IN VITRO REGULATION OF RIBONUCLEOTIDE REDUCTASE FROM RHIZOBIUM MELILOTI

by

Diane L. Laverty

A THESIS

submitted to

University of Houston

in partial fulfillment of
the requirements for the
degree of

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I wish to extend my sincere thanks and deepest appreciation to Dr. Joe R. Cowles, my advisor, for his guidance and advice so generously given during my graduate training. Dr. Cowles' thoughtful ideas and criticisms concerning my program were a source of constant encouragement and essential for its successful completion.

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ABSTRACT

Ribonucleotide reductase from Rhizobium meliloti was purified 8- to 10-fold, yielding a specific activity of 3250 nmoles dGDP formed/mg protein/hr. The optimum concentrations of dihydrolipoate, B_{12} coenzyme, GDP and GTP were 20 mM, 30 uM, 0.2 mM and 1.6 mM, respectively. Although Mg^{++} and ATP were not required for enzyme activity as has been reported in other systems which reduce ribonucleoside diphosphates, these compounds did under certain conditions effect the rate of ribonucleotide reduction. In general, the addition of Mg^{++} at optimum GDP or GTP concentrations did not effect ribonucleotide reductase activity, whereas the addition of ATP was inhibitory. At one-half optimum substrate concentrations Mg^{++} and Mg^{++} plus ATP stimulated GDP and GTP reduction. When Ca^{++} was substituted for Mg^{++} essentially no effect was observed on reductase activity at optimum substrate concentrations; the substitution of Mn^{++} for Mg^{++} inhibited enzyme activity. At one-half optimum substrate concentrations, Ca^{++} stimulated enzyme activity, while Mn^{++} inhibited reductase activity slightly.

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IN VITRO REGULATION OF RIBONUCLEOTIDE REDUCTASE FROM RHIZOBIUM MELILOTI

INTRODUCTION

The reduction of ribonucleotides to deoxyribonucleotides is catalyzed by ribonucleotide reductase. The enzyme has been studied extensively in two bacterial systems, Escherchia coli and Lactobacillus leichmannii, and reported in various other tissues and cells. In general, two classes of ribonucleotide reductase have evolved. One class, represented by E. coli, reduces ribonucleotides at the diphosphate level and requires Mg^{++} and ATP for reduction (75). The other class, represented by L. leichmannii, reduces ribonucleoside triphosphates and requires B_{12} coenzyme for reduction (18).

Ribonucleotide reductase has been reported in Rhizobium meliloti and various other Rhizobium species. The B_{12} coenzyme-dependent reductase system in R. meliloti is unique in that the enzyme has a physiological affinity for certain ribonucleoside di- and triphosphates. This is in contrast to the high specificity for only ribonucleoside diphosphates in the E. coli system and only ribonucleoside triphosphates in the L. leichmannii system. In addition, the R. meliloti system is not dependent on Mg^{++} and ATP as are other systems capable of reducing ribonucleoside diphosphates.

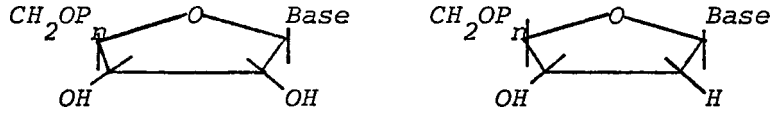
The requirement for Mg^{++} in the E. coli reductase system has been shown to be due to its promotion of subunit binding of the reductase. ATP has been reported to function as a positive allosteric effector. Although Mg^{++} and ATP are not required in the L. leichmannii system, reports have shown that those compounds are capable of inhibiting or

stimulating reductase activity.

Preliminary results with the R. meliloti system have shown that additions of these effectors inhibited GTP reduction under certain conditions, while reduction was stimulated with the GDP substrate. The purpose of this investigation was to analyze the effects of Mg^{++}*, added individually or with ATP, on ribonucleotide reductase activity. Other cations also were studied in the presence or absence of ATP to determine their effect on activity. In addition, an alternate method of batch purification of ribonucleotide reductase was developed.*

REVIEW OF LITERATURE

In 1950, isotopes studies indicated that the transformation of RNA to DNA was not direct (42). From in vivo experiments incorporating labeled nucleotides into rats, the conversion was reported to be from an acid soluble ribosyl derivative to a deoxribosyl derivative. The reduction at the nucleoside or nucleotide level was shown to occur without rupturing the glycosidic linkage (79). In 1959, incorporation studies of ^{14}C-cytidine in chick embryos placed the reductive step at the ribonucleotide level as indicated below (72).



Pn = Pyrophosphate
or Triphosphate

Establishment of Ribonucleotide Reductase Activity.

Escherchia coli. The first demonstration of in vitro ribonucleotide reduction was reported in crude extracts from *E. coli* (78). This extract catalyzed the transformation of CMP to dCMP in the presence of Mg^{++} and ATP; the enzyme involved was designated ribonucleotide reductase. With the removal of endogenous nucleotides on a Dowex-2 column, additions of NADPH increased ribonucleotide reduction, indicating a hydrogen donor requirement (77). Further studies established that CDP was the preferred substrate and that it was reduced to dCDP without any cleavage of the phosphate bonds. Furthermore, under conditions of CDP reduction, neither CMP nor CTP served effectively as substrates.

Novikoff hepatoma. Moore et al. (60, 61) reported that crude extracts of Novikoff ascites hepatoma catalyzed the reduction of CMP in the presence of Mg^{++} and ATP. Later, Fe^{+++} was found to stimulate enzyme activity (62). The cell - free extracts also required NADPH or a NADPH - regenerating system (59, 63). As with E. coli B, ribonucleoside diphosphates were the preferred substrate.

Lactobacillus leichmannii. In 1964, Blakley and Barker (17) reported that cell - free extracts of L. leichmannii reduced CMP to dCMP in the presence of B_{12} coenzyme, ATP, Mg^{++}, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and 2-mercaptoethanol. Reduced lipoic acid later was utilized as the reductant and replaced the glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and 2-mercaptoethanol requirement (16). Upon purification (38), the ribonucleotide reductase lost its ability to reduce mono- and diphosphate ribonucleosides, and consequently became very specific for the ribonucleoside triphosphates (30). The system no longer required Mg^{++} and ATP for activity.

Other Systems. Ribonucleotide reductase has been characterized in several animal tissues and bacterial cells since the initial establishment of enzyme activity. Each system is generally categorized as having requirements similar to either the E. coli B or the Lactobacillus leichmannii ribonucleotide reductase systems. The systems studied that reduce ribonucleoside diphosphates and require ATP and Mg^{++} for activity as does the E. coli B system include: regenerating rat liver (52), Ehrlich ascites cells (60), chick embryos (72, 73), Chinese Hamster fibroblasts (66), cerebral tissues (58), rabbit bone marrow

(46), leukemic mouse spleen (33), calf thymus (3), Yaba Poxvirus tumor and normal monkey tissues (37), Bacteriophage T-4 infected E. coli cells (8, 12), rat hepatomas (60, 29), mouse embryo cells (67), Carcinoma ascites cells (48), and human bone marrow and leukocytes (32, 34).

The B_{12} coenzyme-dependent ribonucleoside triphosphate systems similar to the L. leichmannii system include: Euglena gracilis (36), Rhizobium species (27), Lactobacillus acidophilus (16), Clostridium sticklandii and Clostridium tetanomorphum (1), Corynobacterium Nephridii, Pseudomdnas stutzeri, Micrococcus denitrificans, and Thermus aquaticus (35).

Purification of Ribonucleotide Reductase.

E. coli. Ribonucleotide reductase from cell-free extracts of E. coli has been purified and separated into two nonidentical subunits, B1 and B2. The individual subunits, which were 240-and 500 -fold purified, respectively, did not have ribonucleotide reductase activity (23). In 1969, Brown et al. (20) reported B1 to have a molecular weight between 160,000 and 200,000 daltons, and B2 to have a molecular weight of 78,000 daltons. Further experiments indicate that the B2 subunit was a non-heme iron protein containing two atoms of iron per molecule (21, 22). The B1 subunit, which has the capacity to bind allosteric effectors, appeared to be a dimer, consisting of two polypeptide chains of equal size (25). The ribonucleotide reductase complex contained B1 and B2 in equal molar amounts and had a sedimentation coefficient of 9.7S (24).

L. leichmannii. In 1966, Goulian and Beck (39) reported a 570-fold purification of ribonucleotide reductase from L. leichmannii.

Orr et al. (69) and Panagou et al. (72) also have obtained a highly purified enzyme. Panagou reported the enzyme to be a single polypeptide chain monomer with a molecular weight of 76,000 daltons. An additional six-fold purification with a high percentage of recovery of the ribonucleotide reductase has been obtained with affinity chromatography utilizing a 5'-deoxyadenosylcobalamin agarose absorbent (85).

Related Systems. In Euglena gracilis, the B_{12} coenzyme-dependent ribonucleotide reductase has been purified approximately 17-fold. The molecular weight of this enzyme, determined by sucrose density gradient centrifugation, was approximately 145,000 daltons (36). Another B_{12} coenzyme-dependent reductase from R. meliloti has been purified approximately 20-fold (27).

A ribonucleoside diphosphate system, E. coli phage-T4, has been essentially purified to homogeneity by affinity chromatography utilizing an ATP- and dATP-substituted sepharose absorbent (10).

In general, most mammalian reductase systems have resisted extensive purification. For example, the ribonucleotide reductase from Novikoff hepatoma extracts has been purified only 19-fold (59). The reductase from rabbit bone marrow however, has been purified approximately 57-fold (46). The purified enzymes from both systems were fractionated into separate protein fractions, which individually did not have reductase activity.

Reaction Mechanism.

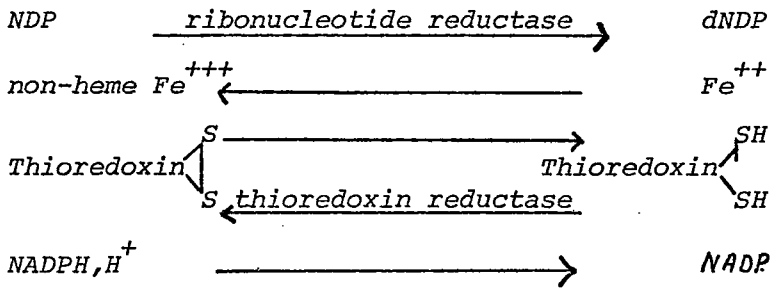
Ribonucleoside diphosphate systems. Early in the study of ribonutide reductase in E. coli, dihydrolipoate was utilized as the reductant. This compound, however, was required at much higher levels than

was expected for a naturally required reductant. A small protein has been isolated from E. coli extracts, which with NADPH substituted for dihydrolipoate (62). Laurent et al. (56) suggested that this heat stable protein, thioredoxin, functioned as an electron carrier between NADPH and the substrate. Moore et al. (64) also isolated a protein fraction from E. coli, thioredoxin reductase, which catalyzed the reduction of thioredoxin by NADPH. The enzyme contained two molecules of FAD (81). Thioredoxin and thioredoxin reductase have molecular weights of 12,000 (56) and 66,000 daltons (81), respectively. Another thioredoxin protein has been isolated and identified from E. coli infected with phage T4 (13, 11).

