B<sub>12</sub> Coenzyme-dependent Ribonucleotide Reductase in Rhizobium Species and the Effects of Cobalt Deficiency on the Activity of the Enzyme<sup>1</sup>

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This investigation revealed that the ribonucleotide reductases in extracts of Rhizobium leguminosarum, R. trifolii, R. phaseoli, R. japonicum, and R. meliloti 3DOal (inactive in nitrogen fixation) are dependent upon B<sub>12</sub> coenzyme for activity. Rhizobium and certain Lactobacillus species are the only two groups of organisms known to contain B<sub>12</sub> coenzyme-dependent ribonucleotide reductases. Extracts of cobalt-deficient R. meliloti cells assayed in the presence of optimum B<sub>12</sub> 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 deoxysteribonucleosides 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<sub>12</sub> was required for the growth of certain Lactobacillus species and that vitamin B<sub>12</sub> could be replaced by the addition of deoxyribonucleotides to the medium. Unless adequate vitamin B<sub>12</sub> 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<sub>12</sub> was involved in the reduction of ribonucleotides to deoxyribonucleotides. More recent experiments (3, 4) have shown that vitamin B<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> coenzyme requirement.

For some time, certain strains of L. leichmannii and L. acidophilus were the only organisms known to have a B<sub>12</sub> coenzyme-dependent ribonucleotide reductase (6). The decision to examine Rhizobium species for B<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> 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 Nitrigen Co. R. meliloti 3DOal, 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 manitol 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
potassium phosphate buffer (pH 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 (pH 7.3), frozen in solid CO₂, 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 pH 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 × g, and the bacteroid pellet was washed with 0.05 M potassium phosphate buffer (pH 7.3). After washing 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 70% distilled water, 30% potassium phosphate buffer in the proportions described for preparation of the nodule extracts. After freezing in solid CO₂, 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 × 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 dihydrodipotato (Sigma Chemical Co.), 10 μmoles of B₁₂ coenzyme (a gift of L. Mervyn of Glaxo Laboratories), 50 μmoles of potassium phosphate buffer (pH 7.3), and an appropriate amount of enzyme. The reaction mixture was 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 chloroacetic acid and later with diphenylamine by the procedure of Blackey (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 dihydrodipotato and B₁₂ 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. B12 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 B12 coenzyme.

Since the ribonucleotide reductase activities in extracts of *R. meliloti* 3DOal 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 monoo-, di-, and triphosphate reduction, catalyzed with extracts of *R. meliloti* 3DOal 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* 3DOal and *R. japonicum* catalyzed the reduction of guanosine diphosphate and adenosine diphosphate more rapidly than guanosine or adenine mono- or triphosphates.

In preliminary experiments, it was observed that the relative affinities for B12 coenzyme by the enzyme from *R. meliloti*, *R. meliloti* 3DOal, and *R. japonicum* varied considerably. The apparent $K_m$ values for B12 coenzyme of the extracts from *R. meliloti*, *R. japonicum*, and *R. meliloti* 3DOal 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 B12 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 B12 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...
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 alleviated in cobalt-deficient medium. Increasing the concentration of deoxyribose to $15 \times 10^{-3} \text{ M}$ and increasing each

![Figure 1](https://example.com/figure1.png)  
*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.*

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 $15 \times 10^{-3} \text{ M}$ and increasing each

![Figure 2](https://example.com/figure2.png)  
*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 0.60 to 0.07 mg of protein from crude extracts of cells grown with and without cobalt, respectively. Abbreviation: dGTP, deoxyguanosine triphosphate.*

<table>
<thead>
<tr>
<th>Supplement to the cobalt deficient medium</th>
<th>Generation time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.2</td>
</tr>
<tr>
<td>Thymidine</td>
<td>5.5</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>5.2</td>
</tr>
<tr>
<td>Deoxyribose</td>
<td>5.4</td>
</tr>
<tr>
<td>Deoxyribose, adenine, guanine, uracil,</td>
<td>4.4</td>
</tr>
<tr>
<td>and cytosine</td>
<td></td>
</tr>
<tr>
<td>Deoxyadenosine, deoxyguanosine,</td>
<td>4.8</td>
</tr>
<tr>
<td>deoxycytidine, and thymidine</td>
<td></td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*The media contained supplements, as indicated, at the following concentrations: deoxyribose, $5.0 \times 10^{-4} \text{ M}$; thymine, deoxyadenosine, deoxyguanosine, and deoxycytidine, each at $3.75 \times 10^{-4} \text{ M}$; adenine, guanine, uracil, and cytosine, each at $1.25 \times 10^{-5} \text{ M}$; and cobalt as cobalt chloride, 24 µg/liter.*
base to $3.75 \times 10^{-4}$ 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.

**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

![Image of electron micrographs 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.](http://jb.asm.org/)

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

<table>
<thead>
<tr>
<th>Supplement to the cobalt deficient medium</th>
<th>Reductase activity $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>458</td>
</tr>
<tr>
<td>Deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine $^c$</td>
<td>316</td>
</tr>
<tr>
<td>Thymidine $^c$</td>
<td>321</td>
</tr>
<tr>
<td>Deoxyadenosine $^c$</td>
<td>389</td>
</tr>
<tr>
<td>Cobalt chloride $^d$</td>
<td>43</td>
</tr>
</tbody>
</table>

$^a$ 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 $3.75 \times 10^{-4}$ M.

$^d$ The concentration of cobalt was 24 μg/liter.
the ribonucleotide reductase apoenzyme. Under conditions of cobalt deficiency where insufficient B12 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 B12 compounds may function in several metabolic sites in the metabolism of Rhizobium species. In addition to the established role of B12 coenzyme in the ribonucleotide reductase, it is known (9) that R. meliloti contains a B12 coenzyme-dependent methylmalonyl coenzyme A mutase. Obviously, additional research is necessary to identify more precisely the various biochemical sites where B12 compounds function in the metabolism of Rhizobium species.

ACKNOWLEDGMENTS

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LITERATURE CITED


