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Title: SOME PROPERTIES OF RIBONUCLEOTIDE REDUCTASE IN RHIZOBIUM SPECIES

Abstract approved: Harold J. Evans

Ribonucleotide reductase from Rhizobium meliloti has been partially purified and characterized. The enzymatic reduction of ribonucleotides to deoxyribonucleotides is dependent upon B_{12} coenzyme and dihydrolipoate.

B_{12} coenzyme was more effective than B_{12} coenzyme analogues in the reductase reaction. The affinity of the R. meliloti reductase system for B_{12} coenzyme was approximately ten-fold less than that of Lactobacillus, the only reductase system reported to be B_{12} coenzyme dependent.

Certain guanosine, adenosine and cytidine phosphates were effective substrates in the reductase reaction. The uridine phosphates were reduced very slowly. The optimum concentrations of the different ribonucleotides as substrates were considerably different. Ribonucleoside diphosphates, in general, were effective at lower

substrate concentrations and had lower K_m values than the respective ribonucleoside mon- or triphosphates. The rates of reduction of ribonucleotides at optimum substrate concentrations were not significantly stimulated by magnesium and ATP.

Dihydrolipoate supplied the electrons for ribonucleotide reductions and the reductase system appears to be specific for this reductant. In preliminary experiments, extracts of R. meliloti contained a thioredoxin system which may function as the natural electron donor for ribonucleotide reduction.

B_{12} coenzyme-dependent ribonucleotide reductases have been identified in other species of Rhizobium. Reductase activity in the extracts of legume nodules also was dependent on B_{12} coenzyme. The amount of reductase activity in the Rhizobium species and in nodules was related to the apparent growth rate of the bacteria.

Cultures of R. meliloti grown on a mineral medium deficient in cobalt had a slower rate of growth than those grown on a medium containing cobalt. Cells from the deficient cultures contained a substantially greater reductase apoenzyme activity than those grown with adequate cobalt. Supplementing the cobalt deficient medium with deoxyribose compounds did not result in an increase in growth rate to that of normal cells nor cause the apoenzyme activity to be repressed.

Some Properties of Ribonucleotide Reductase in *Rhizobium* Species

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LIST OF ABBREVIATIONS USED

dAMP	deoxyadenosine monophosphate
CMP	cytidine monophosphate
GTP	guanosine triphosphate
dTTP	thymidine triphosphate
UDP	uridine diphosphate
mM	millimolar
mμmole	millimicromole
μg	microgram
B_{12} coenzyme or DBCC	5'-deoxyadenosyl 5, 6-dimethyl-benzimidazolylcobamide
FAD	flavin-adenine dinucleotide
$L(S)_2$ or L(SS)	lipoate
$L(SH)_2$	dihydrolipoate
Mg^{++}	magnesium ions
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
Tris-Cl	tris(hydroxymethyl)aminomethane-hydrochloride
DEAE-cellulose	diethylaminoethyl-cellulose
Enz	ribonucleotide reductase
PVP	polyvinylpyrrolodone

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SOME PROPERTIES OF RIBONUCLEOTIDE REDUCTASE IN *RHIZOBIUM* SPECIES

INTRODUCTION

Reichard and Rutberg (78) in 1960 reported that crude extracts from *Escherichia coli* catalyzed the reduction of ribonucleotides to deoxyribonucleotides. The enzyme responsible for the reduction was ribonucleotide reductase. Ribonucleotide reductases also have been found in several other organisms (4, 64, 74), and the enzyme is suggested to be important in *in vivo* deoxyribonucleotide synthesis and therefore in DNA synthesis.

In 1964 Blakley and Barker (23) provided evidence that the ribonucleotide reductase system from *Lactobacillus leichmannii* was stimulated by B_{12} coenzyme. A later investigation (21) established an absolute B_{12} coenzyme requirement for the reductase system from this organism. Of the ribonucleotide reductase systems studied, *L. leichmannii* and certain other species of *Lactobacillus* are the only organisms known to contain a B_{12} coenzyme-dependent reductase (20). The extent that the B_{12} coenzyme-dependent reductases are present in plants, animals and micro-organisms is still unanswered.

Evans and associates (6, 60) have reported a cobalt requirement for symbiotically grown leguminous plants and for various

species of Rhizobium grown in pure culture. Cobalt deficiency in Rhizobium meliloti cells results in a substantial decrease in B_{12} coenzyme concentration (50) and the formation of morphologically elongated cells (31). These observations suggested that abnormal cell morphology was related to a role of B_{12} coenzyme in the cell division process. It was postulated (30) that R. meliloti contained a B_{12} coenzyme-dependent ribonucleotide reductase and that limited B_{12} coenzyme synthesis under conditions of cobalt deficiency interfered with the synthesis of deoxyribonucleotides, which subsequently led to reduced DNA synthesis and cell division.

The primary purpose of this research was to determine whether R. meliloti cells contained ribonucleotide reductase and if so, to determine certain characteristics of the enzyme. After B_{12} coenzyme-dependent ribonucleotide reductase was demonstrated in extracts of R. meliloti, the occurrence and properties of the enzyme from other Rhizobium species and from nodules and roots of legumes were investigated. Studies also were conducted on the effect of cobalt deficiency on the activity of ribonucleotide reductase.

REVIEW OF LITERATURE

Deoxyribose Synthesis In Vivo

Incorporation of labeled cytidine (40, 79) into the RNA and DNA of rats was the first direct evidence that the ribose moiety of ribonucleosides was converted into the deoxyribose moiety of deoxyribonucleosides without cleavage of the glycosidic linkage. In later reports uridine-^{14}C was shown to be incorporated into the thymidylic acid moiety of DNA by E. coli cells infected with T-1 bacteriophage (7). Pyrimidine- and adenine-requiring mutants of Neurospora incorporated labeled ribonucleosides into RNA and DNA (62, 63). From studies on incorporation of cytidine-^{14}C into RNA and DNA of chick embryos (71, 75), it was suggested that the reduction of ribose occurred in the form of ribonucleotides.

Downing and Schweigert (29), in 1956, suggested that vitamin B_{12} was required for synthesis of the deoxyribose moiety of DNA in L. leichmannii. This suggestion was made in part from evidence of earlier studies that certain species of Lactobacillus cultured on a synthetic medium required vitamin B_{12} from liver extracts for normal growth (46) and that the vitamin B_{12} requirement could be replaced by deoxyribonucleotides. Beck et al. (11, 14) have shown that replenishing the supply of vitamin B_{12} to vitamin B_{12}-deficient cultures of L. leichmannii caused a very rapid increase in

acid-soluble deoxyribosyl compounds followed by a less rapid increase in the DNA to RNA and DNA to protein ratios. More direct evidence (23, 29, 81) to support this involvement of vitamin B_{12} in the synthesis of DNA was provided by the demonstration of the incorporation of thymidine-^{14}C and ribose-1-^{14}C into the DNA of L. leichmannii. Studies (61) on guanosine-^{14}C incorporation into DNA of L. leichmannii cells demonstrated that the incorporation also occurred without cleavage of the glycosidic linkage.

Ribonucleotide Reduction In Vitro

Reichard and Rutberg in 1960 reported that cell-free extracts from E. coli catalyzed the reduction of CMP to dCMP in the presence of Mg^{++} and ATP (78). The reductase system also was stimulated by NADPH, if the crude extracts were treated with a Dowex-2 resin or charcoal (76). CDP was reduced at a faster rate than CMP or CTP. Deoxy-CDP was identified as the first product formed during the ribonucleotide reductase reaction.

Blakley and Barker (23) and Abrams and Duraiswami (3), using cell-free extracts from L. leichmannii, also demonstrated the reduction of CMP to dCMP. The system required ATP, Mg^{++}, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, mercaptoethanol, B_{12} coenzyme, CMP-^{14}C and cell-free extract (23). Later, dihydrolipoate was found (21) to replace completely the requirements for

glucose-6-phosphate, glucose-6-phosphate dehydrogenase and mercaptoethanol. The rate of reduction of either CDP or CTP was more than two-fold than for CMP. The ribonucleotide reductase system from L. leichmannii was specific for B_{12} coenzyme and had an apparent K_m for B_{12} coenzyme of 8.6×10^{-7} M. The demonstration of a B_{12} coenzyme requirement for the ribonucleotide reductase system from L. leichmannii provided a biochemical basis for interpretation of earlier in vivo experimental evidence suggesting a role of vitamin B_{12} in DNA synthesis.

Ribonucleotide reductase systems also have been studied in chick embryos (72, 74), calf thymus (4), Ehrlich ascite cells (4), and Novikoff ascite cells (64, 65). These in vitro systems have requirements similar to those of the E. coli B reductase system. Fe^{++}, however, stimulated the Novikoff reductase system (65), and NADH rather than NADPH was claimed to be specific for the calf thymus and Ehrlich systems (4). The NADH specificity of the calf thymus reductase was not confirmed by Moore and Hurlbert (64).

Purification of Ribonucleotide Reductase. E. coli, L. leichmannii and Novikoff ascite cells have been the primary sources of material utilized in the purification of ribonucleotide reductase. Properties of the partially purified reductase from extracts of E. coli B and Novikoff ascite cells were very similar. These two systems, however, differ in several respects from the partially

purified reductase system of L. leichmannii. A discussion of the purification and characteristics of each system is given below.

Cell-free extracts from E. coli B were separated into two protein fractions (A and B), and each was purified 100-200 fold (17, 73). Both fractions were required for the catalysis of CMP reduction, but only fraction B was necessary for CDP reduction. The requirements for CDP reduction catalyzed by fraction B were similar to those for the crude system with the exceptions that Mg^{++} was absolutely required, and the system was highly specific for dihydrolipoate as the reductant.

Fraction B also catalyzed the reduction of uridine, adenosine and guanosine diphosphates (16, 53). The rates of reduction of these compounds were more rapid than those of the respective ribonucleoside mono- or triphosphates. Because fraction B catalyzes the reduction of all four ribonucleoside diphosphates, the question was raised whether fraction B was composed of one enzyme with low ribonucleotide specificity or four enzymes with marked specificity.

Fraction A and B were further purified by Reichard (73). Fraction A chromatographed similar to CMP kinase and was suggested to be composed primarily of phosphokinases. Fraction A then was not essential for CDP reduction. Fraction B was separated into two protein components (B_1 and B_2). These components were shown to be non-identical subunits and both were required for CDP

reduction. The more purified system continued to exhibit a high specificity for dihydrolipoate as the reductant, though this compound was needed at concentrations considerably above physiological levels. It was suggested that dihydrolipoate was not the physiological reductant and participated in the reductase system as a model reductant (66). The highly purified E. coli B reductase system failed to reveal a B_{12} coenzyme requirement (43).

Moore and Hurlbert (64) fractionated extracts from Novikoff ascite cells into three protein fractions, all of which were required for CDP reduction. Requirements for the partially purified reductase system from Novikoff cells were similar to the E. coli B system. The Novikoff system, however, continued to be stimulated by the addition of $FeCl_3$. It was concluded that the characteristics of the E. coli B and Novikoff reductases were similar although not identical (68).

Partially purified extracts from L. leichmannii catalyzed the reduction of ribonucleoside triphosphates at a greater velocity than either the ribonucleoside mono- or diphosphates (2, 24). It was suggested later (20) that the small amount of reductase activity measured with either the ribonucleoside mono- or diphosphates was due to ribonucleoside triphosphate contamination. The partially purified ribonucleoside triphosphate reductase system from L. leichmannii was no longer stimulated by ATP and Mg^{++}, but some stimulation in

the rate of CTP reduction by Mg^{++} and ATP was reported by Abrams (2) and Goulian and Beck (37). The partially purified system of L. leichmannii was specific for certain dithiol reductants and absolutely dependent on B_{12} coenzyme (37, 83). Naturally occurring B_{12} coenzyme analogues, 5'-deoxyadenosyladenylcobamide and 5'-deoxyadenosylbenzimidazolylcobamide, did not completely substitute for B_{12} coenzyme in the reductase reaction (20, 37). A highly purified reductase was obtained from extracts of L. leichmannii and exhibited similar requirements to the partially purified system (36, 37).