The first evidence for a thioredoxin-thioredoxin reductase system in mammalian systems was obtained by Herrman and Moore (43) using Novikoff ascites hepatoma tissues. Regenerating rat liver also has been found to contain a thioredoxin-thioredoxin reductase system which is very similar in properties to the Novikoff hepatoma system (50). Heterologous cross reactions utilizing E. coli ribonucleoside diphosphate reductase and Novikoff hepatoma thioredoxin-thioredoxin reductase have been confirmed (43, 56), but no cross reaction between E. coli thioredoxin-thioredoxin reductase and Novikoff hepatoma ribonucleotide reductase has been observed.

The mechanism of hydrogen transfer from the reductant to the substrate appears similar for the E. coli and most mammalian systems. Using tritiated water, the sulfhydral moieties of either reduced thioredoxin or dihydrolipoate become rapidly labeled (51). The tritium label was shown to be transferred to the 2' position of the ribose

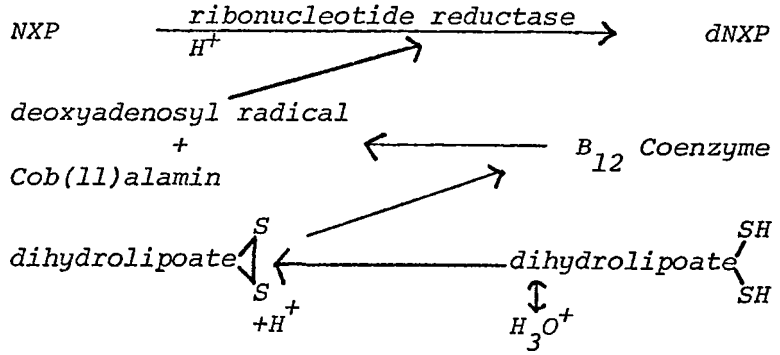
moiety of the substrate and, subsequently, replaced the hydroxyl ion during the reduction process. Brown et al. (21) has suggested that the non-heme iron of the B2 protein (E. coli) participates in this reduction process as an intermediate between the reductant and substrate. A summary of hydrogen transfer is as follows:



B_{12} Coenzyme-Dependent Systems. The earliest evidence connecting vitamin B_{12} derivatives to ribonucleotide reduction was obtained by successfully replacing the vitamin B_{12} requirement in Lactobacillus with deoxyribonucleotides (49). Beck et al. (6) also reported that the intracellular pool of deoxyribosyl compounds in L. leichmannii decreased under vitamin B_{12} deficiencies and increased in vitamin B_{12} additions. The evidence for a B_{12} coenzyme (5, 6 dimethylbenzimidazole cobamide) requirement in ribonucleotide reduction was first demonstrated in 1964 (17). At that time, Blakley proposed that the reductant donated a hydrogen which was carried by B_{12} coenzyme to the substrate.

Vitols et al. (83) have isolated two protein fractions from L. leichmannii, that have similar properties to the E. coli thioredoxin-thioredoxin reductase system and will substitute for dihydrolipoate in the L. leichmannii ribonucleotide reductase assay. These fractions probably represent the physiological hydrogen donor (70).

The L. leichmannii ribonucleotide reductase appears to catalyze first a unique intramolecular hydrogen exchange between tritiated water and the 5' methylene group in the coenzyme before reducing the substrate. The tritium was found located in a non-exchangeable position of the 5' methylene group rather than the expected substrate (16). The reaction then preceeded by slowly degrading the B_{12} coenzyme to Cob(11) alamin and 5' deoxyadenosine. At first, the Cob(11) alamin was believed to bind to the active center of the enzyme and function as a reactive intermediate (41). In 1973, Tomao and Blakley (82) reported direct spectrophotometric observation of a homolytic cleavage of the carbon-cobalt bond which resulted in the formation of a stabilized adenosyl radical. The radical is believed to interact with a thiol group from the enzyme or the reductant and subsequently, rearranges to produce 5' deoxyadenosine. The radical-thiol complex is postulated to be the intermediate reducing agent. This mechanism probably exists in other B_{12} coenzyme-dependent systems. A summary of the reaction mechanism is as follows:



Regulation of Ribonucleotide Reductase Activity.

Nucleotide effector. The function of ATP and Mg^{++} was originally believed to be associated with phosphokinase activity in the conversion of mono- or diphosphate to the corresponding di- or triphosphate ribonucleosides (18, 77). Holmgren et al. (45), however, suggested that ATP was an activator in the E. coli system because CDP reduction was stimulated by ATP additions. In 1966, Larrson and Reichard (53) reported that ATP appeared to function as an allosteric effector. With highly purified preparations of B1 and B2 from E. coli, additions of 1.0 mM ATP greatly increased the affinity of the enzyme for the CDP substrate (55). In contrast, ATP specifically reduced the apparent Km for GDP (54). Studies from the binding of radioactive nucleotides indicated that the B1 subunit, individually or in combination with B2, bound with allosteric effectors (25). Thus, B1 served as the regulatory part of the protein complex.

Two classes of binding sites have been proposed for protein B1. The first class contains two separate binding sites; one binds dATP, and the other, ATP, dATP, dGTP, and dTTP. The second class also has two binding sites, one contains a high affinity for ATP, and the other has an affinity for ATP and dATP. This type of binding resulted in the formation of a specific protein-effector complex that contributed to substrate specificity as well as regulation for enzyme activity.

Sedimentation coefficients for the ribonucleotide reductase were reported to be directly related to the presence of stimulatory and inhibitory effectors (24). In the presence of dATP, a negative effector, the B1 and B2 complex sedimented at 15.5 S. With ATP or dTTP,

positive effectors, the coefficient sedimented near 9.7 S, which was the value assigned to the active complex in the absence of all effectors. The 15.5 S complex, which was enzymatically inactive, was suggested to be a dimer of the active form.

ATP also has been postulated to function as an activator for cytidine reduction in rabbit bone marrow (46), cerebral tissues (58), Novikoff ascites hepatoma (59), and regenerating rat liver (52). These systems have been shown to contain an optimal ATP concentration above 1.0 mM. Yaba poxvirus tumor reductase activity (37), however, is inhibited by ATP concentrations above 0.25 mM.

Prime effectors in the L. leichmannii reductase system were defined as the most active positive effector of the nucleotides tested (5). ATP, in the presence of Mg^{++}, stimulated reduction, but did not produce the abrupt early rise indicative of a prime effector for any of the nucleotide triphosphate substrates. Since the reduction of CTP was approximately linear in relation to ATP concentration, Beck suggested that ATP possibly was undergoing conversion to dATP, which was a prime effector for CTP reduction. When other triphosphate nucleotides were utilized as substrates, activity of the enzyme was inhibited by ATP, individually or in the presence of Mg^{++}.

Euglena gracilis (36) and Rhizobium species (27) were not reported to utilize ATP either as an allosteric or a prime effector.

Divalent Cation Effectors. Early E. coli ribonucleotide reductase assays had shown a dependency for Mg^{++} at a 20.0 mM optimal concentration (77). The substitution of other cations, Mn^{++} at 5.0 mM, Ca^{++} at 20.0 mM and Fe^{++} at 10.0 mM gave 70, 80, and 60% respectively

of the activity that was obtained with Mg^{++}. Higher concentrations of these cations produced strong inhibition. Through purification procedures, Mg^{++} was found to function in the formation of the active complex of the subunits B1 and B2 (23). Later studies indicated that high concentrations of the monovalent cations Na^{+}, K^{+}, or NH_4^{+} could replace the Mg^{++} requirement (20). Whereas the optimum concentration for Mg^{++} was 10.0 mM, a concentration of 450 mM was required for Na^{+} to obtain approximately the same level of activity.

Mg^{++} has not been shown to be required by the phage T4-induced ribonucleotide reductase system (8); however there is a two- to three-fold stimulation of the enzyme activity with additions of the cation.

Most mammalian systems also require Mg^{++} for ribonucleotide reductase activity. The rabbit bone marrow ribonucleotide reductase has shown to require 10.0 mM for optimum activity. Two separate protein fractions are obtained upon purification in the absence of Mg^{++} (46). Mg^{++} was not shown to promote binding of the protein fractions, however, as it does with the E. coli ribonucleotide reductase.

The addition of divalent cations to the L. leichmannii ribonucleotide reductase system resulted in a complex situation of stimulatory and inhibitory effects varying with the substrates utilized. The reduction of CTP was stimulated 52 to 58% by additions of Mg^{++} in the presence of ATP (5). Mn^{++} had the same effect as Mg^{++}, but Ca^{++} was not as active. In contrast, the reduction was strongly inhibited by additions of these cations without ATP. Other divalent cations, Co^{++}, Fe^{++}, Ni^{++}, Zn^{++}, and Cd^{++}, inhibited all enzymatic activity. These cations also produced precipitates in the reaction mixtures.

Beck suggested that Mg^{++} probably produced a change in the physical state of the protein causing the conformation to tighten in such a manner that nucleotides could not bind to the active site. The presence of positive effectors, specific for a triphosphate nucleoside substrate, possibly prevented Mg^{++} from exerting this type of influence on the protein.

Mg^{++} was not required for either Euglena gracilis (36) or Rhizobium meliloti (27), and high concentrations of the cation inhibited activity.

In addition to Mg^{++} requirements, the mammalian systems, Novikoff ascites hepatoma (63), rabbit bone marrow (46), Yaba poxvirus tumor (37), and regenerating rat liver (52) have shown an Fe^{++} stimulation. Ribonucleotide reductase activity in rabbit bone marrow was stimulated by ferrous ions approximately 1.5 to 3.0-fold. Under certain conditions (64) ferrous ions have been found to stimulate activity more than ferric ions. In Novikoff ascites hepatoma extracts, Moore suggested that the reversibly bound ferrous ions form a part of the active site of mammalian ribonucleoside diphosphate reductase.

MATERIALS AND METHODS

Culture Maintainence.

Cultures of *Rhizobium meliloti*, strain F-28, were obtained from Dr. Harold E. Evans of Oregon State University. The bacteria were maintained in flasks containing 60 mls of a mannitol base media (28). The consistuents were the following per liter: K_2HPO_4, 1.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.36 g; $CaSO_4 \cdot 2H_2O$, 0.13 g; $FeCl_3$, 4.0 mg; $CoCl_2 \cdot 6H_2O$, 20.19 mg; mannitol, 3.0 g; and yeast extract, 1.0 g. The cultures were incubated at 30° on a rotary shaker in the dark.

