STUDIES ON THE ALLOSTERIC REGULATION OF RIBONUCLEOTIDE REDUCTASE OF *RHIZOBIUM MELILOTI*

by

William J. Farley

A THESIS

submitted to

University of Houston

in partial fulfillment of
the requirements for the
degree of

Masters of Science

December 1975

Acknowledgments

I wish to convey my deepest thanks to my advisor, Dr. Joe R. Cowles, for his guidance and assistance in helping me complete my research problems. Without his constant encouragement this thesis would not have been possible.

Additional thanks must also be expressed to the members of my committee, Dr. Donald Hendrix, Dr. Thomas Spring, and Dr. S. Venketeswaran for the advise and instruction they provided during my graduate studies.

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ABSTRACT

Ribonucleotide reductase was purified from Rhizobium meliloti, Strain F-28, by ammonium sulfate fractionation and chromatography on Sephadex G-200 and DEAE-cellulose. The enzyme was purified at least 25-fold by these procedures. The purified enzyme was capable of reducing the 5'-diphosphates of guanosine, cytidine, and adenosine (GDP, CDP, and ADP). Reduction of uridine diphosphates (UDP) could not be clearly established by the assay procedure. The allosteric regulation of the reduction of the three ribonucleoside diphosphate substrates by deoxyribonucleoside triphosphates was determined using the purified enzyme. The rate of GDP reduction was decreased by all four deoxyribonucleoside triphosphates at concentrations of 1×10^{-6} M or greater. Deoxy-GTP and dTTP were the most effective negative effectors reducing GDP reduction by 80 and 60% at concentrations of 5×10^{-4} M and 5×10^{-5} M, respectively. ADP reduction was stimulated 300% by dGTP at 1×10^{-6}M. Deoxy-ATP and dTTP however were negative effectors. The rate of CDP reduction was stimulated 300% by dATP at 5×10^{-6} M. Deoxy-TTP at low concentrations (5×10^{-8} M) stimulated CDP reduction 180%. Deoxy-GTP at 1×10^{-5} M was a negative effector, while dCTP had no influence on CDP reduction.

A suggested pattern for the in vivo ribonucleotide reduction based on the results of this study would be as

follows: the initial conversion of GDP to dGDP which requires no positive effectors. Deoxy-GTP then stimulates the reduction of ADP to dADP. Deoxy-ATP in turn stimulates the formation of dCDP from CDP. Although it has not been demonstrated at this time UDP reduction should be stimulated by dCTP. A universal negative inhibitor was not found.

Ribonucleotide diphosphate reductase was inhibited by Mg^{++} at concentrations of 1 to 32 mM. This was shown not to be the result of Mg^{++} precipitation of dihydrolipoate from the reaction mixtures as was expected.

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Introduction

The infection of alfalfa roots by Rhizobium meliloti is one of several examples of free-living rhizobia which can invade the roots of leguminous plants and establish a symbiotic association capable of fixing atmospheric N_2 (95, 38). One of the observed changes which occurs during nodulation and the establishment of symbiosis is the transformation of the small, rod-shaped bacteria into large, distored, extramembrane-bound organisms known as bacteriods. The bacteriods within the nodule then utilize plant photosynthetate as energy sources for the reduction of N_2 to NH_3. Dilworth and Williams (32) observed a substantial decrease in DNA and RNA content per bacteroid during the early stages of lupine nodule development. Sutton (97) reported a decrease in DNA content of lupine bacteroids as compared to tne free-living culture of a fast-growing species of Rhizobium lupini. It appears then that a decreased DNA content may be an essential feature in establishing and maintaining symbiosis. The factors which are regulating DNA synthesis in the bacteroids of developing nodules are yet to be determined and are a consideration in this thesis.

Ribonucleotide reductase has been established in several organisms (39, 56), including Rhizobium meliloti (30, 31), and has been clearly established as a regulatory enzyme in DNA synthesis and cell division (35, 41, 61).

For example, in sea urchin eggs the embryo will not develop past the 4-8 blastomere stage unless ribonucleotide reductase is induced. The enzyme activity is maximal 5 hr after fertilization and the increase in activity closely parallels the rise in DNA synthesis (77). In addition, Elford (35) has shown that ribonucleotide reductase activity is more closely correlated with the growth rate of rat tumors than is either thymidylate synthetase or thymidine kinase, two other enzymes associated with DNA synthesis.

In 1961 Reichard, using chicken embryo extract, demonstrated that the reduction of guanosine monophosphate was stimulated by pyrimidine deoxyribonucleotides, but was inhibited by purine deoxyribonucleotides (88). Later work with ribonucleotide reductase system of E. coli (65, 66) revealed that deoxyribonucleoside triphosphates and ATP were in fact allosteric regulators of ribonucleotide reductase activity according to the terminology of Monod, Wyman, and Changeux (71). The allosteric regulation of ribonucleotide reductase has been described in Lactobacillus leichmannii (4), T_4 phages (9), and numerous mammalian systems (33, 86).

In light of the correlation between levels of ribonucleotide reductase and DNA synthesis in other organisms and tissues, one explanation to account for the reduced DNA content in the bacteroids of developing nodules could be reduced pool sizes of deoxyribonucleotide substrates needed in DNA synthesis. These pools could be controlled in part through the allosteric regulation of ribonucleotide reductase.

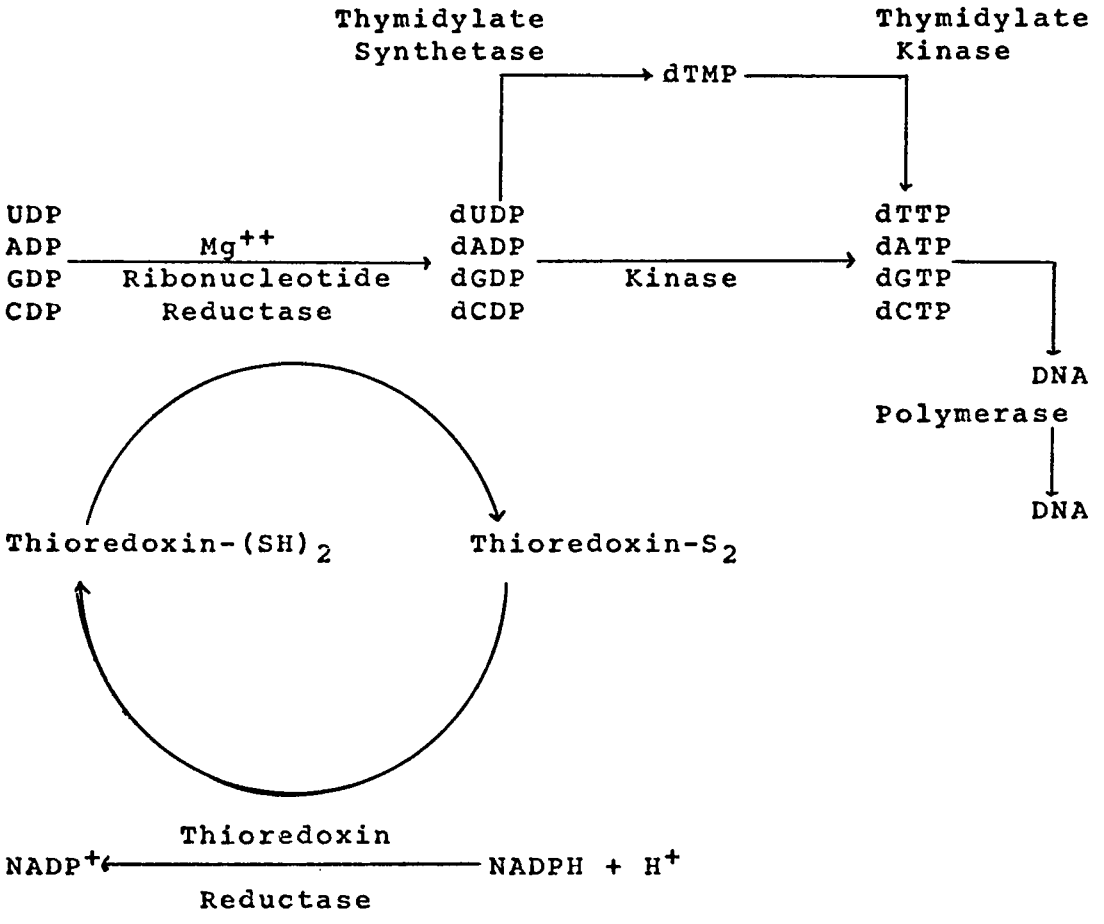
Other factors, such as Mg^{++} concentration, may also have a role in regulating reductase activity. This has been established in the B_{12} coenzyme-requiring L. leichmannii reductase system (4). Although Mg^{++} is not absolutely required for activity, it has been shown to control not only the level of reductase activity but also to influence the allosteric regulatory pattern of the enzyme.

The objective of this research is to obtain additional purification of ribonucleotide reductase of Rhizobium meliloti F-28 and to determine it's pattern of allosteric regulation.

REVIEW OF LITERATURE

Evidence for Ribonucleotide Reduction and it's Relationship to DNA Synthesis.

Replenishment of the deoxyribonucleotides required for DNA synthesis has been shown to occur almost exclusively through the direct reduction of ribonucleotide precursors. The only reported exception to this method involves the salvage pathways in auxotrophic organisms which re-utilize DNA fragments for DNA synthesis (20). The direct reduction of ribonucleotides with the retention of the N-glycoside bond between the base and ribose-5-phosphate was first proposed by Hammarsten in 1950 from studies on ^{15}N-labeled cytidine incorporation into DNA of rat liver (53). The actual conversion of cytidine to deoxycytidine and it's subsequent incorporation into the DNA of bacteria and mammalian tissue was demonstrated by Ross and Schweigert in 1953 (90). The enzyme which catalyzes the reduction of ribonucleotides to deoxyribonucleotides, ribonucleotide reductase, was first demonstrated in cell-free extracts of chicken embryo (84). The enzyme was later established in *E. coli*. (89, 87). The over-all relationship of ribonucleotide reductase to DNA synthesis is illustrated in the following scheme (85, 64):



According to the above scheme the ribonucleoside diphosphates are first converted to deoxyribonucleoside diphosphates. The reduction of all of the ribonucleotides can be catalyzed by a single ribonucleotide reductase. With the exception of dUDP the ribonucleotides are converted directly to deoxyribonucleoside triphosphates. Deoxy-UDP is believed to be de-phosphorylated to dUMP and then methylated to give dTMP. The methylation reaction is catalyzed by thymidylate synthetase. Deoxy-TMP is phosphorylated to dTTP by thymidylate kinase and used along with the other deoxyribonucleotides in DNA synthesis which is catalyzed by DNA

polymerase.

Ribonucleotide Reductase

The ribonucleotide reductase systems that have been studied so far can be separated into two classes depending on whether the reductase system requires B_{12} coenzyme for activity. The systems which do not require B_{12} coenzyme are called Class I reductases. The reductases of this class normally reduce only ribonucleoside diphosphates and require Mg^{++} and a reductant for activity. In some cases enzymatic activity can be stimulated by Fe^{++} or Fe^{+++}. The distinquishing characteristic of Class II reductases is an absolute requirement for B_{12} coenzyme. Ribonucleoside triphosphates are the preferred substrates for this class of enzyme although the diphosphates can sometimes be utilized. The enzyme systems require a reductant for activity but do not have an absolute requirement for Mg^{++}. The natural reductant for both classes for enzyme is NADPH. The hydrogen transfer system is a thioredoxin/thioredoxin reductase. This system has been isolated from *E. coli*. (67, 74), *L. leichmannii* (80, 102, 5), and various other organisms and tissues (56). Dihydrolipoate and dithiothreitol are effective *in vitro* substitutes for the natural physiological reductant system (17).

Class I Reductases

As previously mentioned the most distinquishing feature of the Class I reductases is the lack of dependence on B_{12} coenzyme. The preferred substrates for Class I enzymes

are almost always ribonucleoside diphosphates. In fact, the enzymes are often referred to as ribonucleoside diphosphate reductases (EC 1.17.4.1). Mg^{++} is often required for subunit binding and thus for enzyme integrity. The component involved in the final steps of ribonucleotide reduction (the B_{12} coenzyme step in Class II reductases) is an organic radical maintained by a non-heme iron (3). The majority of the Class I reductases are found in eucaryotic tissue (39) and include specifically wheat (75), Novikoff tumors (73), mouse spleen (41), yeast (69), sea urchin (77), rabbit bone marrow (58), chinese hamster ovary (16), and re-generating rat liver (62). The only known Class I enzymes isolated from procaryotes are those from E. coli., T_4 phage, and T_5 and T_6 phages (37).

Escherichia coli- The most extensively studied Class I ribonucleotide reductase is that of E. coli. The enzyme has been purified to homogenetiy by Thelander (99) and shown to consist of two essential subunits, B_1 and B_2, held together by Mg^{++}. The B_1 subunit has a molecular weight of 160,000 daltons and contains two polypeptide chains of identical molecular weight but with slightly different amino acid composition. The B_2 subunit has a molecular weight of 78,000 daltons and consists of two identical polypeptide chains. The B_1 subunit contains the allosteric binding sites. These sites are divided into two classes with two sites each. The first class (h-sites) has a high affinity for dATP and also binds dGTP, dTTP, and ATP. These sites control the substrate specificity of the enzyme. The second class (l-sites)

has a lower affinity for dATP and also binds ATP. This class controls the over-all level of enzymatic activity (24). The B_2 subunit possesses one non-heme iron atom per chain. This non-heme iron is needed for the integrity of the organic radical that is essential for enzymatic activity. The enzyme is therefore inhibited by hydroxyurea which destroys the organic radical or by chelating agents, such as 8-hydroxyguinoline, that remove the iron atoms and thus result in the loss of the radical (22).

The active site on the enzyme is located between the B_1 and B_2 subunits. The reaction is belived to occur via a ping-pong mechanism involving a reversible flow of electrons from thioredoxin to three disulfide groups on the B_1 subunit (100). The organic radical, in conjunction with the sulfhydryl groups on the B_1 subunit, is believed to be responsible for the actual reduction of the substrate; apparently there is no oxidation or reduction of the iron moiety in ribonucleotide reductase (3).

The reduction rate of each substrate has been shown to be controlled by allosteric effectors. These effectors are deoxyribonucleoside triphosphates and to a lesser extent deoxyribonucleoside diphosphates. The type of effect each effector has on a given substrate is shown in the following table (39):

Effector	Substrate			
	UDP	CDP	GDP	ADP
dTTP	+	+	+++	++
dCTP				
dGTP			+	+++
dATP	+,-	+,-	--	--
ATP	++	++		

The degree of positive stimulation is prepresented by plus (+) signs and the degree of negative stimulation by minus (-) signs. The effect of dATP on UDP and CDP reduction depends on the effector concentration.

In E. coli. ribonucleotide reduction starts with the reduction of pyrimidine diphosphates, UDP and CDP. The reduction of these substrates is stimulated by ATP (65). The dTTP formed from dUDP stimulates additional UDP and CDP reduction and is the prime stimulator of GDP reduction and to a lesser extent ADP reduction. Deoxy-GTP increases the conversion rate of ADP to dADP. Deoxy-ATP stimulates pyrimidine reduction at concentrations of 1×10^{-6} M or less but is a general negative effector for all substrates at higher concentrations (66). The amount of stimulation of in vitro ribonucleotide reduction by the prime effectors can be illustrated as follows: The reduction rates for CDP, UDP, and GDP were 600, 35, and 736 nmoles/mg protein·hr^{-1}, respectively, in the absence of effectors and 3710, 245, and 2730 nmoles/mg protein·hr^{-1}, respectively, in the presence of prime effector (65, 66).

The complexity of the pattern of regulation in E. coli can be illustrated by dATP which stimulates UDP and CDP reduction at low concentrations and inhibits all reduction at high concentrations. Brown et al (23) found that the inhibition by dATP was due to binding of dATP to l-sites which results in dimerization of the enzyme. ATP was shown to reverse the inhibition of dATP by displacing it from the l-sites. Deoxy-TTP, which does not bind to l-sites, will enhance pyrimidine reduction at low dATP concentrations, but will result in greater inhibition at high dATP concentrations.