Natural Reductant. A heat stable protein was isolated from fraction A of E. coli B which in the presence of NADPH substituted for dihydrolipoate in the E. coli B reductase system (66, 67). This protein fraction exhibited properties similar to those of one of the proteins required for CDP reduction in the Novikoff reductase system (64). Laurent et al. (58) suggested that the heat stable protein from E. coli B, thioredoxin, functioned as the electron carrier between NADPH and the ribonucleoside diphsophate. Another protein, similar to one of the two heat-labile protein fractions in the Novikoff system, was purified from fraction B of E. coli B (66, 77). The flavoprotein, referred to as thioredoxin reductase (69), catalyzed the reduction of thioredoxin by NADPH. The investigators (66) were unable, however, to show an absolute requirement for thioredoxin

reductase in the E. coli B reductase system because the most purified sample of fraction B was contaminated with the flavoprotein. An absolute requirement for thioredoxin reductase was demonstrated in the Novikoff reductase system.

Thioredoxin from E. coli B has a molecular weight of 12,000 and is composed of a single-chain polypeptide containing one molecule of cystine (58). Reduced thioredoxin will function as a nonspecific reductant in other systems (82). Thioredoxin reductase from E. coli B has a molecular weight of 65,800 and contains two molecules of FAD per molecule of enzyme (82). The enzyme is capable of accepting four electrons per FAD. It has been proposed that two electrons are accepted by FAD and the other two by a disulfide group (87). Thioredoxin reductase appears to be absolutely specific for the reduction of thioredoxin (69, 82).

Originally it was suggested that the ribonucleotide reductase from L. leichmannii did not contain a thioredoxin system (24). However, the addition of NADPH and E. coli B thioredoxin- thioredoxin reductase (37, 83) to the L. leichmannii reductase system replaced the requirement for dihydrolipoate. The E. coli thioredoxin system also was more efficient than dihydrolipoate in the L. leichmannii system.

Two protein fractions have been isolated from L. leichmannii that exhibit properties similar to those of thioredoxin and thioredoxin

reductase from E. coli B (20, 70). The concentration of thioredoxin in L. leichmannii cells was reported to be sufficient to support the synthesis of deoxyribonucleotides. Consequently the thioredoxin system was proposed as the physiological electron donor for the ribonucleoside triphosphate reductase reaction.

The mechanism of hydrogen transfer in the ribonucleotide reductase reaction has been studied primarily with tritiated water. In the presence of tritiated water the sulfhydryl moities of either reduced thioredoxin or dihydrolipoate were rapidly labeled (54). In the E. coli B reductase system, tritium labeled hydrogen was incorporated into one of the two non-exchangeable positions on the 2' carbon of the deoxyribose moiety of the dCDP during CDP reduction. A hydride ion apparently displaces the hydroxyl moiety on the 2' carbon. Similar results (25, 35) were reported from experiments in which the L. leichmannii reductase system was utilized. Additional studies (85) on the mechanism of hydrogen transfer in the L. leichmannii ribonucleotide reductase reaction revealed that the reductant activates ribonucleotide reductase by: (1) reducing a disulphide bridge in the protein and (2) supplying the hydride ion for ribonucleotide reduction.

Cofactors. B_{12} coenzyme was suggested to function in the ribonucleotide reductase reaction as an intermediate hydrogen transferring agent between reductant and product (20, 37). Studies (1,

42) with the L. leichmannii reductase system showed that the tritium from tritiated water was transferred to a non-exchangeable position on the B_{12} coenzyme molecule. At least one of the hydrogen atoms in the 5' position of the adenosyl moiety of B_{12} coenzyme was labeled (42). It was demonstrated (10) that the tritium from B_{12} coenzyme (DBCC-5'-^{3}H) was exchanged with the hydrogen from water during the reductase reaction. This transfer was dependent upon substrate, reductase and dihydrolipoate (10, 42), even though the reduction of ribonucleotides was not essential. The rate of hydrogen exchange between B_{12} coenzyme and water was demonstrated to be considerably greater than the rate of transfer between B_{12} coenzyme and product. This explains why tritium from B_{12} coenzyme was transferred to water rather than to the product as shown in the following scheme (10):

1. $L(S^3H)_2 + H_2O \underset{\text{fast}}{\overset{\text{fast}}{\rightleftharpoons}} L(SH)_2 + {}^3H_2O$

2. $L(S^3H)_2 + DBCC + Enz \underset{\text{fast}}{\rightleftharpoons} L(SS)\text{-}Enz\text{-}DBCC\text{-}5'\text{-}{}^3H + {}^3H^+$

3. $L(SS)\text{-}Enz\text{-}DBCC\text{-}5'\text{-}{}^3H + GTP \xrightarrow{\text{slow}} dGTP + L(SS) + DBCC + Enz$

In the B_{12} coenzyme-independent reductase systems, it seemed

unlikely that hydride ions were generated from the sulfhydryl moities of dihydrolipoate or reduced thioredoxin without the involvement of an intermediate hydrogen carrier (27, 54). It was suggested recently (27) that protein B_2 from E. coli B was contaminated with a non-heme component that may function as an intermediate hydrogen carrier. After removal of heavy metals, the protein fraction failed to function as a ribonucleotide reductase. Activity was restored by adding small amounts of $Fe(NH_4)_2(SO_4)_2$. Brown et al. (27) have speculated that the non-heme iron in the E. coli B reductase system functions in a manner analogous to B_{12} coenzyme in the L. leichmannii system.

Regulation of Ribonucleotide Reductase Activity. Initially, ATP and Mg^{++} were postulated (24, 76) to be necessary for the phosphokinase reaction which converted either ribonucleoside mono- or diphosphates to ribonucleoside di- or triphosphates. The latter ribonucleotides were presumed to be more effective than the former as substrates in the reductase system. ATP and Mg^{++} also stimulated the rate of CDP reduction in the E. coli B reductase system even though CDP was more effectively reduced than either CMP or CTP (76). Holmgren et al. (43) suggested in 1965 that ATP functioned in the E. coli system as an activator of fraction B. ATP appears to function in the reductase reaction as an allosteric effector causing the reductase to exhibit a greater affinity for CDP (55).

Deoxy-ATP, on the other hand, inhibited the rate of CDP reduction. Other ribonucleoside and deoxyribonucleoside triphosphates also stimulated the rate of CDP reduction (56, 57).

In the E. coli B reductase system, ATP at 1 mM and dTTP at 10^{-2} mM were equally effective at stimulating the reduction of CDP or UDP (57) and were postulated to be the physiological activators of pyrimidine reduction. ATP, but not dTTP, effectively reverses the inhibitory effect of dATP which suggests that ATP and dTTP interact with the enzyme at two different allosteric sites. Ribonucleoside diphosphates also stimulate activity, but they are less effective than the triphosphates (56, 57). Deoxy-TTP and dGTP have been suggested as the physiological activators of purine reduction (56). The E. coli B reductase system was postulated to have only one reductase which is capable of catalyzing the reduction of several substrates. The rate of reduction of the various substrates is controlled by allosteric activators and inhibitors. Brown et al. (26) have postulated that allosteric sites are located on protein B_1.

The L. leichmannii reductase system also is controlled by allosteric factors (15, 36). In this system dATP stimulated CTP reduction, dCTP stimulated UTP reduction and dGTP stimulated CTP reduction (15). The rate of GTP reduction was only slightly stimulated by dTTP (20). Prime activators in the L. leichmannii reductase system were reported (9, 84) to: (1) alleviate the

non-linear reaction rate which occurs when either substrate or B_{12} coenzyme concentrations were limited, (2) increased the affinities of substrates and B_{12} coenzyme for the reductase and (3) prevented Mg^{++} from inhibiting the rate of ribonucleotide reduction.

Through the use of a sucrose density gradient, magnesium was shown to be required for formation of a complex between protein B_1 and B_2 (26). It was postulated, therefore, that the function of Mg^{++} in the E. coli B reductase system is to form an active enzyme complex between protein B_1 and B_2.

Syntheses of ribonucleotide reductase in E. coli $_{15T}$-(a thymineless mutant) and L. leichmannii were reported (12, 18, 19) to be subject to feedback inhibition and repression. Ribonucleotide reductase synthesis in these micro-organisms could be derepressed by omitting thymine from the growth medium (13, 18). Goulian and Beck (38) have suggested that dTTP is the actual repressor of reductase synthesis. Deficient concentrations of either vitamin B_{12} or deoxyribonucleosides in the growth medium of L. leichmannii resulted in a depression of ribonucleotide reductase synthesis (32, 38). Ghambeer and Blakley (30, 32, 33) have suggested that cultures of L. leichmannii accumulate deoxyribosyl compounds which result in a striking decrease in ribonucleotide reductase activity in older cultures.

Cobalt and B_{12} Coenzyme Deficiency. Ahmed and Evans (5)

in 1960 reported that cobalt was required for the growth of soybean plants under symbiotic conditions. Trace amounts of cobalt added to a purified nutrient solution greatly increased the total weight and nitrogen content of deficient plants. Other metals could not substitute for cobalt. It was suggested that cobalt was involved in the nitrogen fixation process and probably was required in the metabolism of the rhizobia within the nodules (6). It later was demonstrated to be required for the normal growth of various Rhizobium species grown in pure culture (60).

Vitamin B_{12} and B_{12} coenzyme are synthesized in the nodules of legumes (6, 47). B_{12} coenzyme also has been identified in cells from pure cultures of R. meliloti (50). Cultures of R. meliloti grown on a cobalt-deficient medium do not contain measurable quantities of B_{12} coenzyme (48, 49). The concentration of B_{12} coenzyme in R. meliloti cells was shown to be directly correlated with the concentration of cobalt in the nutrient medium.

Cultures of L. leichmannii grown on a medium with deficient concentrations of vitamin B_{12} or deoxyribonucleosides contained many elongated cells which were non-viable (11, 14). The cells in these cultures had decreased DNA to RNA and DNA to protein ratios. R. meliloti cultures grown on the cobalt deficient medium also contain many morphologically elongated cells (31). It was suggested (14, 31) that the morphological abnormality of R. meliloti and L. leichmannii in B_{12} deficient media is due to decreased DNA synthesis.

MATERIALS AND METHODS

Culture of the Organisms

Cultures of Rhizobium meliloti F-28, Rhizobium leguminosarium C-56, Rhizobium phaseoli K-17, Rhizobium trifolii K-4, and Rhizobium japonicum A-72 were kindly supplied by Dr. Joe Burton of the Nitrogen Co. Rhizobium meliloti 300al, a strain of R. meliloti ineffective in fixing nitrogen, was a gift from Dr. 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 (28). R. japonicum was maintained and cultured on a medium containing arabinose and glycerol (28).

The bacteria were usually grown in a series of one-liter flasks containing 400 ml of sterilized medium with a 0.25% inoculum. The cultures were incubated in the dark at 30° on a rotary shaker. Larger quantities of R. meliloti, R. japonicum and R. meliloti 3D were obtained by inoculating ten liters of sterilized medium in 12-liter carboys with a 1% inoculum. These were maintained at 30° and were aerated with air filtered through sterile cotton and dispersed with a fritted-glass sparger. Cultures of R. meliloti also were grown in one-liter flasks containing a mineral medium deficient in cobalt. Preparation of the cobalt-deficient medium and treatment of the glassware was described by Kliewer et al. (51). A culture of

R. meliloti was transferred from the mannitol medium to a 50-ml flask containing 20 ml of the cobalt-deficient medium. Successive transfers were made from the cobalt-deficient medium to two 50-ml flasks; one of which contained the deficient medium and the other the deficient medium supplemented with cobalt (24 μg per liter). When the rate of cell growth on the cobalt-deficient medium was noticeably less than that on the supplemented medium, the deficient culture was used to inoculate the one-liter flasks. The effects of cobalt-deficiency were studied, using twelve one-liter flasks containing the cobalt-deficient mineral medium. Four of these flasks contained only the deficient medium, four were supplemented with deoxyribonucleosides (Sigma Chemical Co.), and to the other flasks was added 24 μg of cobalt per liter.

Growth of the bacteria was monitored by measuring the absorbance of the culture at 660 mμ in a Bausch and Lomb colorimeter. Cells normally were harvested when the absorbance reached 0.70 to 0.85. Cultures grown on the cobalt-deficient medium were harvested when the absorbance reached 0.35 to 0.45.

