JOURNAL OF BACTERIOLOGY, Mar. 1969, p. 1460-1465
Vol. 97, No. 3

Printed in U.S.A.

B_{12} Coenzyme-dependent Ribonucleotide Reductase in *Rhizobium* Species and the Effects of Cobalt Deficiency on the Activity of the Enzyme[1]

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Received for publication 25 November 1968

This investigation revealed that the ribonucleotide reductases in extracts of *Rhizobium leguminosarum*, *R. trifolii*, *R. phaseoli*, *R. japonicum*, and *R. meliloti* 3DOa1 (ineffective in nitrogen fixation) are dependent upon B_{12} coenzyme for activity. *Rhizobium* and certain *Lactobacillus* species are the only two groups of organisms known to contain B_{12} coenzyme-dependent ribonucleotide reductases. Extracts of cobalt-deficient *R. meliloti* cells assayed in the presence of optimum B_{12} coenzyme showed a 5- to 10-fold greater ribonucleotide reductase activity than comparable extracts from cells grown on a complete medium. Furthermore, cobalt-deficient cells were abnormally elongated and contained reduced contents of deoxyribonucleic acid. The addition of purified deoxyribonucleosides to cobalt-deficient cultures of *R. meliloti* failed to alleviate deficiency symptoms.

Nutritional experiments by Kitay et al. (13, 14) provided some of the initial evidence that vitamin B_{12} was required for the growth of certain *Lactobacillus* species and that vitamin B_{12} could be replaced by the addition of deoxyribonucleotides to the medium. Unless adequate vitamin B_{12} was available, *L. leichmannii* failed to effectively incorporate ribosyl compounds into deoxyribonucleic acid (DNA; 10). These experiments provided the first indirect evidence that vitamin B_{12} was involved in the reduction of ribonucleotides to deoxyribonucleotides. More recent experiments (3, 4) have shown that vitamin B_{12} deficiency in *L. leichmannii* results in a reduced rate of growth, derepression of ribonucleotide reductase synthesis, a decrease in DNA to ribonucleic acid (RNA) and DNA to protein ratios, and the production of morphologically elongated cells.

Characterization of the ribonucleotide reductases in cell-free extracts of *L. leichmannii* (5, 12) and *Rhizobium meliloti* (8) has demonstrated that the reductases from these organisms are dependent on B_{12} coenzyme for activity. Investigations of the properties of ribonucleotide reductase in extracts of *Escherichia coli* (23, 24), Novikoff hepatoma cells (21), and various other organisms (1, 20, 22), however, have failed to reveal a B_{12} coenzyme requirement.

For some time, certain strains of *L. leichmannii* and *L. acidophilus* were the only organisms known to have a B_{12} coenzyme-dependent ribonucleotide reductase (6). The decision to examine *Rhizobium* species for B_{12} coenzyme-dependent ribonucleotide reductase was based on earlier investigations (15, 19) showing that cobalt is required for growth of these organisms and that cobalt deficiency results in a striking decrease in the B_{12} coenzyme content of cells.

The purpose of this paper is to report that several *Rhizobium* species in pure culture and the bacteroids from nodules of certain leguminous plants contain B_{12} coenzyme-dependent ribonucleotide reductases. The effects of cobalt deficiency on the activity of the ribonucleotide reductase in cell extracts and on the morphology and other properties of *R. meliloti* cells also are reported.

MATERIALS AND METHODS

Culture of the organisms. Cultures of *R. meliloti* F-28, *R. leguminosarum* C-56, *R. phaseoli* K-17, *R. trifolii* K-4, and *R. japonicum* A-72 were kindly supplied by Joe Burton of the Nitragen Co. *R. meliloti* 3DOa1, a strain of *R. meliloti* ineffective in fixing nitrogen, was a gift from L. W. Erdman of the U.S. Department of Agriculture. All of the species, except *R. japonicum*, were maintained and normally grown on a mannitol medium (9). *R. japonicum* was maintained and cultured on a medium containing arabinose and glycerol (9). The bacteria were grown in shake cultures with 1-liter flasks or in aerated 10-liter carboys (8). Cells were harvested by centrifugation when the

[1] Oregon Agricultural Experiment Station paper no. 2531.

absorbance at 660 nm ranged between 0.75 and 0.85 optical density (OD) units (dry weight of cells from 400 ml of media ranged from 195 to 225 mg).

