STUDIES ON RIBONUCLEOTIDE REDUCTASE MUTANTS OF *RHIZOBIUM MELILOTI*

A Thesis

Presented to

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

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by

Verna M. Lewis

December 1974

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ABSTRACT

Optimal reaction concentrations were determined for ribonucleotide reductase of Rhizobium meliloti 3DOal, an ineffective strain in nitrogen fixation. The optimal substrate concentration for GMP, GDP, and GTP was 0.4, 0.1, and 0.4 mM, respectively. Dithiothreitol was the most effective reductant and had an optimal concentration of 20 mM. The optimal B_{12} coenzyme concentration was 35 μM. Ribonucleotide reductase activity of R. meliloti 3DOal was approximately 2-fold less than in R. meliloti F-28, a strain effective in nitrogen fixation, when both were assayed at their optimal reactant concentrations.

Sixteen mutants of R. meliloti F-28 were isolated after treatment with N-methyl-N'-nitro-N-nitrosoguanidine. The growth rates and ribonucleotide reductase activity of most of these organisms were significantly different than that of the prototrophic strain. Two mutants, R. meliloti HP-31 and R. meliloti LZ-26, contained only 10% of the ribonucleotide reductase activity of R. meliloti F-28. These two mutants caused nodule formation on alfalfa plants, and the nodules were capable of acetylene reduction.

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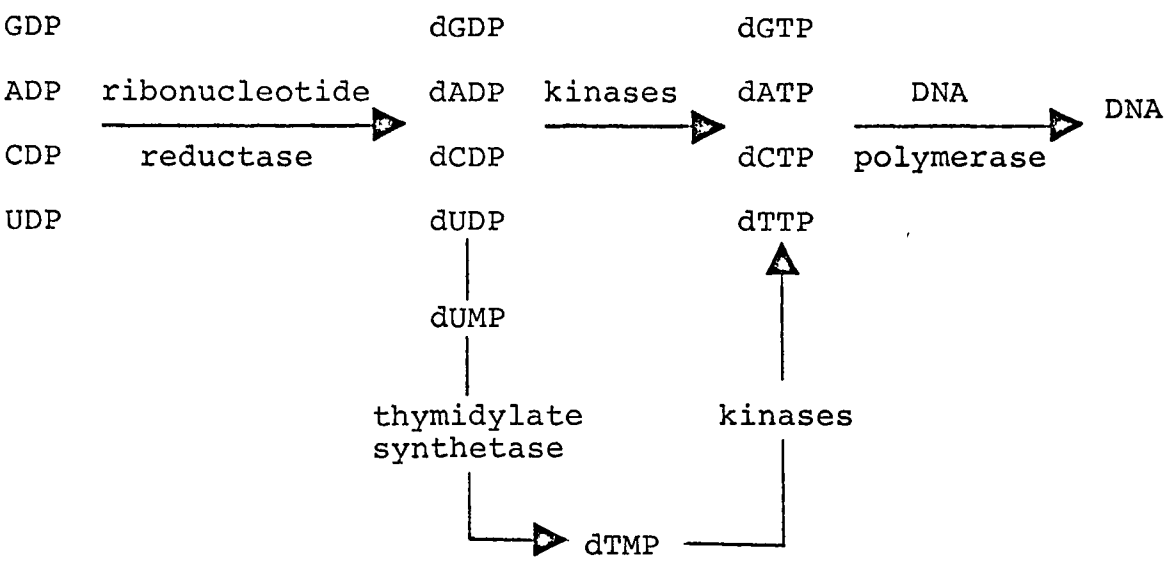
INTRODUCTION

The general pathway of DNA biosynthesis has been elucidated in E. coli (31, 103, 105) and shown to involve four major enzymes: ribonucleotide reductase, thymidylate synthetase, deoxyribonucleoside kinase, and DNA polymerase. The sequence of reactions is according to the following scheme:



The initial enzymatic reaction, reduction of ribonucleotides to deoxyribonucleotides, has been shown to be a rate-limiting step in DNA synthesis. For example, King and van Lancker (80) reported ribonucleotide reductase activity could not be detected in normal rat liver but appeared 24 hr after partial hepatectomy. The induction

of ribonucleotide reductase was inhibited by actinomycin D, which also inhibited the development of regenerating liver. Noronha et al. (117) also reported the absence of ribonucleotide reductase activity in cell-free extracts of Arbacia punctulata eggs. Reductase activity, however, was detected 5 hr after fertilization and paralleled the exponential rise in DNA synthesis during cleavage. The induction of ribonucleotide reductase was shown to be necessary to sustain development beyond the 4 to 8 blastomere stage.

Initial studies on ribonucleotide reductase activity in Rhizobium species showed that R. meliloti 3DOa1, which is an ineffective nitrogen fixing strain, contained 4- to 5-fold less ribonucleotide reductase activity than R. meliloti F-28, which is effective in nitrogen fixation (42), even though the two organisms had similar growth rates in free-living cultures.

The mechanism of establishing an effective symbiosis in leguminous plants is not understood. Neither are the metabolic changes that occur in the symbiot as well as the host tissue upon infection and symbiosis. Ribonucleotide reductase may play an important role in the establishment of symbiosis since the DNA content in rhizobia is reported (46) to decrease during early symbiosis, and

ribonucleotide reductase is known to be a rate-limiting step in DNA synthesis. An organism with comparatively low ribonucleotide reductase activity such as R. meliloti 3DOa1 may not be able to maintain sufficient levels of deoxyribonucleotides for continued DNA synthesis under the complexity of symbiosis and, therefore, could not develop the capacity to fix atmospheric nitrogen.

The objectives of this study were: (1) to determine if the low level of ribonucleotide reductase activity in R. meliloti 3DOa1 represented a true level of enzyme activity or whether it represented a change in enzyme structure or configuration as determined by new reaction requirements or optima and (2) to elucidate whether ribonucleotide reductase is essential in the establishment of effective symbiosis as determined by obtaining ribonucleotide reductase deficient mutants and testing the ability of these organisms to establish effective symbiosis.

REVIEW OF LITERATURE

The concept that the deoxyribose of deoxyribonucleic acid stems from the ribose of a ribonucleoside or ribonucleotide originated from studies with labeled ribosyl pyrimidines (68). In vivo experiments with rats showed that ribonucleosides were transformed into deoxyribonucleosides without rupturing the glycosidic linkage (68, 129). Incorporation studies of ^{14}C-cytidine in chick embryo cells, diluted with pools of nonlabeled deoxycytidine and deoxycytidine monophosphates, indicated the reductive step occurred at the ribonucleotide level (126).

Ribonucleotide Reductase of Escherichia coli

In vitro ribonucleotide reduction was first demonstrated in crude extracts of Escherichia coli by Reichard et al. in 1961 (128). The extract catalyzed the reduction of CMP to dCMP in the presence of ATP and Mg^{++}; the enzyme was designated ribonucleotide reductase. After treatment of the extract with either charcoal or Dowex 2, a NADPH requirement also was demonstrated. Further purification of the cell-free extract showed the enzyme fraction was no longer active with NADPH but required reduced lipoate (124). These studies also demonstrated that CDP

was reduced at a much faster rate than CMP or CTP, which indicated that the ribonucleoside diphosphates were the preferred substrates.

Later, Moore and Reichard (103) reported a heat-stable protein from cell-free extracts of E. coli which replaced the requirement for reduced lipoate when coupled with NADPH and another protein fraction. Laurent et al. (90) characterized the heat-stable protein as a low molecular weight electron carrier between NADPH and the ribonucleoside diphosphates; the carrier was designated thioredoxin. The active component in the other protein fraction from E. coli was called thioredoxin reductase and was shown to catalyze the reduction of thioredoxin by NADPH. Further studies revealed the enzyme was a flavoprotein with a molecular weight of 66,000 daltons.

Studies on a highly purified ribonucleotide reductase fraction showed the enzyme to consist of two non-identical subunits, proteins B1 and B2 (26). Neither subunit alone had enzymatic activity. Based on ultracentrifugation measurements, B1 (7.8S) and B2 (5.5S) formed a catalytically active complex in the presence of magnesium ions (27). This complex had a sedimentation coefficient of 9.7S and contained equimolar amounts

of the two subunits. Brown et al. (31) determined the molecular weight of B1 and B2 to be 160,000 to 200,000 and 78,000 daltons, respectively. Further studies showed that the B2 subunit contains two atoms of iron, and that B1 consisted of a dimer containing two identical subunits (28, 29, 30).

Ribonucleotide reductase activity and substrate specificity in E. coli is modulated by deoxyribonucleoside triphosphates (28). Studies on the binding of deoxyribonucleotide effectors indicated that only B1, either alone or in combination with subunit B2, contained effector binding sites. Two classes of binding sites, h and l, have been proposed for B1. Binding of effectors to the h-sites influences substrate specificity, while the l-sites are primarily involved in the regulation of overall enzyme activity. The first class (h-sites) consists of two separate binding sites; one has a high affinity for dATP and the other a high affinity for ATP, dATP, dGTP, and dTTP. The second class (l-sites) also has two binding sites; one has a low affinity for dATP and the other binds only ATP and dATP.

Ribonucleotide Reductase of Lactobacillus leichmanii

Indirect evidence has long implicated vitamin B_{12} derivatives in the enzymic formation of deoxyribonucleotides. The earliest evidence demonstrated that the addition of deoxyribonucleosides to the growth medium of certain lactobacilli eliminated the nutritional requirement for vitamin B_{12} (81, 82, 137, 146). Beck et al. (13) reported that replenishing vitamin B_{12} in deficient cultures of L. leichmanii resulted in a substantial increase in the intracellular pool size of deoxyribosyl compounds. Direct evidence of cobamide involvement in the enzymic synthesis of deoxyribonucleotides was first reported in cell-free extracts of L. leichmanii (23). The extract catalyzed the reduction of CMP to dCMP only in the presence of B_{12} coenzyme. The reaction also required ATP, Mg^{++}, and dihydrolipoate (3, 23). Later studies on a more purified fraction revealed that GTP reduction was considerably greater than GMP or GDP reduction (24). ATP and Mg^{++} were not required for GTP reduction in the purified fraction. The enzyme in L. leichmanii consists of a single polypeptide chain with a molecular weight of 76,000 daltons (63, 120).

Although dihydrolipoate proved to be a more ef-

fective reductant than other thiols tested (12, 143), the thioredoxin system from E. coli was found to be the most efficient. Orr and Vitols (119) later isolated a NADPH-linked thioredoxin-thioredoxin reductase system from L. leichmanii.

Ribonucleotide reductase of L. leichmanii catalyzes a unique intermolecular hydrogen-exchange reaction between tritiated water and the 5'-methylene moiety attached to cobalt in the coenzyme (5'-deoxyadenosylcobalamin) (67). The hydrogen at this position is considered to be an intermediate in both the hydrogen-exchange reaction and ribonucleotide reduction (67, 141). Direct spectrophotometric observations (141) revealed a homolytic cleavage of the carbon-cobalt bond occurred which formed a stabilized adenosyl radical. The radical is postulated to interact with a thiol group from the enzyme or the reductant and slowly rearrange to yield 5'-deoxyadenosine and a sulfur radical. The deoxyadenosyl radical-thiol system is considered the immediate reducing agent in ribonucleotide reduction.