Growth of Plants

Soybean (Glycine max Merr var. Merrit) and alfalfa (Medicago sativa var. Dupre) seeds were inoculated with commercial preparations of R. japonicum and R. meliloti, and were planted in pots

containing perlite. The plants were cultured in the green house and were provided with a nitrogen-free nutrient solution (5). Supplemental fluorescent lights were used for 14 hours each day. Nodules were harvested from the soybean and alfalfa plants 20 and 44 days, respectively, after the seeds were planted.

Soybean seeds that were not inoculated were planted in a wood flat containing perlite and grown in a green house without supplemental light. The seedlings were harvested five days after planting.

Preparation of Extracts

Rhizobium cells were harvested by centrifugation and washed twice in 0.05 M potassium phosphate buffer (pH 7.3). The cells then were suspended in an equal volume of 0.05 M potassium phosphate buffer (pH 7.3) and broken in a French pressure cell at approximately 6.4 tons per square inch and at a temperature of 0-6°. In some cases cells were frozen in solid CO_2 and then broken in an Eaton press at a pressure of approximately 10.2 tons per square inch. The cell macerates after thawing were centrifuged for 15 minutes at 32,000 x g, and the supernatant was used as the crude extract.

Crude extracts from soybean and alfalfa nodules were prepared using the same techniques. The nodules were harvested, washed free of excess debris and rinsed with distilled water. Usually 15 gm of acid-washed insoluble polyvinylpyrrolodone (PVP) (59)

(Polyclar AT from General Aniline Corp.) and 60 ml of 0.05 M potassium phosphate buffer (pH 7.3) containing 300 mM ascorbic acid were added to a cold mortar containing 30 gm of nodules (52). The nodules were macerated and squeezed through a single layer of bolting cloth. The PVP-nodule solid debris was resuspended and re-macerated in 60 ml of the potassium phosphate buffer containing ascorbic acid. The brei from the two extractions was centrifuged for 15 minutes at 32,000 x g, and the bacteroid pellet was washed with 0.05 M potassium phosphate buffer (pH 7.3). After suspending in the same buffer, the bacteroids were broken in a French or Eaton press as described previously.

The root tips from soybean seedlings (3 cm) were removed and placed into a beaker of cold distilled water. The tips were 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. The roots then were cut into small segments and macerated in an Eaton press. The frozen macerate was allowed to thaw in three volumes of the buffer solution used for breaking the cells. After thawing the mixture was centrifuged for 15 minutes at 32,000 x g and the supernatant was used as the crude extract.

Purification

The crude extracts of rhizobia, nodules and roots were purified

by essentially the same procedure. In a typical purification 20 ml of Rhizobium extract containing 25 mg of protein per ml was utilized. The nucleic acid content of the extract was estimated, and then 25 µl of a 2% protamine sulfate solution (pH 7.0) (Eli Lily & Co.) was added dropwise for each mg of nucleic acids (41). During this and all subsequent operations, the extract was maintained at 0 to 4° C. After ten minutes the mixture was centrifuged ten minutes at 14,000 x g and the pellet discarded. Additional saturated ammonium sulfate solution was added to the supernatant to obtain 45% of saturation. After ten minutes the precipitate was collected by centrifugation for ten minutes at 14,000 x g and dissolved in 10 ml of 0.05 M potassium phosphate buffer (pH 7.3). This solution was dialyzed for 12 hours against four liters of 0.005 M potassium phosphate buffer (pH 7.3) and used in the ribonucleotide reductase assay.

Assay Procedure

A complete reaction mixture (0.5 ml) contained: 1 µmole of GTP (Sigma Chemical Co.); 15 µmoles L $(SH)_2$ (Sigma Chemical Co.); 10 mµmoles B_{12} coenzyme (a gift of Dr. L. Mervyn); 50 µmoles potassium phosphate buffer (pH 7.3) and an appropriate amount of enzyme. The reaction mixtures, unless otherwise indicated, were incubated for 30 or 60 minutes at 37° and terminated by placing the tubes in boiling water for three minutes. The solutions

containing purine substrates were treated with chloracetamide and maintained in boiling water for an additional ten minutes (22). The reactions containing pyrimidine substrates were acidified with HCl and treated according to the procedure reported by Blakley (22), with the exception that 0.12 ml of saturated bromine water was added five to seven minutes before the reaction mixtures were treated with 0.1 ml of 5.0 N NaOH. Two ml of diphenylamine reagent (22), prepared from recrystallized diphenylamine (Matheson, Coleman and Bell) were added to each reaction mixture and allowed to incubate for four hours at 50°. The absorbance (595 mμ) of the blue chromagen was measured with a Beckman DU spectrophotometer and concentrations of deoxyribonucleotide estimated from standard curves prepared with deoxyribonucleoside monophosphates according to the procedure of Blakley (22). Standard curves for dAMP and dGMP were similar, as indicated by the observation that 22 mμmoles of either of these deoxyribonucleotides in reactions resulted in an optical density of approximately 0.100. Standard curves of dUMP and dCMP also were similar, but an optical density of 0.100 was equivalent to approximately 50 mμmoles of these deoxyribonucleotides. The assay does not distinguish between the various ribonucleoside phosphates and the lower limit of detection was three mμmoles per reaction.

Other Determinations

The concentration of $L(SH)_2$ which was prepared by reducing lipoate by the method of Gunsalus and Razzell (39) was determined colorimetrically by its capacity to reduce an excess of a standard solution of potassium ferricyanide (80). The concentration of potassium ferricyanide, after reduction with $L(SH)_2$, was determined on the basis of a molar extinction coefficient of 1040 at 420 mμ.

The concentration of B_{12} coenzyme was determined from the millimolar extinction coefficient of 8.0 at 522 mμ (8). The concentrations of two analogues of B_{12} coenzyme, 5'-deoxyadenosyl-5-methoxybenzimidazolylcobamide and 5'-deoxyadenoxyl-2-methyladenylcobamide (kindly supplied by Dr. Lars Ljungdahl) were estimated by the use of the millimolar extinction coefficients, 7.8 at 514 mμ (calculated from data reported (44)) and 9.0 at 375 (8), respectively. Since only a very small quantity of the 5'-deoxyadenosyl-2-methyladenylcobamide was available, the extinction coefficients for 5'-deoxyadenosyladenylcobamide and 5'-deoxyadenosyl-2-methyladenylcobamide coenzymes were assumed to be identical.

Phosphatase, GTP:GMP phosphotransferase and ATP:AMP phosphotransferase activities in the extracts were measured under conditions similar to those used to determine the ribonucleotide

reductase activity. The only exception was that N-tris-methyl-2-aminoethane sulfonic acid buffer (100 mM, pH 7.3) was used in the phosphatase assay mixtures instead of potassium phosphate buffer. Phosphatase activity was determined by the rate of liberation of phosphate from ribonucleoside triphosphate by the method of Bertani et al. (16). GDP and ADP concentrations resulting from phosphotransferase activity were assayed on aliquots of the boiled reactions by adding phosphoenol pyruvate, an excess of crystalline pyruvate kinase, and then determining the amount of pyruvate formed by the method of Kachmar and Boyer (45). Concentrations of ADP or GDP were estimated from control reactions containing known concentrations of ADP or GDP.

An estimate of the nucleic acid concentration in the crude extract was determined on the basis of absorbance at 260 and 280 mμ (86). All protein concentrations were estimated by the biuret procedure (34) unless otherwise indicated.

RESULTS

Initial Investigations

Initially it was necessary to establish if cell-free extracts from R. meliloti contained ribonucleotide reductase activity. The in vitro reductase activity was demonstrated in preliminary experiments by measuring the rate of GTP reduction using a colorimetric assay similar to that described by Blakley (22). The cell-free extracts of R. meliloti used to catalyze this reduction were purified with protamine sulfate and ammonium sulfate to remove large amounts of deoxyribose compounds. These endogenous compounds interferred with the detection of the small quantities of deoxyribonucleotides formed by the ribonucleotide reductase reaction.

The essential components of the ribonucleotide reductase reaction were established (Table I). Ribonucleotide reductase activity was demonstrated using GTP as the substrate, dihydrolipoate as the reductant and B_{12} coenzyme as a cofactor. The reaction rates were proportional to the protein added to the reaction mixture during the incubation period of one hour. It also was shown that the ribonucleotide reductase activity in cells varied with the age of the culture (Table II). The largest quantity of enzyme (determined from a plot of the data) appeared to be in cultures of R. meliloti harvested at an optical density of approximately 0.75. These preliminary

Table I. Requirements for the Ribonucleotide Reductase Reaction.

The complete system in a final volume of 0.5 ml contained; 15 μmoles $L(SH)_2$; 5 μmoles GTP, 8 mμmoles B_{12} coenzyme; 50 μmoles potassium phosphate buffer (pH 7.3) and 0.6 mg protein (0-55% $(NH_4)_2SO_4$ fraction). Reaction mixtures were incubated 1 hour at 37°.

Omission from complete system	dGTP formed (mμmoles/mg protein/hr)
None	110
GTP	9
B_{12} coenzyme	2
Dihydrolipoate	0
Enzyme (boiled enzyme added)	0

Table II. The Effect of Culture Age and Growth on the Specific Activity of Ribonucleotide Reductase.

In the ribonucleotide reductase assay the complete system in a final volume of 0.5 ml contained: 9 μmoles $L(SH)_2$, 1 μmole GTP, 8 mμmoles B_{12} coenzyme; 50 μmoles potassium phosphate buffer (pH 7.3) and 0.25 to 0.50 mg protein (0-55% $(NH_4)_2SO_4$ fraction). Reaction mixtures were incubated 1 hour at 37°. The cultures were grown in one liter flasks containing 400 ml of medium (see Materials and Methods).

Age of culture	Growth		Ribonucleotide reductase activity
(hrs)	Absorbance (OD at 660 mμ)	Wet weight of cells (gms)	(mμmoles dGTP/mg protein/hr)
13.5	0.36	0.31	303
15.5	0.63	0.58	363
17.5	0.91	0.91	294
20.5	1.10	1.20	186

investigations provided sufficient information so that a more systematic study could be conducted on the properties of ribonucleotide reductase in R. meliloti.

Additional Purification of the Enzyme

During the initial investigation a 30 to 45% ammonium sulfate fraction of the crude extract was shown to contain the highest specific reductase activity. Similar fractions were used for chromatography of the reductase on DEAE-cellulose. A DEAE-cellulose column (2.4 X 22.0 cm) was prepared, and equilibrated with 200 ml of 0.05 M Tris-Cl buffer, (pH 7.5) containing 1 mM 2-mercaptoethanol according to a procedure similar to that of Goulian and Beck (37). In a typical experiment 15 ml of the dialyzed ammonium sulfate fraction was added to the column, and subsequently eluted with a linear gradient of Tris-Cl buffer (pH 7.5). The buffer gradient containing 1 mM 2-mercaptoethanol was prepared by placing 150 ml of 0.18 M buffer in the mixing chamber and an equal volume of 0.28 M solution in the reservoir. The ten ml fractions containing appreciable protein were dialyzed against 0.005 M potassium phosphate buffer (pH 7.3) and then assayed for ribonucleotide reductase activity. As shown by Figure 1, most of the protein was present in fractions 10 to 13, and the major peak of ribonucleotide reductase activity was eluted in fractions 12 to 15. A summary of the