In the experiments in which the effects of cobalt deficiency were investigated, *R. meliloti* was grown in 1-liter flasks containing cobalt-deficient medium prepared by the procedures of Kliewer et al. (16). In these experiments, *R. meliloti* was transferred from the maintenance medium to a 50-ml flask containing 20 ml of the cobalt-deficient medium. Successive transfers then were made to pairs of 50-ml flasks, one of which contained the deficient medium and the other the same medium supplemented with 24 μg of cobalt per liter. When the rate of cell growth was noticeably decreased in the cobalt-deficient flasks, these flasks were used to inoculate the cultures in cobalt deficiency experiments. In the experiments in which the effects of cobalt deficiency on ribonucleotide reductase were studied, twelve 1-liter flasks, each containing 400 ml of the cobalt-deficient mineral medium, were prepared. Four flasks received no additions, four were supplemented with deoxyribonucleosides (Sigma Chemical Co., St. Louis, Mo.), and the other flasks were supplied with 24 μg of cobalt per liter. Cells were harvested when the absorbance at 660 nm reached 0.35 to 0.45 OD units (dry weight of cells from 400 ml of media ranged from 30 to 45 mg). All experiments were run in duplicate or triplicate.

Growth of plants. Soybean (*Glycine max* Merr var. Merrit) and alfalfa (*Medicago satava* var. Dupre) seeds were inoculated with commercial preparations of *R. japonicum* and *R. meliloti* (supplied by Joe C. Burton) and were planted in pots of perlite. The plants were cultured in the greenhouse and were provided with a nitrogen-free nutrient solution (2). Supplemental fluorescent lights were used for 16 hr each day. Nodules from soybean and alfalfa plants were harvested 20 and 44 days, respectively, after planting. Soybean seeds that were not inoculated were germinated in a wooden flat containing perlite, grown for 5 days in a greenhouse without supplemental light, and then harvested.

Preparation of extracts. Cells from cultures of the *Rhizobium* species were harvested by centrifugation, washed, and then broken in a French press by procedures previously described (8). The crude extracts were treated with 25 μliters of 2% protamine sulfate solution (*p*H 7.0; Eli Lilly & Co., Indianapolis, Ind.) for each microgram of nucleic acid in the extract. After 10 min, the mixture was centrifuged for 10 min at 14,000 $\times$ *g* and the pellet was discarded. Saturated ammonium sulfate solution (enzyme grade, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.), adjusted to *p*H 7.0, was added to the supernatant solution to obtain 30% saturation. After 10 min the precipitate was collected by centrifugation for 10 min at 14,000 $\times$ *g*, and additional saturated ammonium sulfate solution was added to the supernatant solution to obtain 45% saturation. After 10 min the precipitate was collected by centrifugation for 10 min at 14,000 $\times$ *g* and dissolved in 10 ml of 0.05 M potassium phosphate buffer (*p*H 7.3). This solution was dialyzed for 12 hr against 4 liters of 0.005 M potassium phosphate buffer (*p*H 7.3) and was used for ribonucleotide reductase assays. Cells harvested from the cobalt-deficient medium were washed in 0.05 M potassium phosphate buffer (*p*H 7.3), frozen in solid CO_2, and broken in an Eaton press at a pressure of approximately 10.2 tons per square inch. After thawing and centrifugation, the supernatant fluid was used in assays.

Soybean and alfalfa nodules used for the preparation of bacteroid extracts were harvested, washed with tap water, and rinsed with distilled water. Usually, 15 g of acid-washed polyvinylpyrrolodone (PVP; Polyclar AT from General Aniline & Film Corp., New York, N.Y.) and 60 ml of 0.05 M potassium phosphate buffer containing 200 mM ascorbate (adjusted to *p*H 7.3) were added to a cold mortar containing 30 g of nodules (17, 18). The nodules were macerated and squeezed through a single layer of bolting cloth. The mixture of PVP and solid nodule debris was resuspended and remacerated in 50 ml of the potassium phosphate buffer and ascorbate. The brei from the two extractions was centrifuged for 15 min at 32,000 $\times$ *g*, and the bacteroid pellet was washed with 0.05 M potassium phosphate buffer (*p*H 7.3). After suspending in the same buffer, the bacteroids were broken in a French or Eaton press and the crude extract was used in assays.