Other Ribonucleotide Reductase Systems

Ribonucleotide reductase activity was demonstrated

in extracts of mammalian tissues by Moore and Hurlbert in 1960 (106, 107). Crude extracts of Novikoff ascites hepatoma catalyzed the reduction of CMP in the presence of ATP and Mg^{++}. A partially purified extract of Novikoff hepatoma required CDP, ATP, Mg^{++}, Fe^{+++}, and reduced lipoate for the reduction of CDP to dCDP (104). Ribonucleoside diphosphates were the preferred substrates.

Ribonucleotide reductase has been recently demonstrated and partially characterized in plant tissues. Muller et al. (111) have reported CDP and ADP reduction in wheat embryos in the presence of a dithiol and iron. Ribonucleotide reductase also has been demonstrated in soybean callus and pea root tissues (65).

In general, the ribonucleotide reductases studied can be grouped into two classes. The basic characteristic of Class I is the reduction of ribonucleoside diphosphates in the presence of ATP and Mg^{++}. The mammalian and plant systems are generally grouped with E. coli in this class. Specifically included are: chick embryos (125, 126), Ehrlich ascites cells (107), regenerating rat liver (89), cerebral tissues (100), Chinese hamster fibroblasts (112), rabbit bone marrow (70), calf thymus (2), leukemic mouse spleen (54), Yaba poxvirus tumor and normal monkey tissues (62),

rat hepatomas (51, 107), bacteriophage T-4 infected *E. coli* cells (19, 20), mouse embryo cells (116), Carcinoma ascites cells (79), human bone marrow and leukocytes (53, 55), wheat embryos (111), soybean callus and pea root tissues (65).

Class II ribonucleotide reductase has an absolute requirement for B_{12} coenzyme and can reduce ribonucleoside triphosphates. Organisms in this class include: *Rhizobium* species (42), *Euglena gracilis* (61), *Clostridium sticklandii* and *Clostridium tetanomorphum* (1), *Lactobacillus acidophilus* (21), *Corynebacterium nephridii*, *Pseudomonas stutzeri*, *Micrococcus denitrificans*, and *Thermus aquaticus* (60).

N-methyl-*N*'-nitro-*N*-nitrosoguanidine as a Mutagenic Agent

The classes of chemicals that will induce genetic mutations in bacterial, plant, and mammalian systems vary greatly. Chemicals of this capacity include such diverse compounds as nitrogen mustard, azaserine, mitomycin C, hydroxylamine, nitrous acid, and diazomethane. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was added to the list of chemical mutagens in 1960 when Mandel and Greenberg (95) demonstrated the presence of *E. coli* auxotrophs following MNNG treatment. These auxotrophs were identified as colonies which failed to grow on a

glucose-salts medium when replicated from a complete medium. Approximately 10 percent of the surviving colonies were auxotrophs. These organisms required amino acids, vitamins, purines, and pyrimidines for normal growth. Adelberg et al. (4), using E. coli K12, showed that MNNG induced at least one mutation per treated cell under optimal conditions permitting over 50 percent survival. One reported (4) disadvantage of using MNNG as a mutagen is the high probability of multiple site mutations.

MNNG has been successfully used as a mutagenic agent in a variety of organisms, including: RNA virus (134), Aerobacter aerogenes (11), Coprinus lagopus (101), Escherichia coli (140), Salmonella typhimurium (50), Saccharomyces cerevisiae (96, 133, 149), Schizosaccharomyces pombe (92), Streptomyces coelicolor (44), Aspergillus nidulans (5), Neurospora crassa (94), Anacystis nidulans (9), Drosophila melanogaster (25), and Arabidopsis thaliana (57, 58, 110, 142). MNNG also has been reported to produce non-chromosomal mutations in Chlamydomonas reinhardi (59), and Euglena gracilis (98, 99), in addition to producing chromosome breakage in Triticales (77) and Vicia faba (56, 77).

The effectiveness of MNNG as a mutagenic agent in

mammalian and plant tissues varies. Ehling et al. (48) reported MNNG to have low potency as a mutagen in male mice. Parkin et al. (121), however, showed that MNNG produced a dose-related response, indicating the mutagen was effective at high doses. MNNG also was reported to be an effective mutagen in mammalian tissue culture cells (41). In higher plants, MNNG has been reported to be an effective mutagen in Arabidopsis thaliana (57, 58, 110, 142), whereas in barley, the mutagen's effectiveness has been inconsistent (49, 123).

The optimal conditions for MNNG mutagenesis in bacterial cells varies. Adelberg et al. (4) showed that the optimal conditions for MNNG mutagenesis in E. coli K12 consisted of treating logarithmic phase cells for 15 to 30 min at concentrations of 30 and 100 µg/ml. The optimal production of mutants in Aerobacter aerogenes occurred after 60 min of treatment at 1 mg/ml or 15 min at 4 mg/ml (11). MNNG also induced mutations in Salmonella typhimurium following 20 min of treatment at a final concentration of 5 µg/ml (50).

The mutagenic action of MNNG is believed to be through the alkylation of DNA (52, 76, 91, 93). The in vitro reaction of MNNG with DNA has been shown to yield 7-methylguanine as the principal product of muta-

genesis (91, 99). Very often, however, the mutagenic properties of MNNG have been attributed to the formation of diazomethane and nitrocyanide in neutral and alkaline solutions and to nitrous acid in an acidic medium (52). McCalla et al. (99) showed that the amount of methylation of DNA by labeled MNNG increased with increasing hydroxyl ion concentrations, presumably, through the production of diazomethane. On the other hand, Zimmermann et al. (149) demonstrated that at low pH, MNNG was shown to decompose into nitrous acid which, in turn, caused the deamination of adenine. Apparently, the mutagenic properties of MNNG, under acidic conditions, are through the production of nitrous acid which, in turn, cause deamination of the DNA. Under basic conditions, the DNA is alkylated via diazomethane, a breakdown product of MNNG.

Biological Nitrogen Fixation in Leguminous Plants

The first significant indications that leguminous plants fix nitrogen was reported in 1886-88 when Hellriegel and Wilfarth (138) observed that legumes which bore characteristic nodules on their roots assimilated elemental nitrogen. The root nodules were suggested to be essential for fixation since plants

without nodules did not assimilate nitrogen. In 1888, Beijerinck (14) isolated a bacterium in pure culture, which was able to cause nodule formation on soybean roots; this organism later was named Rhizobium japonicum.

The initial response leading to infection and nodule formation includes an increase in bacterial cell division in the vicinity of the host plant roots (34, 73). The small rodlike bacteria are believed to enter the plant roots via the root hairs and soon become encased in an infection thread. Jordan (73) observed that the initial naked mucoid infection thread in alfalfa nodules migrates between the cell walls of the cortical cells (middle lamella), and at various locations, a tubular sheath grows from the infection thread into the cell wall and plasma membrane. Vesicles formed in this sheath rupture to liberate the bacteria into the host cytoplasm. The bacteria then become enclosed in a membrane to form the bacteroids. Bacteroid formation is marked by the formation of an elaborate intracytoplasmic membrane system within the bacteroids by invagination of the plasma membrane (34). Sometime during the entry of the infection thread into the cortical region, cells of the host plant undergo rapid cell division to give rise to the plant nodules.

Based on cross-reactive antigenic studies, the Rhizobium-legume association is specifically compatible. Charudattan et al. (39) reported the presence of cross-reactive antigens between three species of rhizobia and eight host plants. The cross-reactive antigens were absent between rhizobia and eight non-host plants. The common antigens among hosts and bacteria were not related to the specificity of compatible Rhizobium-legume associations. The occurrence of cross-reactive antigens, however, was postulated to be related to the host's tolerance of nodule bacteria in legumes.

A number of metabolic changes in the bacteria accompany nodule and bacteroid development. These include: the synthesis of large quantities of leghaemoglobin and poly-β-hydroxybutyrate (7, 84, 135), a decrease in the amount of nucleic acids and proteins/bacteroid (46), and a shift in the cytochrome and pigment composition (6, 8, 16).

Smith (135) showed that leghaemoglobin was present only in cells which contained bacteroids. The red pigment is a myoglobin-like haemoprotein of at least two molecular forms (135). These forms differ in molecular weight and amino acid composition in soybean nodules

(15). Leghaemoglobin has been suggested to be the principal carrier of oxygen in the bacteroids (147). Scholander (132) demonstrated that oxygen diffusion through the membrane of a cell saturated with haemoglobin was much faster than the diffusion of nitrogen in the same system. The facilitated diffusion was proposed to be an important way in which oxygen could be translocated through the bacteroid to the bacteria under conditions of low oxygen partial pressures.

Poly-β-hydroxybutyrate is reported to make up as much as 40 percent of the dry weight of bacteroids (84) and was suggested as a possible energy reserve to be used in generating reducing power for nitrogen fixation. β-hydroxybutyrate dehydrogenase, which dehydrogenates β-hydroxybutyrate, has been reported in bacteroids (15, 84). Klucas and Evans (84) used benzyl- or methylviologen to couple the dehydrogenation of β-hydroxybutyrate to the reduction of nitrogen in bacteroid extracts. Later work, however, indicated that the actual contribution of β-hydroxybutyrate as an active reductant source was minor (145).

Dilworth and Williams (46) reported that the DNA, RNA, and protein content in lupin bacteroids were 13, 40, and 35% lower in six-week-old nodules than in one-

week-old nodules. The protein but not the RNA and DNA content increased again in older nodules. Bergersen (16), however, reported that the total nucleic acid content in soybean bacteroids did not change during nodule development.

Appleby et al. (6, 8) have shown that cultured R. japonicum contain cytochromes b, c, o, a, a_3, and a pigment identified as P-428. The mature bacteroids, on the other hand, did not have cytochromes a, o, and a_3 but instead contained cytochromes c_{552} and pigments P-450, P-420, and P-428. These changes, apparently, are necessary for functional electron transport in the bacteria under symbiotic conditions (reduced O_2 levels).

Rhizobium species have not been demonstrated to fix nitrogen in free-living cultures (15). These cultured cells, apparently, are incapable of synthesizing the total nitrogen complex.

Nitrogen Fixation in Cell-Free Extracts

In vitro nitrogen fixation activity was first reported by Carnahan et al. (36, 37) using cell-free extracts of Clostridium pasteurianum. Similar success has since been reported with Azotobacter vinelandii by Nicholas and Fisher (35, 115), Rhodospirillum rubrum

and blue-green algae by Schneider et al. (131), Chromatium by Arnon et al. (35), Bacillus polymyxa by Grau and Wilson (64), and in soybean bacteroids by Koch, Evans, and Russell (87).

Active cell-free extracts of soybean bacteroids were prepared in an ascorbate-polyvinylpyrrolidone medium under anaerobic conditions (87). The extract catalyzed the reduction of both acetylene and nitrogen in the presence of sodium dithionite and an ATP-generating system. Partial purification of the bacteroid nitrogenase system showed the enzyme also catalyzed the evolution of hydrogen during the course of nitrogen fixation (86). The rate of nitrogen fixation by bacteroid nitrogenase was strongly inhibited by hydrogen.