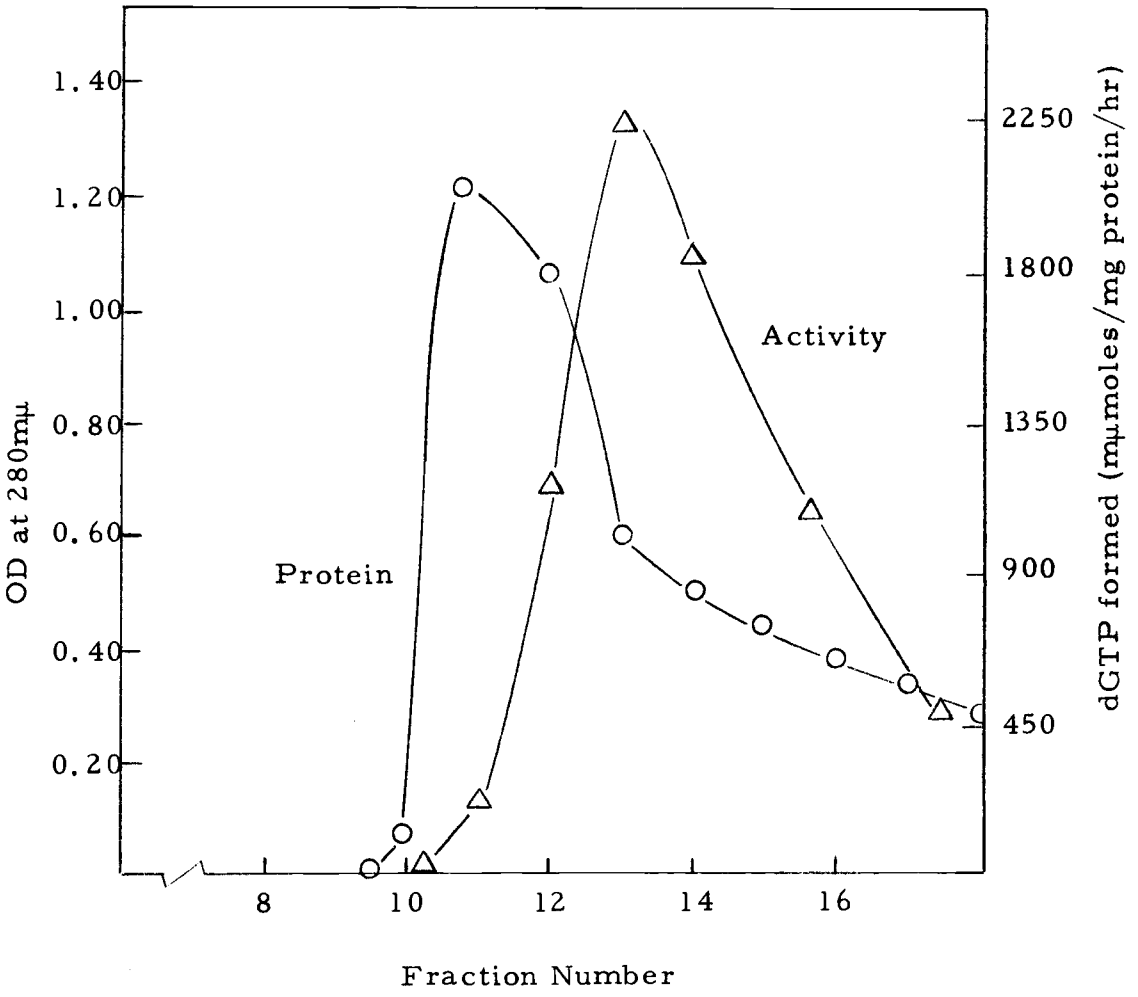

Figure 1. Partial Purification of Ribonucleotide Reductase on a DEAE-cellulose Column. Details of the experiment are presented in the text.

purification (Table III) shows that the peak fraction (fraction 13) catalyzed the reduction of 2262 mμmoles of GTP per mg of protein per hour and was purified about 20-fold. This preparation, referred to as DEAE eluate, was used in all subsequent experiments unless otherwise indicated.

A series of preliminary experiments was conducted in an attempt to further purify the enzyme. Additional fractionation by ammonium sulfate and by chromatography on columns of hydroxyl apatite, Bio-Gel P-100 or P-150 did not appreciably purify the reductase more than that obtained by DEAE-cellulose purification.

Linearity of Reaction

It was demonstrated that dGTP formation was essentially linear with incubation time for a period of 30 minutes (Figure 2). The rate of dGTP formation also was proportional to the amount of protein added (Figure 3).

Cobamide Coenzyme Requirement

The concentration of B_{12} coenzyme necessary for saturation of ribonucleotide reductase in extracts of R. meliloti is illustrated in Figure 4. Saturation of the enzyme is approached at a B_{12} coenzyme concentration of 24 μM which is approximately three-fold the concentration reported to saturate the ribonucleotide reductase from

Table III. Summary of Purification of Ribonucleotide Reductase from R. meliloti.

Details of the purification procedure are presented in the text.

Fraction	Volume (ml)	Protein (mg)	Specific activity* (units/mg protein)	Total activity* (units x 10^{-3})
Crude extract	50	1250	112	140
Protamine sulfate supernatant**	54	902	87	78
$(NH_4)_2SO_4$ ppt. (30-45%)	15	129	372	48
DEAE eluate (fraction 13)	12	6	2262	14

*One unit of activity is defined as the amount of enzyme necessary to catalyze the synthesis of 1 mμmole of dGTP per hour in the standard assay.

**This fraction was not dialyzed before it was assayed.

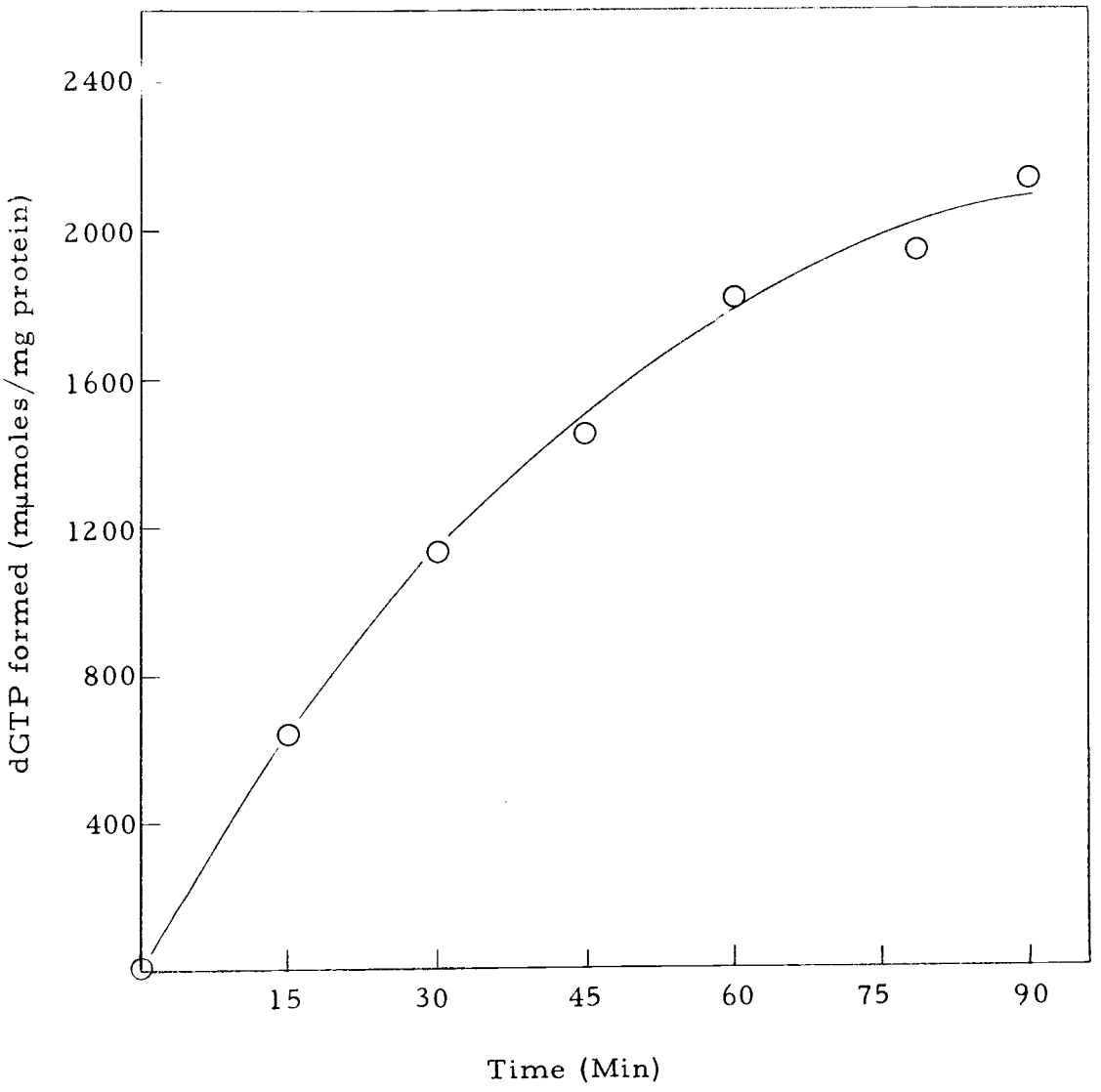

Figure 2. Ribonucleotide Reductase Activity as a Function of the Period of Incubation. The complete system in a final volume of 0.5 ml contained: 15 μmoles $L(SH)_2$; 1 μmole GTP; 10 mμmoles B_{12} coenzyme; 50 μmoles potassium phosphate buffer (pH 7.3) and 36 μg protein (DEAE eluate). The period of incubation at 37^{o} was varied as indicated.

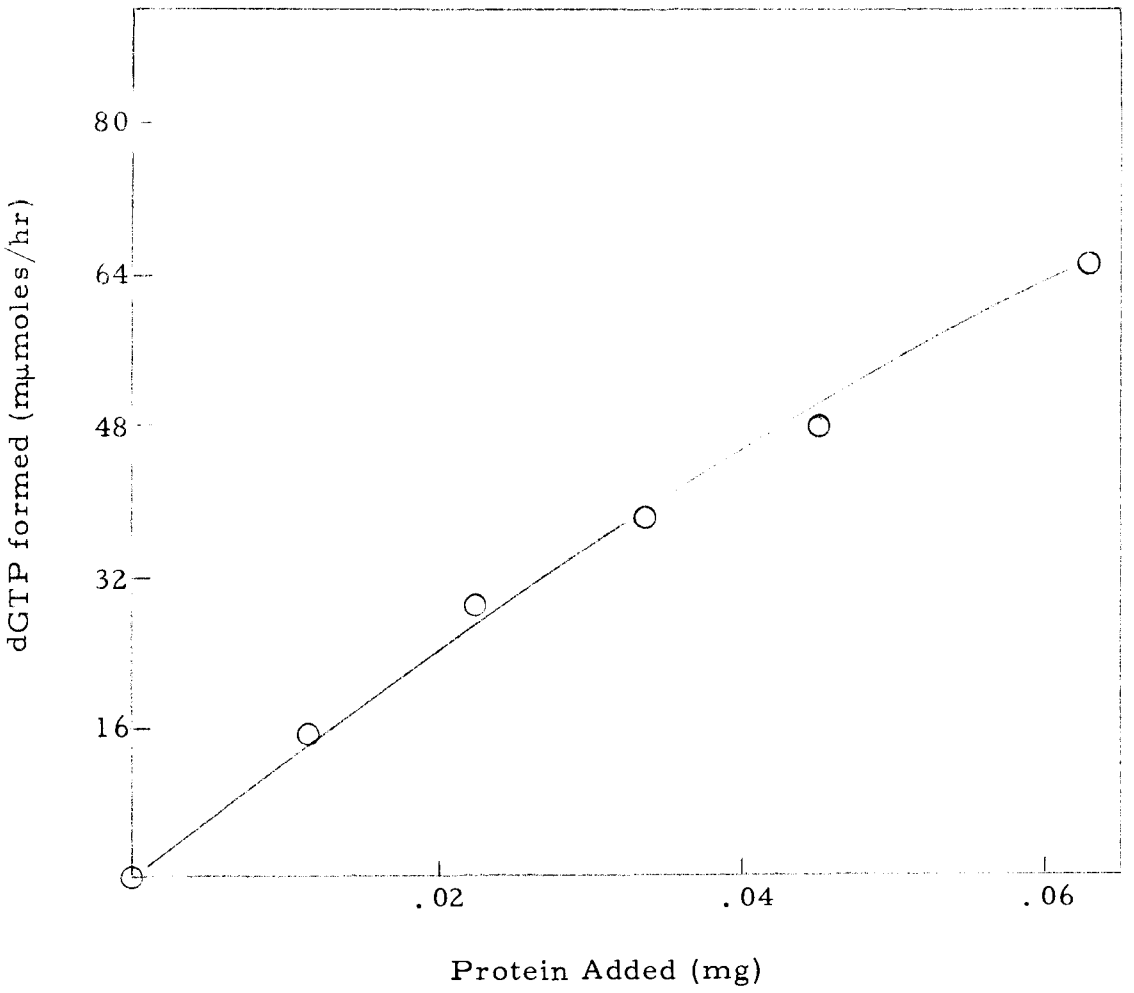

Figure 3. Ribonucleotide Reductase Activity as Related to Protein Concentration. The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except that the protein concentrations were varied as indicated. The period of incubation was 30 minutes at 37°.