There are two other points to mention in regards to in vitro regulation of ribonucleotide reductase in E. coli. The first is that allosteric effectors are believed to function by lowering the Km and increasing the Vmax of the reactions. For example, the Km of GDP decreases from 8 X 10^{-4} M in the absence of a positive effector to 2 X 10^{-5} M in it's presence. The second point is that Brown (24) has demonstrated that substrate vs. velocity curves are hyperbolic both in the absence and presence of effectors which suggests that, at least in vitro, positive homotrophic cooperativity does not occur.

Blakley (17) and Brown (21) have shown that the in vitro levels of ribonucleotide reductase are far below the rates needed for in vivo DNA synthesis and may reflect the need for a multi-enzyme complex for maximal activity. Biswas (13, 14) demonstrated that de novo synthesis of ribonu-

cleotide reductase in E. coli. is repressed by thymine derivatives and that a 10-fold increase in enzyme levels can be achieved by growing E. $coli_{15\ T^-}$ in thymineless medium.

T_4 Bacteriophages- Upon infection of E. coli with T_4 phage a new ribonucleotide reductase enzyme and a new thioredoxin molecule are synthesized within the bacteria (11). The synthesis of the new ribonucleotide reductase and thioredoxin has been shown to be under the control of the phage genome (98, 107). The reductase enzyme has a molecular weight of 225,000 daltons and contains equimolar amounts of two polypeptide chains, α and β, with molecular weights of 85,000 and 35,000, respectively. The native enzyme is believed to have a subunit structure of α_2-β_2 in which α_2 and β_2 correspond, respectively, to the B_1 and B_2 subunits of E. coli. In constrast to E. coli, the phage enzyme does not require Mg^{++} for enzyme integrity but acivity is stimulated 2- to 3-fold by 7mM Mg^{++}. Each polypeptide chain of the β_2 subunit contains a non-heme iron molecule and the enzyme is inhibited by hydroxyurea and 8-hydroxyguinoline. The reduction of the diphosphate substrates is dependent upon NADPH, T_4 thioredoxin, and E. coli thioredoxin reductase (9).

Competitive studies have shown that the enzyme possesses one allosteric site and one catalytic site. Regulation is similar to that of E. coli except that (10): 1) dATP (5×10^{-6} M) is the prime stimulator of pyrimidine reduction, 2) there is no general inhibitor in the bacteriophage system,

and 3) the only detectable negative inhibition is dATP inhibition of GDP reduction. The absence of a general inhibitor is presumed to result from a lack of the 1-type sites on the T_4 stimulated enzyme as compared to that found in uninfected E. coli. At optimum effector concentrations (5×10^{-6} to 5×10^{-5} M) the maximum rate of reduction for all substrates is between 68,000 to 82,000 nmoles/mg protein·hr^{-1}. These in vitro rates result in the synthesis of 50 molecules of T_4 DNA per cell per 20 min which is similar to the amount of DNA synthesized in the host at the time of a normal burst.

Eucaryotes- Except for a few examples such as wheat (75), broad bean (78), and slime mold (12) almost all ribonucleotide reductases from eucaryotic organisms have been isolated from mammalian tissue. The highest levels of enzyme activity have been demonstrated in tissues such as rabbit bone marrow (58), mouse spleen (41), and tumors (35) which contain a more rapid rate of in vivo DNA synthesis or in tissues such as regenerating rat liver (62) which have been artifically induced to proliferate. Examples of CDP reduction rates (nmoles/mg protein·hr^{-1}) in the presence of ATP include : 2.0 for mouse spleen (41), 17.0 for regenerating liver (62) and rabbit bone marrow (58), 4.0 for brain tumors (70), and 40.0 for Novikoff tumors (73). CDP reduction in normal rat liver on the other hand is only 0.70 nmoles/mg protein·hr^{-1}.

The ribonucleotide reduction pattern for the eucaryotic

system is similar to that of E. coli (65, 66). The enzymes from the various tissues vary in terms of Fe^{++} and Mg^{++} requirements and in the case of rabbit bone marrow evidence has been presented that the enzyme consists of two essential subunits (58). As in E. coli the diphosphates are the preferred substrates.

The enzyme activity is regulated by allosteric effectors in the range of 1×10^{-5} to 1×10^{-4} M and in some cases naturally occurring inhibitors may be present (28). Variations in the allosteric patterns suggest that the reductase from mammal tissues differ somewhat from that of E. coli. These variations include: 1) the failure of ATP to reverse dATP inhibition in regenerating rat liver (63), 2) maximal stimulation of ADP reduction by GTP and not by dGTP in mouse spleen (41), and 3) the failure of positive effectors to alter the Vmax of ADP reduction in Novikoff tumors (73). In addition, the role of dCTP, which is believed by some investigators (16) to be a dominant factor in the relationship between ribonucleotide reductase, deoxyribonucleotide pools, and DNA synthesis varies in different tissues. For example, in Novikoff tumors (73) as in E. coli. (65) dCTP did not affect the reduction of any substrate. On the other hand, in brain tissue (70) and monkey tumors (45), dCTP inhibits the reduction of CDP while in mouse spleen (41) it is the prime stimulator of CDP reduction.

Cory (27) has observed that during the purification of reductase from Ehrlich tumor cells there was a change in the

ratio of CDP to ADP reduction rates and has shown that the reduction of each is specifically inhibited by periodate-oxidized AMP and dimethylsulfoxide, respectively. Since he was able to demonstrate that the reduction of each substrate was controlled by a common mechanism he suggested the presence of either different catalytic sites on the same enzyme or a common allosteric subunit which regulates two or more separate catalytic subunits.

Ribonucleotide reductase activity of regenerating rat liver (62) and Novikoff tumors (34) has been shown to be associated with a membrane fragment. This fragment also contained DNA polymerase, thymidine kinase, and deoxycytidine deaminase activity. The nature and origin of the membrane fragment, however, has not been determined.

Class II Reductases

The Class II reductases are characterized by an absolute requirement for B_{12} coenzyme (59, 6, 7, 19). This class of enzymes is not inhibited by hydroxyurea or 8-hydroxyguinoline. The enzyme systems do not have an absolute requirement for Mg^{++} although in some cases Mg^{++} can stimulate activity. Some of the reductases in this class reduce ribonucleoside diphosphates while others prefer, or require, ribonucleoside triphosphates. Examples of diphosphate reductase systems include: Rhizobium meliloti (30), Bacillus megaterium (106), and Corynebacterium nephridi (56). The remainder of the B_{12} coenzyme-requiring systems reduce triphosphates (EC 1.17.4.2.) and include L. leichmannii and

Thermus X-1 (91). The only eucaryotic organisms reported to have a B_{12} coenzyme-requiring reductase are the algae Euglena gracilis and Astasia longa (56) and the fungus Pithomyces charatum (96).

Lactobacillus leichmannii- L. leichmannii is the most studied Class II ribonucleotide reductase system. The enzyme has been purified to homogeneity by Panagou (82) and consists of a single polypeptide chain with a molecular weight of approximately 76,000 daltons. The single polypeptide chain has no tendency to form a multi-subunit complex either in the presence or absence of effectors or substrates (25). The enzyme is believed to possess one catalytic site per molecule which is capable of reducing all four ribonucleoside triphosphate substrates (4).

The allosteric regulation of the L. leichmannii system was first demonstrated with studies on CTP reduction (47). Maximal stimulation of CTP reduction occurred in the presence of 8×10^{-3} M ATP and 16 mM Mg^{++}. Under these conditions ATP was shown to decrease the Km for CTP from 9.5×10^{-3} to 1.7×10^{-3} M (48). Deoxy-ATP was shown to effectively substitute for ATP and produce even higher rates of reduction. Thus dATP was suggested to be the actual prime effector for CTP reduction.

Beck in 1966 (4) showed that the physical state of the enzyme is controlled by a complex interaction between substrates, allosteric effectors, and divalent cations such as Mg^{++}. When each substrate was assayed in the presence and absence

of it's prime effector the following data were obtained:

Substrate	Effectors				
	none	dGTP	dATP	dCTP	dTTP
	(μmoles/mg protein·hr^{-1})				
GTP	13.6				32.8
ATP	11.6	32.8			
CTP	9.8		32.7		
UTP	1.2			10.8	

The reaction assays contained 16 mM Mg^{++} and, when present, effectors at a concentration of 4 X 10^{-4} M.

Assuming that *in vitro* studies correlate with *in vivo* activity and that the bacteria function with an internal Mg^{++} concentration of 6 to 8 mM ribonucleotide reduction would proceed as follows. In the absence of effectors GTP would be the most actively reducted substrate. At dGTP concentrations of 1 to 5 X 10^{-4} M or greater ATP reduction would be maximally stimulated. In a similar manner dATP would stimulate the production of dCTP which in turn would stimulate dUTP production. Deoxy-TTP which is formed from dUTP would inhibit GTP reduction at concentrations greater than 1 X 10^{-3} M.

There are two conflicting views as to how many and what type of effector sites are present on the enzyme. Chen *et al* (25) have concluded that there is only one allosteric site for which all effectors and even the substrate must complete. On the other hand, Follman and Hogenkamp (56, 40)

have presented evidence for the existence of one catalytic site and two different types of allosteric sites. One type would regulate substrate binding while the other would regulate the affinity for B_{12} coenzyme. Regardless of which of these views is correct the enzyme has no quaternary structure (25, 82) and allosteric control can not be discussed in terms of interaction between subunits or protomers, but only in terms of site-site interaction on the same polypeptide chain.

The *in vivo* enzymatic activity is controlled by internal concentrations of thymine derivatives which repress synthesis of the apoprotein (49). De-repression is achieved by either depriving the bacteria of B_{12} coenzyme, which indirectly increases the concentration of thymidine, or by first removing all folate from the bacteria and then growing them in thymineless medium (8, 42). Since *in vitro* reduction of CTP is 8-9 times higher than that required for *in vivo* DNA synthesis Blakley (17) has suggested that the growth conditions normally employed result in de-repression of the apoprotein.

Euglena gracilis- Ribonucleotide reductase was first isolated from *Euglena gracilis* in a partially purified state by Gleason and Hogenkamp (43) and was later purified to homogeneity by Hamiliton (52). The enzyme has a molecular weight of approximately 400,000 daltons and consists of 4 subunits of 100,000 daltons each. The enzyme reduces ribonucleoside triphosphates. Mg^{++} at concentrations below 2 mM is

slightly stimulatory while at higher concentrations it is inhibitory. In the absence of effectors, GTP and ATP are the most actively reduced substrates with specific activities of 3340 and 3180 nmoles/mg protein·hr^{-1}, respectively. CTP and UTP under the same conditions have specific activities of 1810 and 1020 nmoles/mg protein·hr^{-1}, respectively. Deoxy-TTP was shown to be a strong negative inhibitor of all substrates except UTP and is thus the dominant effector in Euglena gracilis. Using the purified enzyme Hamilton (52) was unable to show any stimulation of purine reduction, which was in contrast to the results with the partially purified extract (43). Hamilton suggested that this was due to a loss of a subunit during purification. CTP reduction on the other hand was stimulated 1.5-fold by ATP (1×10^{-4} M) or by dATP (2×10^{-6} M). The Km for CTP increased from 0.09 mM in the presence of dATP to 0.15 mM in the presence of dTTP. This is in agreement with the 4-fold decrease in enzyme activity caused by dTTP.

Thermus X-1- Ribonucleotide reductase has been isolated from the extreme thermophile Thermus X-1 in which growth and reductase activity are optimal at 70 C (91). The enzyme has a molecular weight of 80,000 daltons and reduces ribonucleoside triphosphates. Activity is only slightly stimulated by Mg^{++}. The specific activities of GTP, CTP, UTP, and ATP are 11.2, 7.8, 1.7, and 1.3 µmoles/mg protein·hr^{-1}, respectively. The only strong allosteric effector of the enzyme is dGTP which stimulates ATP reduction 4-fold. This stimu-

lation was found to be the result of a temperature-dependent conformational change with an optimum at 70 C. A general inhibitor was not domonstrated for the Thermus X-1 reductase. Deoxy-GTP, however, has been shown to inhibit the reduction of CTP and UTP. This means that dGTP, which is the product of the most actively reducted substrate, stimulates the reduction of the poorest substrate, ATP, and inhibits the reduction of CTP and UTP.

Role of B_{12} coenzyme in the Allosteric Regulation of Ribonucleotide reductase.

The actual role of B_{12} coenzyme in ribonucleotide reductase enzymes has been reviewed by Hogenkamp and Sando (56) and involves a reversible homolytic cleavage of the carbon-cobalt bond producing a pair of organic radicals. One of these radicals reacts with a dithiol to yield an enzyme bound reducing agent capable of the direct reduction of the substrate (1, 55). This radical, which functions in electron transport, is equivalent to the organic radical in Class I reductases which is maintained by the presence of tightly bound non-heme iron. Orme-Johnson (79) has identified the active radical as 5'-deoxyadenosyl and evidence has been presented that the reduction occurs without inversion at the 2' carbon. This excludes the classical bimolecular nucleophilic substitution of the hydroxyl group by a hydride ion (39).

Vitols et al (103), using the L. leichmannii reductase, have demonstrated that the Km for B_{12} coenzyme is dependent

on both the concentration and type of substrate used. It was found that the Km for B_{12} coenzyme was lower in the presence of high substrate concentrations that at low substrate concentrations. At low substrate concentrations only the catalytic site is occuppied and the reaction proceeds slowly with a high Km for B_{12} coenzyme. At high substrate concentrations the allosteric site becomes occuppied and induces a conformational change which lowers the Km and increases the reaction rates.

It was also demonatrated that, besides lowering the Km for the substrate and increasing the Vmax, the stimulatory effects of deoxyribonucleotides may be due in part to the lowering of the Km for the coenzyme. For example, when ATP was used as substrate at 2 mM concentration the Km for B_{12} coenzyme changed from 8.3×10^{-6} M in the absence of positive effectors to 5.7×10^{-7} M in the presence of 1 mM dGTP. Thus, due to the ability of positive effectors to lower the Km of B_{12} coenzyme, a greater degree of stimulation in reaction velocity by deoxyribonucleotides will be observed at low, rather than high, coenzyme concentrations.

Relationship of In Vivo Levels of Ribonucleotide Reductase to DNA Synthesis.

The significance of ribonucleotide reductase in DNA synthesis can best be determined by monitoring enzyme activity during the life cycle of various organisms. The growth rate of a series of rat hepatomas was shown by Elford (35) to be directly related to *in vivo* levels of ribonu-

cleotide reductase. The activity of two other enzymes involved in DNA synthesis, thymidylate synthetase and thymidine kinase, did not parallel the growtn rate of the tumors. Similar results were obtained by Cory (29) using Ehrlich ascites tumor cells. In these experiments, ribonucleotide reductase was the only enzyme which correlated with the rate of thymidine incorporation into DNA. Noronha (77) has demonstrated in sea urchin eggs that, of the major enzymes involved in DNA synthesis, ribonucleotide reductase was the only enzyme whose synthesis was induced de novo upon fertilization. The increased synthesis of the enzyme closely paralleled the rise in DNA synthesis and was essential for developement of the fertilized egg past the 8-cell blastomere stage.

Turner (101) provided evidence in mouse fibroblasts (L-cells) that not only does the rise in ribonucleotide reductase activity coincide with increased DNA synthesis but also that enzyme activity declines as DNA synthesis decreases. In yeast cells (69), however, reductase activity delcines prior to the peak of DNA synthesis. Reductase activity in regenerating rat liver (92) was observed to peak after the decline in DNA synthesis. In both of these cases, however, the rise in ribonucleotide reductase activity preceded and was essential for increased DNA synthesis.