The purified bacteroid nitrogenase is reported (83) to consist of two separate components; the first component contained iron and molybdenum in the ratio of 20:1, and the second contained iron but only a trace of molybdenum. Bergersen et al. (18), using Sephadex G-200 filtration, reported molecular weights of 182,000 and 51,000 daltons for the Mo-Fe and Fe-proteins, respectively. The Mo-Fe protein contained nine atoms of iron and one atom of molybdenum per molecule of protein. The Fe-protein contained one

atom of iron per molecule of protein. Both protein components were necessary for nitrogenase activity.

Studies on cell-free extracts of soybean root nodules have revealed only fragmentary details of the natural electron source or transport chain(s) operating during symbiotic nitrogen fixation. Klucas et al. (84) reported that the electrons for nitrogen fixation could be supplied by an $NADH_2$-generating system in the presence of benzylviologen, an electron carrier. Benzylviologen could be replaced, in part, by a crude factor and flavin nucleotide (88). Koch et al. (86) also identified a nodule bacteroid component containing nonhaem iron and acid-labile sulfur. Further studies revealed the component was an electron carrier but not a flavoprotein (148). Later studies showed the nonhaem iron protein was similar to the ferridoxin from Azotobacter (88). The component, however, did not function as a cofactor in the phosphoroclastic breakdown of pyruvate or in the photochemical reduction of NADP. A second electron transport factor has been isolated, which appears to be analogous to azotoflavin from Azotobacter (88). The two proteins were reported to transfer electrons from spinach chloroplasts lacking photosystem II to soybean nodule nitrogenase. Phillips

et al. (122) demonstrated that Rhizobium japonicum produced the factors which transfer electrons to nitrogenase in the low oxygen conditions of the leguminous root nodules.

MATERIALS AND METHODS

Culture Maintenance and Growth

Cultures of Rhizobium meliloti F-28 and Rhizobium meliloti 3DOal were kindly supplied by Dr. Harold J. Evans of Oregon State University. Stock cultures were maintained on agar slants or plates. The media contained in a liter: 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.36 g $MgSO_4 \cdot 7H_2O$, 0.13 g $CaSO_4 \cdot 2H_2O$, 4.0 g $FeCl_3$, 20.19 mg $CoCl_2 \cdot 6H_2O$, 3.0 g mannitol, and 1.0 g yeast extract (43). Prior to the growth of large quantities of cells, the organisms were grown in 250 ml flasks containing 60 ml of liquid media. The cultures were grown at 30° in the dark on a gyrotory shaker (New Brunswick Scientific Company). Cells for experimental purposes were grown in one-liter flasks containing 500 ml of media. A 0.5% inoculum was used and the cells were harvested at an absorbance of 0.75 to 0.90 (660 nm) unless otherwise indicated.

Cell Extraction and Enzyme Preparation

Rhizobium cells were harvested in a Sorvall refrigerated centrifuge (GS-3 rotor) and washed twice in 50 mM potassium phosphate buffer (pH 7.3). The cell paste was resuspended in an equal volume of phosphate

buffer, and the cells were lysed in a cold French pressure cell at approximately 10 tons per 2 in diameter. The homogenate was centrifuged 15 min at 27,000 x g, and the supernatant was used as the crude extract.

Enzyme Purification

The crude extracts of R. meliloti F-28 and R. meliloti 3DOa1 were brought to 30% saturation by slowly adding (dropwise) enzyme grade ammonium sulfate (pH 7.3) (Sigma Chemical Co.). After stirring for an additional 10 min, the mixture was centrifuged 10 min at 12,000 x g. The resulting supernatant was brought to 50% saturation with saturated ammonium sulfate, stirred and centrifuged as above. The pellet was resuspended in 10 to 12 ml of 50 mM potassium phosphate buffer (pH 7.3) and dialyzed for 12 hr against 3 liters of 5 mM potassium phosphate buffer (pH 7.3) containing 1 mM 2-mercaptoethanol. The dialyzed preparation was chromatographed on a DEAE-cellulose column (Whatman DE-32). The column (2.4 x 15 cm) was prepared under pressure and equilibrated with 50 mM potassium phosphate buffer (pH 7.3). The protein was eluted with a linear KCl gradient (0.05 - 0.35 M). Two hundred ml of 50 mM potassium phosphate buffer (pH 7.3) containing 1 mM 2-mercaptoethanol and 50 mM KCl was added to the mixing

chamber, and 150 ml of 50 mM potassium phosphate buffer (pH 7.3) containing 1 mM 2-mercaptoethanol and 350 mM KCl was added to the reservoir. The eluate was collected in 10 ml fractions at approximately 1.5 ml per min. Peak fractions were pooled and dialyzed 3 hr against 2 liters of 5 mM potassium phosphate buffer (pH 7.3) containing 25% polyethylene glycol. The concentrated sample (approximately 10% of the original sample size) was used as the partially purified fraction unless otherwise indicated.

Ribonucleotide Reductase Colorimetric Assay

Ribonucleotide reductase activity of R. meliloti F-28 and R. meliloti 3DOal was determined by the colorimetric assay procedure described by Blakley (22). The complete reaction mixtures contained in a final volume of 0.5 ml: 50 µmoles potassium phosphate buffer (pH 7.3), 0.8 µmole GTP or 0.24 µmole GDP, 15 µmoles dihydrolipoate, 10 nmoles B_{12} coenzyme, and the cell-free extract. Approximately 250, 150, or 50 µg of the crude, ammonium sulfate-fractionated, or partially purified extract was used, respectively. After a 30 min incubation period at 37°, the reactions were stopped by boiling (3 min). Four-tenths ml of 0.5 M chloroacetamide was added to each tube, and the mixtures were boiled for an additional 10 min. After the solutions had cooled, 2.0 ml of di-

phenylamine reagent, prepared from recrystallized diphenylamine (Baker Chemical Co.), were added, and the mixtures were incubated 4 hr at 50°. The color intensity was measured on a spectrophotometer (Hitachi Perkin-Elmer) at 595 nm. The deoxyguanosine phosphates formed were determined from a standard curve prepared from either dGMP, dGDP, or dGTP (Sigma Chemical Co.).

Ribonucleotide Reductase Radioactive Assay

Measurements of ribonucleotide reductase activity of R. meliloti F-28 mutants and comparative measurements on the prototrophic strain were determined by a radioactive assay procedure described by Moore (102). The complete reaction mixtures in a final volume of 0.2 ml contained: 7.5 µmoles potassium phosphate buffer (pH 7.3), 0.08 µmole GDP, 0.5 µCi ^{14}C-GDP, 3.75 nmoles B_{12} coenzyme, 6 µmoles dihydrolipoate and 150 µg of the cell-free extract. Following a 30 min incubation period at 37°, the reactions were stopped by boiling (2 min). After the solutions cooled, 10 µl Tris-HCl buffer (pH 8.5) and 10 µl alkaline phosphatase (Sigma Chemical Co.) were added, and the mixtures were incubated 60 min at 37°. Following the incubation period, 10 µl of deoxyguanosine carrier were added, and the tubes were placed in the freezer for approximately

40 min. The reaction mixtures were thawed and centrifuged 20 min in a table-top centrifuge at approximately 2,000 x g. Thirty µl of each sample were spotted on Whatman's 3 mm chromatographic paper and chromatographed (descending method) for 10 hr in a solvent containing 0.75 ml EDTA (0.25 M), 18.75 ml NH_4OAc (pH 9.5) (5 M), 75.0 ml saturated NaB_4O_7 and 205.5 ml C_2H_5OH (95%) (102). UV-absorbing areas were marked using a short-wave mineral lamp. Strips for each sample were cut into 0.5 inch sections and counted in a liquid scintillation spectrometer. The scintillation cocktail consisted of 5 g PPO and 0.3 g POPOP per liter of toluene.

Plant Growth

Alfalfa seeds (Medicago species) were germinated in 8-in plastic pots containing Perl-Gro horticulture perlite. The seedlings were thinned to approximately 40 seedlings per pot and grown in the greenhouse under supplementary florescent lamps to provide a 14-hr day. The plants were irrigated with a nitrogen-free nutrient solution four times weekly and watered on the remaining days. The nutrient solution contained in a liter: 0.8 g $Ca_3(PO_4)_2$, 0.5 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.1 g $FeCl_3 \cdot 2H_2O$, 2.77 mg H_3BO_3, 1.78 mg $MnCl_2 \cdot 4H_2O$,

0.104 mg $ZnCl_2$, 0.125 mg $NaMoO_4$, and 0.054 mg $CuCl_2 \cdot 2H_2O$.

The plants were inoculated with laboratory-grown cultures of R. meliloti F-28, R. meliloti 3DOal, R. meliloti HP-31, R. meliloti LZ-26, R. meliloti LJ-22, and R. meliloti JF-26 1 to 2 weeks after germination.

Acetylene Assay for Nitrogenase Activity

The production of ethylene from acetylene was measured using a Perkin-Elmer 900 gas chromatograph equipped with a hydrogen flame detector according to the methods of Koch and Evans (85). A stainless steel column (5' x 1/8") was packed with 80 - 100 mesh Porapak sieve material. The column temperature was 35°, and the flow rate of the carrier gas (nitrogen) was 30 ml/min. The ethylene and acetylene peaks were identified by comparison of retention times with those of known standards. Ethylene concentrations were based on an ethylene standard curve.

The nodules, from the 25 largest six-week-old alfalfa plants per pot, were harvested and placed in 18 ml test tubes. The tubes were sealed with rubber serum caps and flushed with argon. Six-point-three ml of argon were removed and replaced with 4.5 ml of oxygen and 1.8 ml of acetylene. A 0.5 ml sample was with-

drawn at zero time, and two 0.5 ml aliquots were withdrawn at 15 min intervals for 1 hr and analyzed for ethylene production; the samples were incubated at room temperature. After completion of the acetylene assay procedure, the nodules were weighed. Also, the average height of the plants were determined based on the distance between the first and last internodes.

Other Preparations and Determinations

R. meliloti F-28 was cultured in a synthetic medium (17) (Table I) for mutagenic treatment. Solutions of the mutagenic agent, N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Co.), were prepared in the dark and used immediately. Dihydrolipoate was prepared according to Gunsalus and Razzell (66). The concentration of dihydrolipoate was determined based on its capacity to reduce a known excess of a standard solution of potassium ferricyanide (E_M^{420} = 1040) (130). The concentration of B_{12} coenzyme was determined from an absorbance at 522 nm (E_{mM}^{522} = 80) (10). Protein concentrations were determined from absorbance at 260 and 280 nm (144).

Enzyme preparations were conducted at 0° to 4°, and the fractions were stored at -10°. All reagents used in the purification, mutagenesis, and assay pro-

Table I. Bergersen's Medium for Rhizobium meliloti.