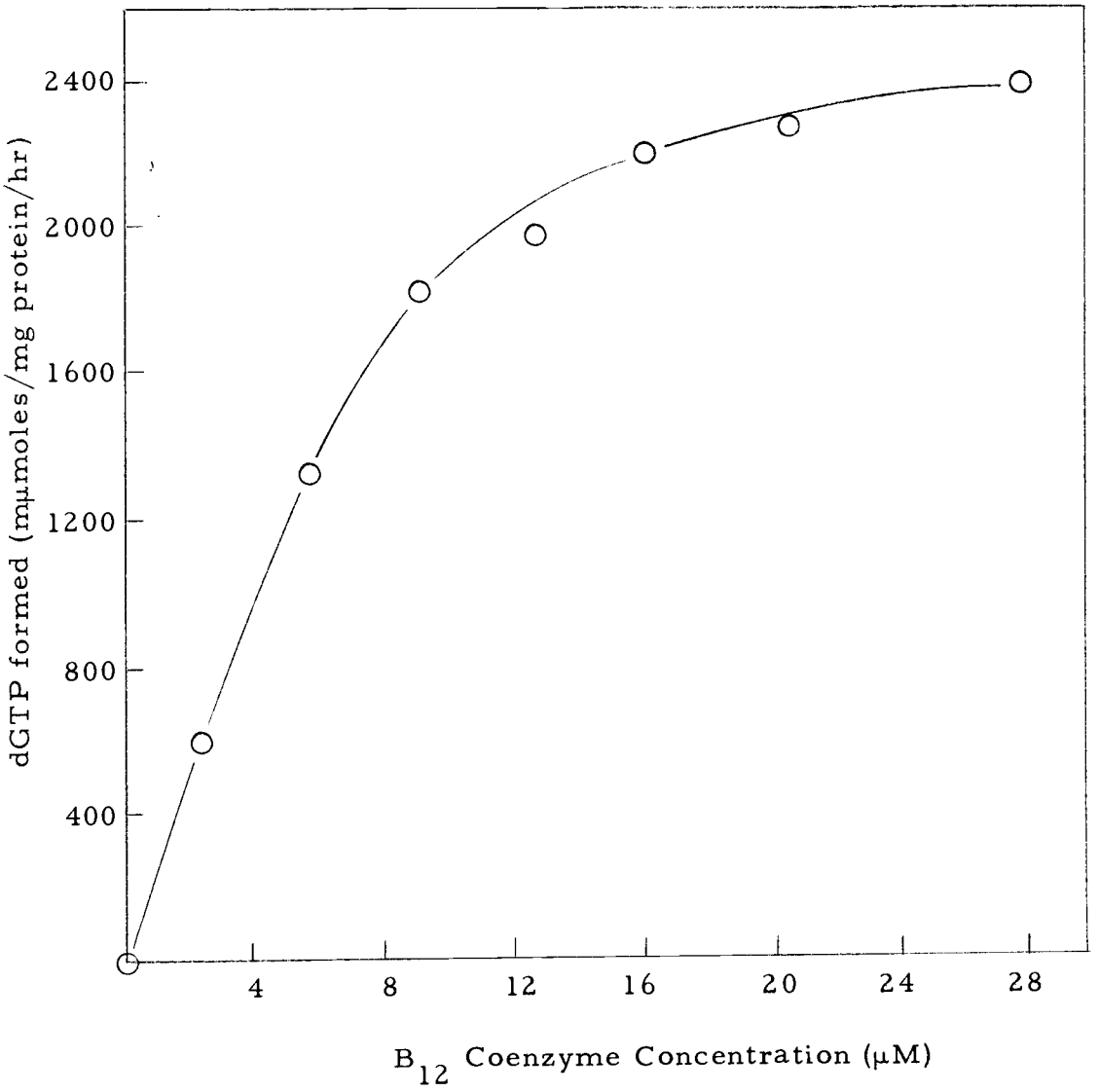

Figure 4. The Effect of B_{12} Coenzyme Concentration on Ribonucleotide Reductase Activity. The reaction mixtures were the same as the complete reaction mixture described in Figure 2 except that the B_{12} coenzyme concentration was varied as indicated. The period of incubation was 30 minutes at 37°.

L. leichmannii (21). From a Lineweaver-Burk plot of the data in Figure 4, an apparent K_m value for B_{12} coenzyme was calculated to be 5.5 μM. The substitution of 5'-deoxyadenosyl-5-methoxybenzimidazolylcobamide (20 μM) or 5'-deoxyadenosyl-2-methyladenylcobamide (20 μM) for B_{12} coenzyme resulted in a marked decrease in ribonucleotide reductase activity. The rates of ribonucleotide reductase reaction with B_{12} coenzyme, 5'-deoxyadenosyl-5-methoxybenzimidazolylcobamide and 5'-deoxyadenosyl-2-methyladenylcobamide were 2370, 1212 and 644 mμmoles per mg protein per hour, respectively.

Substrates

In most experiments GTP was used as the substrate for the ribonucleotide reductase reaction. Saturation of the enzyme with GTP was achieved at a concentration of about 2 mM (Figure 5). The concentrations of GTP used in the L. leichmannii system (20, 24) (7.8 and 10.0 mM) were inhibitory in the R. meliloti system. None of the other ribonucleotides, when tested a 2 mM, functioned as effectively as GTP (Table IV). The rates of ADP and CDP reduction were 72 and 48%, of the GTP reduction rate, while the remainder of the ribonucleotides were reduced at rates less than 35% that of GTP. In all of these experiments the ribonucleoside diphosphates were better substrates than the corresponding ribonucleoside

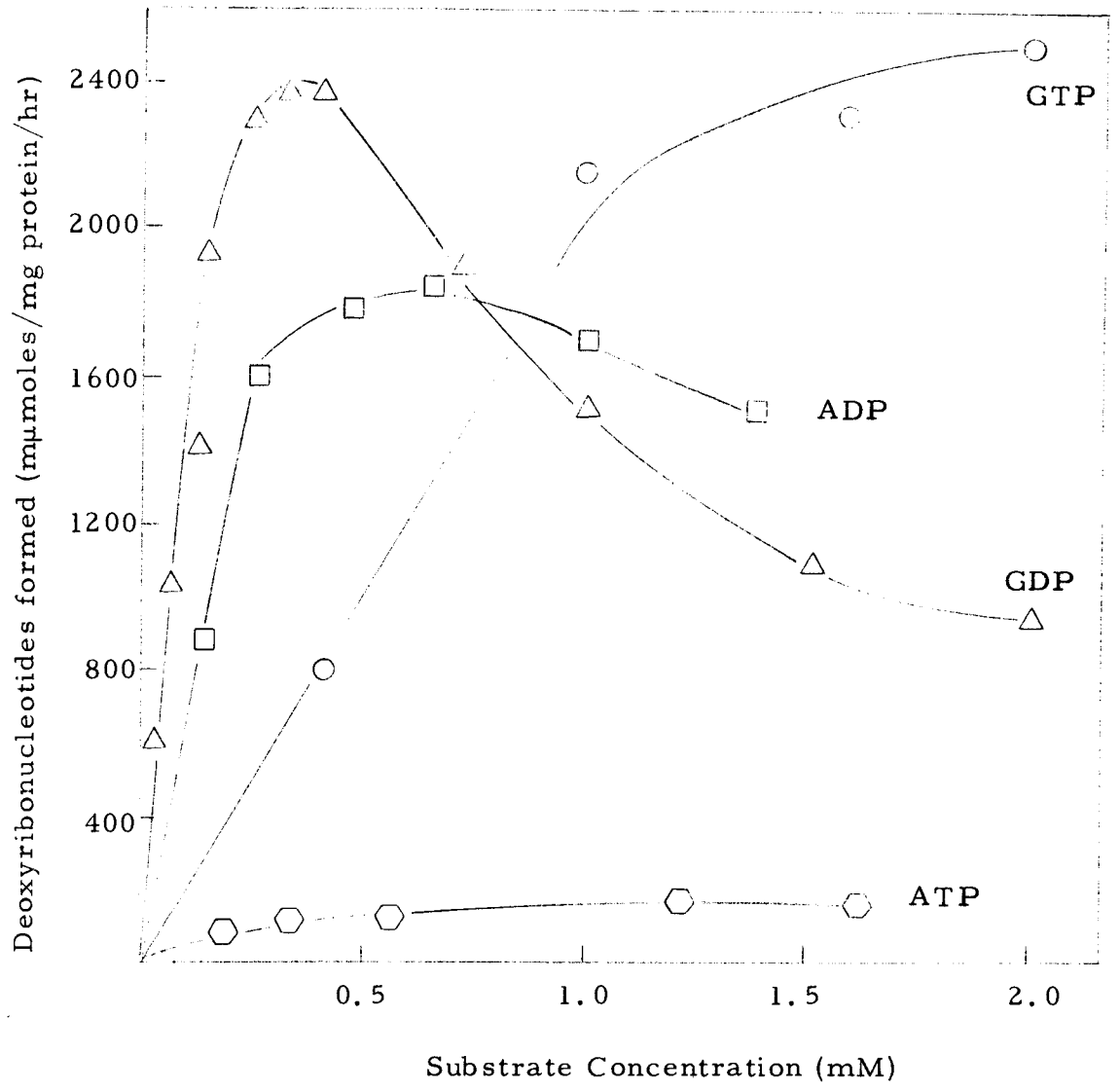

Figure 5. A Comparison of the Effectiveness of Different Purine Ribonucleotides as Substrates for the Ribonucleotide Reductase Reaction. The reaction mixtures contained the components as listed for the complete reaction mixture in Figure 2 except the ribonucleotides shown were substituted for GTP and the concentration of each ribonucleotide was varied as indicated. The period of incubation was 30 minutes at 37°.

Table IV. Comparison of Various Ribonucleotides Used as Substrates for the Ribonucleotide Reductase Reaction.

The reaction mixtures were the same as described in Figure 2 with the following exceptions: The ribonucleotides as indicated were substituted for GTP and the protein concentration with the pyrimidine substrates was increased from 36 to 54 μg per assay. The concentration of each ribonucleotide was 1 μmole in a total volume of 0.5 ml. The period of incubation for the purine and pyrimidine substrates were 30 and 45 minutes, respectively, at 37°.

Substrate	deoxyribonucleotides formed (mμmoles/mg protein/hr)
GTP	2318
GDP	722
GMP	628
ATP	162
ADP	1672
AMP	15
CTP	581
CDP	1120
CMP	118
UTP	173
UDP	253
UMP	103

monophosphates. Except for GDP, the ribonucleoside diphosphates were more effective as substrates than the corresponding ribonucleoside triphosphates. The data presented in Figure 5 show that the concentration of GTP resulting in maximum reductase velocity was not the optimum concentration for the other ribonucleotide substrates. GDP, for example, was reduced at less than 40% the rate of GTP when both were tested at 2 mM. If the concentration of GDP was reduced to 0.3 mM, then the rate of reductase reaction was comparable to that observed for GTP at a concentration of 2 mM.

Since GDP and ADP were effective substrates for the reductase reaction at low concentrations, it was possible that part of the reductase activity observed when ribonucleoside triphosphates were used as substrates was a result of (a) enzymes in the partially-purified extracts which catalyzed the formation of ribonucleoside diphosphates from the ribonucleoside triphosphates or (b) ribonucleoside diphosphate contamination in the ribonucleoside triphosphates. The partially-purified extracts were shown to contain enzymes which formed inorganic phosphate from GTP or ATP. The phosphatase activity, under conditions similar to those used in the reductase reaction, caused 0.92 μmoles of phosphate to be liberated per mg of protein per hour. The synthesis of GDP or ADP, however, could not be demonstrated when GTP or ATP were used under these conditions. The ribonucleoside triphosphates apparently were not being

enzymatically converted to the diphosphates and the diphosphates being used in the reductase reaction. On the other hand, thin layer chromatography of GTP and ATP showed that these substrates were contaminated with GDP and ADP. GTP contained approximately 8% GDP and ATP contained about 4% ADP.