Allosteric Regulation of In Vivo Ribonucleotide Reductase

Allosteric regulation of in vivo DNA synthesis has been

studied in an effort to correlate the *in vitro* allosteric studies with the *in vivo* fluctuations in deoxyribonucleotide pools. Bjursell and Reichard (16) found that the addition of 1 mM thymidine to synchronized cultures of chinese hamster ovary cells resulted in a drastic decrease in the level of dCTP and a concomitant reduction in DNA synthesis. The inhibition of DNA synthesis could be reversed by the addition of dCTP or by the removal of thymidine. These results were discussed in terms of the *in vitro* allosteric regulation of ribonucleotide reductase in *E. coli*. Thymidine caused an increase in the dTTP pool sizes, which, in the presence of endogenous ATP, inhibited CDP reduction. The levels of dCTP under these conditions then were too low to maintain a normal rate of DNA synthesis. Another example of in vivo regulation involved the hydroxyurea inhibition of reductase activity in moust embryo cells (93). Hydroxyurea resulted in a rapid decline in levels of dGTP and dATP, while the levels of dCTP and particularly dTTP increased. Upon removal of hydroxyurea the large dTTP pool stimulated the synthesis of dGDP which in turn resulted in increased levels of dAGP. This also is in agreement with the *E. coli in vitro* model of allosteric regulation.

In many tissues such as hamster kidney cells (15), regenerating rat liver (92), and synchronized hamster cells (94) the level of dCTP has been reported to be the highest of the deoxynucleotide pools while that of dGTP is the lowest. In regenerating rat liver (92) the different deoxy-

ribonucleotide pools all reached a maximum concentration level at the same time as maximal DNA synthesis occurred and declined as the rate of DNA synthesis decreased. These results might suggest a tight coupling between deoxyribonucleotide pools and DNA synthesis but other observations suggest otherwise. Skoog (94) showed that, in synchronized hamster cells, only the level of dCTP peaked at the same time as DNA synthesis. In addition, synchronized L_{929} cells were shown by Adams (2) to contain larger pool sizes at the end of S-phase than during DNA synthesis. The situation is futher complicated by results with sea urchin eggs (50) and synchronized hamster cells (94) which showed that there were measurable deoxyribonucleotide pools in cells not involved in DNA synthesis. The studies with sea urchin eggs also showed that the drop in the level of dCTP occurred at a faster rate than it's utilization in DNA synthesis. Based on these results Nordenskjold (76) has suggested that the pool sized of deoxyribonucleotides do not regulate DNA synthesis *per se* even though they are essential for synthesis.

The present assay methods however only measure total pool sizes. Gourlie (50) has suggested that DNA synthesis in sea urchin eggs may be regulated by compartmentalization of a selected group of DNA precursors and Werner (105) has presented evidence that even in *E. coli*. there may be a system of using specific precursors for DNA synthesis as compared to those used in DNA repair. The use of exogenous pools over endogenous pools might explain the results of

Colby (26) who failed to show any change in the deoxyribonucleotide pools of growing, inhibited, or transformed chicken fibroblasts.

MATERIALS AND METHODS

Culture of the Organism

Rhizobium meliloti, strain F 28, was supplied by Dr. Harold Evans of Oregon State University. Cells were maintained in 250 ml flasks on 50 ml of liquid mannitol media and were grown at 30° in the dark on a gyrotary shaker. The stock media, stored in a dark-brown bottle at 4°, contained: 100 g K_2HPO_4; 13 g $CaSO_4$; 36 g $MgSO_4.7H_2O$; 100 g autolyzed yeast extract; and 300 g mannitol. One liter of the basic media contained: 6.5 g stock media, 0.10 ml $FeCl_3$ (4%, w/v) and 0.04 ml $CoCl_2$ (0.06%, w/v). For experimental purposes the cells were grown in two 12-liter carboys each containing 10-liters of media. Each carboy was inoculated with 50 ml of a one-day old maintenance culture and grown in the dark at 30°. Airation was supplied through sterile cotton filters. The cells were harvested in a Sharples centrifuge when the cultures reached an absorbance (660 nm) of 0.7 to 0.8, usually 12 to 16 hr. after inoculation.

Preparation of Cell-free Extract

The harvested cells were washed three times (10 min. at 12,000 X g) in 50 mM potassium phosphate buffer, pH 7.3. The cell paste, which usually weighted 20 to 25 g, was resuspended in an equal volume ot 50 mM potassium phosphate

buffer, pH 7.3, containing 1.0 mM β-mercaptoethanol. The resuspended cells were broken in a French pressure cell at 10 tons of pressure on a 2" diameter ram. The cell brei was adjusted to approximately 110 ml with the extraction buffer and centrifuged 15 min. at 27,000 X g. The supernatant, ofter adjustment to a final protein concentration of 20 mg/ml with the extraction buffer, was used as the crude extract.

Fractionation with Ammonium Sulfate

The crude extract was fractionated with ice-cold saturated ammonium sulfate, pH 9.0 (Schwarz/Mann, enzyme grade). In the first step, sufficient ammonium sulfate solution was added dropwise to the stirring protein solution to give a final concentration of 25% of saturation. The mixture was stirred an additional 10 min. and then centrifuged 10 min. at 14,000 X g. After the resulting supernatant was brought to 45% ammonium sulfate it was stirred and re-centrifuged. The 45% pellet was resuspended in 20 to 25 ml of 50 mM potassium phosphate buffer, pH 7.3, containing 1.0 mM β-mercaptoethanol.

Chromatography on Sephadex G-200

Sephadex G-200 (10 g in 800 to 900 ml water) was activated by heating 5 hr at 90° to 100°. After activation the mixture was allowed to settle 20 min. and the liquid siphoned off to remove the fines. The particles were resuspended in

800 to 900 ml of 50 mM potassium phosphate, pH 7.3, containing 50 mM potassium chloride and 1.0 mM β-mercaptoethanol (phosphate buffer I) and the fines were removed three additional times. The Sephadex was poured into a 1000 ml funnel attached to the top of a 2.5 X 100 cm column filled with phsphate buffer I and allowed to settle to a height of 85 cm. The column was washed with 3 liters of phosphate buffer I using a flow rate of 10 to 12 ml/hr. The void volume was determined with 1.5 ml of Blue Dextran (1%, w/v), containing 10% (w/v) sucrose. The ammonium sulfate fraction was layered onto the gel and allowed to pentrate the bed before applying the buffer. Approximately eighty 5-ml fractions were collected and the fractions with the peak of ribonucleotide reductase activity were pooled and dialyzed 12 to 18 hr. at 0.4° against 4 liters of 50 mM potassium phosphate buffer, pH 7.3, containing 1.0 mM β-mercaptoethanol.

Chromatography on DEAE-cellulose

DEAE-cellulose was prepared according to the Whatman Data Manual with final equilibration in 50 mM potassium phosphate buffer, pH 7.3, containing 1.0 mM β-mercaptoethanol. The column (2.5 X 10 cm) was washed with 300 ml of the same buffer. The dialyzed protein sample was layered on the column and eluted with a linear salt gradient consisting of 200 ml of phosphate buffer I in the mixing chamber and 150 ml of phosphate buffer I containing 0.35 M KCl in the reservoir. Ten ml fractions were collected at a flow rate of 1.3 ml/min.

The two or three tubes with the highest specific activity were pooled and the enzyme was stored under nitrogen at 0-4° in 0.60 ml fractions.

Colorimetric Assay for Ribonucleotide Reductase Activity

Ribonucleotide reductase activity during purification was monitored by the diphenylamine assay of Blakley (18). The complete reaction mixture contained in a final volume 0.5 ml: 15 µmoles dihydrolipoate; 10 nmoles B-12 coenzyme; 200 nmoles GDP; 50 µmoles potassium phosphate, pH 7.3; and 100-200 µg enzyme. The reaction mixtures were incubated 30 min. at 37° and terminated by boiling for 3 min. Four-tenths ml of chloroacetamide solution was added and the reactions were boiled an additional 10 min. After cooling, 2.0 ml of diphenylamine reagent was added and the solutions were incubated 4 hr. at 50°. Color intensity was determined spectrometrically (595 nm) and the deoxyguanosine formed was measured from a standard curve. An absorbance of 0.10 corresponded to 35 nmoles of deoxyguanosine.

Radioactive Assay for Ribonucleotide Reductase Activity

The radioactive assay was patterned from that of Moore (72). A typical reaction mixture contained in a final volume of 0.20 ml: 3.75 nmoles B_{12}-coenzyme; 7.5 µmoles potassium phosphate buffer, pH 7.3; 6 µmoles dihydrolipoate; 10-80 nmoles GDP; 0.05 µCi ^{14}C-GDP; and 3 to 5 µg enzyme. The specific activities of the radioactive substrates were:

^{14}C-U-GDP, 534 mCi/mM; ^{14}C-U-CDP, 477 mCi/mM; ^{14}C-U-UDP, 510 mCi/mM; and ^{14}C-U-ADP, 487 mCi/mM. In order that adequate response to both positive and negative effectors could be detected the concentration of the unlabeled substrates in the reaction mixtures, which lacked effectors, were adjusted so that one-third to one-half of the radioactivity in the origianl substrate appeared in the product peak after incubation. Details on the allosteric effector concentrations are given with the individual graphs. The reaction mixtures were incubated 30 min. at 37° unless stated otherwise. The reactions were terminated by boiling for 2 min. After cooling to room temperature, 10 µl of 1 M Tris-Cl buffer, pH 8.5, and 10 µl of alkaline phosphatase (144 units/ml) were added and the reactions incubated 2 hr. at 37°. The tubes were again boiled for 2 min. and, after cooling, 10 µl of 20 mM deoxynucleoside carrier was added. The samples were centrifuged 20 min. at 5,000 X g and 30 µl of the supernatant was applied to Whatman 3MM chromatography paper. The papers were eluted for 10 hr. by descending chromatography in Reichard's solvent: ethanol-5M ammonium acetate, pH 9.5-saturated sodium tetraborate-0.5 M EDTA (11:1:4:0.02) (83). When ADP was used the volume of ethanol was reduced to 3.3 volumes (72). The deoxyribonucleoside and ribonucleoside spots were located with a UV mineral lamp. The paper was cut along the path of the sample. Each strip was then cut into ½" segments and the radioactivity determined in a Parkard Tri-Carb 3003 Spectrophotometer. The

counting cocktail contained 0.3 g POPOP and 5.0 g PPO per liter of scintillation grade toluene.

Other Methods

Diphenylamine (Mathison, Coleman, Bell) was recrystallized from ethanol prior to preparing the complete reagent; 10 g of diphenylamine was dissolved in 250 ml of 95% ethanol and was recrystallized by slowly adding water. The crystals were dried for 3 to 4 days and then stored in a beaker wrapped with aluminum foil. The diphenylamine was prepared by dissolving 2.0 g of the crystals in 100 ml of glass-distilled acetic acid and 2.75 ml of concentrated sulfuric acid.

The chloroacetamide solution was prepared by dissolving 0.24 g of 2-chloroacetamide, (Baker Chemical CO.), in 5 ml of 250 mM potassium phosphate buffer, pH 7.3.

The dihycrolipoate was prepared according to Gunsalus and Razzell (51) and the concentration of B_{12} coenzyme was determined according to Cowles and Evans (30).

The protein concentration of all fractions was determined by the absorbance at 280 and 260 nm (104). In addition, the protein of the crude and ammonium sulfate fractions was checked by the Biuret method (46) and the active fractions from the DEAE-cellulose column were checked by a modified Lowry method (54). Both methods correlated well with the 280/260 determinations.

RESULTS

Purification of Ribonucleotide Reductase

The purification procedures used to purify ribonucleotide reductase of R. meliloti consisted of batch purification with ammonium sulfate and chromatography on Sephadex G-200 and DEAE-cellulose columns. A typical profile from the Sephadex G-200 column (Figure 1.) yielded a single peak of enzyme activity which eluted with the major protein peak. The maximum total activity was 5700 nmoles/ml protein·hr^{-1}. The average specific activity from the major portion of the enzyme peak was 3000 nmoles/mg protein·hr^{-1}. Elution of the enzyme peak (Fraction 37) occurred about 15 ml past the void volume of the column. The most active fractions from the Sephadex G-200 column were pooled, dialyzed, and chromatographed on a DEAE-cellulose column (Figure 2.). The ribonucleotide reductase activity eluted between KCl concentration of 0.13 to 0.17 M. Initially the maximum specific activity from the DEAE-cellulose column was about 12,000 nmoles/mg protein·hr^{-1}, however, in one case the maximum activity was 20,000 to 30,000 nmoles/mg protein·hr^{-1}. The peak fractions from the DEAE-cellulose column were pooled for futher experimentation.