Bacteria Growth.

To obtain large quantities of cells, a 0.60% inoculum of the bacteria was added to 10-liter carboys of sterilized media. Air was filtered through sterile cotton and bubbled through the carboys which were kept at 30° and in the dark. The bacterial growth was monitored by absorbance measurements at 660 nm on a Beckman DB spectrophotometer. The cells were harvested at an absorbance of 0.75 - 1.00 O.D. units, usually 15 - 16 hours after inoculation.

Preparation of Extracts.

Rhizobium cells were harvested with a Sharples centrifuge and washed twice in 0.05 M potassium phosphate buffer (pH 7.3). The cells were resuspended in an equal volume of buffer and broken in a cold French press at approximately 20,000 lbs./sq. in. The suspension was centrifuged for 15 min. at 21,000 X g, and the supernatant was used as the crude extract. These and all subsequent precedures were conducted at 0° - 4°.

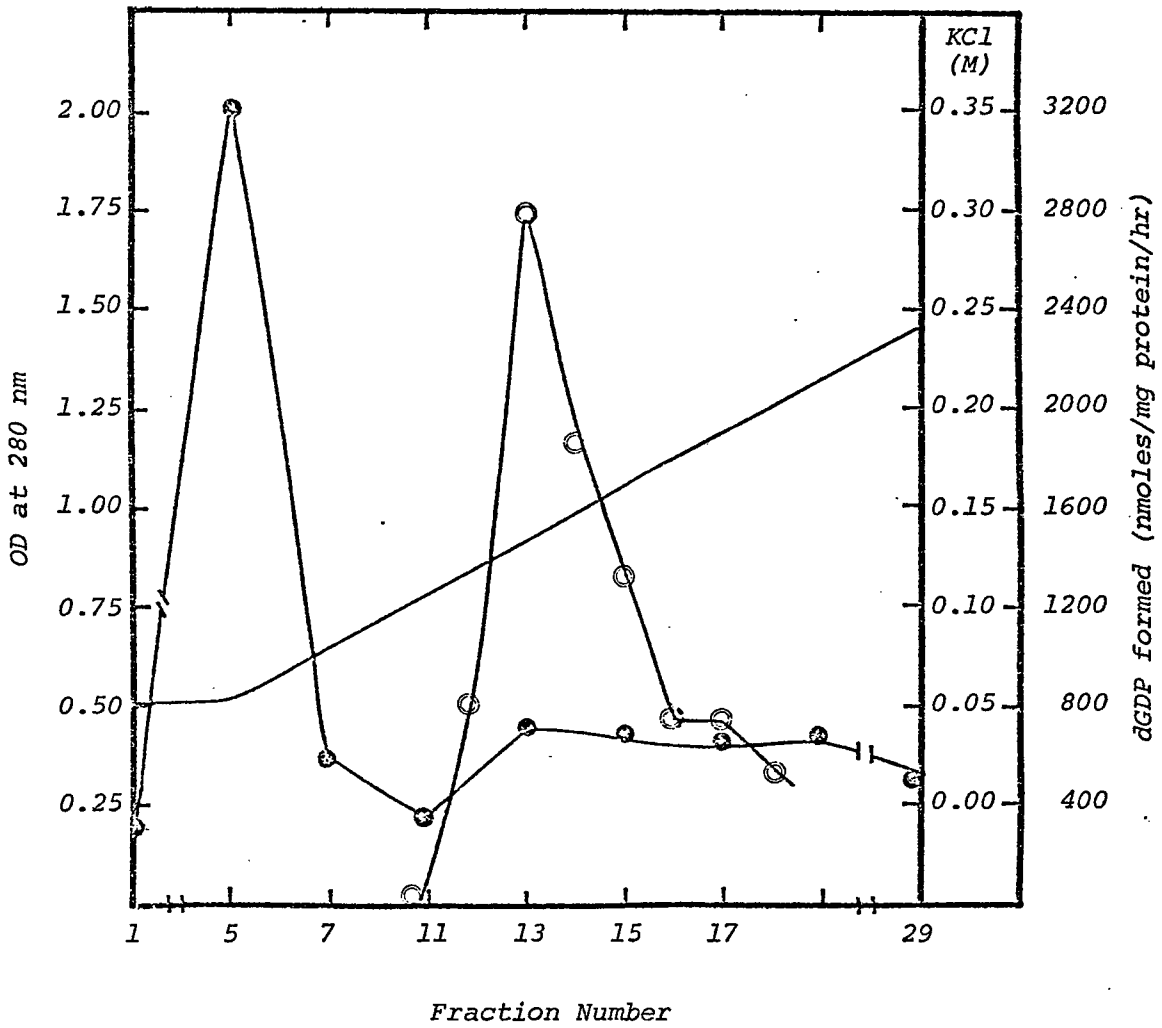
Purification.

All reagents used in the purification and enzyme assays were prepared in glass distilled water. Approximately 50 mls of the crude extract was brought to 30% saturation with saturated ammonium sulfate (pH 7.3) (Sigma, recrystallized from EDTA). After stirring for 10 min., the mixture was centrifuged 10 min. at 14,000 X g. The resulting supernatant was brought to 50% saturation with ammonium sulfate, stirred for 10 min., and centrifuged again for 10 min. at 14,000 X g. The pellet was resuspended in 10 - 12 mls of 0.05 M potassium phosphate buffer (pH 7.3). The solution was dialyzed for 12 hrs. against 4 liters of 5 mM potassium phosphate buffer (pH 7.3) containing 1 mM 2 - mercaptoethanol.

Further purification was obtained using a DEAE-cellulose (Whatman DE - 32 microgranular) column. The column (2.4 X 15 cm) was prepared under pressure and equilabrated with 0.05 M potassium phosphate buffer (pH 7.3). The protein was eluted with potassium phosphate buffer containing a linear potassium chloride gradient (0.05 - 0.35 M). The eluting solutions consisted of: 150 mls of 0.05 M potassium phosphate buffer (pH 7.3), containing 0.05 M potassium chloride, and 1 mM 2-mercaptoethanol in the mixing chamber; and 150 mls of 0.05 M potassium phosphate buffer (pH 7.3), containing 0.35 M potassium chloride, and 1 mM 2-mercaptoethanol in the reservoir. The flow rate of the column was approximately 1.7 mls per min., and 12 ml fractions were collected.

The major peak of ribonucleotide reductase activity was eluted in fractions 10 to 13 (figure 1). The peak fraction was applied to a Sephadex G-25 column (1.5 x 13 cm) and eluted with a 0.05 M potassium

FIGURE 1. Purification Of Ribonucleotide Reductase On A DEAE-cellulose Column. o——o, O.D. at 280 nm; ●——●, reductase activity with GDP as substrate; ——, KCl gradient. A solution containing 150 mgs of protein was placed on the column and eluted in 12 ml fractions. Assays were performed with 0.10 ml aliquots of the fractions.



phosphate buffer (pH 7.3). The protein was collected in 3.5 ml quantities and stored at -10°. This preparation was used in the following experiments unless otherwise indicated.

Assay Procedure.

The complete assay mixture for ribonucleotide reductase contained: 50 umoles of potassium phosphate buffer (pH 7.3); 10 umoles of dihydrolipoate reduced from lipoate (Sigma Chemical Company); 0.1 umoles of GDP (Sigma Chemical Company); 10 nmoles B_{12} coenzyme (Calbiochem); and an appropriate amount of enzyme and water to a final volume of 0.5 mls. The reaction mixtures were incubated at 37° for 30 min. unless otherwise indicated. After the incubation period, the reaction tubes were placed in boiling water for 3 min. Four-tenths ml of 0.5 M chloroacetamide was added to each tube and the mixture boiled for an additional 10 min. After the solutions had cooled, 2.0 mls of diphenylamine reagent, prepared from recrystallized diphenylamine (Baker Chemical Compan) were added, and the mixtures were incubated 4 hrs. at 50°. The amount of color developed was measured on a Beckman DB spectrophotometer at 595 nm. The amount of deoxyguanosine phosphate formed was determined from a standard curve prepared from dGMP (Sigma Chemical Company); 0.10 O.D. units equalled approximately 22 nmoles of deoxyribonucleotides. Specific activity is defined as the amount of deoxyribonucleotides produced per milligram protein per hour.

Other Preparations

The method of Gunsalus and Razzell was utilized for the reduction of lipoate (40). The concentration of dihydrolipoate formed was

determined by its capacity to reduce an excess of a standard solution of potassium ferricyanide on the basis of a molar extinction coefficient of 1040 at 420 nm (80).

The concentration of the B_{12} coenzyme was determined from the millimolar extinction coefficient of 8.0 at 522 nm (4).

The cations, magnesium, manganese, and calcium (Matheson, Cole and Bell Chemical Co.), were prepared as the acetate salts (47) in the effector studies. ATP (Sigma Chemical Co.) was prepared in a stock solution of 10 mM as were the cations.

Protein concentrations were estimated by the Lowry method (57).

RESULTS

Purification by DEAE-cellulose Chromatography.

After the crude extracts were fractionated with ammonium sulfate (30 to 50%), the protein was placed on a DEAE-cellulose column and eluted with a linear KCl gradient (figure 1). The highest specific activity was obtained at a KCl concentration of approximately 0.13 M. After desalting on a Sephadex G-25 column, the peak fractions contained a specific activity of 3,000 to 3,500 nmoles dGDP formed / mg protein / hour, representing an 8- to 10-fold purification. A summary of the purification steps are reported in Table I. Small aliquots of the partially purified enzyme were frozen and later utilized in the following experiments.

The Establishment of Linearity.

The rate of product formation was linear with increasing amounts of protein up to 40 ug per reaction (figure 2). The reaction rate with respect to incubation period also was linear for at least 60 minutes (figure 3). To insure proper kinetics, the reactions were incubated for 30 minutes and normally contained 15 to 30 ug of protein.

The Establishment of Optimum Substrate and Cofactor Concentrations.

The optimum concentration for dihydrolipoate in the reductase reaction was 20.0 mM (figure 4). Higher concentrations resulted in enzyme inhibition. Reductase activity reached a plateau at a B_{12} coenzyme concentration of 30.0 uM (figure 5). Near maximum activity at 20.0 uM was the concentration utilized in the reaction mixtures due

Table I. Summary of Purification of Ribonucleotide Reductase from R. meliloti. The complete reaction mixture contained 50 umoles of potassium phosphate buffer (pH 7.3); 10 umoles $L(SH)_2$; 10 nmoles B_{12} coenzyme; 0.10 umoles GDP; and an appropriate amount of enzyme and water in a final volume of 0.5 ml. Reactions were incubated 30 min. at 37°.