Major Elements	Final Quantity per liter
Na_2HPO_4	0.23 g
KH_2PO_4	0.23 g
$MgSO_4 \cdot 7H_2O$	0.10 g
Mannitol	10.00 g
Na Glutamate	1.10 g
Trace Elements	
$CaCl_2$	0.05 g
H_3BO_3	1.45 mg
$FeSO_4 \cdot 7H_2O$	1.25 mg
$CoSO_4 \cdot 7H_2O$	0.70 mg
$CuSO_4 \cdot 7H_2O$	0.05 mg
$MnCl_2 \cdot 4H_2O$	0.04 mg
$ZnSO_4 \cdot 7H_2O$	1.08 mg
Nitrilo triacetate	0.07 g
Na_2MoO_4	1.25 mg

Table I. Continued.

Vitamins	Final Quantity per liter
Riboflavin	0.02 mg
P-amino benzoic acid	0.02 mg
Nicotinic acid	0.02 mg
Biotin	0.02 mg
Thiamine HCl	0.02 mg
Pyridoxine HCl	0.02 mg
Calcium pantothenate	0.02 mg
Inositol	0.12 mg
pH 6.9	

cedures were prepared with double-distilled, deionized water.

RESULTS

Ribonucleotide Reductase of R. meliloti 3DOa1

The initial measurements of ribonucleotide reductase activity of R. meliloti 3DOa1 were conducted under conditions reported (42) to be optimum for R. meliloti F-28. Under these conditions, ribonucleotide reductase activity in cell-free extracts of R. meliloti 3DOa1 was linear for at least 30 min (Figure 1). The reaction rate also was linear with increasing amounts of protein to approximately 0.25 mg per reaction (Figure 2).

Crude extracts of R. meliloti 3DOa1 and F-28 were fractionated with ammonium sulfate and used to measure the levels of ribonucleotide reductase activity in these organisms at optimal reaction conditions reported for R. meliloti F-28. Ribonucleotide reductase activity in R. meliloti 3DOa1 was 3- to 4-fold less than in R. meliloti F-28 (Table II), which is in agreement with previously reported studies (42).

Since R. meliloti 3DOa1 has the same generation time as R. meliloti F-28 (2.8 hr), it was expected that the two organisms would have similar levels of ribonucleotide reductase activity. Several factors could account for the reduced level of ribonucleotide reductase activity in R. meliloti 3DOa1, including a dif-

FIGURE 1. Ribonucleotide Reductase Activity as Influenced by the Incubation Period. The complete reaction mixtures contained in a final volume of 0.5 ml: 50 umoles potassium phosphate buffer (pH 7.3), 0.2 umole GDP, 15 umoles dihydrolipoate, 10 nmoles B_{12} coenzyme, and 0.25 mg of a crude enzyme preparation. The incubation period at 37° was varied as indicated.

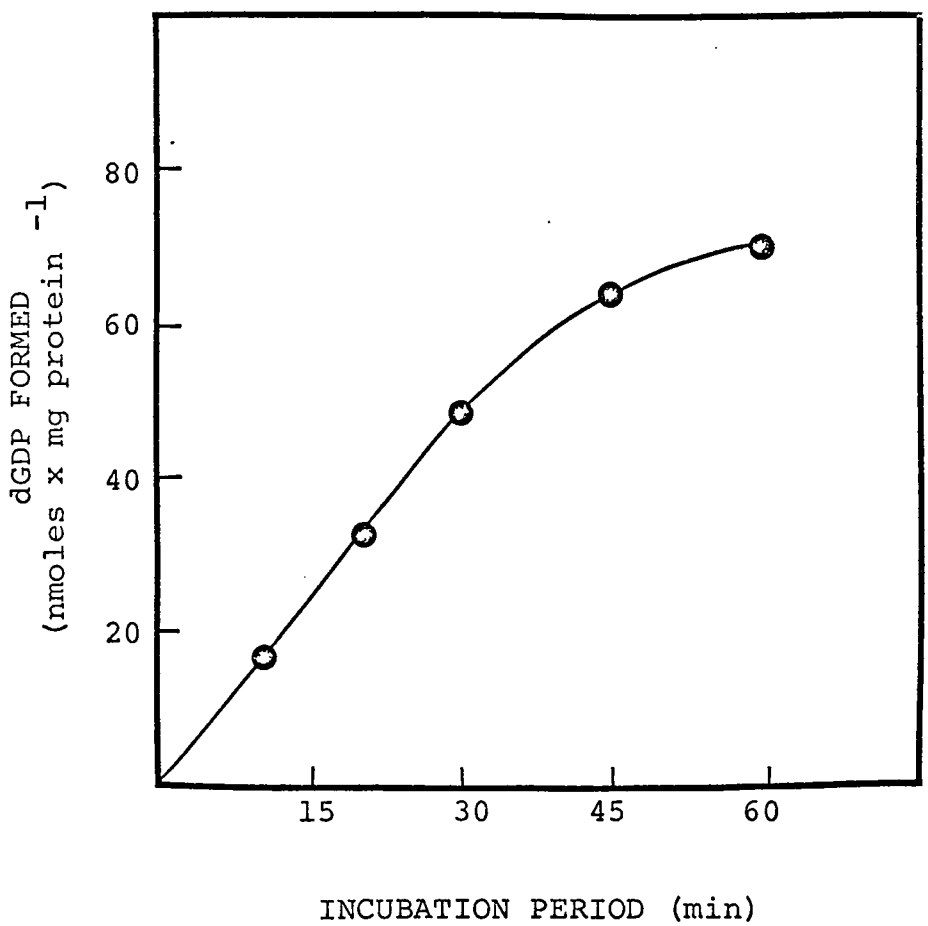


FIGURE 2. The Response of Ribonucleotide Reductase Activity to Increasing Amounts of Protein. The reaction mixtures were as described in the legend of Figure 1 except the amount of protein was varied as indicated, and the period of incubation was 30 min.

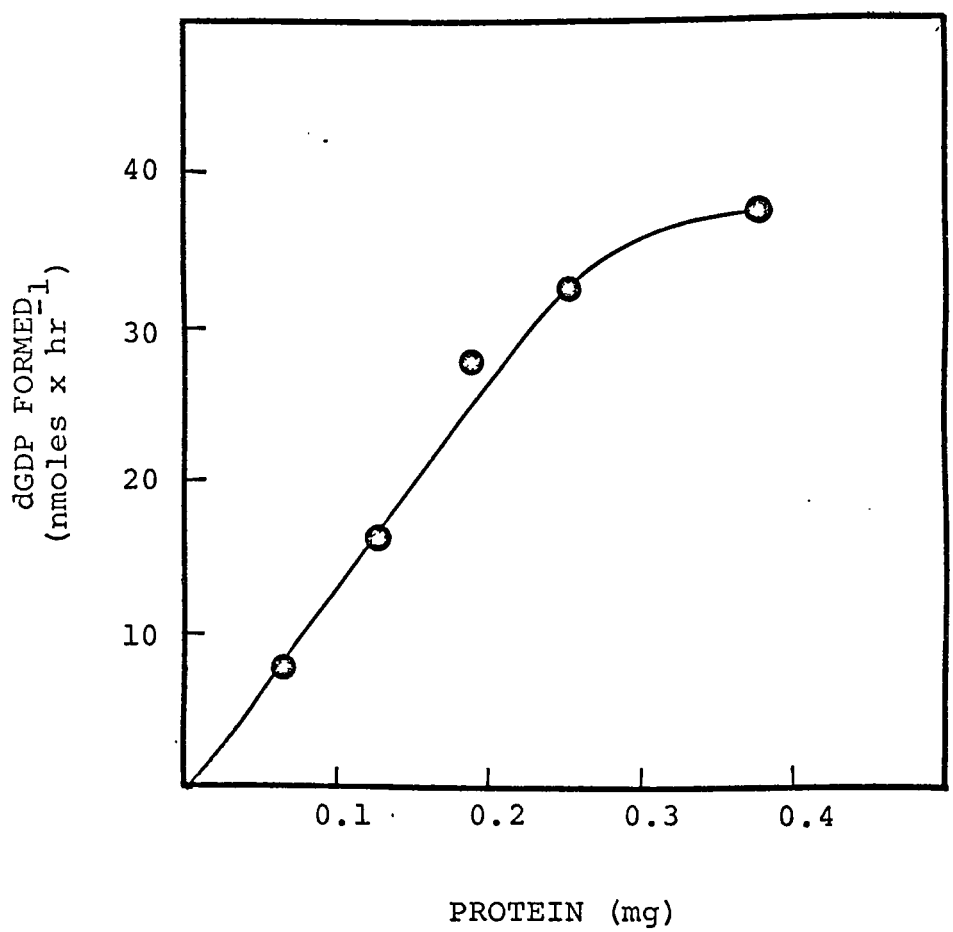


TABLE II. Comparison of Ribonucleotide Reductase Activity in Extracts of *R. meliloti* F-28 and *R. meliloti* 3DOal. The complete reaction mixtures were as described in the legend of Figure 1 except 0.8 µmole GTP was substituted for GDP, 0.15 mg of an ammonium sulfate-fractionated enzyme preparation was used, and the period of incubation was 30 min.

Source of Enzyme	dGTP FORMED (nmoles x mg protein^{-1} x hr^{-1})
R. meliloti F-28	371
R. meliloti 3DOal	118

ference in substrate and cofactor specificity and/or optimal concentrations of reactants. To test these possibilities, extracts from R. meliloti 3DOa1 were fractionated further by DEAE-cellulose column chromatography and used to determine substrate and reductant specificity and the optimal concentrations of substrates and cofactors. A typical protein profile and ribonucleotide reductase activity from a DEAE-cellulose column are shown in Figure 3. The highest reductase activity was obtained at a KCl concentration of approximately 0.25 M. The peak fractions had a specific activity of 400 to 500 nmoles x mg protein^{-1} x hr^{-1} and represents an approximate 10- and 4-fold purification, respectively, over the crude and ammonium sulfate fractions (Table III). Ribonucleotide reductase of R. meliloti F-28 was purified approximately 20-fold over the crude extract by DEAE-cellulose chromatography (42). As seen in Table III only 10 to 15% of the total reductase activity was recovered in the three peak fractions (30 ml).

The peak fractions from the DEAE-cellulose column were used to determine the substrate and reductant specificity and the optimal substrate and cofactor concentrations for the R. meliloti 3DOa1 ribonucleo-

FIGURE 3. Purification of Ribonucleotide Reductase by DEAE-Cellulose Column Chromatography. ●—● O.D. at 280 nm; ○—○ dGTP formed; —— KCl gradient. One hundred and fifty mg of protein was added to the column and eluted in 10 ml fractions with increasing concentrations of KCl to 0.35 M. Ribonucleotide reductase activity was determined as described in Materials and Methods.