Figure 1. Chromatography of Ribonucleotide Reductase on Sephadex G-200

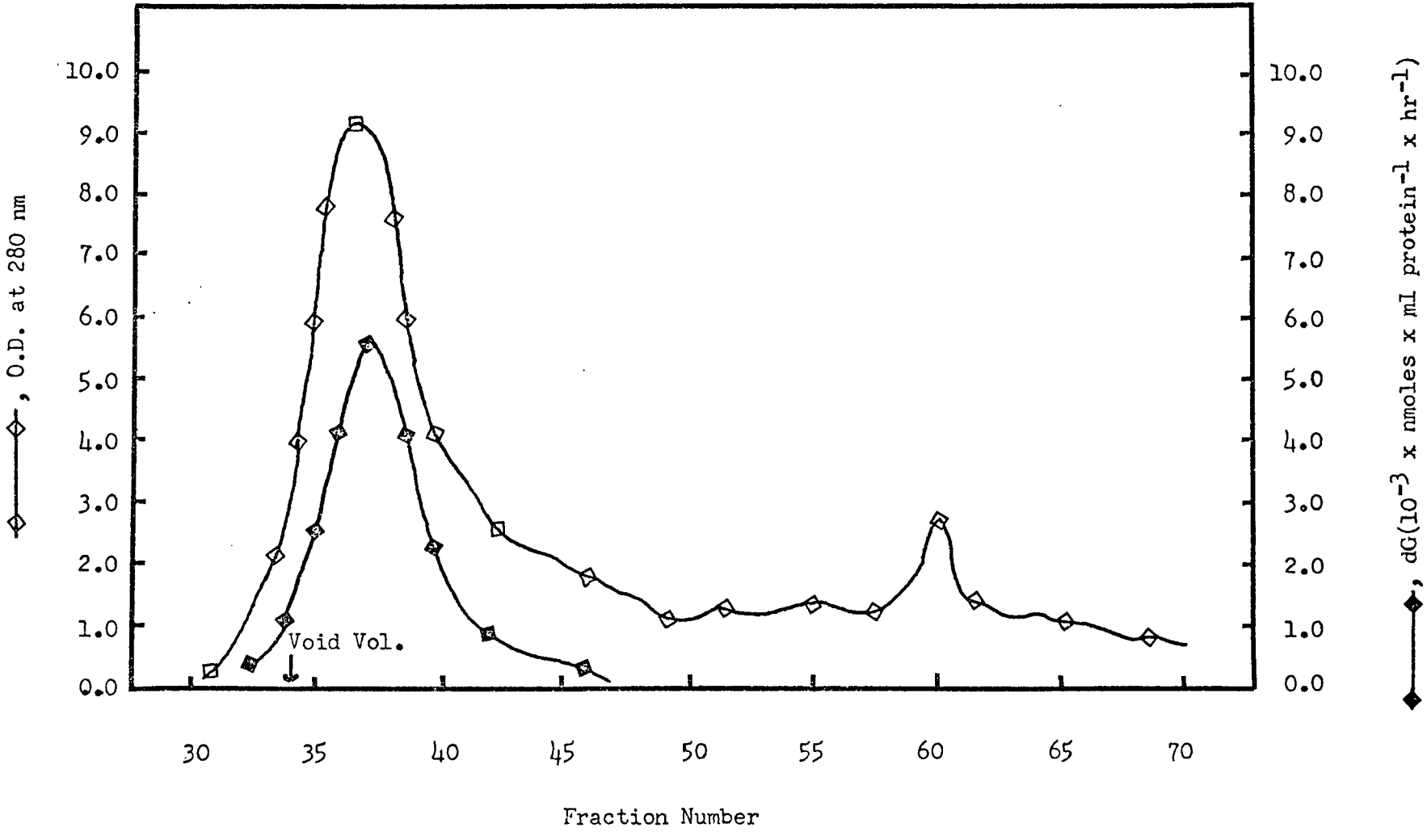


Figure 2. Purification of Ribonucleotide Reductase on DEAE-Cellulose

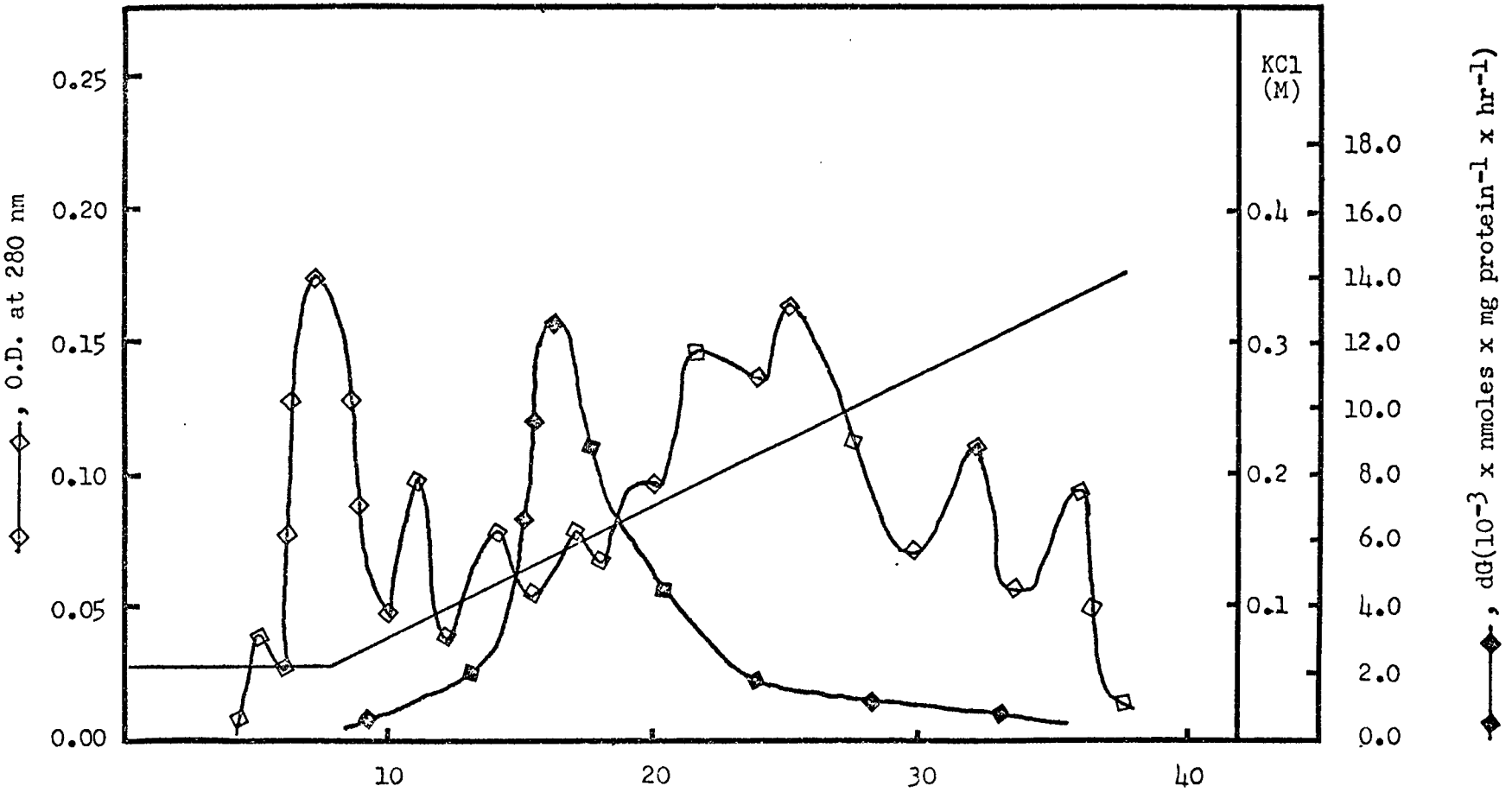


Table 1. Purification of Ribonucleotide Reductase

Fraction	Protein (mg)	Specific Activity (nmoles/mg protein·hr)	Activity (nmoles/hr)
Crude	2370	210	497,700
$(NH_4)_2SO_4$ (25-45%)	220	630	138,600
Sephadex G-200	43.5	3185	138,560
Dialyzed	37.2	2500	93,000
DEAE- Cellulose	7.9	5162	41,040

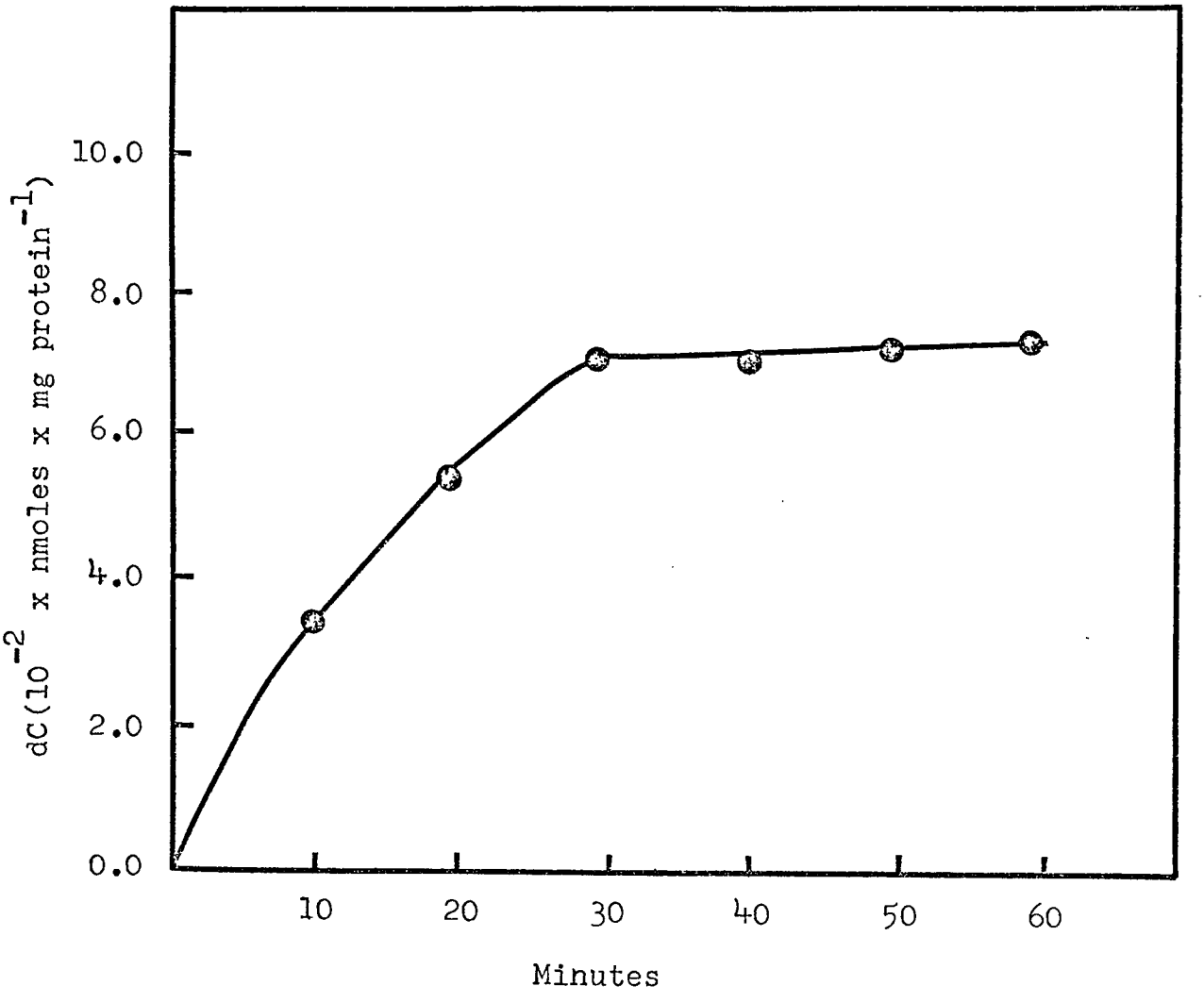
A summary of the purification of ribonucleotide reductase of R. meliloti is shown in Table 1. Typically, 20 liters of cells (20 to 25 g) yielded 2,000 to 3,000 mg of protein with a specific activity of 200 to 250 nmoles/mg protein·hr^{-1}. Fractionation with ammonium sulfate increased the specific activity to 600 to 700 nmoles/mg protein·hr^{-1}. Purification on Sephadex G-200 increased the specific activity 6-fold; however, approximately 16% of the activity was lost during dialysis. The specific activity of the pooled fractions from the DEAE-cellulose column was 5162 nmoles/mg protein·$hr^{-1}$and represented an approximate 25-fold purification. The enzyme from the DEAE-cellulose column seemed to lose activity at two different rates. Approximately 50% of the inital activity was lost 4 to 5 days after fractionation and the remainder was lost at a rate of 5 to 10% per month.

Effect of Time on CDP Reduction

The influence of the incubation period on the rate of substrate reduction was tested with each substrate using the purified enzyme. Deoxyribonucleotide formation increased with increasing periods of incubation for 30 min. This was therefore the incubation period routinely used. The profile shown for CDP reduction is an example of the response of deoxyribonucleotide formation to incubation periods (Figure 3.). Similar curves were obtained for the other substrates.

Figure 3.

The Effect of Time on the Reduction of CDP. Reactions were under standard conditions at 0.035mM CDP and 0.003mg protein.



Allosteric Regulation of Ribonucleotide Reduction

Studies on the allosteric regulation of ribonucleotide reductase of R. meliloti were conducted using deoxyribonucleoside triphosphates and the purified enzyme from the DEAE-cellulose column. The optimum concentration for each ribonucleotide was determined prior to determining the allosteric pattern for each substrate. The substrate concentrations actually used were those that enabled both stimulation and inhibition to be detected. The concentrations for a given substrate sometimes changes as a result of the loss of activity over the period of time each was studied. The effector range of 10^{-8} to 10^{-3} M was chosen for this study and includes the concentrations used in published studies on all ribonucleotide reductase systems.

Effector Studies on GDP Reduction

Guanosine diphosphate (GDP), in the absence of effectors, was reduced at a rate faster than that for any of the other ribonucleotides examined. The reduction rate ranged from 4,000 to 30,000 nmoles/mg protein·hr^{-1} depending on the age of the enzyme and the original activity of the extract (Figures 4-7). The optimum substrate concentration for GDP (0.4 mM) was used unless stated otherwise. As seen in the figures, all deoxyribonucleoside triphosphates inhibited GDP reduction. The strongest negative effector was dGTP (Figure 4) which inhibited GDP reduction 70 to 80% at 5 X 10^{-4} M.

Figure 4.

The Effect of dGTP on the Reduction of GDP. Reactions were under standard conditions at 0.40mM GDP and 0.003mg protein.

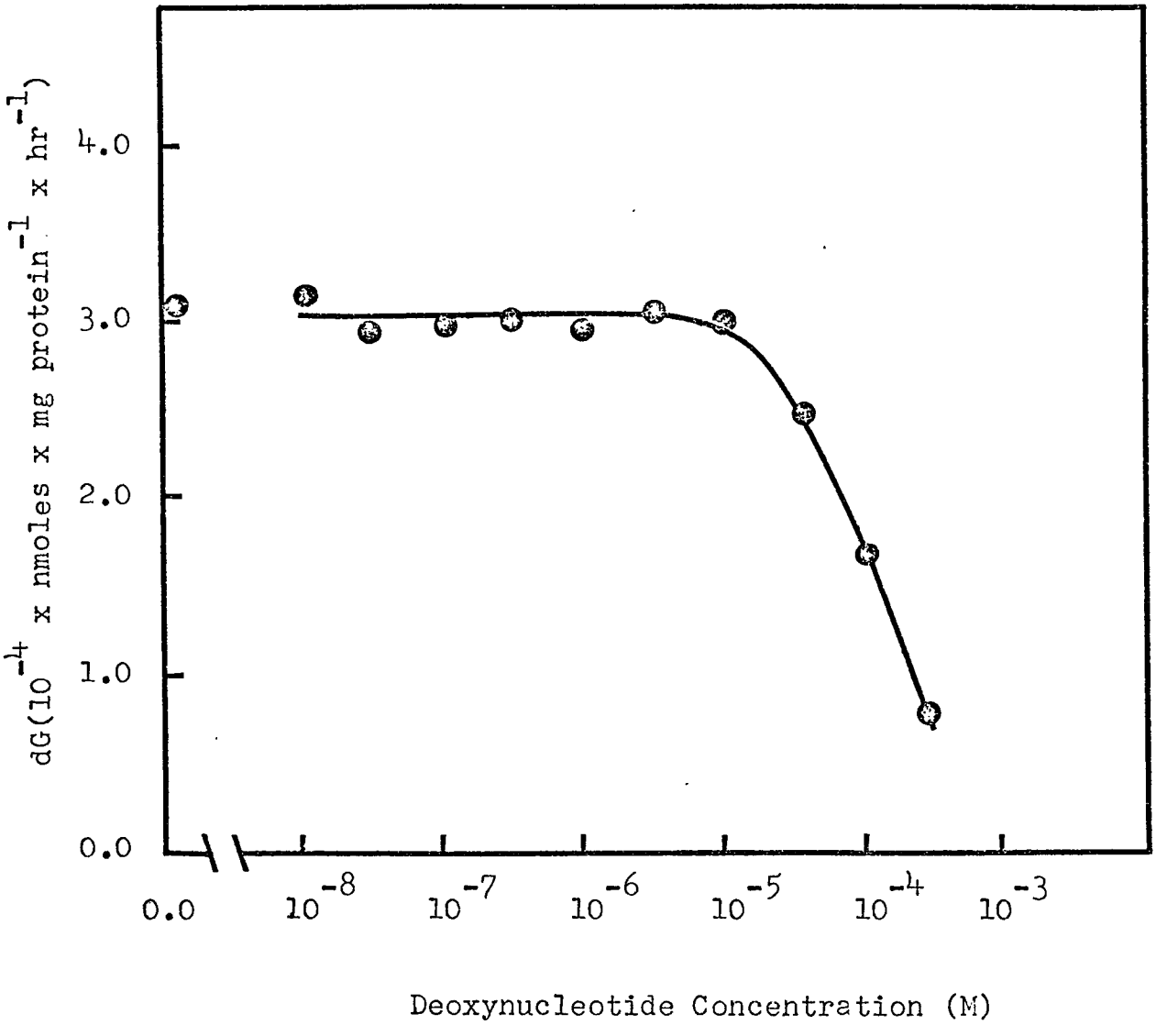


Figure 5.

The Effect of dTTP on the Reduction of GDP. Reactions were under standard conditions at 0.10mM GDP and 0.003mg protein.