Fraction	Volume	Protein	Specific Activity	Total Activity	Recovery
	(ml)	(mg)	(unit[a]/mg protein)	(unit X 10^{-3})	%
Crude	51	679	368	250	100
Ammonium sulfate fractionation	11	158	588	93	37
DEAE cellulose chromatography	12	6	2692	16	6.4
Sephadex G-25 chromatography	11	5	3258	16	6.4

[a] *One unit of activity is defined as the amount of enzyme necessary to catalyze the synthesis of 1 nmole of dGDP/hr. in the standard assay.*

FIGURE 2. Ribonucleotide Reductase Activity As Influenced By Protein Concentration. The reaction mixtures were the same as described for the complete reaction mixture in Table 1 with the exception of the indicated protein concentrations. The period of incubation was 30 min. at 37°.

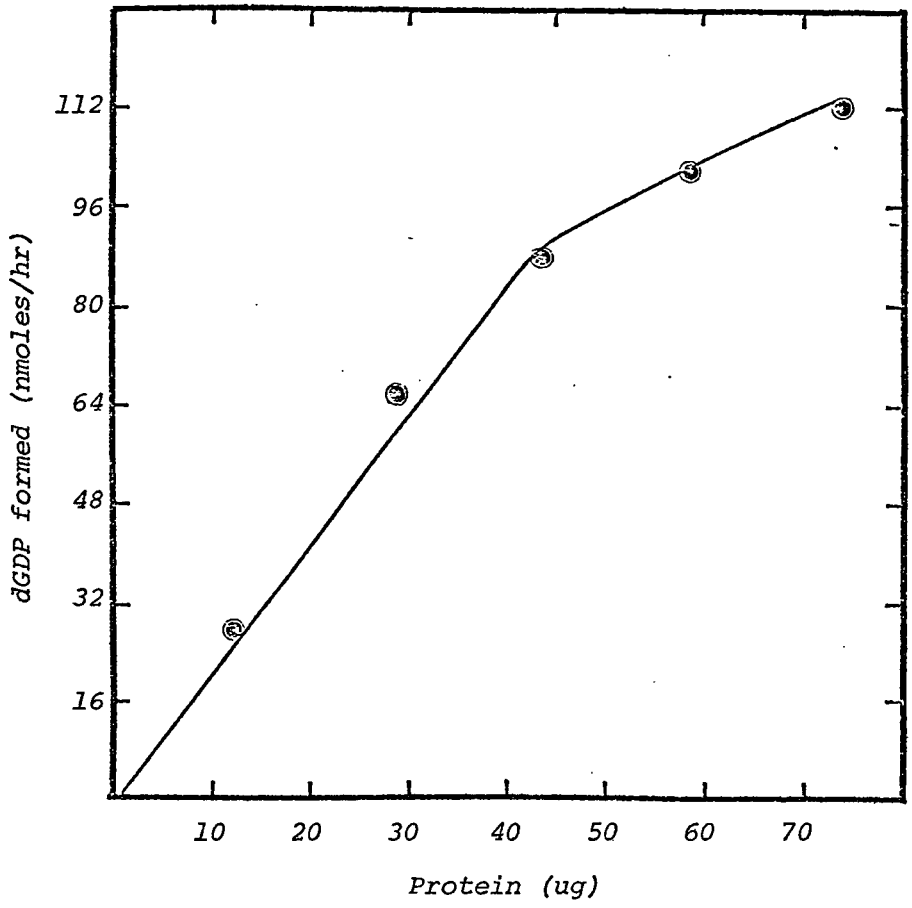


FIGURE 3. Ribonucleotide Reductase Activity As Influenced By The Incubation Period. The reaction mixtures were the same as described for the complete reaction mixture in Table 1 with the exception of the indicated incubation periods.

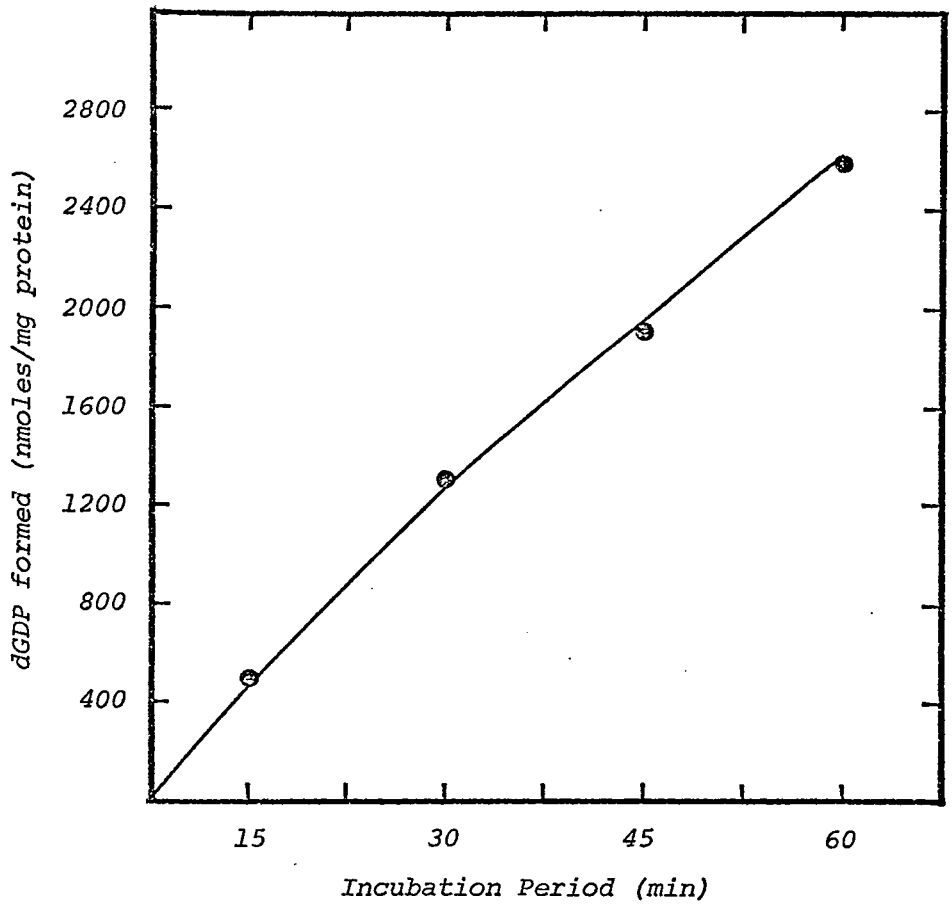


FIGURE 4. The Effect Of Dihydrolipoate Concentration On Ribonucleotide Reductase Activity. The reaction mixtures were the same as the complete reaction mixture described in Table I except that $L(SH)_2$ concentrations were varied as indicated.

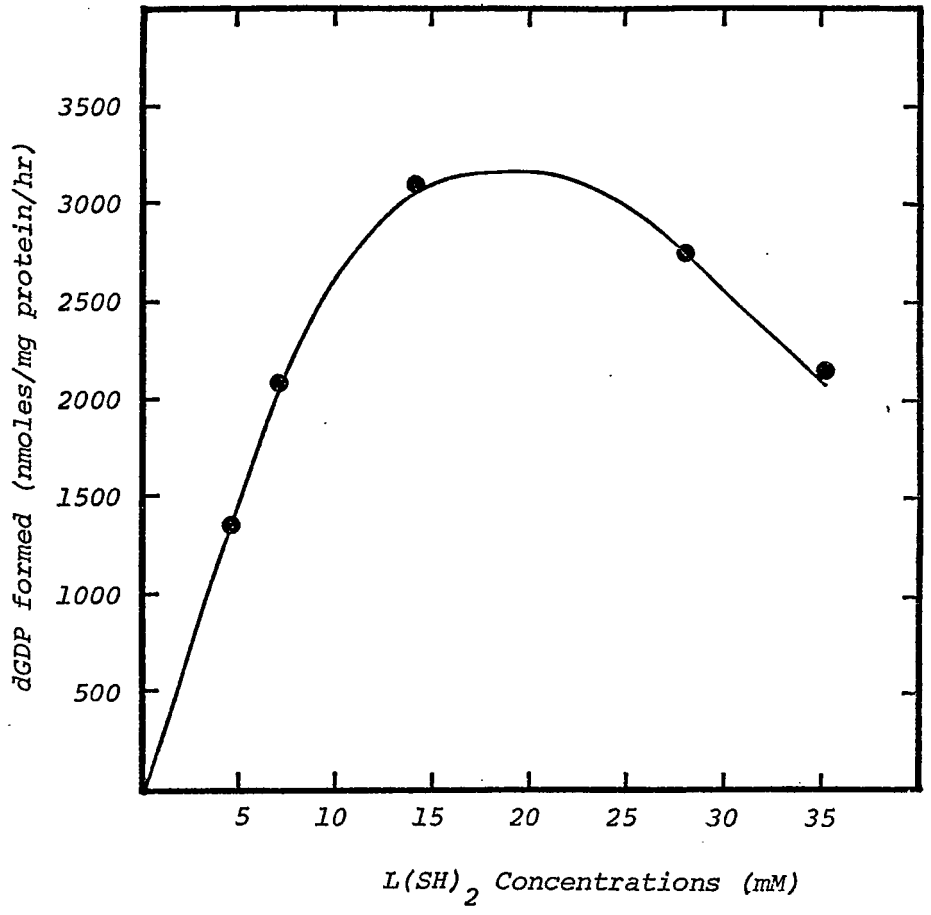
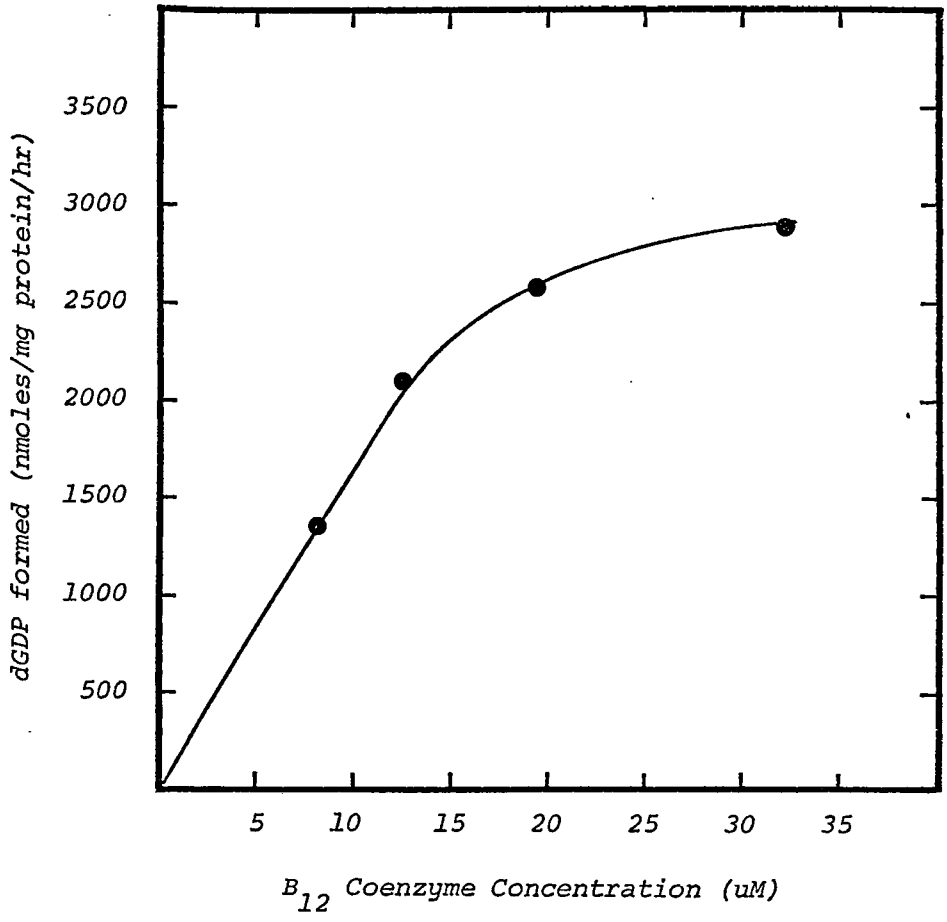


FIGURE 5. The Effect Of B_{12} Coenzyme Concentration On Ribonucleotide Reductase Activity. The reaction mixtures were the same as the complete reaction mixture described in Table 1 except that B_{12} coenzyme concentrations were varied as indicated.



to the limited supply available. The optimum substrate concentrations for GTP and GDP were 1.6 and 0.2 mM, respectively (figure 6). Again concentrations greater than the optimum levels inhibited enzyme activity.