The root tips from soybean seedlings (terminal 3 cm) were removed and placed into a beaker of cold distilled water. The tips of roots were cut into small segments and added to an equal weight of a mixture consisting of PVP, ascorbate, and potassium phosphate buffer in the proportions described for preparation of the nodule extracts. After freezing in solid CO_2, the root segments were broken in an Eaton press. The frozen material was allowed to thaw in three volumes of the PVP-ascorbate-potassium phosphate buffer solution used for breaking the bacterial cells and was centrifuged for 15 min at 32,000 $\times$ *g*. The supernatant liquid was used in the ribonucleotide reductase assays.

Ribonucleotide reductase assay. A complete reaction mixture (0.5 ml) contained 1 μmole of guanosine triphosphate (Sigma Chemical Co.), 15 μmoles of dihydrolipoate (Sigma Chemical Co.), 10 nmoles of B_{12} coenzyme (a gift of L. Mervyn of Glaxo Laboratories), 50 μmoles of potassium phosphate buffer (*p*H 7.3), and an appropriate amount of enzyme. The reaction mixtures were incubated for 1 hr at 37 C, and the reactions were terminated by placing the tubes in boiling water for 3 min. The reaction mixtures were treated with chloroacetamide and later with diphenylamine by the procedure of Blakley (7). The absorbance of the reaction mixtures was measured with a Hitachi 139 spectrophotometer, and the concentration of deoxyribonucleotides was estimated from standard curves prepared with deoxyguanosine monophosphate or deoxyadenosine monophosphate. The assay procedure was not sufficiently sensitive to accurately measure less than 3 nmoles of deoxyribose in 0.5 ml.

Other determinations. The methods used to determine the concentrations of dihydrolipoate and B_{12} coenzyme have been reported previously (8).

The nucleic acid content of crude extracts was estimated from measurements of absorbance at 260 and 280 nm (25). All protein concentrations were estimated by the biuret procedure (11). The generation times were calculated from changes in absorbance of the culture at 660 nm during the logarithmic phase of growth (0.1 to 0.35 OD units of culture).

For the preparation of the electron micrographs, cells from cultures of *R. meliloti* were placed on 150 mesh copper grids coated with Formvar. The cell suspension was allowed to settle for 1 min and the majority of the drop was removed with filter paper. After the grid had dried, a drop of 1% sodium phosphotungstate was placed on the grid for 1 min and then removed with filter paper. The dried grids were examined with a Philips EM 300 electron microscope operating at 60 kv.

RESULTS

Ribonucleotide reductase in Rhizobium species and in legume nodules. B_{12} coenzyme-dependent ribonucleotide reductase activity was observed in five *Rhizobium* species and in the bacteroids from soybean and alfalfa nodules (Table 1). With the exception of *R. meliloti* 3DOal, which is ineffective in nitrogen fixation, the activities of the enzyme in extracts of the different bacteria were correlated in a general way with the growth rates of the organisms. The highest specific activity of the enzyme was observed in extracts of *R. meliloti*, an organism showing the lowest generation time, and the lowest activity of the reductase was observed in extracts of *R. japonicum*, an organism which exhibits the slowest growth rate. Both reductase activities and rates of growth of the other *Rhizobium* species were intermediate between those of *R. meliloti* and *R. japonicum*. Although the ribonucleotide reductase activities in bacteroids from both soybean and alfalfa nodules were relatively low, activity was clearly dependent upon B_{12} coenzyme.

TABLE 1. *Ribonucleotide reductase activities in extracts of Rhizobium species and legume nodule bacteroids*[a]

Source of enzyme[b]	Reductase activity in complete system[c]	Reductase activity in complete system minus B_{12} coenzyme[c]	Generation time in log phase (hr)
R. meliloti	346	2	2.8
R. meliloti 3DOa1	80	2	2.8
R. trifolii	155	3	4.0
R. phaseoli	152	3	3.7
R. leguminosarum	150	<1	3.9
R. japonicum	38	<1	10.2
Soybean nodule bacteroids	7	<1	
Alfalfa nodule bacteroids	31	<1	

[a] Each reaction mixture contained extracts purified with protamine sulfate and ammonium sulfate.

[b] The concentration of protein in each reaction mixture was 0.1 to 0.5 mg of bacterial protein and 1.0 to 1.8 mg of bacteroid protein.

[c] Expressed as nanomoles of deoxyguanosine triphosphate per milligram of protein per hour.

