurring simultaneously with the specific incorporation of Se, forming seleno amino acids (25, 26). This substitution of Se for S may be at least partly responsible for the existence of radioactively labeled Se in both subunits and may further complicate stoichiometric determi-

nations. In this regard, the amino acid composition deduced from the DNA sequence of the two hydrogenase components shows 10 and 11 methionines in the large and small hydrogenase subunits, respectively.

Sequencing of the genes for the two *B. japonicum* hydrogenase subunits did not reveal a TGA codon in the gene sequence for either protein component (L. A. Sayavedra, G. K. Powell, H. J. Evans, and R. O. Morris, Proc. Natl. Acad. Sci. USA, in press). Therefore, there is no evidence for involvement of a TGA codon (UGA in the mRNA) for incorporation of selenocysteine into the protein, a phenomenon that has been established for incorporation of selenocysteine into formic dehydrogenase and glutathione peroxidase (3).

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