Effector Studies.

The R. meliloti system is unique in its ability to utilize both di- and triphosphate ribonucleosides as substrates. Reduction, however, is not dependent on Mg^{++} and ATP, as are other systems that utilize ribonucleoside diphosphates. Preliminary experiments (27) have shown that low, equal molar concentrations of Mg^{++} and ATP either stimulated or inhibited R. meliloti ribonucleotide reductase activity, depending on the particular substrate and its concentration. Therefore, it is possible that Mg^{++} and ATP have a regulatory effect in regard to utilization of the substrates by the reductase.

To examine this possibility, GTP and GDP were utilized as substrates at optimum and one-half optimum concentrations. One-half optimum was defined as the concentration of substrate that resulted in one-half the maximum activity. The optimum and one-half optimum GTP concentrations were 1.6 and 0.4 mM, respectively, while the concentrations for GDP were 0.1 and 0.06 mM (figure 6).

Mg^{++} and ATP Effects. The addition of Mg^{++} or ATP to the reaction mixture, individually, did not appreciably effect the rate of GTP reduction at optimum GTP concentrations (figure 7). The addition of the compounds in a 2:1 ratio (Mg^{++}:ATP), however, decreased the rate of GTP reduction, especially at 3.0 mM Mg^{++} and 1.5 mM ATP concentrations. Increasing concentrations of Mg^{++} did not effect reductase activity at

FIGURE 6. The Effect of GDP and GTP Concentrations On Ribonucleotide Reductase Activity. □——□ *, GDP substrate;* ●——●*, GTP substrate. The reaction mixtures contained the components listed in Table 1 for the complete reaction except the ribonucleotides shown in the indicated concentrations were substituted for the GDP component. The incubation period was 30 min. at 37°.*

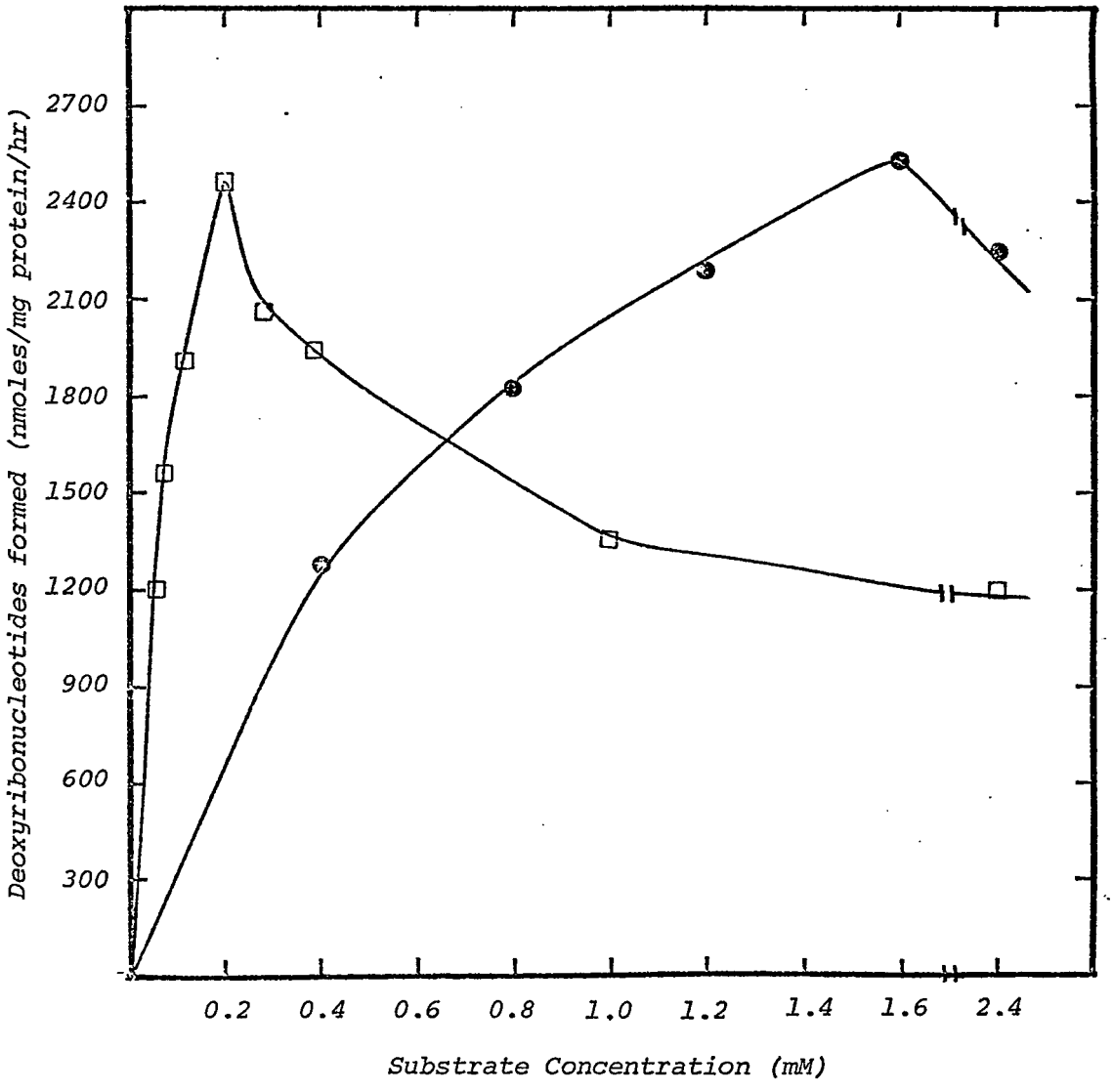
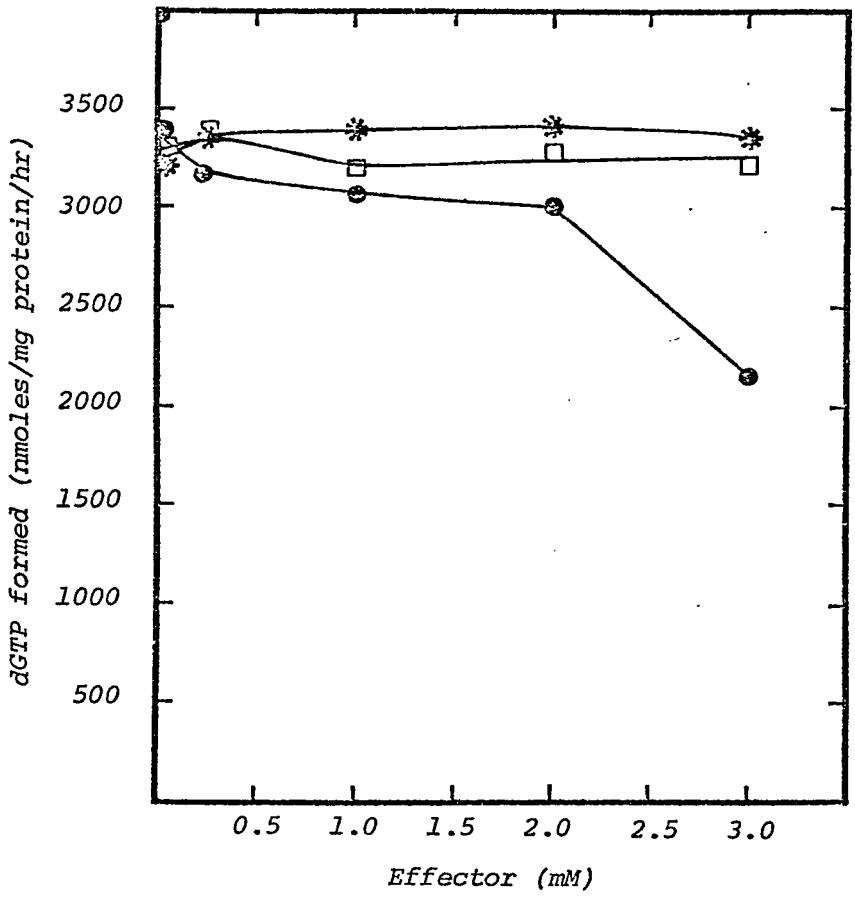


*FIGURE 7. The Effect of Mg^{++}, ATP, and Mg^{++} Plus ATP On Ribonucleotide Reductase Activity At Optimum GTP Concentrations. The effectors are: *——*, Mg^{++}; □——□, ATP; and ●——●, Mg^{++} plus ATP effectors, the ATP concentration is one-half the value represented on the abscissa.*



optimum substrate levels of GDP (figure 8). ATP, however, inhibited activity at concentrations of 1.0 mM and greater. The inhibition at 1.0 mM was approximately 20% and at 3.0 mM approximately 40%. Mg^{++} plus ATP in combination also inhibited GDP reduction. Mg^{++} did appear to prevent the inhibitory effect of ATP at 1.0 mM but not at higher ATP concentrations.

In contrast to the optimum levels of substrate, the rate of GTP reduction at half optimum levels was enhanced by ATP additions (figure 9). Mg^{++} at 1.0 mM and the combination of Mg^{++} and ATP at 0.2 mM and 0.1 mM, respectively, stimulated GTP reduction 30 to 40%. ATP concentrations above 0.1 mM had a slightly inhibitory effect. When GDP was utilized at half optimum concentrations, Mg^{++} and ATP, individually, enhanced activity 10 to 15% (figure 10). The effector combination of 1.0 Mg^{++} and 0.5 mM ATP produced approximately a 15% stimulation.