Since the ribonucleotide reductase activities in extracts of *R. meliloti* 3DOa1 and *R. japonicum* were low, assays were conducted to determine whether substrates other than guanosine triphosphate might function more effectively than guanosine triphosphate in the enzyme system from these organisms. The rate of adenosine and guanosine mono-, di-, and triphosphate reduction, catalyzed with extracts of *R. meliloti* 3DOa1 or *R. japonicum*, was more than twofold less than the reduction rate of either guanosine triphosphate or guanosine diphosphate in assays containing *R. meliloti* enzyme. Extracts of *R. meliloti* 3DOa1 and *R. japonicum* catalyzed the reduction of guanosine diphosphate and adenosine diphosphate more rapidly than guanosine or adenosine mono- or triphosphates.

In preliminary experiments, it was observed that the relative affinities for B_{12} coenzyme by the enzyme from *R. meliloti*, *R. meliloti* 3DOa1, and *R. japonicum* varied considerably. The apparent K_m values for B_{12} coenzyme of the extracts from *R. meliloti*, *R. japonicum*, and *R. meliloti* 3DOa1 were 7.6, 51.6, and 51.8 μM, respectively.

Since no clear-cut demonstration of a cobalt requirement for growth of higher plants has been demonstrated, it was of interest to investigate the properties of the ribonucleotide reductase in extracts of the tips of roots of young soybean seedlings that were grown without inoculation with *Rhizobium*. These extracts catalyzed the reduction of guanosine triphosphate at a rate of 3 to 5 nmoles per mg of protein per hr. A statistical analysis of replicated experiments indicated that the addition of B_{12} coenzyme to the assays did not significantly stimulate the rate of reduction. Since the activities of the root extracts were quite low it is necessary to utilize an assay more sensitive than the colorimetric procedure to determine conclusively whether B_{12} coenzyme is involved in the ribonucleotide reductase system from soybean roots.

Cobalt deficiency. The omission of cobalt from the purified medium used for the culture of *R. meliloti* resulted in a marked decrease in the rate of growth of the organism (Fig. 1). After a period of 20 hr, the culture lacking added cobalt apparently obtained sufficient cobalt impurities

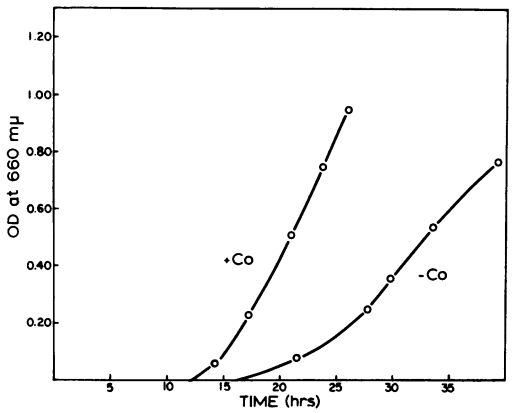

FIG. 1. *Growth rates of R. meliloti on a purified culture medium lacking cobalt and on a comparable medium to which we added 24 μg of cobalt per liter.*

from the glass culture vessels and other sources to support some growth. In previous experiments it has been shown that polypropylene culture vessels provide considerably less contaminating cobalt than glass vessels, and thus under these conditions cobalt deficiency in *R. meliloti* was maintained for periods of 35 hr or more.

The specific activity of ribonucleotide reductase in extracts of *R. meliloti* cells from cultures grown on the cobalt-deficient medium was 5- to 15-fold greater than that of comparable extracts of cells from cultures grown with the complete medium (Fig. 2). Since the activity of the reductase was measured with adequate B_{12} coenzyme in the assay mixture, the high activity represents an increase in the amount of apoenzyme in the deficient cells.

The vitamin B_{12} requirement of *L. leichmannii* is alleviated considerably by the addition of deoxyribonucleosides to cultures (3, 4, 14). This information and the results in Fig. 2 showing high ribonucleotide reductase activity in cells from cultures deficient in cobalt suggested that the products of the ribonucleotide reductase reaction may serve as repressors of the synthesis of the ribonucleotide reductase apoenzyme. Therefore, experiments were designed to determine whether certain deoxyribose compounds might substitute for cobalt in the metabolism of *R. meliloti*. In these experiments, the bacteria were cultured on the cobalt-deficient medium to which we added cobalt or deoxyribose compounds (Table 2). It is clear from these results that none of the additions appreciably substituted for cobalt as a growth factor for *R. meliloti*. An electron micrograph of normal cells and cobalt-deficient cells grown on a medium supplemented with deoxyadenosine, deoxyguanosine, deoxycytosine, and thymidine shows that these additions failed to prevent the occurrence of elongated cells (Fig. 3). With the exception of the addition of cobalt, the shortest generation time was observed from a combined addition of deoxyribose, adenine, guanine, uracil, and cytosine to the cobalt-deficient medium. Increasing the concentration of deoxyribose to 15×10^{-5} M and increasing each