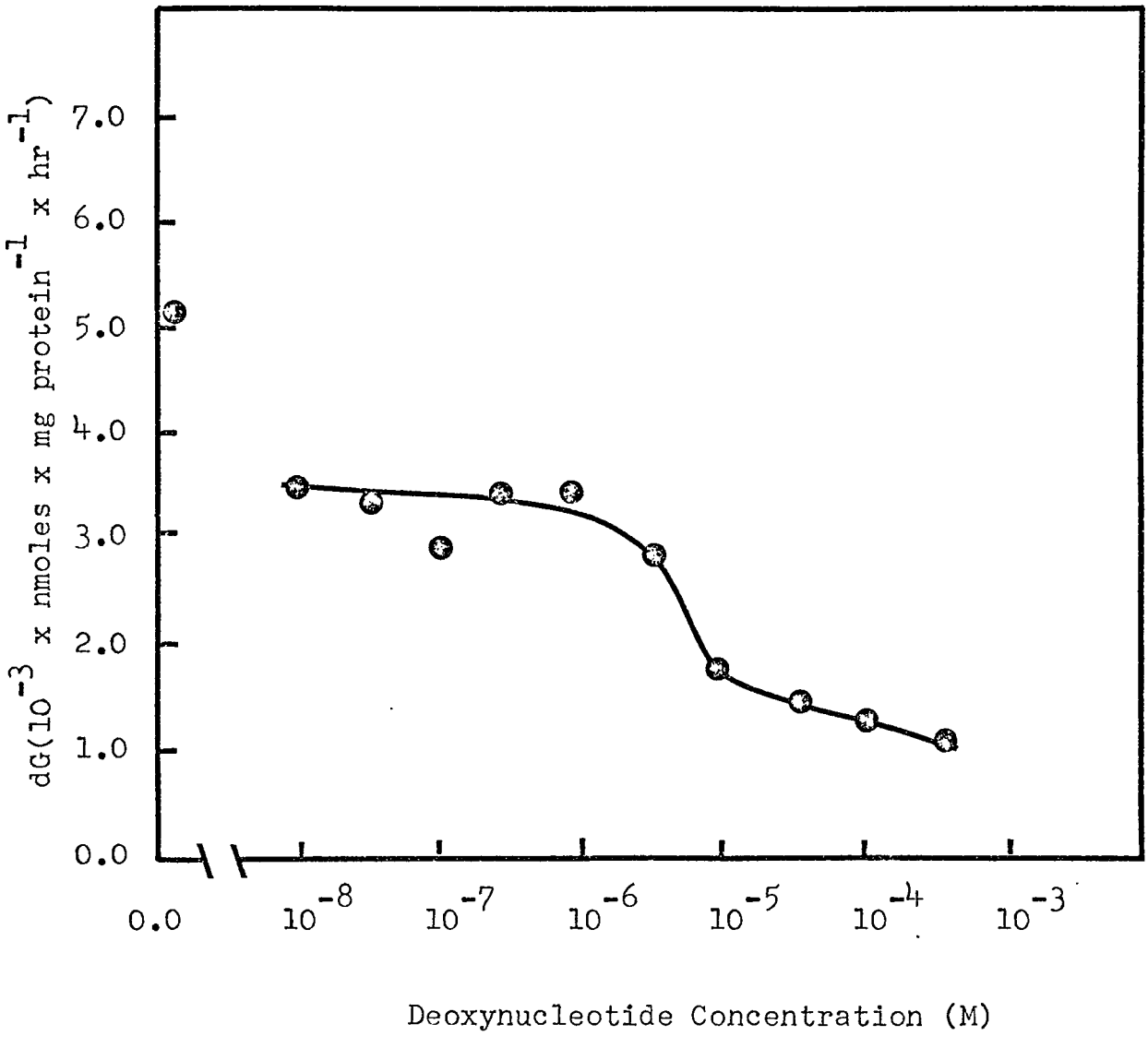
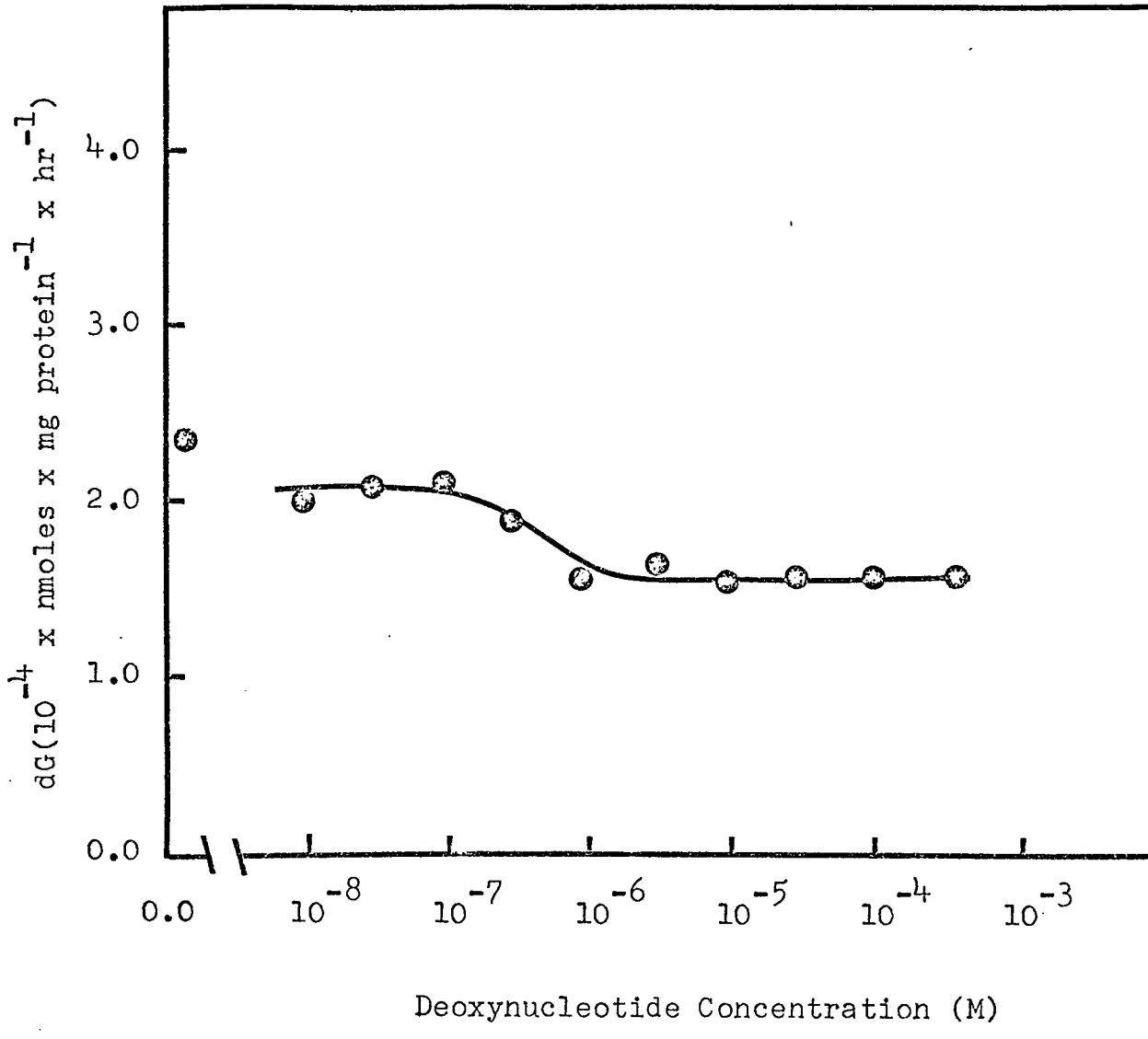


Figure 7.

The Effect of dATP on the Reduction of GDP. Reactions were under standard conditions at 0.40mM GDP and 0.003mg protein.



Concentrations less than 1 X 10^{-5} M however had no effect on GDP reduction. Deoxy-TTP (Figure 5) produced approximately 60% inhibition at 5 X 10^{-5} M concentration while concentrations less than 1 X 10^{-6} M had very little effect on enzyme activity. The most gradual loss in reductase activity was produced by dCTP (Figure 6). The greatest inhibition was at 5 X 10^{-4} M, the highest concentration used. The weakest negative effector of GDP reduction was dATP (Figure 7). There was only about a 25% loss in activity between 5 X 10^{-7} and 1 X 10^{-6} M. Futher increases produced no more inhibition.

Effector Studies on ADP Reduction

The rate of adenosine diphosphate (ADP) reduction varied from 400 to 1000 nmoles/mg protein·hr^{-1} which was only 5 to 10% of that of GDP reduction. The optimum substrate concentration of ADP was 30 to 60 μM. ADP reduction was stimulated by dGTP and inhibited by dTTP and dATP. Deoxy-GTP stimulated ADP reduction 250% at concentrations of 1 X 10^{-6} M or greater (Figure 8). Concentrations as small as 1 X 10^{-8} M caused slight stimulation over the control. The effect of dCTP on ADP reduction did not follow a smooth curve (Figure 9). The reduction rate was slightly stimulated by 1 X 10^{-8} M to 5 X 10^{-8} M dCTP while 1 X 10^{-7} M to 5 X 10^{-7} M dCTP inhibited activity. ADP reduction was again stimulated by 1 X 10^{-6} M to 5 X 10^{-6} M dCTP before the final inhibition by concentrations above 1 X 10^{-5} M. Although this

Figure 8.

The Effect of dGTP on the Reduction of ADP. Reactions were under standard conditions at 0.06 mM ADP and 0.003 mg protein.

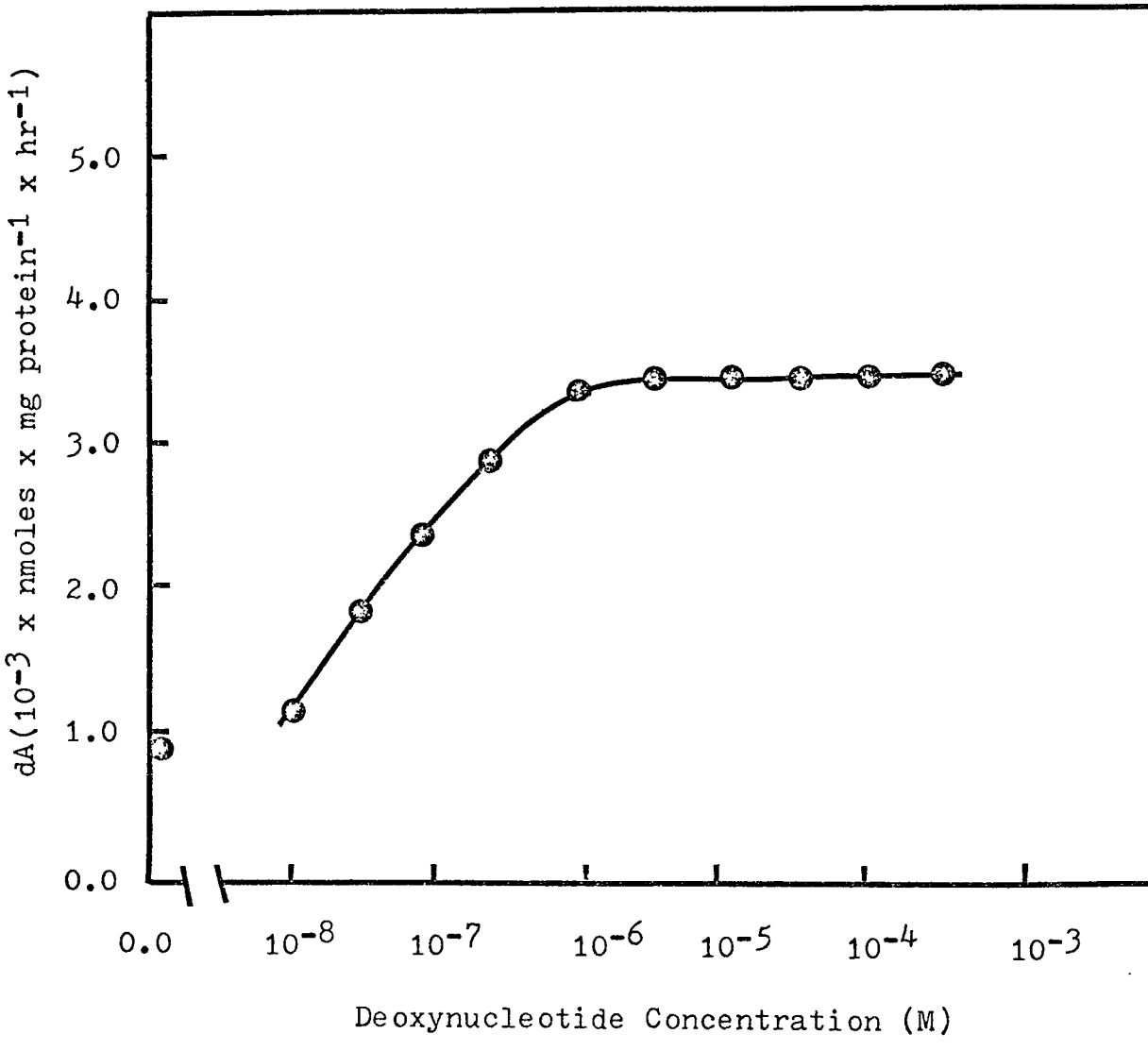


Figure 9.

The Effect of dCTP on the Reduction of ADP. Reactions were under standard conditions at 0.03mM ADP and 0.0086mg protein.

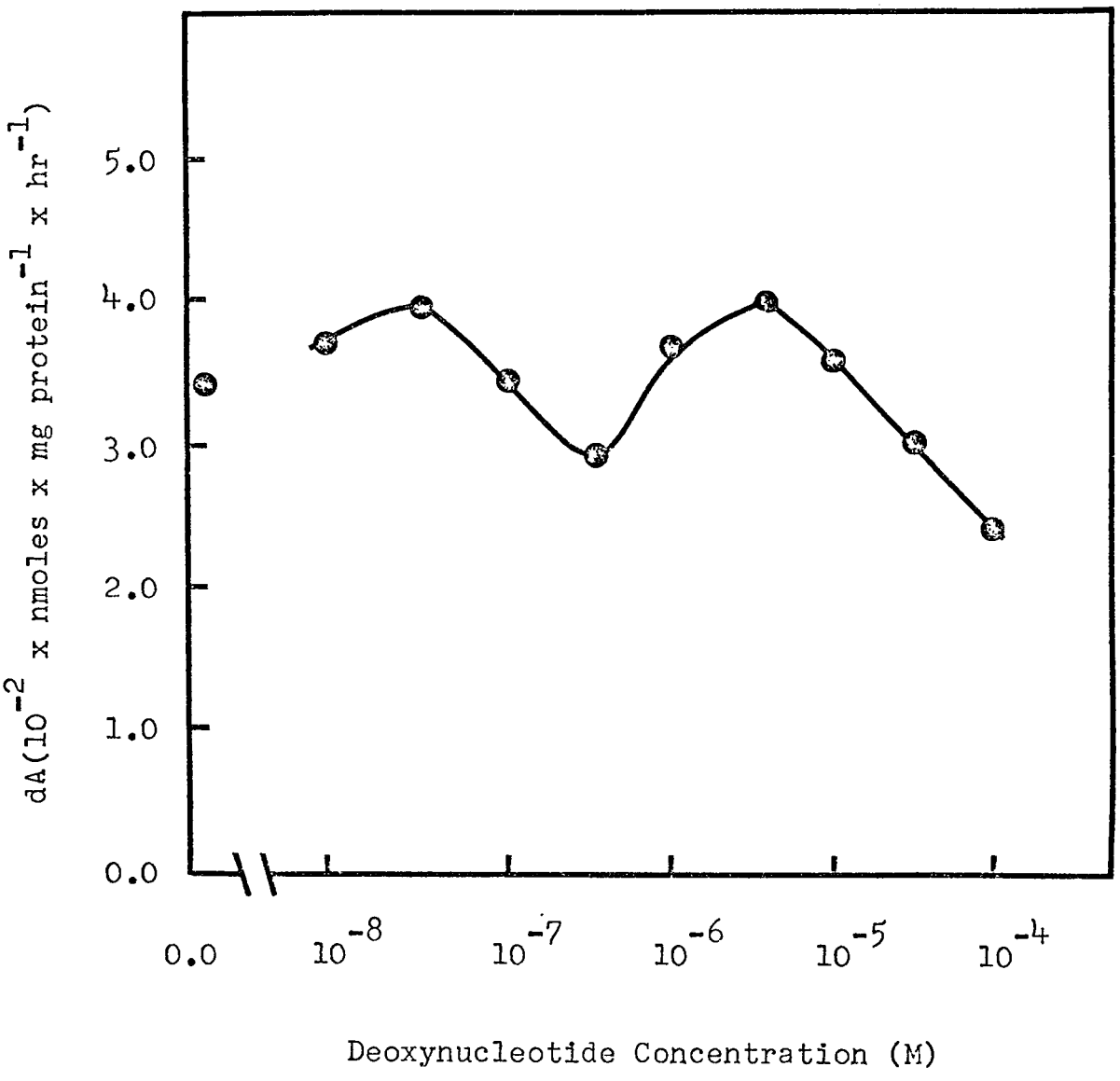


Figure 10.

The Effect of dTTP on the Reduction of ADP. Reactions were under standard conditions at 0.03mM ADP and 0.0086mg protein.