Other Divalent Cations and ATP. Since Mg^{++} and ATP did have stimulatory and inhibitory effects on the rate of guanosine reduction, it was of interest to determine if other divalent cations alone or in combination with ATP had similar effects on GTP and GDP reduction. As illustrated in figure 11, Ca^{++} and Ca^{++} plus ATP did not effect the rate of GTP reduction at optimum GTP concentrations. Mn^{++} and Mn^{++} plus ATP, however, did cause some inhibition even at low concentrations. The greatest inhibitory effect was by Mn^{++} at 3.0 mM. The rate of GDP reduction at optimum substrate levels was inhibited approximately 30% by Mn^{++}, individually and combined with ATP (figure 12). Ca^{++} or Ca^{++} plus ATP again showed no appreciable effect on enzyme activity.

In contrast to the effect at optimum substrate concentrations, Ca^{++} and Ca^{++} plus ATP produced a significant stimulation in the rate

*FIGURE 8. The Effect Of Mg^{++}, ATP, And Mg^{++} Plus On Ribonucleotide Reductase Activity At Optimum GDP Concentrations. The effectors are: *——*, Mg^{++}; □——□, ATP; and ●——●, Mg^{++} plus ATP effectors, the ATP concentration is one-half the value represented on the abscissa.*

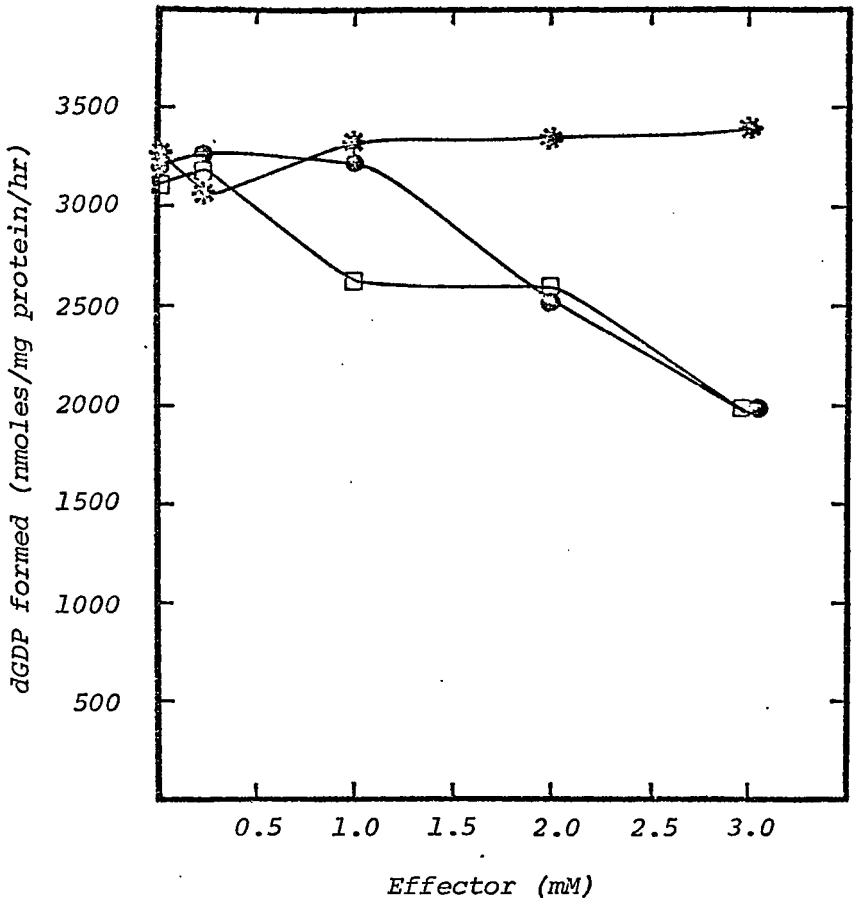


FIGURE 9. The Effect Of Mg^{++}, ATP And Mg^{++} Plus ATP On Ribonucleotide Reductase Activity At One-half Optimum GTP Concentrations. The effectors are: ✱——✱, Mg^{++}; □——□, ATP; and ●——●, Mg^{++} plus ATP effectors, the ATP concentration is one-half the value represented on the abscissa.

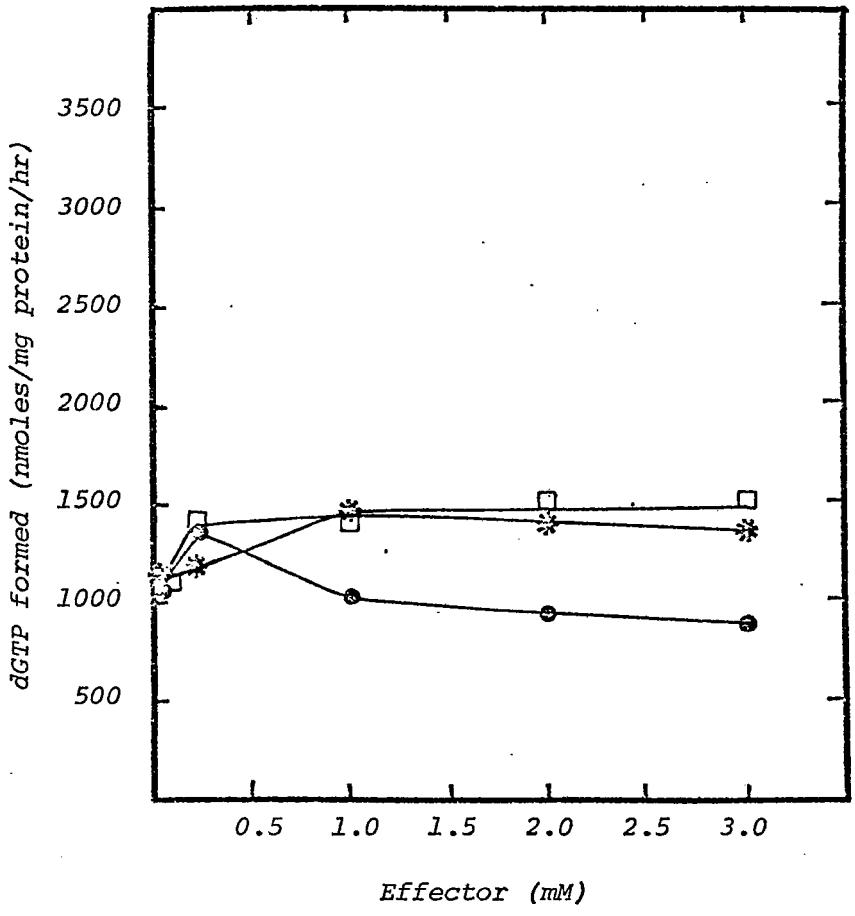


*FIGURE 10. The Effect Of Mg^{++}, ATP, And Mg^{++} Plus ATP On Ribonucleotide Reductase Activity At One-half Optimum GDP Concentrations. The effectors are: *——*, Mg^{++}; □——□, ATP; and ●——●, Mg^{++} plus ATP effectors, the ATP concentration is one-half the value represented on the abscissa.*

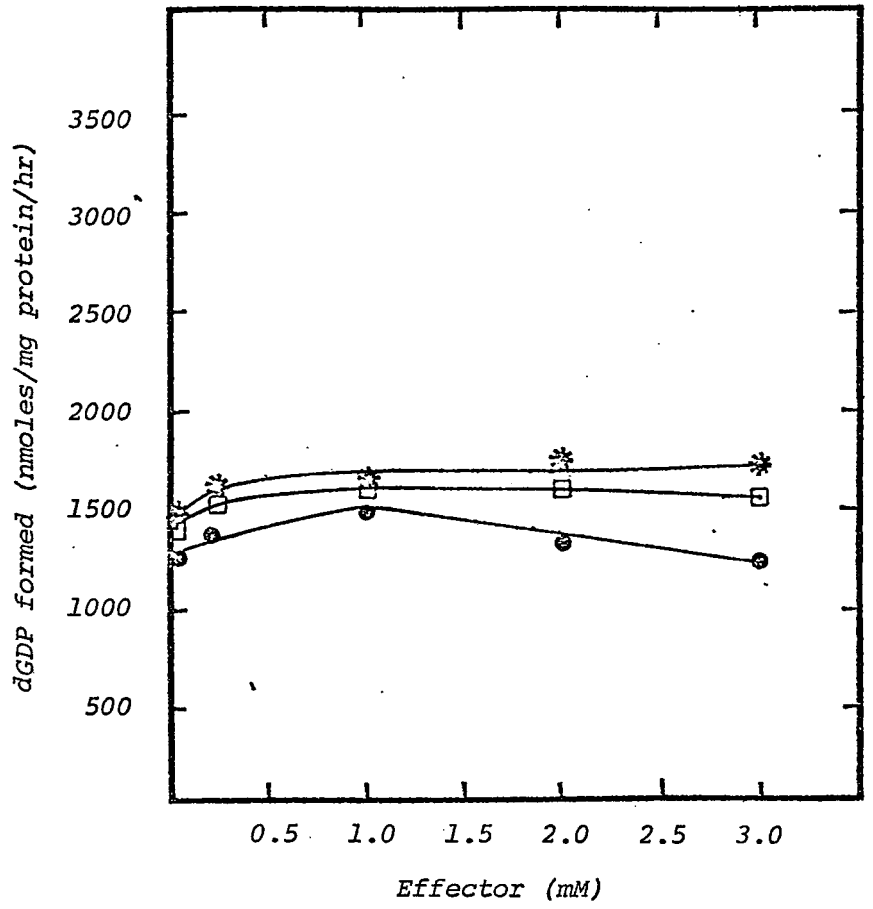


*FIGURE 11. The Effect Of Ca^{++}, Ca^{++} Plus, Mn^{+++} And Mn^{+++} Plus ATP On Ribonucleotide Reductase Activity At Optimum GTP Concentrations. The effectors are □——□, Ca^{++}; *——*, Ca^{++} plus ATP; o——o, Mn^{++}; and ●——●, Mn^{++} plus ATP. In the experiments containing the Ca^{++} plus ATP and Mn^{++} plus ATP effectors, the ATP concentration is one-half the value represented on the abscissa.*