A series of estimated K_m and V_{max} values calculated from plots of substrate concentration divided by reaction rate against substrate concentration is presented in Table V. Guanosine, adenosine and cytidine diphosphates exhibit lower K_m values than the corresponding ribonucleoside mono- or triphosphates. The K_m for GMP and AMP are relatively high in comparison to the K_m of other ribonucleotides tested. Thin-layer chromatography of the ribonucleoside monophosphates revealed no measurable contamination by ribonucleoside di- or triphosphates. It seems unlikely, therefore, that the increased velocity obtained at very high concentrations of GMP or AMP was due to impurities in these substrates. Guanosine mono- di- and triphosphates were all effective substrates in the reductase system at optimum concentrations. Comparable V_{max} values were obtained with each of these ribonucleotides, but the apparent K_m values were strikingly different (Table V). The V_{max} for each of the respective three adenosine and cytidine phosphates were different. Ribonucleotide reductase activity was very low when the uridine phosphates were used as substrates, and

Table V. Comparison of Kinetic and Optimum Substrate Concentration Data for Different Ribonucleotides.

The reaction mixtures each in a final volume of 0.5 ml were as described in Figure 2 (complete system) except the various ribonucleotide were substituted for GTP at substrate concentrations necessary to make the indicated determinations. Four substrate concentrations in the following ranges were used for determining the K_m and V_{max} values: GDP, ADP and CMP, 0.05 to 0.35 μmoles; GTP, ATP and CDP, 0.15 to 1.0 μmoles; GMP and CTP, 0.6 to 3.2 μmoles and AMP to 10 μmoles. The K_m and V_{max} values were determined from a plot of substrate concentration divided by reaction rate against substrate concentration.

Substrates	K_m (mM)	V_{max}*	Optimum substrate concentration** (mM)
GTP	0.76	3510	2.0
GDP	0.16	3560	0.4
GMP	10.51	4010	15.0
ATP	0.23	208	1.2
ADP	0.14	2280	0.6
AMP	9.90	304	20.0
CTP	1.63	1160	4.0
CDP	0.24	1987	1.4
CMP	0.42	279	0.6

*mμmoles deoxyribonucleotide formed/mg protein/hr.

**Concentration of substrate necessary for saturation of the enzyme.

therefore an accurate determination of K_m and V_{max} values for these ribonucleotides was not feasible with the method used.

Effects of Mg^{++} and ATP

The rate of GTP reduction in a complete reaction mixture is inhibited by adding Mg^{++} and ATP (Table VI). ATP is reduced at a much slower rate than GTP and therefore may decrease the rate of GTP reduction by competing with it in the reductase reaction. In addition concentrations greater than 2 mM of either GTP or ATP without Mg^{++} also were inhibitory. The rate of ATP or ADP reduction also was inhibited by the addition of Mg^{++} and ATP, but these additions stimulated the rate of reduction of GDP, GMP, or AMP (Table VI). When optimum concentrations of GDP and GMP were used (Table VII), the effects of added Mg^{++} and ATP were minimized. The stimulatory effect of ATP and Mg^{++} very likely is due to a requirement for phosphokinases (76) which catalyze the synthesis of ribonucleosides di- or triphosphates from ribonucleotide mono- and diphosphates, respectively. Since certain concentrations of ribonucleotides inhibit the enzyme, the possibility that kinase activity could effectively reduce the concentration of a particular ribonucleoside phosphate by converting it to some other ribonucleoside phosphate must be considered.

Table VI. The Effect of Mg^{++} and ATP on the Rate of Reduction of Guanosine and Adenosine Phosphates.

The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except equal amounts of Mg^{++} and ATP were added at the indicated concentrations and the substrates listed were substituted for GTP. The concentration of all the substrates was 2 mM. The period of incubation was 30 minutes at 37^{o}.

Substrate	Deoxyribonucleosides formed (mμmoles/mg protein/hr) Concentration of Mg^{++} and ATP (mM)			
	None	0.5	1.0	2.0
GTP	2240	1662	1466	612
GDP	373	1049	1689	1235
GMP	567	1679	1782	1561
ATP	62	25	3	0
ADP	1421	920	908	664
AMP	10	767	693	536

Table VII. The Effect of Mg^{++} and ATP on the Rate of Reduction of Guanosine Phosphates at Optimum Concentrations.

The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except: (1) GDP (0.3 mM) and GMP (15 mM) were substituted for GTP, and (2) Mg^{++} (1.0 mM) and ATP (1.0 mM) were added to the complete mixture as indicated. The period of incubation was 30 minutes at 37° .

Reductase system	Deoxyguanosine phosphates formed (mμmoles/mg protein/hr)
Complete (GTP)	2162
Complete, Mg^{++} and ATP	1588
Complete (GDP)	2362
Complete, Mg^{++} and ATP	2274
Complete (GMP)	2437
Complete, Mg^{++} and ATP	2852

Reductant

Vitols and Blakley (83) have reported that dihydrolipoate is the most effective electron donor for the ribonucleotide reductase system from L. leichmannii and, consequently it was used as the reductant in the system from R. meliloti. From Figure 6 it can be seen that the R. meliloti reductase was saturated with $L(SH)_2$ at a concentration of 30 mM. The concentration of $L(SH)_2$ reported (21, 83) to saturate the L. leichmannii system also was 30 mM. From a comparison of several other reductants (Table VIII), it is obvious that $L(SH)_2$ was the most effective reductant.

In some preliminary experiments in Dr. Evans' laboratory, Mr. Peter Wong isolated fractions from R. meliloti that contain thioredoxin and thioredoxin reductase. When these components and NADPH were substituted for $L(SH)_2$ in reaction mixtures of the type described in Table V, rates of GTP reduction were observed that were approximately 50% of the rate obtained when $L(SH)_2$ was utilized in the system. The thioredoxin system in R. meliloti requires further investigation.

Ribonucleotide Reductase in Various Rhizobium Species and Nodules

B_{12} coenzyme-dependent ribonucleotide reductase activity was found in several Rhizobium species (Table IX) and in the bacteroids

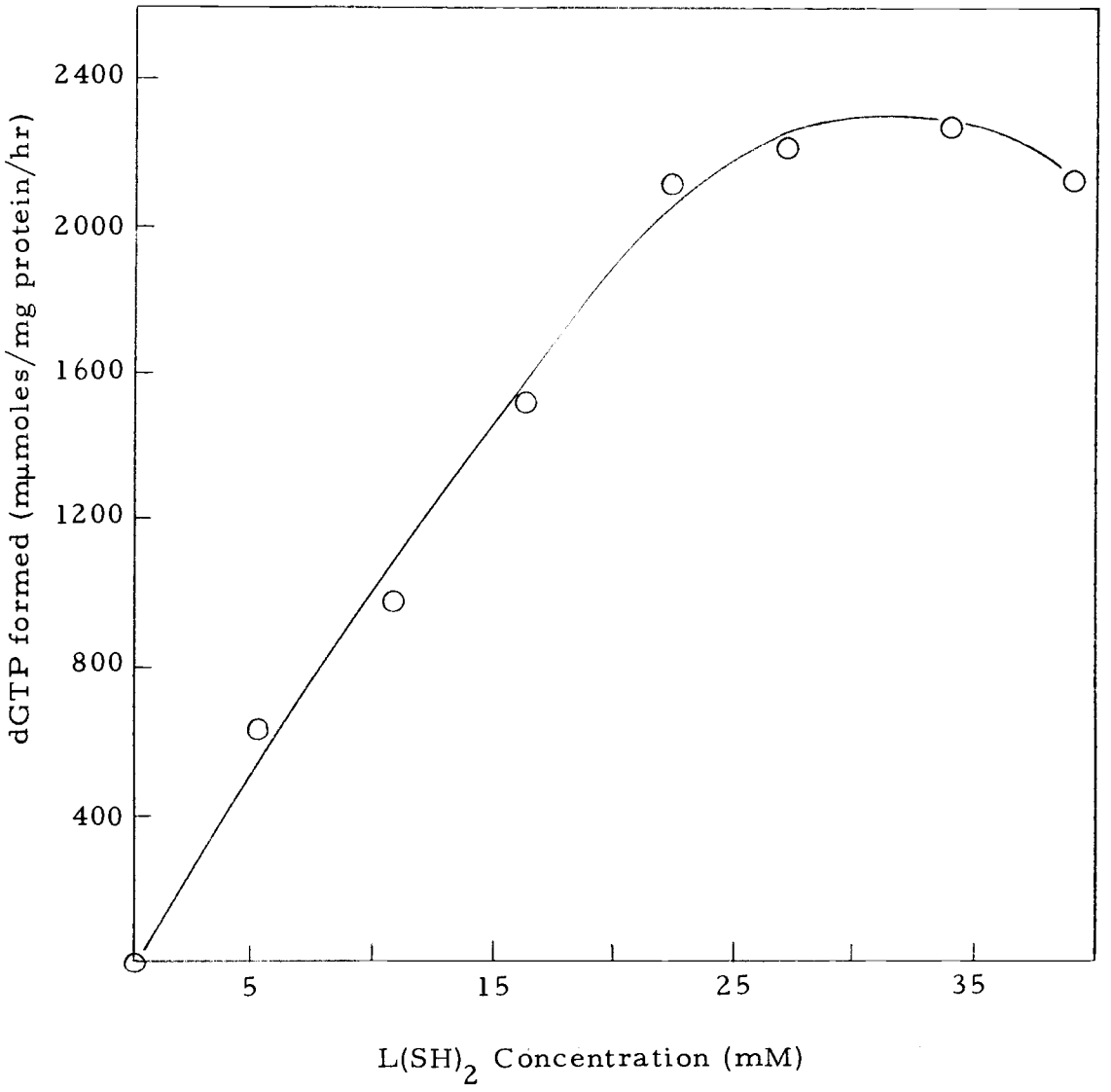

Figure 6. The Effect of Dihydiolipoate Concentration on Ribonucleotide Reductase Activity. The reaction mixtures contained components as listed for the complete reaction mixture in Figure 2 except that the $L(SH)_2$ concentration was varied as indicated. The period of incubation was 30 minutes at 37^{o}.

Table VIII. The Effectiveness of Various Reductants in the Ribonucleotide Reductase Reaction.

The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except that the reductants indicated were substituted for $L(SH)_2$. The concentration of each reductant was 15 μmoles per reaction (0.5 ml) and the period of incubation was 30 minutes at 37°.

Reductants	dGTP formed (mμmoles/mg protein/hr.)
Dihydrolipoate	2410
Dithiothreitol	398
Dithioerythritol	166
Glutathione	146
Cysteine	72
2-Mercaptoethanol	50

Table IX. Ribonucleotide Reductase Activities and Generation Times of a Series of Rhizobium Species.

The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except 0.1 to 0.5 mg or protein from extracts of the indicated Rhizobium species (30-45% $(NH_4)_2SO_4$ fraction) were substituted for the DEAE-fractionated extracts from R. meliloti. The reaction mixtures were incubated 1 hour at 37°. The generation times were calculated from changes in absorbance of the cultures at 660 mμ during the logarithmic phase of growth (0.1 to 0.4 OD of culture).

Species	Reductase activity in complete system (mμmoles dGTP/mg protein/hr)	Reductase activity in complete system minus B_{12} coenzyme (mμmoles dGTP/mg protein/hr)	Generation time in log phase (hrs)	Generation time* prior to harvest (hrs)
R. meliloti	346	2	2.8	6.2
R. meliloti-3D	80	2	2.8	6.3
R. trifolii	155	3	4.0	7.6
R. phaseoli	152	3	3.7	9.8
R. leguminosarium	150	< 1	3.9	8.0
R. japonicum	38	< 1	10.2	14.8

*This represents the approximate rate of growth just prior to harvest (0.65 to 0.80 of culture).

isolated from soybean and alfalfa nodules (Table X). The ribonucleotide reductase activity in general is correlated with the apparent growth of the bacteria. The rate of GTP reduction catalyzed with extracts of R. meliloti was eight- to ten-fold greater than the rate of GTP reduction catalyzed with extracts of R. japonicum (Table IX). The generation time for R. meliloti was approximately four-fold greater than that of R. japonicum. The other Rhizobium species had reductase activities and generation times intermediate between R. meliloti and R. japonicum. The reductase activity in the bacteroids of young soybean and alfalfa nodules also was six- to ten-fold less than in extracts from pure cultures of R. japonicum and R. meliloti, respectively.