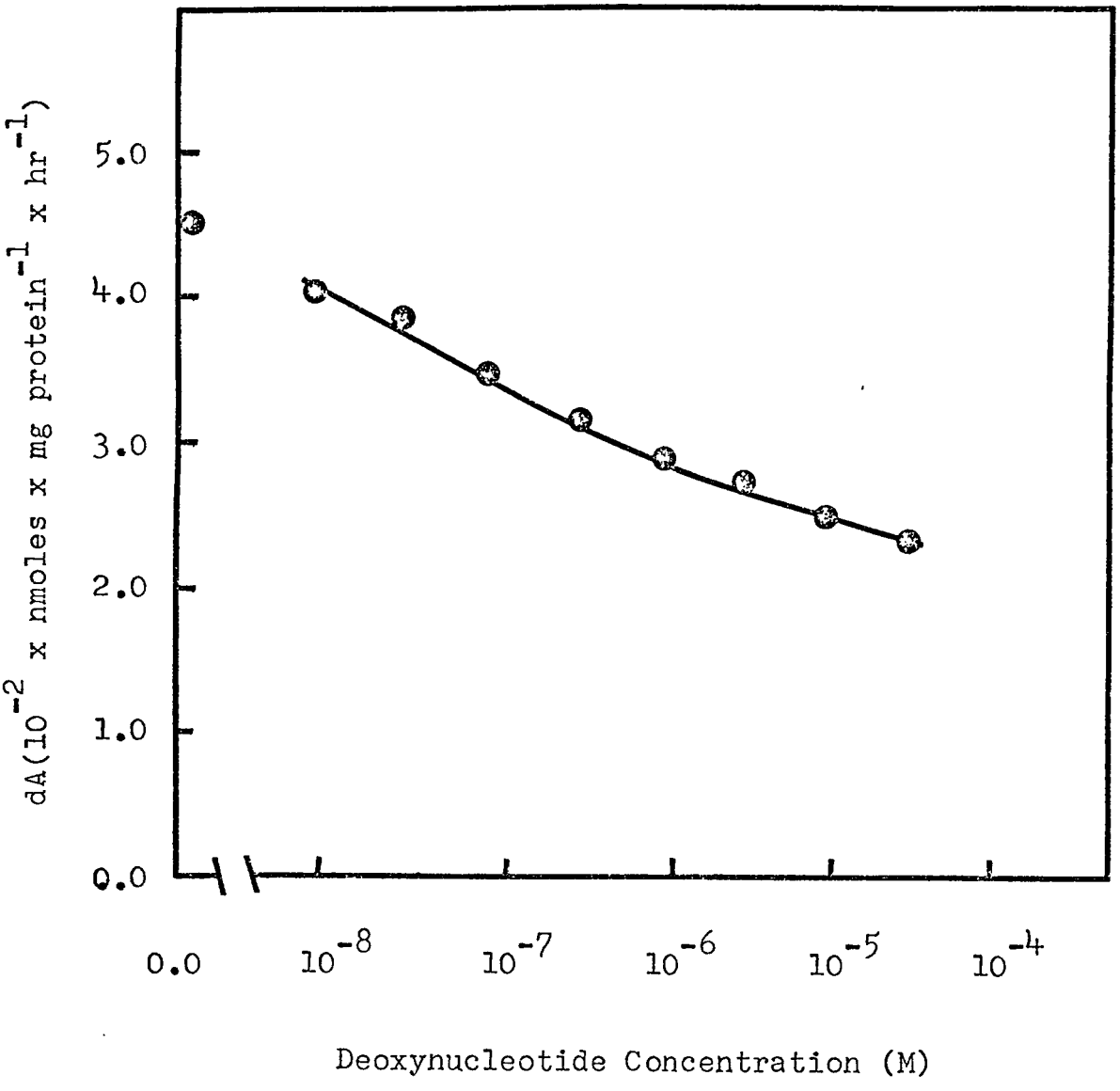
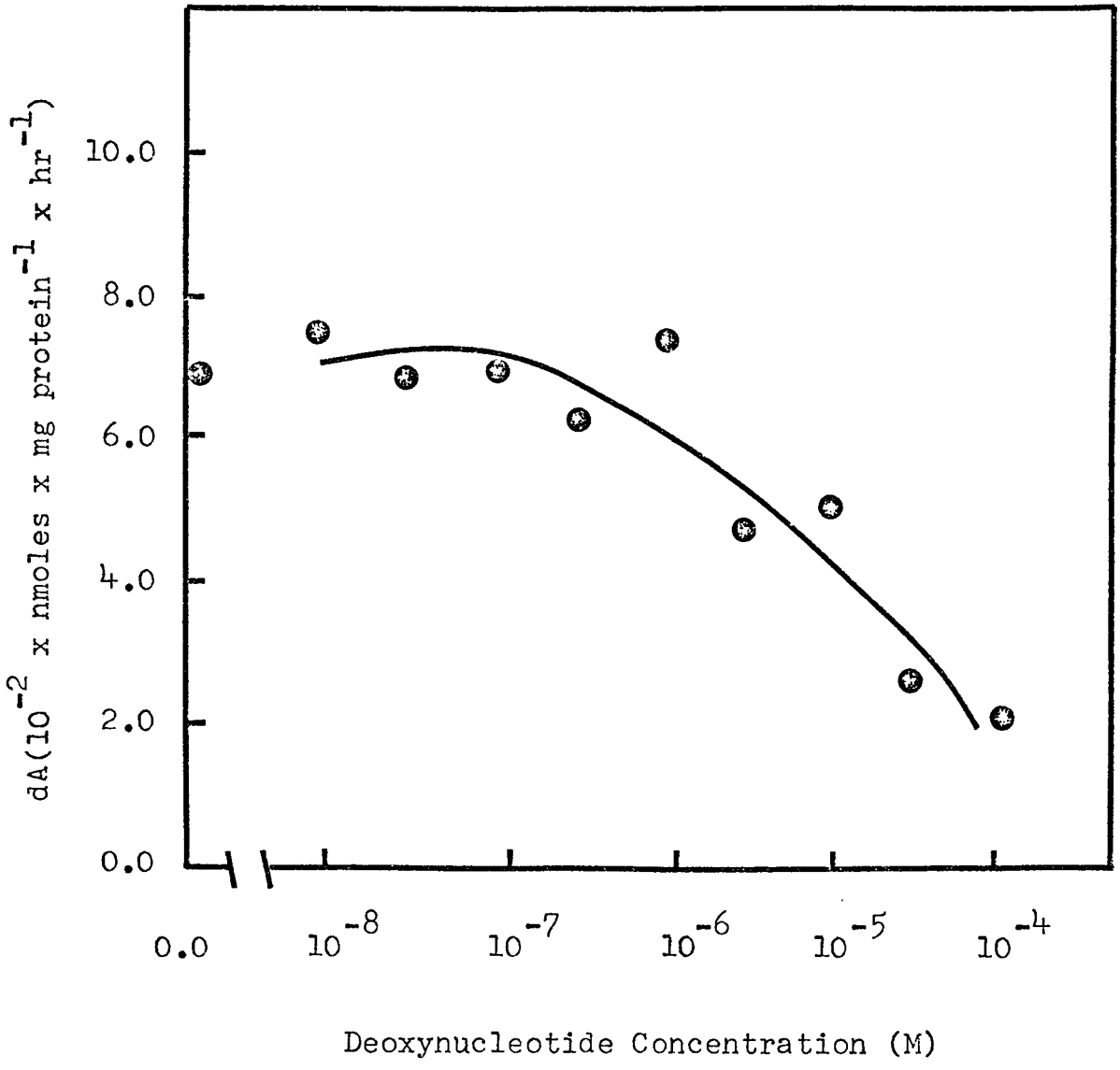


Figure 11.

The Effect of dATP on the Reduction of ADP. Reactions were under standard conditions at 0.06mM ADP and 0.004mg protein.



type of pattern is unique for a single effector regulating a single substrate it was reproducible. Increasing concentrations of dTTP inhibit ADP reduction (Figure 10). The greatest hinhbition (40%) occurred at 5 X 10^{-5} M dTTP. Deoxy-ATP caused a 70% inhibition of ADP reduction at 5 X 10^{-5} M (Figure 11). Deoxy-ATP concentrations less than 10^{-7} M did not effect the rate of ADP reduction.

Effector Studies on CDP Reduction

The rate of CDP reduction in the data shown was consistantly about 1000 nmoles/mg protein·hr^{-1}. The optimum concentration of substrate was 35 to 80 μM. CDP reduction was stimulated maximally by dATP and slightly by dTTP. Deoxy-GTP was a negative effector while dCTP has no effect at all. CDP reduction, over the control, was stimulated by all concentrations of dATP with a 300% increase at 5 X 10^{-6} M (Figure 12). The pattern was again unusual but reproducible. Deoxy-TTP (Figure 13) at low concentrations (5 X 10^{-8} M) stimulated CDP reduction almost 200%, but, at concentrations of 10^{-6} M or greater, slight inhibition occurred. Deoxy-GTP (Figure 14) produced a 40 to 50% inhibition of CDP reduction at concentrations of 10^{-5} M. Concentrations between 10^{-8} M and 10^{-6} M were without effect. Deoxy-CTP (Figure 15) had no influence on CDP reduction.

Effector Studies on UDP Reduction

Attempts to establish the allosteric regulation of UDP

Figure 12.

The Effect of dATP on the Reduction of CDP. Reactions were under standard conditions at 0.0875mM CDP and 0.0086mg protein.

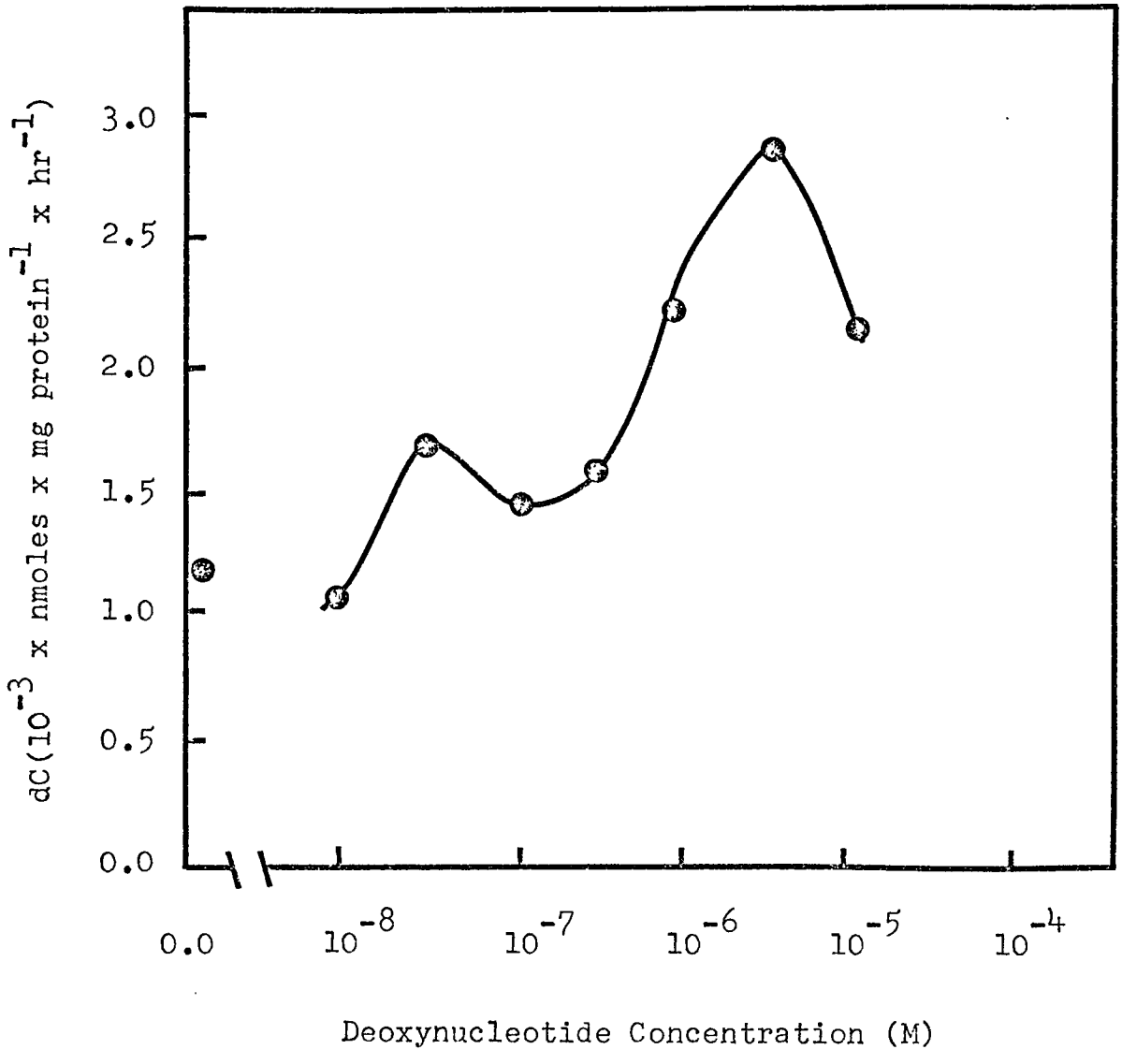


Figure 13.

The Effect of dTTP on the Reduction of CDP. Reactions were under standard conditions at 0.0875mM and 0.0086mg protein.

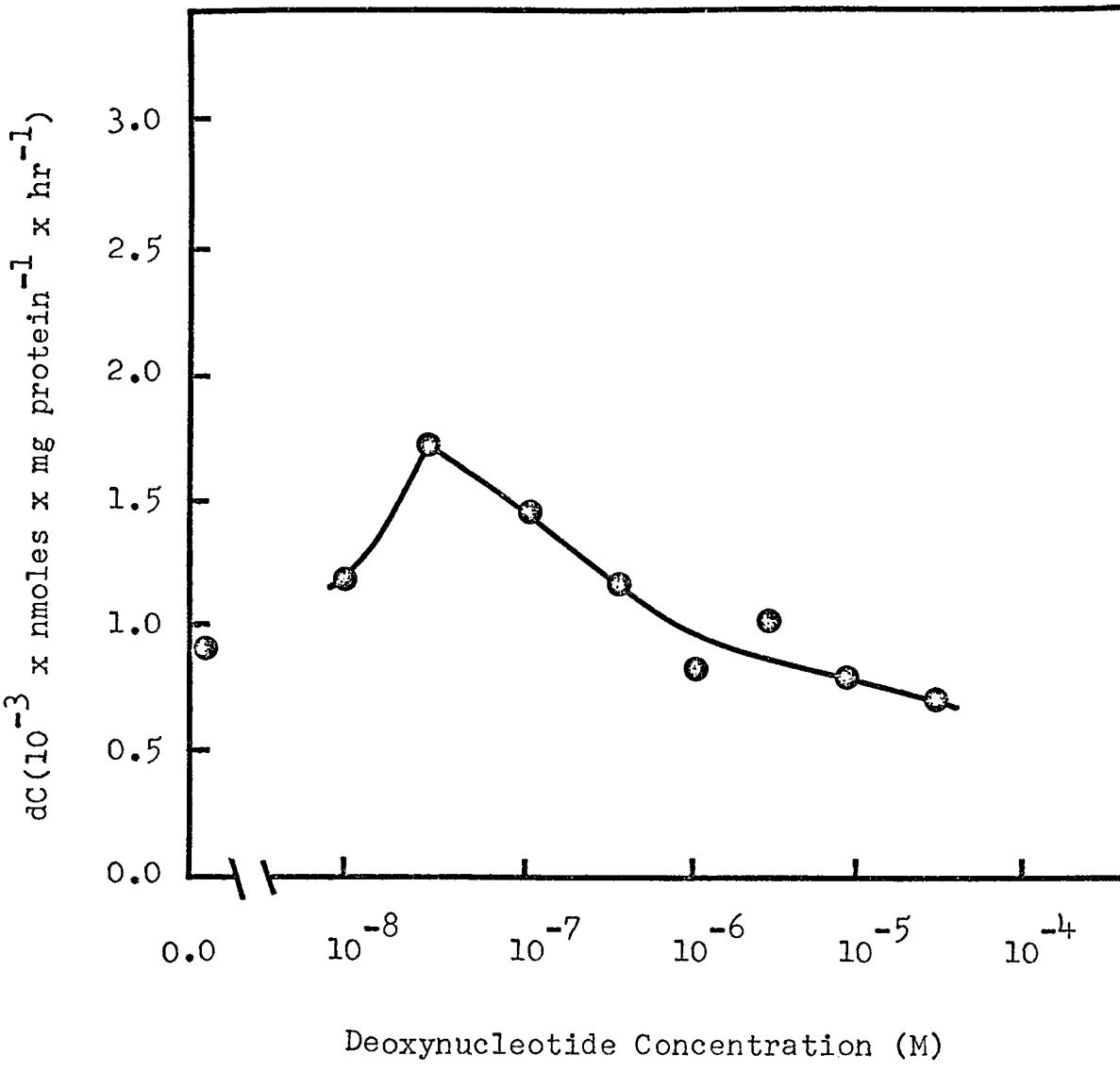


Figure 14.