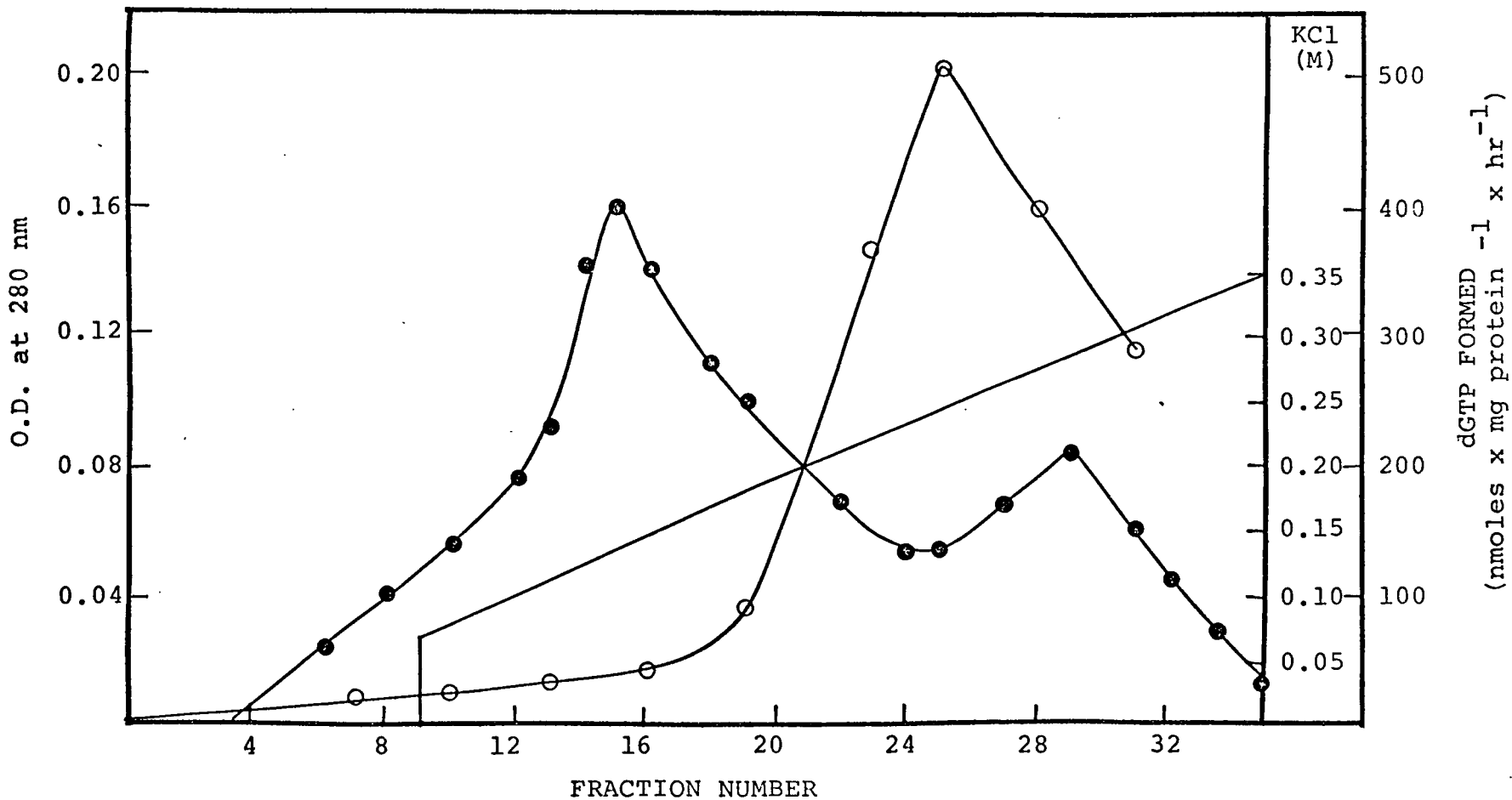


TABLE III. A Summary of the Purification of Ribonucleotide Reductase of R. meliloti 3D0al. Ribonucleotide reductase activity was determined as described in Materials and Methods.

Fraction	Volume (ml)	Concentration (mg/ml)	Total Protein (mg)	Specific Activity[a] (units/mg protein)	Total Activity[a] (units x 10^{-3})
Crude	35.0	25.5	893.0	48	42.8
$(NH_4)_2SO_4$	14.0	9.0	127.0	133	16.9
DEAE	2.5	3.0	7.5	500	3.8

[a]One unit is defined as the formation of one nmole of dGDP per hour.

tide reductase system. The optimal substrate concentrations for GMP, GDP, and GTP were 0.4, 0.1, and 0.4 mM, respectively (Figure 4). This compares to a 0.4 and 1.6 mM GDP and GTP optima reported for R. meliloti F-28. As with the R. meliloti F-28 ribonucleotide reductase system, the optimal concentration for GDP was considerably lower than for GTP or GMP, indicating a greater affinity for this substrate. Substrate concentrations greater than optimum inhibited reductase activity of R. meliloti 3DOal. Supra-optimal concentrations also were inhibitory in the R. meliloti F-28 reductase system for GDP but not for GTP or GMP. A comparison of the reduction rates of the three guanosine substrates in R. meliloti F-28 and 3DOal are shown in Table IV. The data for R. meliloti F-28 were taken from Cowles and Evans (42). The concentration of substrates used was based on the GTP optimal for both enzymes; activities at optimal GDP concentrations are in parenthesis. The major difference in these enzymes is the inability of GDP to be reduced at comparable rates to GTP at optimal concentrations in R. meliloti 3DOal and the apparent effectiveness of GMP in R. meliloti 3DOal as compared to R. meliloti F-28.

The effects of increasing concentrations of vari-

FIGURE 4. The Effect of Increasing Concentrations of Guanosine Phosphates on Ribonucleotide Reductase Activity of R. meliloti 3DOal. The complete reaction mixtures contained in a final volume of 0.5 ml: 50 µmoles potassium phosphate buffer (pH 7.3), 15 µmoles dihydrolipoate, 10 nmoles B_{12} coenzyme, 0.06 mg of protein (DEAE eluate), and varying concentrations of substrate. The period of incubation was 30 min at 37°.

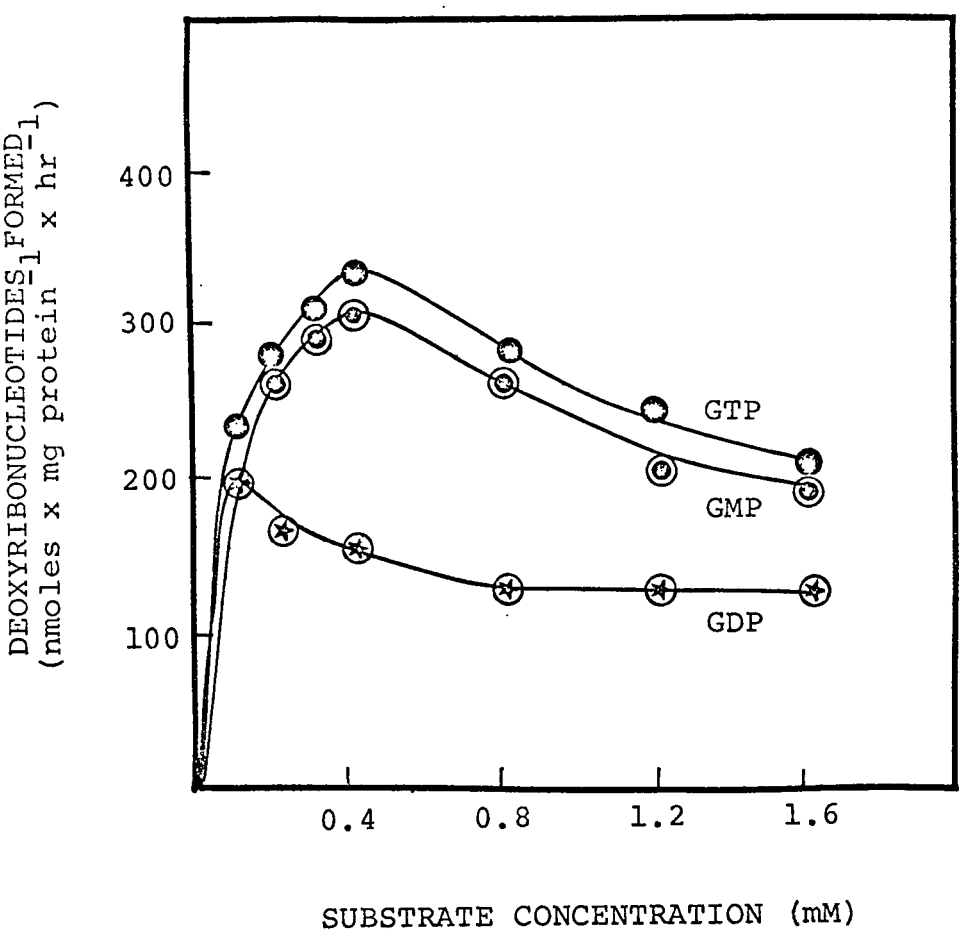


TABLE IV. Comparison of the Reduction Rates of Guanosine Phosphates by R. meliloti 3DOal to those Reported for R. meliloti F-28 (42). The reaction conditions for R. meliloti 3DOal were as described in the legend of Figure 4 except the concentration of guanosine phosphates was 0.4 mM. The concentration of each ribonucleotide for R. meliloti F-28 was 2.0 mM. GDP reduction at optimal concentrations are enclosed in parenthesis.

Substrates	DEOXYRIBONUCLEOTIDES FORMED ($nmoles \times mg\ protein^{-1} \times hr^{-1}$)	
	R. meliloti F-28	R. meliloti 3DOal
GTP	2318	333
GDP	722 (2400)	160 (200)
GMP	628	310

ous reductants on reductase activity are shown in Figure 5. The optimal concentrations of dithiothreitol and dihydrolipoate were 20 and 40 mM, respectively. Although an optimum for 2-mercaptoethanol was not established even at 50 mM, further studies showed that concentrations greater than 50 mM inhibited activity, thus effectively establishing a 2-mercaptoethanol optimum at 50 mM. Dithiothreitol was the most effective reductant, and 2-mercaptoethanol was the least effective. This is considerably different from data reported for the R. meliloti F-28 system. A comparison of the two enzyme systems at a single reductant concentration is shown in Table V. The comparisons were made at 30 mM, which was optimum for dihydrolipoate in the R. meliloti F-28 system and near optimum for dithiothreitol in the R. meliloti 3DOal system. Dihydrolipoate was considerably more effective (6-fold) in R. meliloti F-28 than dithiothreitol, whereas in R. meliloti 3DOal, dithiothreitol was 2-fold more effective than dihydrolipoate at 30 mM and 40% more effective at optimal concentrations (Figure 5). The monothiol, 2-mercaptoethanol, was an ineffective reductant in both enzyme systems.

Maximum ribonucleotide reductase activity was obtained at a B_{12} coenzyme concentration of 35 to 40 μM

FIGURE 5. The Effect of Increasing Concentrations of Reductants on Ribonucleotide Reductase Activity. The reaction mixtures were as described in the legend of Figure 4 except the reductants shown were substituted for dihydrolipoate at the concentrations indicated, and the GTP concentration was 0.4 mM.