A very low rate of GTP reduction was observed with extracts from the root tips of soybean seedlings. A series of replicated experiments were analyzed statistically, and the effect of B_{12} coenzyme was shown to be insignificant. An assay more sensitive than the colorimetric procedure must be used to determine whether B_{12} coenzyme stimulates the reductase system from soybean roots.

The substrate specificities of the ribonucleotide reductases in R. meliloti-3D and R. japonicum were examined to determine if a high ribonucleoside diphosphate specificity could account for the slow rate of reduction of GTP in these organisms. Comparable extracts from R. meliloti also were used in the experiment as a

Table X. Ribonucleotide Reductase in Legume Nodules.

The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except 1.0 mg of alfalfa nodule protein and 1.8 mg of soybean nodule protein (30-45% $(NH_4)_2SO_4$ fraction) were used as a source of enzymes. The reaction mixtures were incubated 1 hour at 37°.

Source of nodules	Reductase activity in complete system (mμmoles dGTP/mg protein/hr)	Reductase activity in complete system lacking B_{12} coenzyme (mμmoles dGTP/mg protein/hr)
Soybean*	6.5	< 1
Alfalfa*	31.3	< 1

*Soybean plants were inoculated with R. japonicum and alfalfa plants with R. meliloti.

reference. The rate of reduction of the purine ribonucleotides catalyzed with extracts of R. meliloti-3D and R. japonicum was considerably less than the rate of GTP or GDP reduction by the R. meliloti reductase system (Table XI). The ribonucleoside diphosphates, GDP and ADP, were reduced at a faster rate in the R. meliloti and R. japonicum reductase systems than the guanosine and adenosine mono- or triphosphates. In addition, the reductase of R. japonicum seems to be less specific for the ribonucleotides than the enzyme from either of the R. meliloti strains. The reductase system from R. meliloti, R. meliloti-3D and R. japonicum also exhibit different affinities for B_{12} coenzyme. In preliminary experiments the apparent K_m values for B_{12} coenzyme of the R. meliloti, R. japonicum and R. meliloti-3D systems (30-45% $(NH_4)_2SO_4$ fraction) were calculated to be 7.6, 51.6 and 51.8 μM, respectively.

Cobalt Deficiency

Cobalt deficiency in cultures of R. meliloti resulted in a decreased rate of cell growth (Figure 7). The generation time for R. meliloti cultures grown on the cobalt-deficient medium was about twice that of comparable cultures supplied with adequate cobalt. Cells from cultures of R. meliloti grown on the cobalt-deficient medium contained 5- to 15-fold greater specific activity of ribonucleotide reductase than cells from cultures grown with the

Table XI. Comparison of Various Ribonucleotides as Substrates for the Ribonucleotide Reductases from R. meliloti, R. meliloti-3D and R. japonicum.

The reaction mixtures were the same as the complete reaction mixture described in Figure 2 with the following exceptions: Extracts from the indicated Rhizobium species and strains (0.09 to 0.24 mg of protein from the 30-45% $(NH_4)_2SO_4$ fractions) were substituted for the DEAE-fractionated extracts from R. meliloti and the ribonucleotides as indicated were substituted for GTP. The reaction mixtures were incubated 1 hour at 37°.

	Deoxyribonucleotides formed (mμmoles/mg protein/hr)		
Substrate	R. meliloti	R. meliloti-3D	R. japonicum
GTP (2.0 mM)	285	54	44
GDP (2.0 mM)	274	170	70
GDP (0.3 mM)	242	45	39
GMP (2.0 mM)	44	33	13
ATP (2.0 mM)	0	0	14
ADP (2.0 mM)	145	68	86
ADP (0.6 mM)	185	31	71
AMP (2.0 mM)	7	27	10

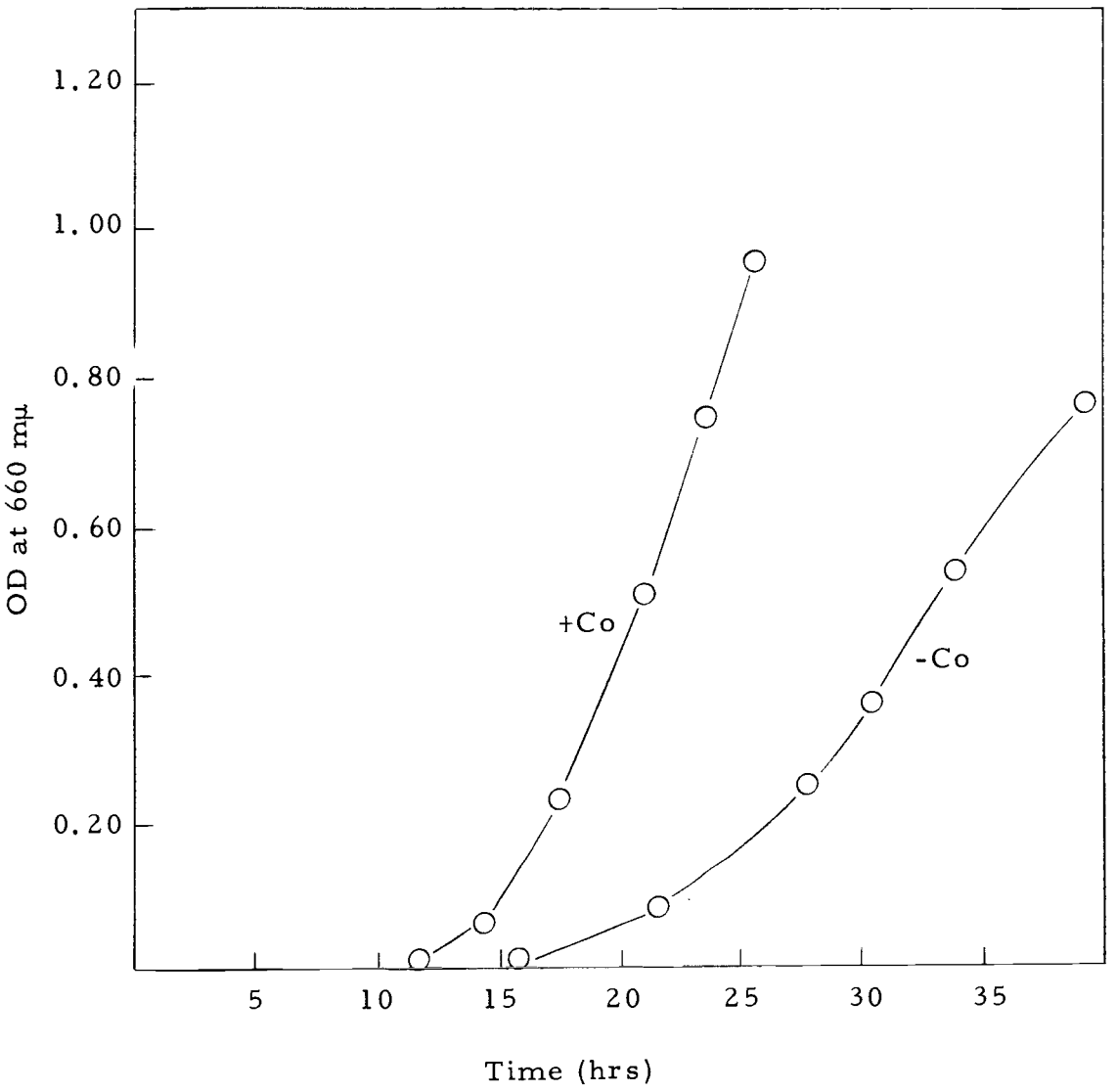

Figure 7. Growth Rates of R. meliloti with and without Cobalt. A purified culture medium lacking cobalt (see Materials and Methods) and a comparable medium to which was added 24 μg of cobalt per liter were utilized.

complete medium (Figure 8). Since the activity of the reductase was measured with B_{12} coenzyme in the assay mixture, the high activity in extracts of cells from the cobalt deficient medium represents an increase in the amount of apoenzyme.

Since there is some evidence (33) to indicate that vitamin B_{12} requirement in L. leichmannii can be partially alleviated by the addition of deoxyribonucleosides and because of the results reported in Figure 8 it is possible that some product of the ribonucleotide reductase may serve as a repressor of the synthesis of the ribonucleotide reductase apoenzyme. Experiments were designed to determine if the addition of certain deoxyribose compounds might substitute for cobalt in the metabolism of R. meliloti. In these experiments the micro-organisms also were cultured on the cobalt-deficient medium. Cobalt or deoxyribose compounds were added to some of the flasks containing the deficient medium (Table XII). It is apparent from these data that none of the additions appreciably substituted for cobalt in the growth of R. meliloti. With the exception of the addition of cobalt, the shortest generation time was obtained when deoxyribose, adenine, guanine, uracil and cytosine were added. In this case the generation time was reduced from 5.2 hours to 4.4 hours. Increasing the concentration of deoxyribose to 15×10^{-5} M and each base to 3.75×10^{-5} M did not further reduce the generation time. In contrast, the addition of cobalt reduced the

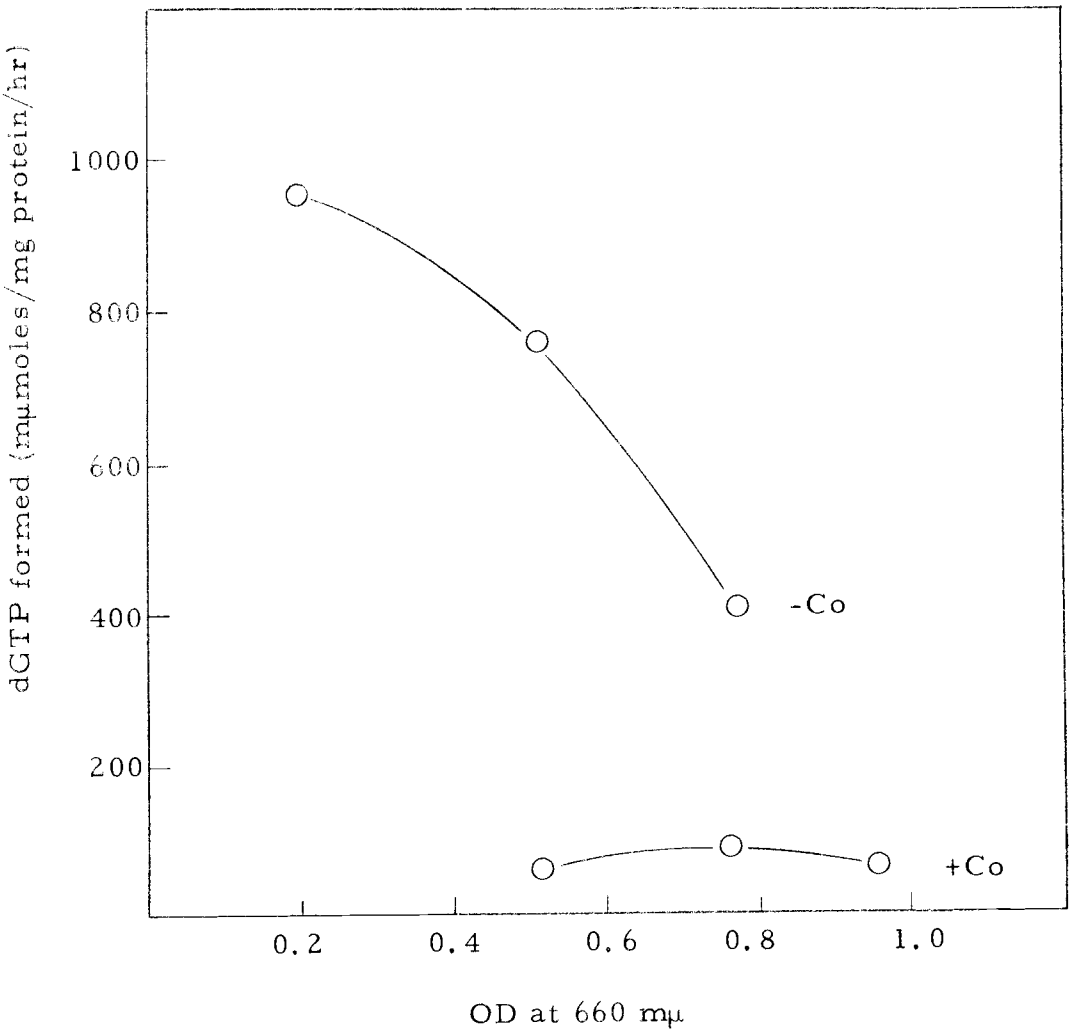

Figure 8. Ribonucleotide Reductase Activity in Cells Grown with and without Cobalt. A purified culture medium lacking cobalt (see Materials and Methods) and a comparable medium to which was added 24 μg of cobalt per liter were utilized. The reaction mixtures were the same as described for the complete reaction mixture in Figure 2 except 0.25 to 0.32 and 0.05 to 0.07 mg of protein from crude extracts of R. meliloti grown with and without cobalt, respectively, were substituted for the DEAE-fractionated extracts from R. meliloti. The reaction mixtures were incubated 1 hour at 37°.