The Effect of dGTP on the Reduction of CDP. Reactions were under standard conditions at 0.035mM CDP and 0.003mg protein.

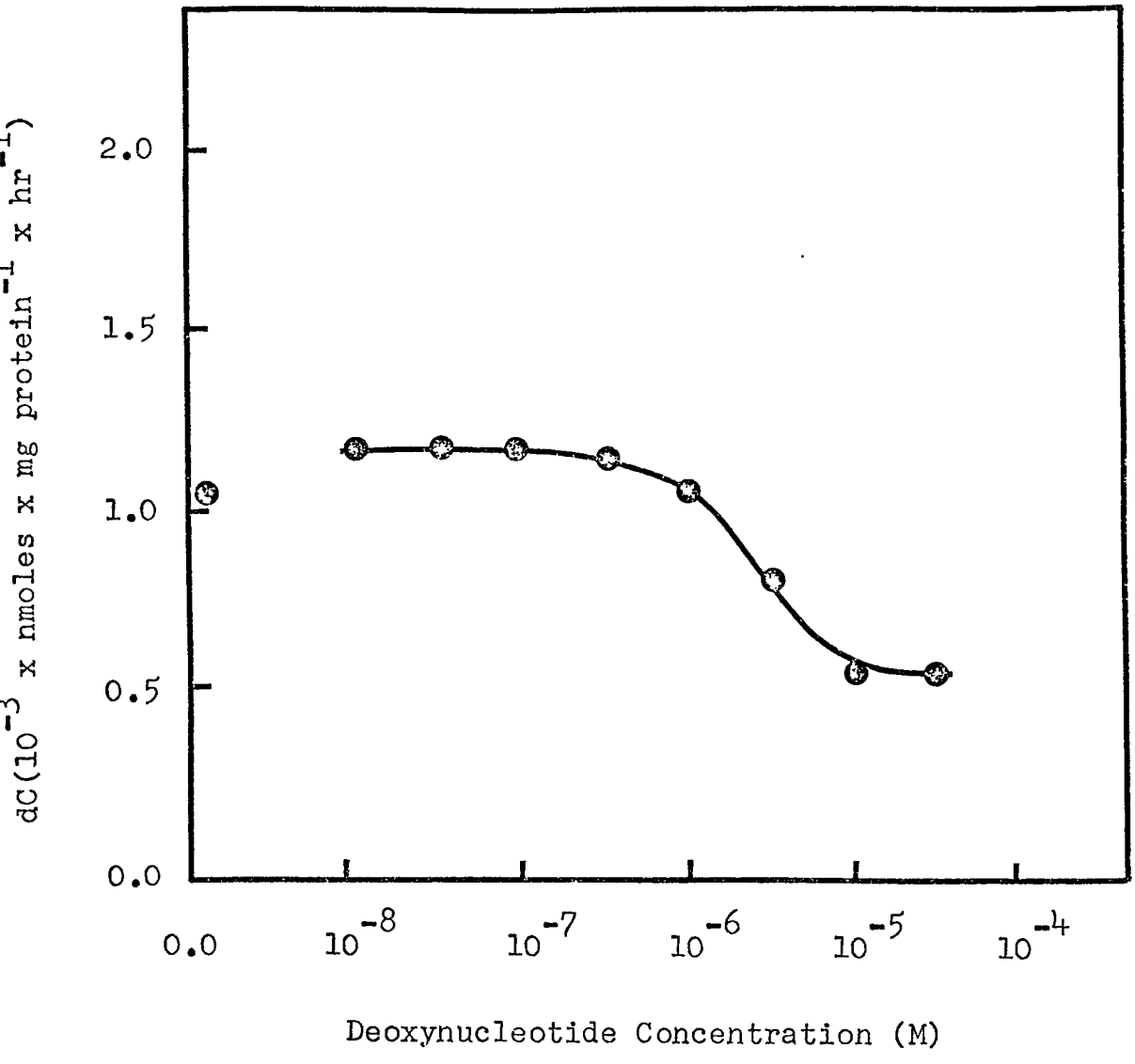
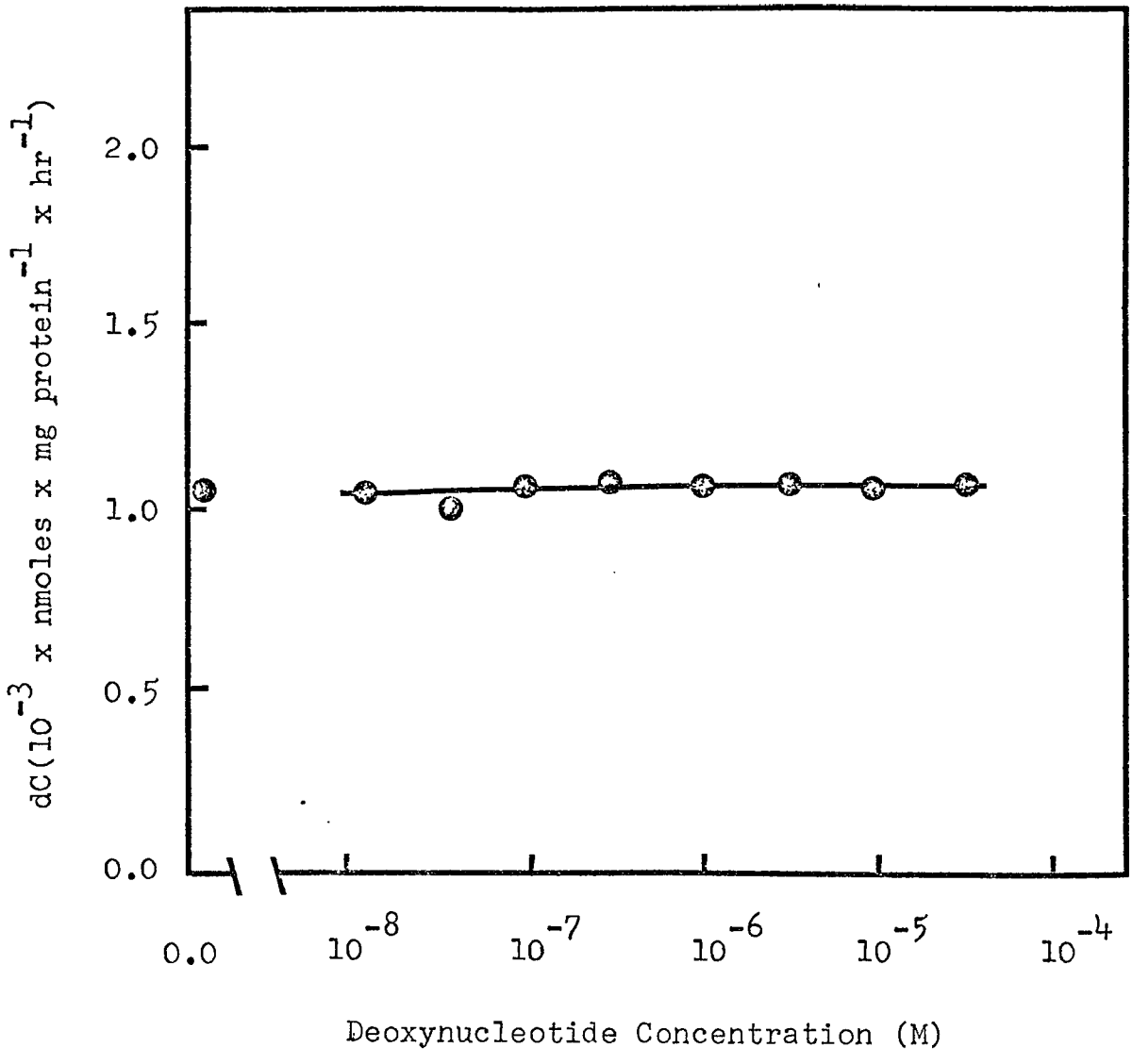


Figure 15.

The Effect of dCTP on the Reduction of CDP. Reactions were under standard conditions at 0.035mM CDP and 0.003mg protein.



were unsuccessful using the standard assay procedure. The difficulty was due in part to the inability to clearly demonstrate UDP reduction. A typical radioactive profile from the chromatographic strip using UDP as substrate is shown in Figure 16. The problem with the profile is that the area on each strip which should contain deoxyuridine has a very low number of counts while two areas slightly closer to the origin (Regions A and B) contain an unusally high number of counts. These radioactive areas located between the substrate and product were not observed with the other substrates. It is possible that these counts represent dUMP or dUDP which were not completely de-phosphorylated to deoxyuridine. This, however, appears unlikely since the blank also contained high counts in these regions. The effect of increasing concentrations of UDP on UDP reduction is shown in Table 2. As expected, the total counts in the uridine peak increased with increasing concentrations of unlabelled substrate. The counts located in the deoxyuridine region, although erratic, appear to indicate that reduction was occurring. However, in light of the high number of counts that are located just prior to the deoxyuridine peak, the evidence is inconclusive as to whether reduction was in fact actually occurring.

The problem with attempts to demonstrate UDP reduction was investigated futher. In order to determine if the radioactive or unlabelled substrates had decomposed or were contaminated, a sample of each was chromatographed independently (Figure 17). Both ^{14}C-UDP and UDP gave identical homogeneous

Figure 16.

Profile of Radioactivity from Chromatographic Separation of Uridine and it's reaction products. The blank and complete contained 0.005mg protein and 0.50 mM UDP. Positions of uridine and deoxyuridine were determined with standards. Blank, ————————; complete, ---------- .

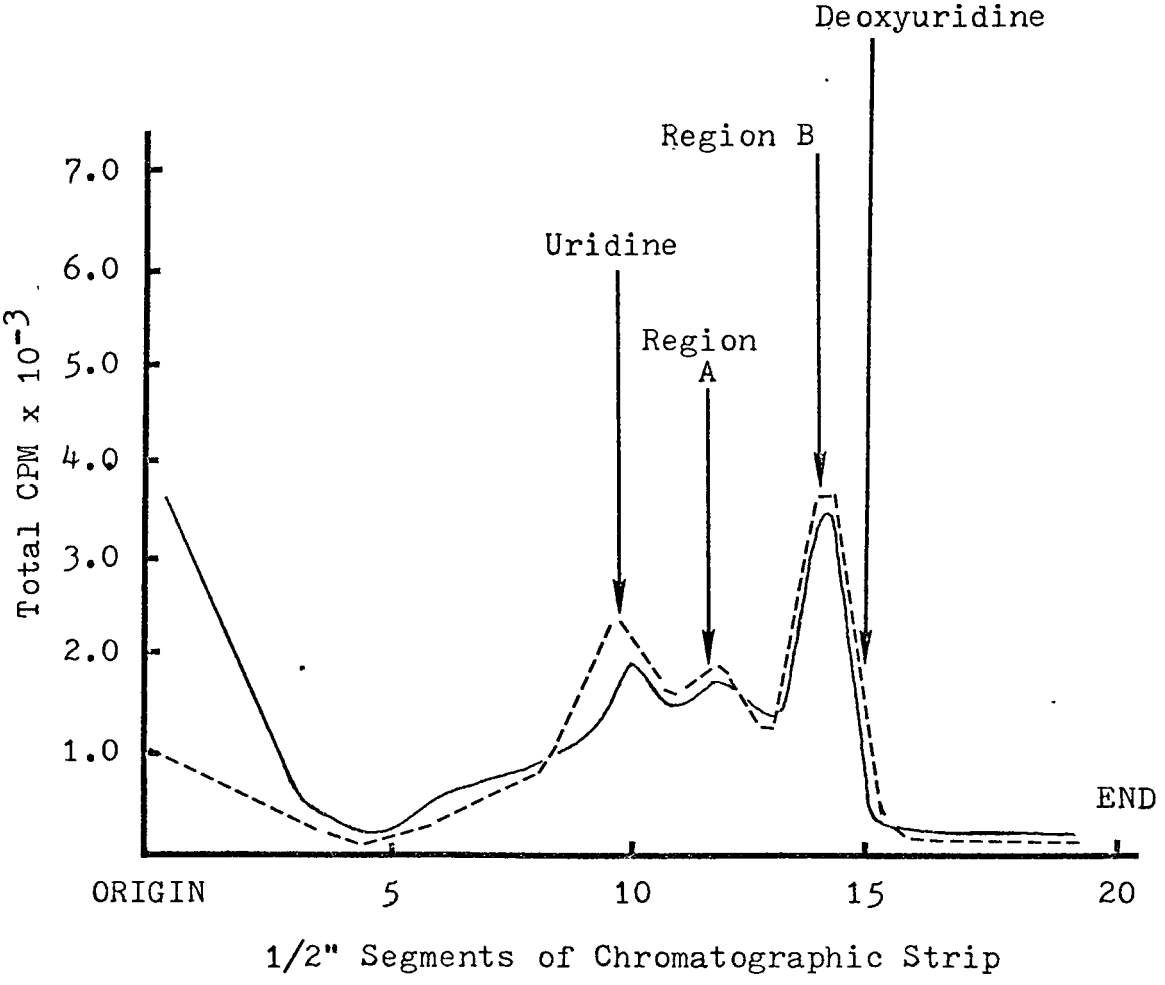
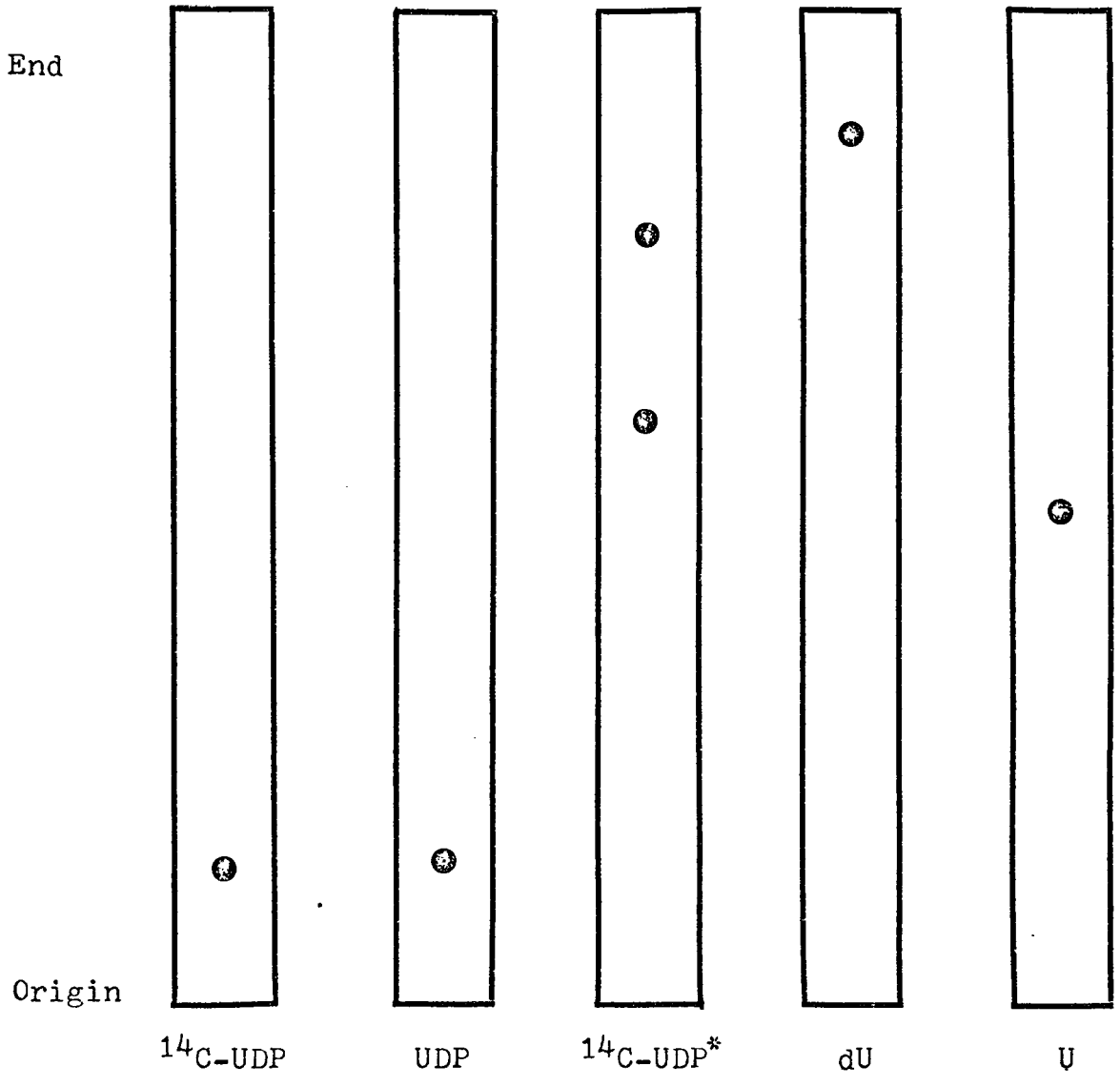


Table 2. Variation in Radioactive Counts from Specific Areas of the Chromatogram Separating Uridine and it's Reaction Products. Each assay contained 0.005 mg protein and UDP as indicated. The areas listed refer to those shown in Figure 16.