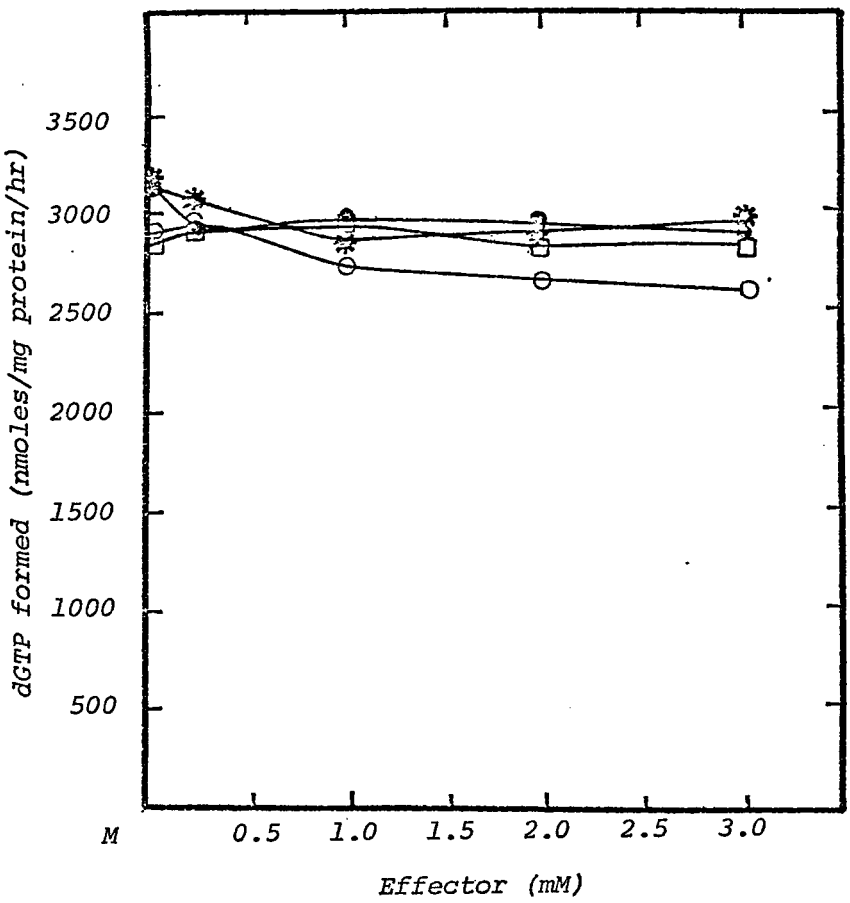
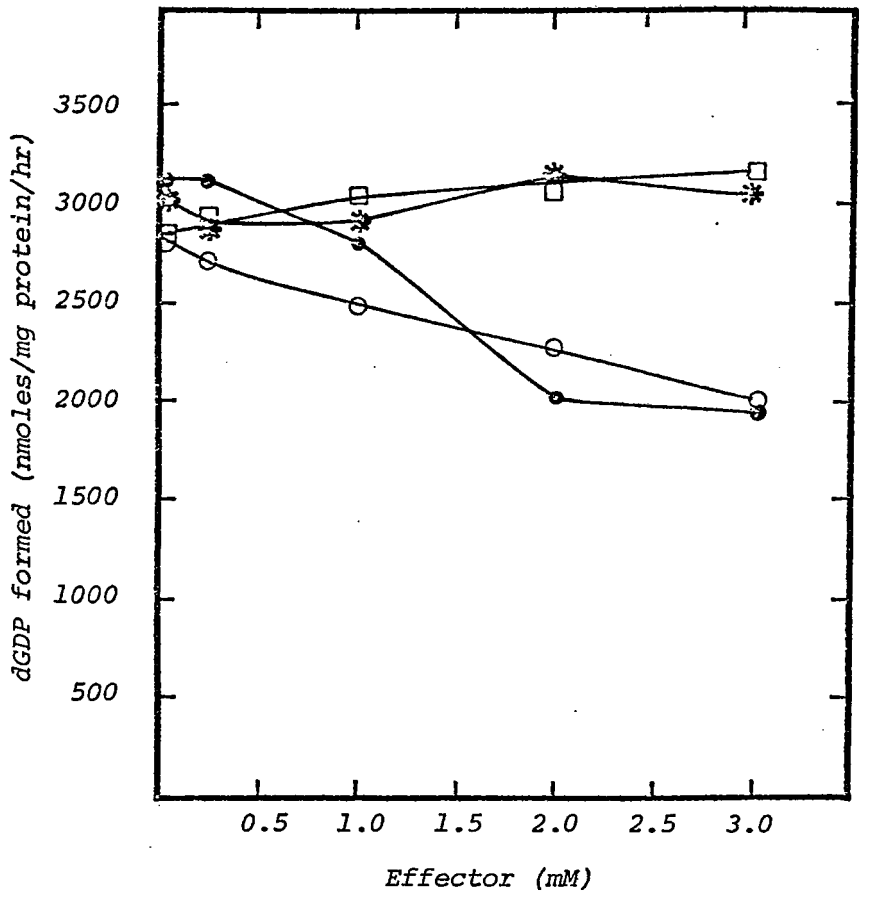


FIGURE 12. The Effect Of Ca^{++}, Ca^{++} Plus ATP, Mn^{++}, and Mn^{++} Plus ATP On Ribonucleotide Reductase Activity At Optimum GDP Concentrations. The effectors are □—□, Ca^{++}; ✱—✱, Ca^{++} plus ATP; ○—○, Mn^{++}; and ●—●, Mn^{++} plus ATP. In the experiments containing the Ca^{++} plus ATP and Mn^{++} plus ATP effectors, the ATP concentrations in one-half the value represented on the abscissa.



of dGTP formation at half optimum substrate levels (figure 13). At 3.0 mM Ca^{++}, there was an increase in enzyme activity of approximately 33%. Mn^{++} and Mn^{++} plus ATP slightly inhibited the rate of GTP reduction. Stimulation was again apparent with Ca^{++} and Ca^{++} plus ATP additions in the rate of GDP reduction at half optimum substrate concentrations. At 3.0 mM Ca^{++}, the amount of dGDP formed was still increasing (figure 14). Activity was again inhibited by Mn^{++} and Mn^{++} plus ATP as it was with GTP as substrate.

Purification with Polyethylene Glycol 6,000.

Initially, experiments with crude extracts from R. meliloti fractionated with ammonium sulfate (Schwarz - Mann enzymatic grade) resulted in a less reductase activity than had been obtained with unfractionated extracts. Thus, an alternate means of batch purification was developed. A 50% solution (w/v) of polyethylene glycol 6,000 (31) was used to experimentally batch fractionate the crude preparation. As shown in Table II, the 2 to 20% fraction resulted in a 2- to 3-fold enrichment of ribonucleotide reductase activity. Figure 15 shows the results of a successful purification with a DEAE-cellulose column of the polyethylene glycol fractionated reductase. Although the protein elution profile from the DEAE-cellulose column is different from that obtained with the ammonium sulfate fractioned extract, the enzyme did elute in a discrete peak. The peak reductase fraction contained a specific activity similar to that reported in figure 1. This technique was as effective as ammonium sulfate as a means of batch purification, but the polyethylene glycol was found to adhere tightly to the reductase

and attempts to separate the protein fraction from the polymer by either DEAE-cellulose chromatography or adding ammonium sulfate were not consistently successful.

FIGURE 13. The Effect Of Ca^{++}, Ca^{++} Plus ATP, Mn^{++}, And Mn^{++} Plus ATP On Ribonucleotide Reductase Activity At One-half Optimum GTP Concentrations. The effectors are □—□, Ca^{++}; ✱—✱, Ca^{++} plus ATP; ○—○, Mn^{++}; and ●—●, Mn^{++} plus ATP. In the experiments containing the Ca^{++} plus ATP and Mn^{++} plus ATP effectors, the ATP concentration is one-half the value represented on the abscissa.

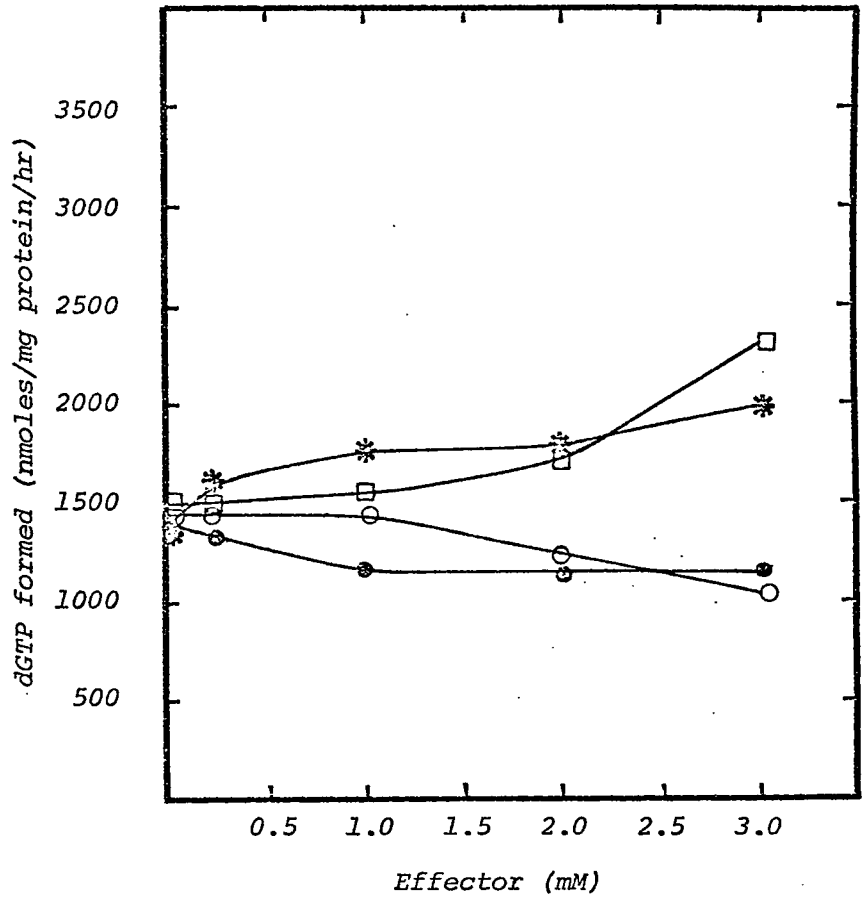


FIGURE 14. The Effect Of Ca^{++} Plus ATP, Mn^{++}, and Mn^{++} Plus ATP On Ribonucleotide Reductase Activity at One-half Optimum GDP Concentrations. The effectors are □——□, Ca^{++}; ✱——✱, Ca^{++} plus ATP; o——o, Mn^{++}; and ●——●, Mn^{++} plus ATP. In the experiments containing the Ca^{++} plus ATP and Mn^{++} plus ATP effectors, the ATP concentrations is one-half the value represented on the abscissa.