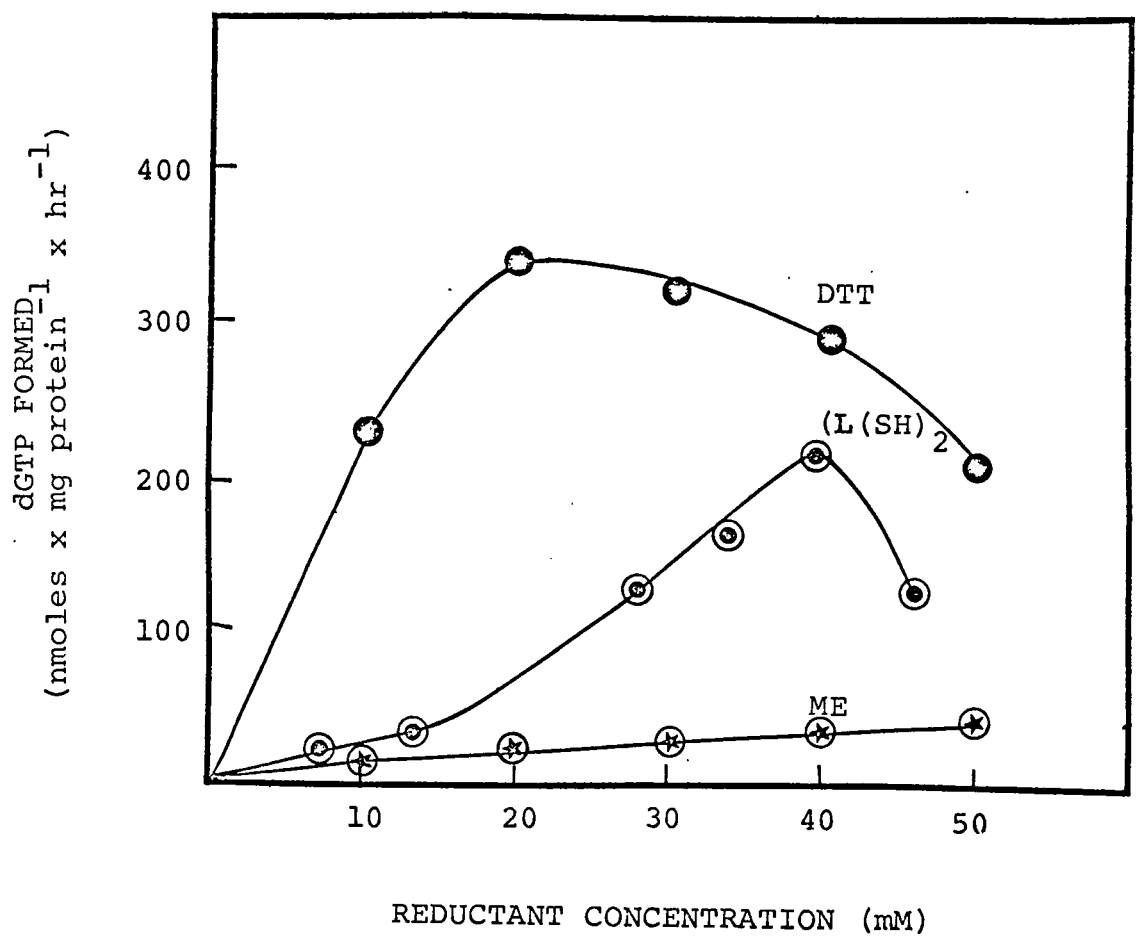


TABLE V. Comparison of the Effectiveness of Various Reductants on Ribonucleotide Reductase Activity of R. meliloti 3DOal to those Reported for R. meliloti F-28 (42). The reaction mixtures for R. meliloti 3DOal were as described in the legend of Figure 4 except the reductants listed were substituted for dihydrolipoate at a concentration of 30 mM (15 µmoles per 0.5 reaction), and the GTP concentration was 0.4 mM.

Reductant	dGTP FORMED (nmoles x mg protein^{-1} x hr^{-1})	
	R. meliloti F-28	R. meliloti 3DOal
Dihydrolipoate	2410	145
Dithiothreitol	398	327
2-Mercaptoethanol	50	33

(Figure 6), which was very similar to the optimal B_{12} coenzyme concentration (30 μM) in R. meliloti F-28.

A comparative summary of the optimal substrate and cofactor concentrations for R. meliloti F-28 and R. meliloti 3DOal are shown in Table VI. The ammonium sulfate-fractionated extracts of R. meliloti 3DOal and F-28 were compared using the established optima for R. meliloti 3DOal and the reported optima for R. meliloti F-28 (Table VII). Although activity in R. meliloti 3DOal increased approximately 2-fold, it was still significantly less than in R. meliloti F-28.

The pH optimum for R. meliloti F-28 and R. meliloti 3DOal were 8.0 and 7.3, respectively (Figure 7). The measurements of ribonucleotide reductase activity in these two organisms had been conducted at pH 7.3.

FIGURE 6. The Effect of Increasing Concentrations of B_{12} Coenzyme on Ribonucleotide Reductase Activity. The reaction conditions were as described in the legend of Figure 4 with the following exceptions: (1) 10 µmoles dithiothreitol was substituted for dihydrolipoate, (2) the GTP concentration was 0.4 mM, and (3) the concentration of B_{12} coenzyme was varied as indicated.

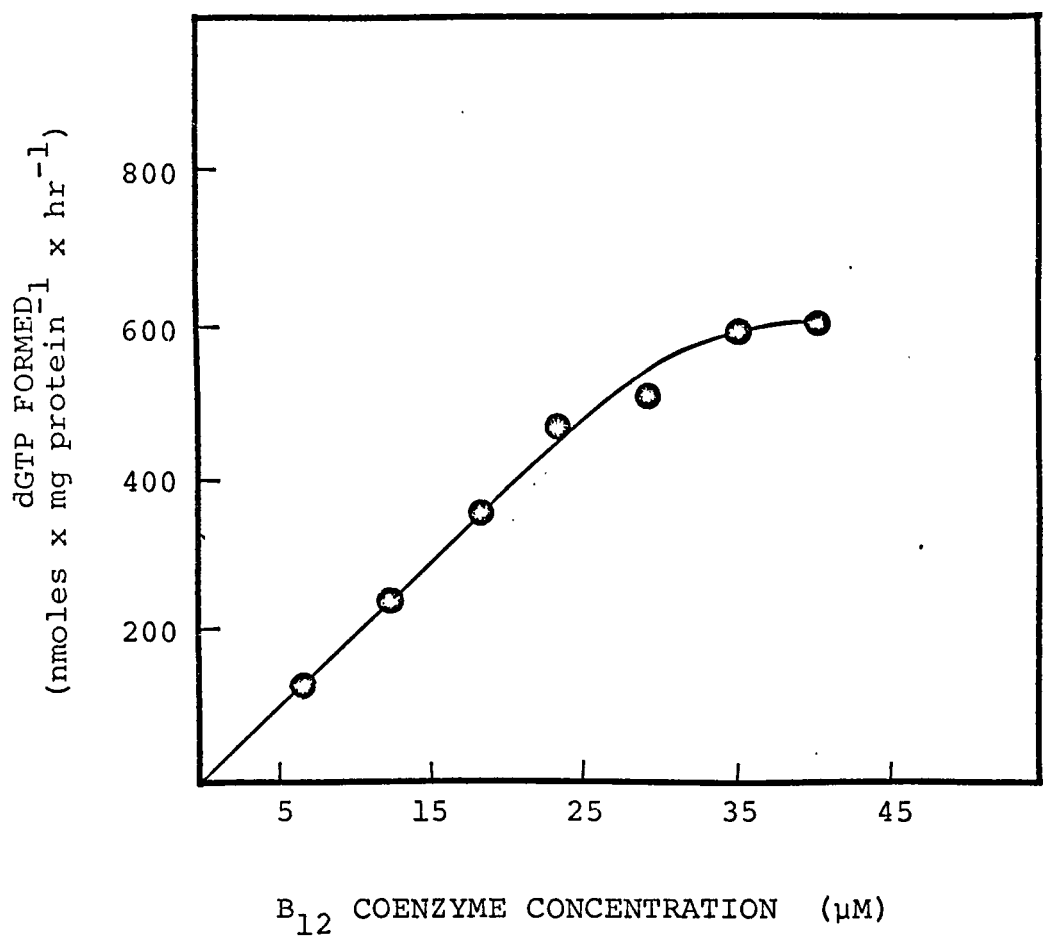


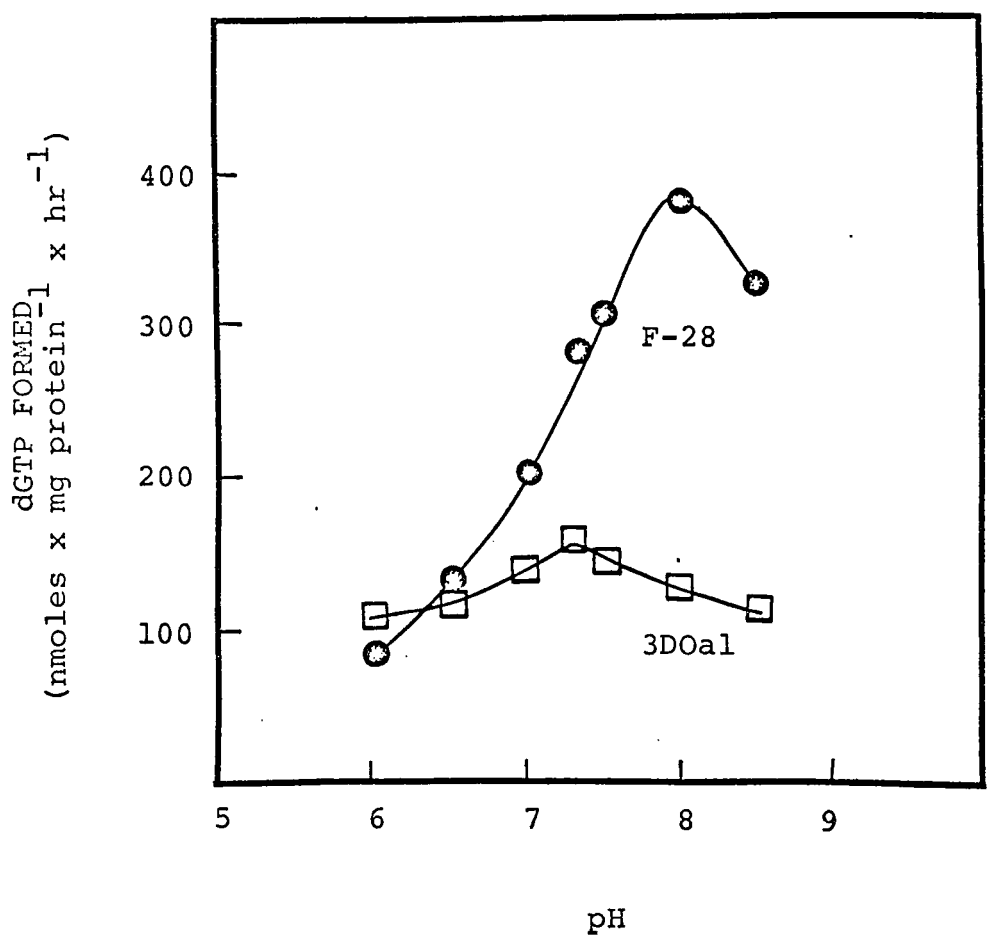
TABLE VI. Optimal Substrate and Cofactor Concentrations for R. meliloti 3DOal and R. meliloti F-28 (42).