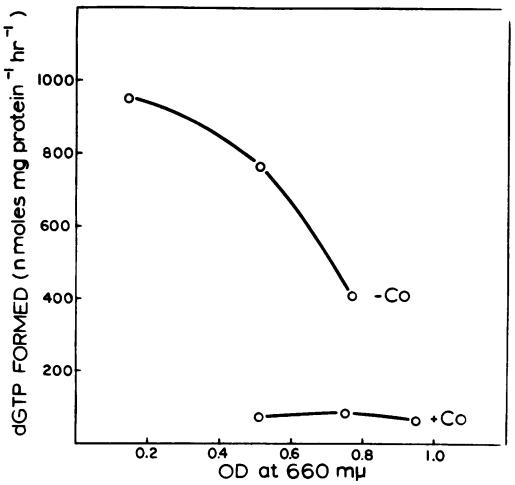

FIG. 2. *Ribonucleotide reductase activities of R. meliloti cells grown on a purified medium lacking cobalt and on a comparable medium to which we added 24 μg of cobalt per liter. Each reaction mixture contained 0.25 to 0.32 and 0.05 to 0.07 mg of protein from crude extracts of cells grown with and without cobalt, respectively. Abbreviation: dGTP, deoxyguanosine triphosphate.*

TABLE 2. *Generation times of R. meliloti grown on cobalt-deficient media supplemented with various compounds*

Supplement to the cobalt deficient medium[a]	Generation time (hr)
None	5.2
Thymidine	5.5
Deoxyadenosine	5.2
Deoxyribose	5.4
Deoxyribose, adenine, guanine, uracil, and cytosine	4.4
Deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine	4.8
Cobalt chloride	2.8

[a] The media contained supplements, as indicated, at the following concentrations: deoxyribose, 5.0×10^{-5} M; thymine, deoxyadenosine, deoxyguanosine, and deoxycytidine, each at 3.75×10^{-5} M; adenine, guanine, uracil, and cytosine, each at 1.25×10^{-5} M; and cobalt as cobalt chloride, 24 μg/liter.

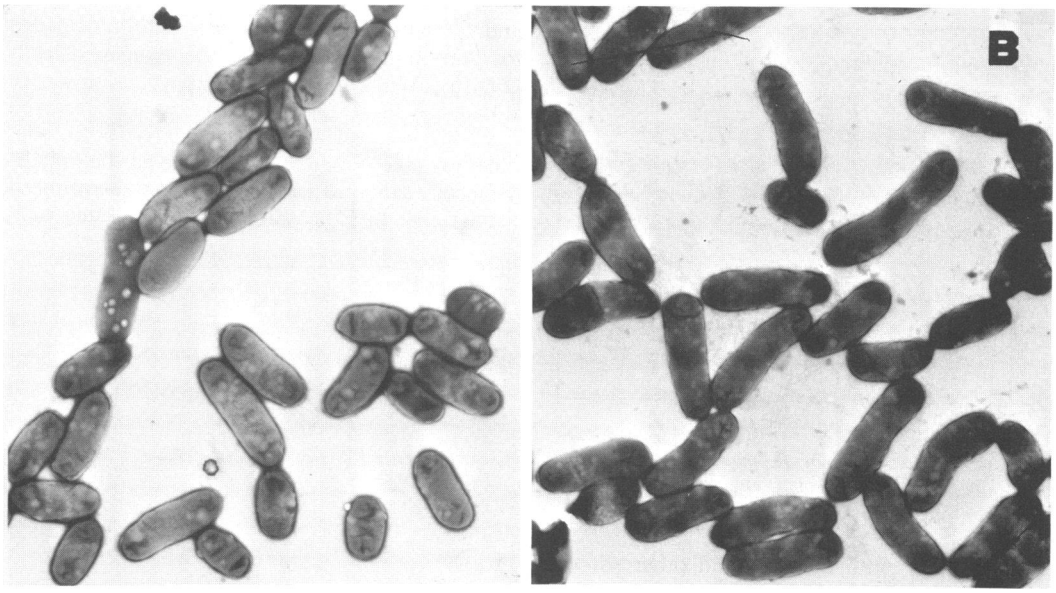

FIG. 3. *Electron microphotographs of normal R. meliloti cells (A) and cobalt-deficient cells grown on media supplemented with deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine (B). See Table 2 for further details.* × *6,860.*