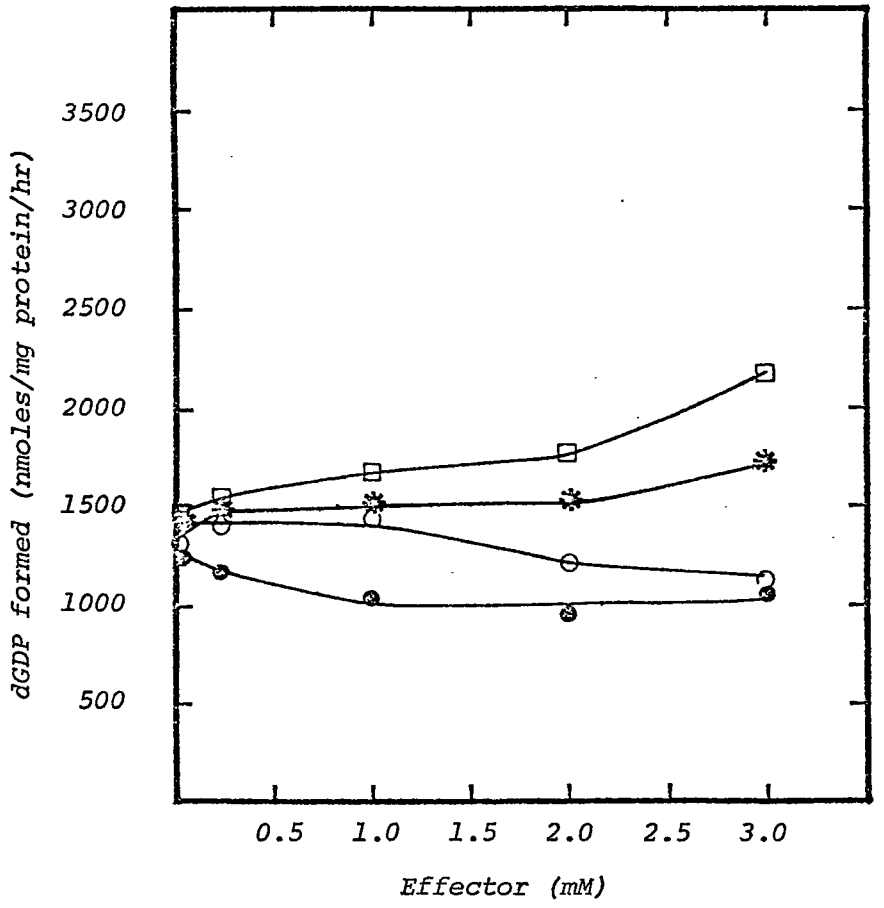
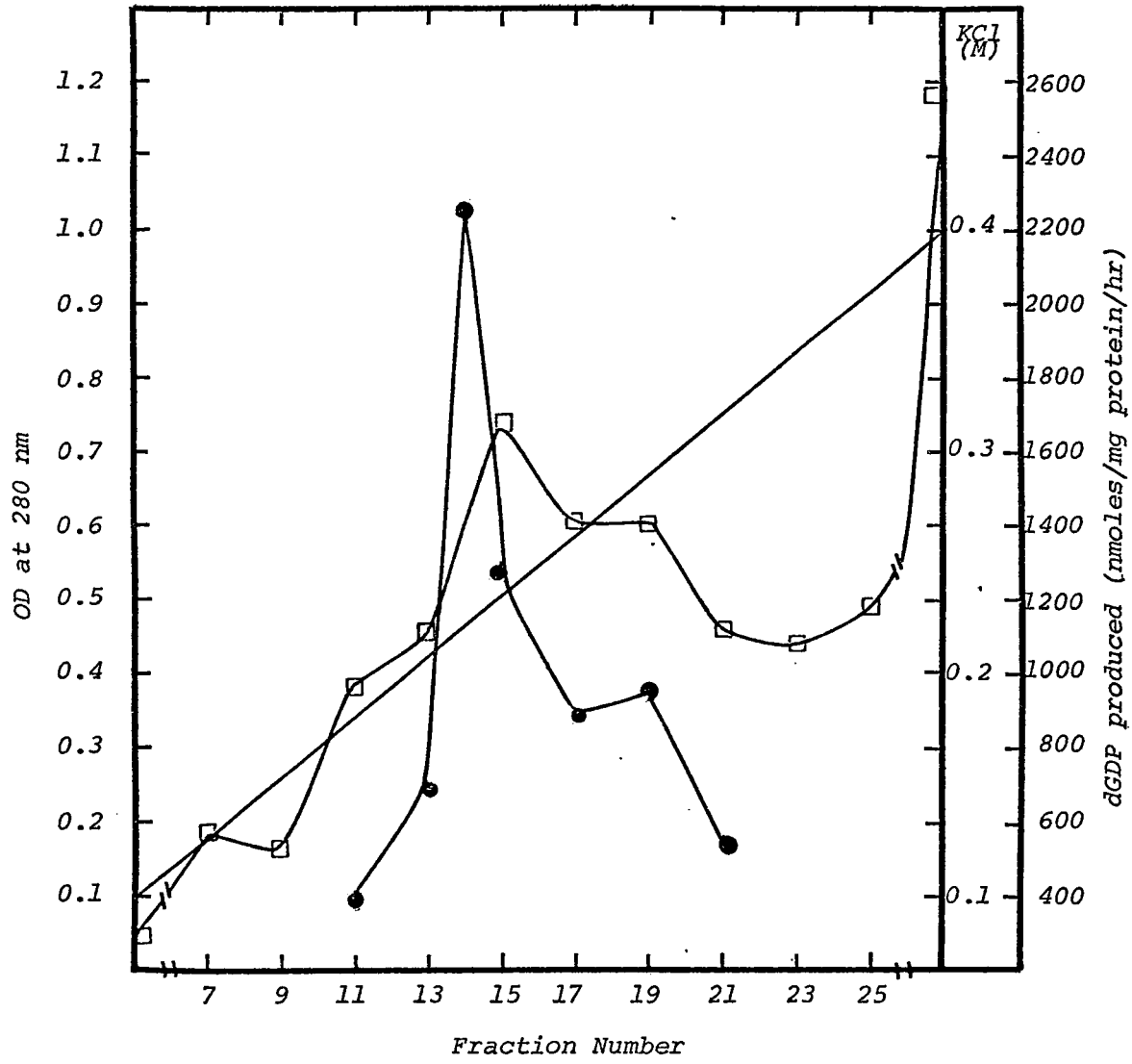


Table II. Purification Of Ribonucleotide Reductase From R. meliloti With Polyethylene Glycol 6,000. The reaction mixture was the complete reaction mixture listed in Table I.

Fraction	Specific Activity (unit[a]/mg protein)	Total Activity (unit X 10^{-3})	Recovery %
Crude	392	229	100
0-2%	302	20	9
2-20%	858	169	73
20-30%	229	38	16
Supernatant	210	20	9

[a] One unit of activity is defined as the amount of enzyme necessary to catalyze the synthesis of 1 nmole of dGDP/hr. in the standard assay.

FIGURE 15. Purification Of Ribonucleotide Reductase Fractionated With Polyethylene Glycol 6,000 On A DEAE-cellulose Column.



□——□, O.D. at 280 nm; ●——● reductase activity utilizing GDP as substrate. Approximately 160 mgs. of protein was placed on the column and eluted in 10 ml fractions. Aliquots of 0.10 ml were assayed for reductase activity.

DISCUSSION

The level of ribonucleotide reductase purification from R. meliloti is considered to be at a minimum for the type of study conducted. Even so, the specific activity obtained from the DEAE-cellulose column was 20 to 30% higher than was previously reported (27). Also, the level of purification must be considered a conservative value since no corrections were made for any loss of enzyme activity during the purification process.

In general, neither Mg^{++}, Mn^{++}, nor Ca^{++}, individually or in combination with ATP, substantially stimulated GTP reduction at optimum substrate concentrations. Reports by Beck (5) showed Mg^{++}, Mn^{++}, and Ca^{++} without ATP would inhibit ribonucleoside triphosphate reduction in the L. leichmannii system. Beck, in fact, extended the concentration of Mg^{++} to 30.0 mM and obtained complete inhibition of all reduction processes. This same effect with Mg^{++} was also shown in the Euglena gracilis triphosphate ribonucleoside reductase system (36).

The rate of GDP reduction at optimum substrate concentrations was not greatly stimulated by the addition of cations, individually or in combination with ATP. The inhibition of GDP reduction by certain of the potential effectors, however, was greater than with GTP reduction.

Mg^{++} and Ca^{++} were particularly effective in stimulating ribonucleotide reductase activity at one-half optimum substrate concentrations. Mn^{++}, however, was a very effective inhibitor.

It is apparent from these results that Mg^{++} and ATP are not required for GDP or GTP reduction, and that in certain instances inhibition occurs when these compounds are added even at relatively small concentrations. Previous reports have shown and were confirmed in this investigation that ATP was reduced at a very insignificant rate. The inhibition by ATP, individually or in combination with a cation, however, may be due to competition between ATP and the substrate for the reactive site on the enzyme.

Although at optimum substrate concentrations cations in low concentrations did not have a substantially regulatory effect, at non-optimum concentrations the effect was significant. Therefore, under in vivo conditions cations may possess a regulatory role in the rate of ribonucleotide reduction. Under these conditions, Mg^{++} and Ca^{++} would have stimulatory and Mn^{++} would have inhibitory roles.

SUMMARY

The ribonucleotide reductase from Rhizobium meliloti was examined as to the effects Mg^{++}, Ca^{++}, and Mn^{++}, individually and combined with ATP, would exert on activity. A summary of the results obtained by this investigation is as follows:

1. *The enzyme used in the experiments was purified 8- to 10-fold and desalted by means of a Sephadex G-25 column.*
2. *The rate of ribonucleotide reduction was linearly correlated with respect to protein concentration and incubation period.*
3. *The optimum cofactor (B_{12} coenzyme) and reductant (dihydrolipoate) concentrations were 30.0 uM and 20.0 mM, respectively.*
4. *The optimum and one-half optimum GTP concentrations were 1.6 mM and 0.4 mM, respectively.*
5. *The optimum and one-half optimum GDP concentrations were 0.1 mM and 0.06 mM, respectively.*
6. *At optimum GTP concentrations, reductase activity was stimulated slightly at low concentrations of Ca^{++} and Mg^{++}. Mn^{++} and ATP inhibited activity. The most detrimental effect on GTP reduction was found with Mg^{++} plus ATP additions.*
7. *At optimum GDP concentrations, reductase activity was stimulated by both Mg^{++} and Ca^{++} at 3.0 mM. Increasing inhibition of activity was produced by increasing Mn^{++} concentrations. The addition of ATP plus the cations did not change the effect exerted by the cation individually.*

8. *A significant increase in reduction was produced by 3.0 mM Ca^{++} at one-half optimum concentrations of both GTP and GDP. Ca^{++} plus ATP also increased enzyme activity, but to a lesser degree than Ca^{++} individually.*
9. *Mn^{++} and Mn^{++} plus ATP inhibited reduction of both GTP and GDP at one-half optimum substrate concentrations.*
10. *A crude extract of ribonucleotide reductase was effectively purified (2- to 3-fold) with polyethylene glycol 6,000.*

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