Substrate or Cofactor	R. meliloti F-28	R. meliloti 3DOal
GTP	1.6 mM	0.4 mM
GDP	0.4 mM	0.1 mM
$L(SH)_2$	30.0 mM	40.0 mM
DTT	——	20.0 mM
B_{12} Coenzyme	30.0 µM	35.0 µM

TABLE VII. Comparison of Ribonucleotide Reductase Activity of R. meliloti F-28 and R. meliloti 3DOa1 at Known Optimal Conditions for each Strain. The concentrations of substrate and cofactors were as reported in Table VI. An ammonium sulfate-fractionated enzyme preparation was used and the reaction mixtures were incubated 30 min at 37°.

Source of Enzyme	dGTP FORMED (nmoles x mg protein^{-1} x hr^{-1})
R. meliloti F-28	381
R. meliloti 3DOa1	196

FIGURE 7. The Effect of pH on Ribonucleotide Reductase Activity of R. meliloti F-28 and R. meliloti 3DOal. The complete reaction mixtures were as described in the legend of Figure 1 except the pH of the phosphate buffer was varied as indicated, 0.8 µmole GTP was substituted for GDP, an ammonium sulfate-fractionated enzyme preparation was used, and the incubation period was 30 min.



Ribonucleotide Reductase Mutants

As previously discussed, R. meliloti 3D0a1 is reported to be ineffective in carrying out biological nitrogen fixation under symbiotic conditions. The reason for the ineffectiveness is unknown, but previous reports (42), including this thesis, have demonstrated a significant difference in ribonucleotide reductase activity in this organism as compared to an effective strain. The second aspect of this thesis reports studies on ribonucleotide reductase deficient mutants obtained from R. meliloti F-28 in an effort to more fully assess the significance of ribonucleotide reductase activity relative to effective symbiosis.

Cultures of R. meliloti F-28 were grown in a synthetic medium and treated with different concentrations of N-methyl-N'-nitro-N-nitrosoguanidine for various lengths of time. The treatment, detection, and isolation procedures for the mutants are described in Table VIII. The treatments which were most effective in the number of mutations produced were 20 µg/ml for 120 min and 30 µg/ml for 60 min. Sixteen colonies, which had reduced or no growth on the synthetic minimal media, were isolated. An approximation of the

TABLE VIII. Treatment, Detection, and Isolation Procedures for *R. meliloti* F-28 Mutants.

1. A viable cell count was performed on an actively dividing culture of *R. meliloti* F-28 grown in a synthetic mannitol medium containing 5 µg/ml of deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine.

2. Cultures of the prototrophic strain were treated with 10, 20, and 30 µg/ml of N-methyl-N-nitro-N-nitrosoguanidine for 30, 60, 120, and 240 minutes (12 cultures in total).

3. A 1/250 dilution of the cells were made in physiological saline and allowed to express overnight.

4. Cells were serially diluted and plated on complete media containing 5 µg/ml of adenosine, guanosine, cytidine, uridine and each deoxyribonucleoside.

5. The plates were incubated for 36 hours at 30° in the dark.

6. Colonies were replica plated on minimal media (complete media minus the deoxyribonucleosides) and incubated 36 hours at 30° in the dark.

7. Colonies which grew on complete media but exhibited reduced or no growth on minimal media were selected.

8. The selected colonies were transferred to complete media with sterile toothpicks and incubated at 30° for 36 hours in the dark.

9. Colonies were again replica plated on minimal media and incubated at 30° for 36 hours in the dark.

10. Those colonies which again displayed reduced or no growth were confirmed as mutants.

growth rate and slime production of each organism is shown in Table IX. R. meliloti HP-31 and R. meliloti LZ-26 were the fastest growing mutants with a generation time of 1.3 and 1.4 hr, respectively. Both organisms, however, produced large quantities of slime, which, undoubtedly, affected the measurement of growth rates. Two other organisms, R. meliloti LJ-22 and R. meliloti FAR-28, produced very little slime and had growth rates of 2.7 and 4.4 hr, respectively. The slowest growing mutants were R. meliloti JV-13 and R. meliloti VM-3 at 5.1 hr, respectively. It is interesting that one produced heavy slime while the other had only slight slime production. The effects on growth as a result of omitting individual deoxyribonucleosides from the media are reported in Table X. The growth of R. meliloti JC-31, VL-22, and HP-31 was significantly affected by the omission of all four deoxyribonucleosides, whereas, the growth of R. meliloti JG-26 and R. meliloti CL-15 was not. R. meliloti Lew-21 and R. meliloti VM-3 were affected by the omission of certain deoxyribonucleosides but not others. Ribonucleotide reductase activity was determined in crude extracts of each organism (Table XI). Ribonucleotide reductase activity of R. meliloti

TABLE IX. Growth Rates and Slime Production of Selected R. meliloti F-28 Mutants. Generation time was determined by monitoring the absorbance at 660 nm in side-arm flasks. Cells were grown in a yeast-extract mannitol media. The amount of slime produced was determined by visual observations relative to the prototrophic strain.

Organism	Generation time in log phase (hr)	Slime production
R. meliloti F-28	2.8	Slight
" LZ-26	1.4	Heavy
" VL-22	2.5	Slight
" JV-13	5.1	Slight
" CL-15	2.9	Slight
" PU-22	2.6	Moderate
" JG-26	3.2	Slight
" JF-25	2.6	Slight
" HS-12	2.4	Slight
" FAR-28	4.4	Slight
" VM-3	5.1	Heavy
" LJ-22	2.7	Slight
" PH-1	2.6	Slight
" MH-28	3.4	Slight

TABLE IX. Continued.

Organism	Generation time in log phase (hr)	Slime production
R. meliloti HP-31	1.3	Heavy
" Lew-21	1.6	Moderate
" JC-31	2.5	Slight

TABLE X. The Effects of Omitting Individual Deoxyribonucleosides on the Growth of R. meliloti F-28 Mutants. The organisms were grown for 30 hr at 30° in synthetic media supplemented with deoxyribonucleosides minus the nucleoside indicated. Absorbance was determined after 30 hr at 660 nm and expressed in Klett units.

Organism	Growth (Klett Units)				
	Complete	-dA	-dG	-dC	-T
R. meliloti JC-31	160	97	90	103	90
" PH-1	189	126	151	120	115
" VM-3	250	160	194	160	255
" Lew-21	179	160	126	110	135
" JG-26	189	135	145	155	151
" MH-28	170	120	140	130	145
" PU-22	165	155	135	155	130
" HS-12	140	100	80	90	100
" VL-22	80	35	56	63	51
" JF-25	174	140	135	126	135
" LZ-26	44	10	23	10	10
" FAR-28	165	140	126	120	105

TABLE X. Continued.

Organism	Growth (Klett Units)				
	Complete	-dA	-dG	-dC	-T
R. meliloti LJ-22	169	120	120	90	97
" CL-15	151	126	155	160	145
" JV-13	145	105	115	115	110
" HP-31	40	10	10	15	15

TABLE XI. Ribonucleotide Reductase Activity of *R. meliloti* F-28 and *R. meliloti* F-28 Mutants. The complete reaction mixtures in a final volume of 0.2 ml contained: 7.5 µmoles potassium phosphate buffer (pH 7.3), 0.5 µCi ^{14}C-GDP, 0.8 µmole GDP, 3.75 nmoles B_{12} coenzyme, 6 µmoles dihydrolipoate, and 0.25 mg of a crude enzyme preparation. The reactions were incubated 30 min at 37°.

Organism	dGDP FORMED (nmoles x mg protein^{-1} x hr^{-1})
R. meliloti F-28	72
" LZ-26	6
" VL-22	29
" JV-13	48
" CL-15	23
" PU-22	13
" JG-26	14
" JF-25	12
" HS-12	53
" FAR-28	60
" VM-3	36
" LJ-22	65
" PH-1	47

TABLE XI. Continued.

Organism	dGDP FORMED (nmoles x mg protein^{-1} x hr^{-1})
R. meliloti MH-28	34
" HP-31	5
" Lew-21	17
" JC-31	55

LJ-22 and R. meliloti FAR-28 was approximately 75% of the activity of R. meliloti F-28, whereas, the activity of R. meliloti HP-31 and R. meliloti LZ-26 was only 10% of the activity in the prototrophic strain. The latter two organisms were selected for more extensive studies.

R. meliloti HP-31 and R. meliloti LZ-26 were unable to grow to a density comparable with R. meliloti F-28 in both the synthetic and yeast-extract mannitol media even though their generation times were 50% of that of R. meliloti F-28 (Table IX). The inability of the organisms to maintain a rapid growth rate may have been attributed to limiting components in the media such as amino acids and deoxyribonucleosides. The synthetic media was again used to determine these limiting factors. The growth of R. meliloti LZ-26 was increased by supplementing the media with 10 µg/ml of proline, alanine, phenylalanine, and 2X the original concentration (5 µg/ml) of deoxyribonucleosides; the growth of R. meliloti HP-31 was increased by supplementing the media with 10 µg/ml of phenylalanine, proline, and the deoxyribonucleosides. After the limiting factors were determined, the yeast-extract mannitol media was used. The media was supplemented with

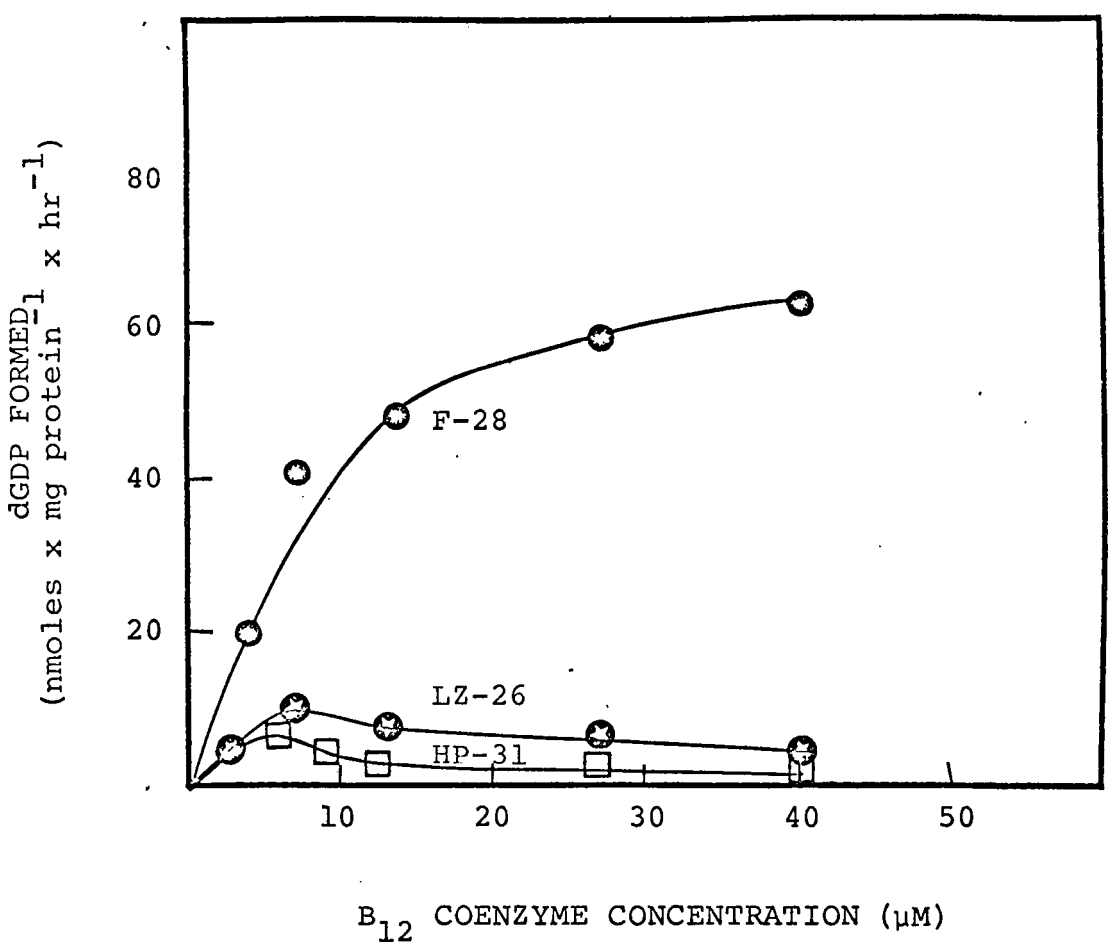
Casamino acids (2.5 g/l), and the organisms grew to a density comparable with R. meliloti F-28. Further studies were conducted to determine if the low level of ribonucleotide reductase activity in R. meliloti HP-31 and R. meliloti LZ-26 resulted from the limiting factors in the media. Extracts of R. meliloti HP-31 and LZ-26, grown in the yeast-extract mannitol media supplemented with Casamino acids, contained only slightly more ribonucleotide reductase activity than unsupplemented media (Table XII).