Table XII. The Generation Time of R. meliloti Grown on Cobalt Deficient Media Supplemented with Various Compounds.

The cobalt deficient medium was supplemented as indicated with the following: deoxyribose, 5.0×10^{-5} M; thymidine, deoxyadenosine, deoxyguanosine and deoxycytidine, 3.75×10^{-5} M; adenine, guanine, uracil and cytosine, 1.25×10^{-5} M and cobalt 24 μg/l. The generation times were calculated from changes in absorbance of the culture at 660 mμ during the logarithmic phase of growth (0.1 to 0.35 OD of culture).

Supplement to the cobalt deficient medium	Generation time (hrs)
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

generation time from 5.2 hours to 2.8 hours. In experiments where the cells were harvested and the ribonucleotide reductase activity was determined, the activity in cells grown on the cobalt-deficient medium supplemented with deoxyguanosine, deoxyadenosine, deoxycytidine and thymidine was about 30% less than the activity in cells grown on the cobalt-deficient medium (Table XIII). Thymidine was as effective in reducing the ribonucleotide reductase activity as the four deoxyribonucleosides. The rate of GTP reduction could not be decreased below 300 mμmoles per mg protein per hour by increasing the deoxyribonucleoside concentration to 10.25×10^{-5} M. It is evident that the additions only partially substituted for cobalt in the growth of this organism.

Preliminary investigations were conducted on the effect of cobalt deficiency on morphological appearance and DNA content of R. meliloti cells. The DNA concentrations in cells grown on the cobalt-deficient medium or the deficient medium supplemented with deoxyribonucleosides or medium supplemented with cobalt were 0.77, 1.46, and 2.08 mg of DNA per gram of cells, respectively. Abnormal cells were observed in R. meliloti cultures grown on the cobalt deficient medium and the medium supplemented with deoxyribonucleosides. The proportion of elongated cells has not been determined.

Table XIII. Ribonucleotide Reductase Activity in Extracts of R. meliloti Grown on Cobalt Deficient Media Supplied with Supplements.

The cobalt deficient medium was supplemented with 3.75×10^{-5} M of the indicated deoxyribonucleosides or with 24 μg of cobalt per liter. The reaction mixtures were the same as described for the complete reaction in Figure 2 except crude extracts (0.1 to 0.2 mg protein) of R. meliloti grown with the indicated supplement was substituted for the DEAE-fractionated extracts from R. meliloti. The reaction mixtures were incubated 1 hour at 37°.

Supplement to the cobalt deficient medium	Reductase activity (mμmoles dGTP/mg protein/hr)
None	458
Deoxyadenosine, deoxyguanosine deoxycytidine and thymidine	316
Thymidine	321
Deoxyadenosine	389
Cobalt chloride	43

DISCUSSION

The experiments reported in this investigation provide evidence that the ribonucleotide reductase from R. meliloti is dependent on B_{12} coenzyme for activity. B_{12} coenzyme-dependent ribonucleotide reductases also were found in other species of Rhizobium and in the bacteroids of alfalfa and soybean nodules. Ribonucleotide reductase from L. leichmannii and from certain other Lactobacillus species are the only reductase systems reported to be dependent on B_{12} coenzyme. Rhizobium species then represent the second group of micro-organisms known to have B_{12} coenzyme-dependent ribonucleotide reductases. Whether B_{12} coenzyme is required for ribonucleotide reductase in the non-symbiotic plant tissues remains to be determined.

Of the B_{12} coenzyme derivatives tested in the R. meliloti system, B_{12} coenzyme appears to be the most effective cofactor. In this respect the ribonucleotide reductase from R. meliloti resembles the enzyme from L. leichmannii (21, 37) in that certain closely related analogues of B_{12} coenzyme can partially substitute for B_{12} coenzyme.

The K_m for B_{12} coenzyme in the ribonucleotide reductase system of R. meliloti was approximately ten-fold greater than the K_m reported for B_{12} coenzyme in the L. leichmannii system. Rhizobia

are known to have the capacity to synthesize B_{12} coenzyme (50) whereas lactobacilli are dependent upon an exogenous source of vitamin B_{12}. One might speculate that the apparent greater affinity of the L. leichmannii ribonucleotide reductase for B_{12} coenzyme is related to the selection of a reductase system in the evolutionary process that could best survive in an environment containing low concentrations of vitamin B_{12}.

Blakley et al. (24) have reported that the ribonucleotide reductase from L. leichmannii is highly specific for ribonucleotide triphosphates and have proposed that the low reaction rates exhibited with ribonucleoside diphosphates may be due to contamination with ribonucleoside triphosphates. In contrast, the ribonucleotide reductases from E. coli (73) and certain other systems (4, 64, 72) are reported to exhibit considerable specificity for ribonucleoside diphosphates. The reductase from R. meliloti at this stage of purity does not show a high substrate specificity (Table IV). It is clear, however, from the data presented (Figure 5 and Table V) that misleading results may be obtained if substrates are compared from data attained at a single concentration. It is unlikely that one could account for the effectiveness of GDP as a substrate (Figure 5) on the basis of contamination by other ribonucleotides since the optimum concentration of GDP is lower than that of either GMP or GTP. The rate of GTP reduction, on the other hand, could be due in part to a

contamination of GDP in the GTP or the conversion of GTP to GDP by enzymes in the partially-purified extracts. The extracts apparently did not contain phosphotransferases and the phosphatase activity did not result in an accumulation of ribonucleoside diphosphates. Since the GTP contained some GDP, this contamination can account for part of the deoxyribonucleotide formation observed when GTP was used as the substrate (Figure 5). The ATP substrates also were contaminated with ADP, and this could account for the apparent ATP reduction.

The high affinity of the R. meliloti reductase system for ribonucleoside diphosphates indicates that the diphosphates are the preferred substrates for this system. Other evidence supporting this conclusion includes: (a) the ineffectiveness of ribonucleoside triphosphates other than GTP as substrates and (b) the high optimum concentrations of AMP and GMP required for the maximum rate of ribonucleotide reduction. The R. meliloti reductase system, in respect to substrate specificity, appears to be similar to the ribonuclotide reductase from E. coli B (73). Further investigation of the ribonucleotide specificity will be necessary after the substrates are purified and a more purified enzyme is available.

The activity of ribonucleotide reductase in L. leichmannii has been reported (33) to reach a maximum and then decrease with increasing age of the cultures. The decline in reductase activity with

age has been attributed to increased concentrations of deoxyribonucleotides (32). A similar pattern of increase and then decrease in activity of the reductase was shown in R. meliloti cultures (Table II). In general, the rate of GTP reduction was positively correlated with the rate of growth of the different species (Table IX). The reductase activity in the bacteroids of nodules was relatively low (Table X) and is another example of the relationship between reductase activity and the growth rate of the organisms. It appears that the synthesis of ribonucleotide reductase is controlled by several factors and further investigation is necessary to understand the control mechanisms.

Although no attempt has been made to extract the maximum amount of ribonucleotide reductase from R. meliloti, the present evidence is consistent with the conclusion that the reductase is present in sufficient quantity to be of physiological importance. In preliminary experiments the DNA contents of samples of R. meliloti cells were measured at different times during the growth of the organism and the rate of incorporation of deoxyribonucleotides into DNA was calculated from these data. The in vitro rate of GTP reduction by a crude extract from a comparable sample of R. meliloti cells was approximately 50% of that required to account for the in vivo synthesis of DNA. Similar measurements and calculations were made with extracts from soybean nodules. The rate of GTP

reduction in extracts from 20-day-old soybean nodules could account for approximately 90% of the in vitro synthesis of DNA in soybean nodules from plants 20 to 28 days old.

Beck and associates (13, 18) reported that the synthesis of ribonucleotide reductase in E. coli $_{15T}$-(a thymineless mutant) and L. leichmannii could be derepressed by omitting thymine from the culture medium. These organisms cultured on a medium lacking thymine had approximately ten-fold greater ribonucleotide reductase activity than cultures grown on a medium supplied with thymine. The increased synthesis of ribonucleotide reductase could be inhibited by preventing protein synthesis or by adding thymine to the medium. A similar derepression occurs in R. meliloti cultures grown under cobalt deficiency (Figure 8). Since it was possible to cause the derepression of ribonucleotide reductase (the apoenzyme) by limiting the cobalt concentration and therefore the concentration of B_{12} coenzyme, it might be possible to prevent the derepression by adding deoxyribose compounds to the cobalt-deficient medium. The ribonucleotide reductase activity in cells grown on the cobalt-deficient medium supplemented with deoxyribonucleosides was reduced about 30% in comparison to the activity in cells grown on the cobalt-deficient medium. The reductase activity in cells grown on the cobalt-deficient medium supplemented with deoxyribonucleosides was still approximately 7-fold greater than the enzyme activity

in normal cells. The amount of deoxyribonucleosides which penetrated into the cells was not determined and may have been very low. However, the slow rate of growth observed with cobalt-deficient cultures may be caused not only by a reduced activity of ribonucleotide reductase but also by other B_{12} coenzyme-requiring reactions.

SUMMARY

Extracts of R. meliloti contain an enzyme which catalyzes the reduction of GTP to dGTP. A summary of the results obtained from an investigation of the properties of ribonucleotide reductase in rhizobia is as follows:

1. Ribonucleotide reductase from cell-free extracts of R. meliloti was purified about 20-fold.
2. The activity of the enzyme was completely dependent upon B_{12} coenzyme. Certain B_{12} coenzyme analogues, however substituted to some extent for B_{12} coenzyme in the reductase reaction. The K_m of the R. meliloti reductase for B_{12} coenzyme was 5.5 μM, which is considerably higher than the comparable K_m of 0.86 μM reported for the reductase from L. leichmannii.
3. Ribonucleotide reductase from R. meliloti does not appear to have a high specificity for substrate. Generally, the ribonucleoside diphosphates were reduced at more rapid rates than the respective mono- and triphosphates. Guanosine mono-, di- and triphosphates, however, were effective substrates at optimum concentrations. Considerable variation was observed in the optimum concentrations of the different substrates for maximum reductase

activity. The enzyme from R. meliloti exhibited the greatest affinity for the ribonucleoside diphosphates.

4. No absolute requirement for magnesium and ATP could be shown for ribonucleotide reduction. Under conditions where GDP, GMP or AMP was used as the substrate the addition of Mg^{++} and ATP resulted in increased enzyme activity. On the other hand these additions inhibited the rates of GTP, ADP and ATP reduction.

5. Several sulfhydryl compounds were tested as possible electron donors for the ribonucleotide reductase reaction. Dihydrolipoate proved to be the most effective reductant. In preliminary experiments NADPH and two protein fractions from R. meliloti substituted for dihydrolipoate as a reductant in the reaction.

6. B_{12} coenzyme-dependent ribonucleotide reductases were found in four other Rhizobium species. The relative activity of reductase in these species seemed to be positively correlated with growth rates.

7. Alfalfa and soybean nodule bacteroids also contained B_{12} coenzyme-dependent ribonucleotide reductase. The activity of the enzyme in the bacteroids of the nodules was considerably less than that in bacteria from pure cultures of R. japonicum and R. meliloti.

8. Cobalt deficiency in R. meliloti resulted in a decreased rate of growth and a striking increase in the activity of the reductase apoenzyme. The addition of compounds containing deoxyribose to the cobalt-deficient medium failed to increase the rate of growth of the organism or decrease the level of apoenzyme activity in the cells.

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