base to 3.75×10^{-5} M failed to further reduce the generation time. In contrast, the addition of cobalt reduced the generation time from 5.2, where no cobalt was added, to 2.8 hr.

In another experiment, *R. meliloti* was cultured in a series of purified media containing either certain ribonucleosides or cobalt chloride (Table 3). The ribonucleotide reductase activity in extracts of cells grown on the cobalt-deficient medium supplemented with a combination of deoxyguanosine, deoxyadenosine, deoxycytidine, and thymidine was about 30% less than that in cells grown on the cobalt-deficient medium (Table 3). The addition of thymidine alone also resulted in a small reduction in the ribonucleotide reductase activity of cell extracts. It is clear, however, that cobalt was the only addition to the deficient medium that resulted in a normal level of ribonucleotide reductase in extracts.

TABLE 3. *Ribonucleotide reductase activity in extracts of R. meliloti grown on cobalt-deficient media with supplements*[a]

Supplement to the cobalt deficient medium	Reductase activity[b]
None	458
Deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine[c]	316
Thymidine[c]	321
Deoxyadenosine[c]	389
Cobalt chloride[d]	43

[a] Each reaction mixture contained crude extract (0.1 to 0.2 mg of protein).

[b] Expressed as nanomoles of deoxyguanosine per milligram of protein per hour.

[c] The concentration of each deoxyribonucleoside was 3.75×10^{-5} M.

[d] The concentration of cobalt was 24 μg/liter.

DISCUSSION

All of the *Rhizobium* species examined contained B_{12} coenzyme-dependent ribonucleotide reductases. Therefore, *Rhizobium* is the second genus of microorganisms in which B_{12} coenzyme-dependent ribonucleotide reductase systems have been identified.

Cobalt is required for *R. meliloti* whether the organism is grown in pure culture (19) or is living in symbiosis with the host plant (2). When *R. meliloti* is cultured under conditions of cobalt deficiency, cells contain a strikingly decreased content of B_{12} coenzyme and produce many abnormally elongated cells (15). Furthermore, the cobalt-deficient cells contain considerably less DNA than cells from cultures supplied with adequate cobalt. Another characteristic of cobalt deficiency in *R. meliloti* is an increased synthesis of the ribonucleotide reductase apoenzyme (Fig. 2). These results strongly suggest that the reduced B_{12} coenzyme content of cells that results from cobalt deficiency causes a lesion in the synthesis of deoxynucleotides. It seems reasonable to expect that some product of ribonucleotide reduction might serve as a repressor of the synthesis of

the ribonucleotide reductase apoenzyme. Under conditions of cobalt deficiency where insufficient B_{12} coenzyme is available for the normal function of the reductase, the synthesis of the apoenzyme would be expected to be derepressed. Beck and Hardy (3) reported a derepression of ribonucleotide reductase synthesis when thymine was omitted in media used for the culture of *L. leichmannii* or *E. coli* 15T (a thymineless mutant). In experiments reported here, thymidine and other ribonucleosides failed to effectively repress the synthesis of the reductase in *R. meliloti*. Also the addition of various ribonucleosides to cobalt-deficient cultures did not prevent the formation of abnormally elongated cells. The possibility exists that the added ribonucleosides and other compounds were not taken up by the cells.

It seems likely that cobalt as a constituent of B_{12} compounds may function in several metabolic sites in the metabolism of *Rhizobium* species. In addition to the established role of B_{12} coenzyme in the ribonucleotide reductase, it is known (9) that *R. meliloti* contains a B_{12} coenzyme-dependent methylmalomyl coenzyme A mutase. Obviously, additional research is necessary to identify more precisely the various biochemical sites where B_{12} compounds function in the metabolism of *Rhizobium* species.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB5185X and by the Oregon Agricultural Experiment Station.

We acknowledge the technical assistance of John Elser in conducting the electron microscopy.

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