The level of ribonucleotide reductase activity in the R. meliloti HP-31 and LZ-26 systems could be, in part, a result of sub-optimum reaction concentrations or specificity as shown with R. meliloti 3DOal. Preliminary studies of substrate and cofactor requirements in the two organisms were determined. The optimal concentration of B_{12} coenzyme in crude extracts of R. meliloti HP-31 and LZ-26 were 5 to 6 μM as compared to 40 μM for R. meliloti F-28 (Figure 8) Supra-optimal concentrations of B_{12} coenzyme inhibited ribonucleotide reductase activity of R. meliloti HP-31 and LZ-26. The effect of various reductants on ribonucleotide reductase activity of R. meliloti F-28, HP-31, and LZ-26

TABLE XII. Ribonucleotide Reductase Activity of *R. meliloti* HP-31 and *R. meliloti* LZ-26 Grown with and without Casamino Acids. Cells were grown in yeast extract-mannitol media with and without Casamino acids (2.5g/l). The reaction conditions were as described in the legend of Table XI.

Organism	dGDP FORMED (nmoles x mg protein^{-1} x hr^{-1})
R. meliloti HP-31	
- amino acids	7.5
+ amino acids	9.4
R. meliloti LZ-26	
- amino acids	6.0
+ amino acids	7.6

FIGURE 8. The Effect of Increasing Concentrations of B_{12} Coenzyme on Ribonucleotide Reductase Activity of R. meliloti F-28, R. meliloti LZ-26 and R. meliloti HP-31. The reaction conditions were as described in the legend of Table XI except the B_{12} coenzyme concentration was varied as indicated.



are reported in Table XIII. Dithiothreitol at 30 mM was the most effective reductant in R. meliloti HP-31 and LZ-26, whereas, dihydrolipoate was substantially preferred in R. meliloti F-28. Dihydrolipoate was approximately 80% as effective as dithiothreitol in R. meliloti HP-31 and LZ-26. Again, the monothiol, 2-mercaptoethanol, was ineffective in all systems. The effect of two concentrations of GDP and GTP on ribonucleotide reductase activity was determined (Table XIV). In all organisms, ribonucleotide reductase activity was highest in the presence of 1.6 mM GTP. Eight-tenths mM GDP was the preferred substrate concentration for GDP reduction in all organisms but was not as effective as GTP at the concentration tested. Essentially no difference was found between the organisms relative to substrate specificity.

Four of the newly obtained mutants, R. meliloti LJ-22, R. meliloti HP-31, R. meliloti LZ-26, and R. meliloti JF-25, along with R. meliloti F-28 and R. meliloti 3DOa1, were used to inoculate alfalfa plants in an effort to determine if the mutants were effective in nodule formation and nitrogen fixation. In general, the mutants had less nitrogenase activity than R. meliloti F-28 (Table XV) The only exception was R.

TABLE XIII. The Effects of Various Reductants on Ribonucleotide Reductase Activity of *R. meliloti* F-28, *R. meliloti* HP-31, and *R. meliloti* LZ-26. The reaction conditions were as described in the legend of Table XI except the reductants indicated were substituted for dihydrolipoate. The concentration of each reductant was 30 mM.

Reductant	dGDP FORMED (nmoles x mg protein^{-1} x hr^{-1})		
	F-28	HP-31	LZ-26
Dihydrolipoate	70.0	6.1	5.3
Dithiothreitol	10.2	7.4	6.4
2-Mercaptoethanol	5.0	1.8	2.4

TABLE XIV. Comparison of GDP and GTP as Substrates for Ribonucleotide Reduction of R. meliloti F-28, R. meliloti HP-31, and R. meliloti LZ-26. The reaction conditions were as described in the legend of Table XI except GTP was substituted for GDP and the substrate concentrations were varied as indicated.

Substrate	DEOXYRIBONUCLEOTIDES$_{-1}$ FORMED$_{-1}$ (nmoles x mg protein^{-1} x hr^{-1})		
	F-28	HP-31	LZ-26
GDP			
0.4 mM	71	20	12
0.8 mM	100	39	40
GTP			
0.8 mM	96	60	31
1.6 mM	246	145	82

TABLE XV. The Effectiveness of R. meliloti Mutants in Nodule Formation and Nitrogen Fixation.

Organism	Growth (cm)	Nodules Formed (no.)	Weight of Nodules (mg)	Ethylene Produced/ 25 plants (μmoles/hr)	Ethylene Produced/ g nodule (μmoles/hr)
R. meliloti F-28	8.5	++	129	9.2	71.5
" 3DOa1	7.1	++	35	1.7	48.0
" JF-25	10.3	++	173	6.8	35.7
" LJ-22	10.1	++	128	5.8	45.3
" LZ-26	7.8	++	108	3.0	26.3
" HP-31	6.9	++	78	5.8	71.5

meliloti HP-31 which had a similar rate of enzymatic activity per gram of nodule as compared to R. meliloti F-28 but had less activity on a per plant basis. Actually, the nitrogenase values varied considerably between certain duplicate samples, which, therefore, makes an accurate interpretation of the data virtually impossible. The plant height for R. meliloti JF-25 and R. meliloti LJ-22 was 15 to 25% greater than for R. meliloti F-28, whereas, the other plants were smaller. R. meliloti 3DOa1, LZ-26, and HP-31 infected plants had at least 15% less nodules than R. meliloti F-28. In addition, up to 35% of the nodules infected with R. meliloti LZ-26 and HP-31 were bleached and did not appear to contain leghemoglobin. R. meliloti JF-25 infected plants contained 30 to 35% more nodules than plants infected with R. meliloti F-28. The deficiencies in plant growth, the smaller quantities of nodules, and the number of bleached nodules on plants infected with R. meliloti HP-31 and LZ-26 as compared to R. meliloti F-28 suggest that these organisms had greater difficulty infecting the alfalfa plants and producing effective bacteroids. These two organisms also had the lowest ribonucleotide reductase activity (Table XI). R. meliloti 3DOa1 had been reported previously to be in-

effective in nitrogen fixation (42).

DISCUSSION

The results reported in this investigation provide evidence that ribonucleotide reductase of Rhizobium meliloti 3DOal has certain properties that differ from those of the R. meliloti F-28 ribonucleotide reductase system (42), in addition to a decreased level of enzymatic activity. The optimal concentration for all guanosine phosphates was at least 4-fold less than those reported for R. meliloti F-28. The rate of GDP reduction at optimal concentrations, however, was approximately 1.5-fold less than GTP at its optimal concentration, and GMP reduction was almost as effective as GTP. Both points differ from the R. meliloti F-28 reductase system. The optimal substrate concentration for GDP was 4-fold less than the GTP or GMP optimum, which suggests that GDP may be the preferred substrate. This would be similar to the R. meliloti F-28 reductase system. The effectiveness of GDP is unlikely to result from contamination of other ribonucleotides since the optimal concentration of GDP is lower than that of either GMP or GTP, whereas, the effectiveness of GTP or GMP as a substrate could be due, in part, to GDP contamination.

One of the most striking differences between the

two ribonucleotide reductase systems is that of reductant preference. Dithiothreitol was the most effective reductant for the R. meliloti 3DOal reductase system, whereas, dihydrolipoate was reported to be the most effective for ribonucleotide reductase activity of R. meliloti F-28. Dithiothreitol was 1.5-fold more effective than dihydrolipoate in the R. meliloti 3DOal system at optimal concentrations for both. Since both dithiothreitol and dihydrolipoate are dithiols, their relative effectiveness in the R. meliloti 3DOal and R. meliloti F-28 reductase systems may be due to structure and size. Dithiothreitol is a four-carbon compound with a molecular weight of 154, whereas, dihydrolipoate is an eight-carbon compound with a molecular weight of 208.

Ribonucleotide reductase activity of R. meliloti 3DOal, measured at the established optima, was still 2-fold less than the activity of R. meliloti F-28. The organisms were previously reported (42) to have similar growth rates, however, necessary deoxyribonucleosides may have been present in the yeast-extract mannitol media which could have stimulated the growth of R. meliloti 3DOal, even with the deficiency in

ribonucleotide reductase activity. The lower level of reductase activity in R. meliloti 3DOal as compared to R. meliloti F-28 could be due to a difference in in vivo allosteric regulation such that the ribonucleotide reductase system in R. meliloti 3DOal is more efficient and not as much enzyme is being synthesized. The low level of activity also could be due to different enzyme configurations such that it would be impossible to obtain similar in vitro activities. Although the mutagenesis procedures for R. meliloti 3DOal were not known, one can deduce that the synthesis of ribonucleotide reductase was altered.

Treatment of R. meliloti F-28 cultures with N-methyl-N'-nitro-N-nitrosoguanidine resulted in the production of sixteen auxotrophs. In general, the mutants had altered ribonucleotide reductase activity and growth rates. In addition, several of the organisms produced large quantities of slime and exhibited amino acid requirements. R. meliloti HP-31 and R. meliloti LZ-26 had the lowest ribonucleotide reductase activity of the mutants isolated and produced the heaviest slime. Possibly, the low level of ribonucleotide reductase activity influenced the increased rate of slime production. The rate of slime

production was not altered by the addition of deoxyribonucleosides to the media.

Since several characteristics of R. meliloti F-28 were altered following treatment with MNNG, mutations at other alleles were possible. In fact, one reported disadvantage of the mutagen is the high probability of multiple site mutations (4). The mutagen also could have affected the synthesis of kinases. Deficiencies in kinases would have affected the level of ribonucleotide reductase activity since ribonucleosides were supplied in the media for the selection process, and ribonucleotides are needed for reduction.

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