(UDP)	Counts Per Minute			
	Uridine	Region A	Region B.	Deoxyuridine
Blank	3384	3188	3650	444
0.50 mM	4544	1918	4181	846
0.75 mM	6710	1343	3901	685
1.00 mM	8264	1480	2907	716
1.25 mM	8409	1878	2714	950

Figure 17

Chromatography of Uridine Diphosphate, Deoxyuridine, and Uridine



(*)-Sample was prepared and treated in the same manner as a complete reaction mixture except that dihydrolipoate, B_{12} coenzyme, and protein were omitted.

dU= deoxyuridine; U= Uridine; UDP= Uridine Diphosphate

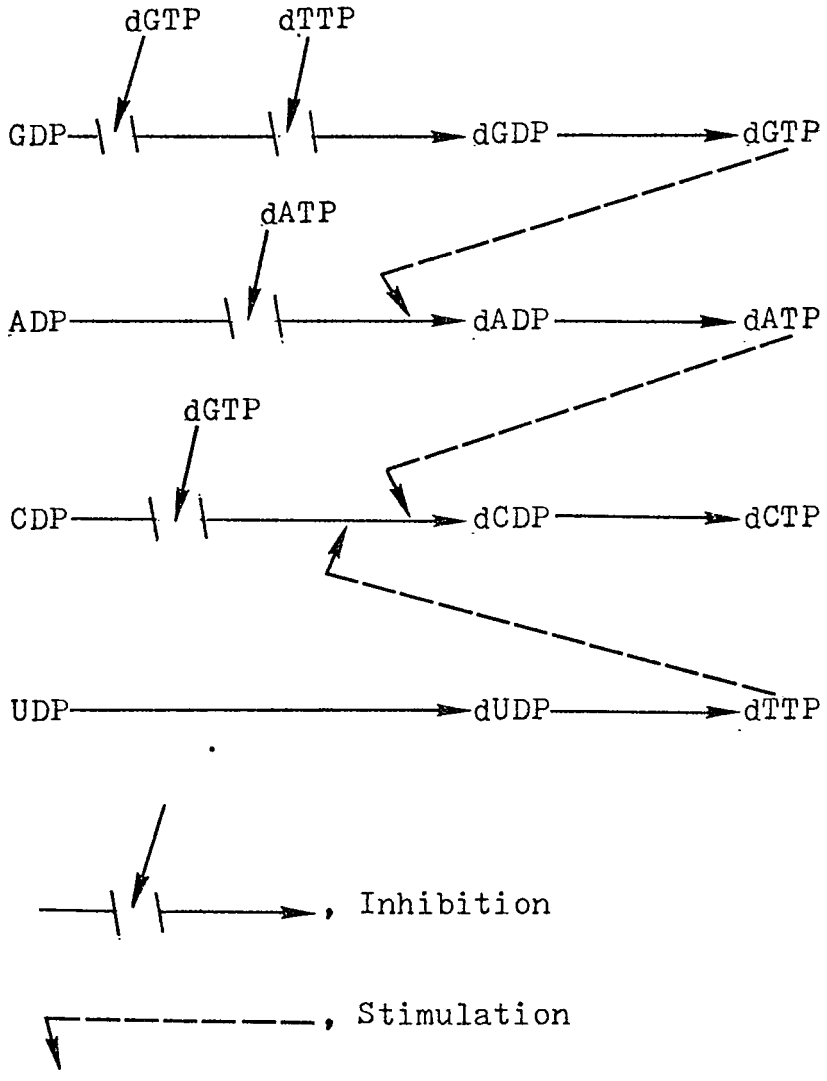
spots located only near the origin as is expected from a ribonucleoside diphosphate. Deoxyuridine and uridine also chromatographed in their proper positions. To determine if the standard assay method was altering the substrate, radioactive UDP and phosphate buffer, pH 7.3, were added to a reaction tube and the volume was brought up to 0.20 ml with water (no extract was added). The mixture was incubated in the same manner as a complete assay. The sample, when chromatographed, gave only two spots of equal radioactivity located between the deoxyuridine and uridine positions. (Figure 17). This was somewhat surprising since, in Figure 16, the complete reaction mixture also had a peak corresponding to uridine. These results make it impossible to determine the allosteric effectors for UDP reduction by this technique.

Overall Pattern of Allosteric Regulation

The proposed scheme for allosteric regulation of ribonucleoside diphosphate reductase of *R. meliloti* is summarized in Figure 18. GDP is the most actively reducted substrate and requires no positive effectors. Deoxy-GTP then stimulates ADP reduction to form dATP which in turn stimulates CDP reduction. It is believed that dCTP would then stimulate UDP reduction. Deoxy-GTP was shown to be a strong negative effector for GDP and CDP reduction, while dATP primarily inhibits ADP reduction. Deoxy-TTP at low concentrations was found to stimulate CDP reduction. A universal negative effector was not found.

Figure 18.

Proposed Scheme for the Allosteric Regulation of Ribonucleotide Reductase of Rhizobium meliloti.



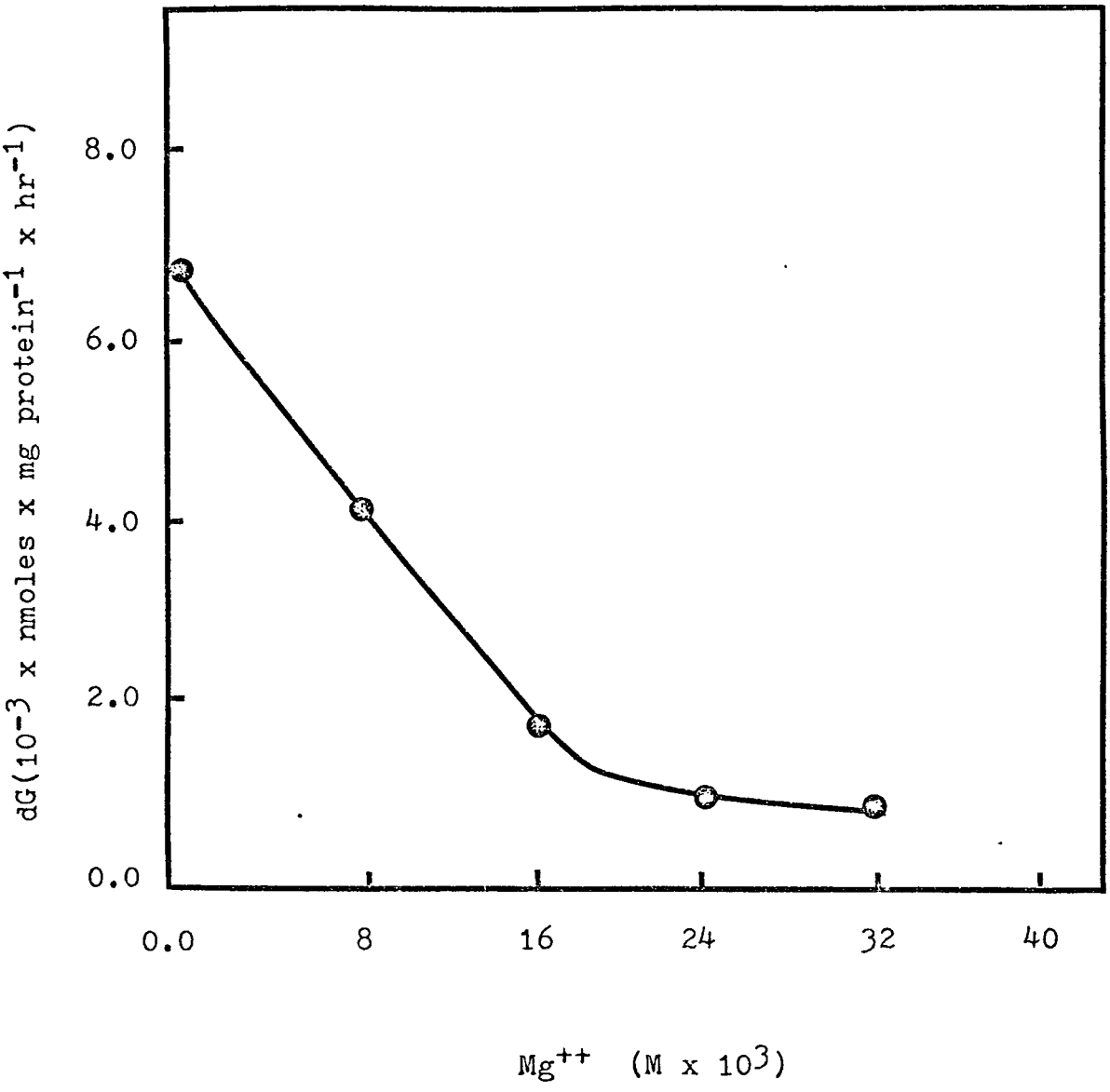
Reduction of GDP in the Presence of Mg^{++}

Magnesium is absolutely required in most Class I ribonucleoside diphosphate reductase systems studied, but an absolute requirement has not been demonstrated for B_{12} coenzyme requiring systems. Mg^{++} however has been shown to affect ribonucleotide reductase activity in R. meliloti (68). The most extensive study of the effects of Mg^{++} on a cobamide-dependent reductase is that of Beck using L. leichmannii (4). He demonstrated an optimal concentration of Mg^{++} for each substrate in the presence of it's prime positive effector. In the case of GDP reduction all effectors were negative in the absence of Mg^{++}, but, in the presence of 5mM Mg^{++}, dTTP became a strong positive effector. In the absence of the positive effector GDP reduction was reported to occur in the presence of Mg^{++} only if enzyme was added to an incubation mixture already containing substrate and Mg^{++}. In contrast to these findings with L. leichmannii, Mg^{++} is a strong inhibitor of ribonucleotide reductase of R. meliloti even when the enzyme was added last (Figure 19). There was a rapid, linear loss in activity to 30% of that of the control using concentration of Mg^{++} from 1 to 16 mM. Mg^{++} concentrations to 32 mM resulted in additional small decreases in reductase activity.

Blakley (17) found that in the L. leichmannii system dihydrolipoate at concentrations over 30 mM, together with a Mg^{++} concentration above 20 mM, resulted in the formation of a white precipitate with a loss in enzyme activity. The

Figure 19.

The Influence of Mg^{++} on the Reduction of GDP. Except for Mg^{++}, reactions were under standard conditions at 0.40mM GDP and 0.005mg protein.



white precipitate was believed to be a combination of dihydrolipoate and Mg^{++} and the reduced concentration of one or both was thought to result in the loss of enzyme activity. A similar precipitate was found at high Mg^{++} concentrations with the reductase system of R. meliloti. To determine if the Mg^{++} was in fact removing dihydrolipoate from the reaction mixture increasing concentrations of dihydrolipoate were added to assays containing 6 mM Mg^{++}. Table 3 shows that Mg^{++} at this concentration resulted in a 25% inhibition of enzyme activity. Adding increasing amounts of dihydrolipoate above the optimum (30 mM) resulted in futher inhibition in reductase activity and was unable to restore the activity back to the control level.

Table 3. The dependence of GDP Reduction on constant Mg^{++} concentration and increasing lipoate concentration. Incubations were under standard conditions with 0.0086mg protein. Complete mixtures contained 30mM lipoate and no Mg^{++}. Mg^{++} concentration in remaining mixtures was 6mM.

Incubation Mixtures	dG Formed
	(nmoles/mg protein · hr)
Complete	4217
Mg^{++}, lipoate (30mM)	3107
Mg^{++}, lipoate (45mM)	1917
Mg^{++}, lipoate (60mM)	1536
Mg^{++}, lipoate (75mM)	1516

DISCUSSION

Ribonucleotide reductase of R. meliloti consistently chromatographed as a single peak of activity during purification. Elution from Sephadex G-200 occurred near the void volume. Therefore, if the enzyme consists of a multisubunit complex it's integrity is probably not maintained by divalent cations. This is in contrast to the enzyme from E. coli which was shown to dissociate when chromatographed on hydroxyapatite (21).

In general, the allosteric pattern of ribonucleotide reductase regulation of R. meliloti is most similar to that of L. leichmannii. It is difficult to make exact comparisons between the R. meliloti reductase and those of other tissues in that most effector studies have been made over a much smaller concentration range. For example, the range of effector concentrations used in the present study was 1×10^{-8} to 1×10^{-4} M while the range reported for L. leichmannii was 1 to 8×10^{-3} M.. In most cases however the optimum effector concentration for the R. meliloti reductase was 10- to 100-fold lower than those reported for L. leichmannii. This difference was most striking in the dGTP stimulation of ADP reduction and inhibition of GDP reduction. Allosteric regulation of ribonucleotide reductase in R. meliloti was shown to differ from that of E. coli in that 1) no general inhibitor was found for the R. meliloti reductase system, 2) CDP reduction in E. coli requires ATP, while in R. meliloti CDP

reduction is stimulated by dATP, 3) whereas dTTP is the prime stimulator of GDP reduction in E. coli, it was shown to inhibit GDP reduction in R. meliloti, and 4) the reduction process in R. meliloti is most probably initiated by the reduction of GDP while in E. coli it is initiated by CDP.

The relative rates of ribonucleotide reduction in R. meliloti were dependent upon whether or not the substrates were assayed in the presence or absence of prime effectors. For example, the relative rates of GDP, ADP, and CDP reduction in the absence of effectors were 5:0.07:1, while in the presence of effectors they were 1.4:1.2:1. In L. leichmannii the relative rates of reduction for GTP, ATP, CTP, and UTP were 11:10:8:1 in the absence of effectors and were 3:3:3:1 in the presence of positive effectors.

The values for the relative rates of UDP reduction and for the allosteric studies on UDP reduction by R. meliloti ribonucleotide reductase are not available due to the inability to adequately demonstrate the formation of dUDP with the current assay prodecure. The major protion of the radioactivity from ^{14}C-UDP was distributed into multiple regions on the chromatograph. The areas were not easily identified and had considerably higher radioactivity than was found in the product region. The breakdown of substrate or possibly product, probably resulted from the repeated boiling steps used in the assay. The solution to this problem must await experiments to determine exactly what parts of the assay

procedure are responsible for the decomposition, if indeed that is what is happening.

Mg^{++} is not required for ribonucleotide reductase activity in R. meliloti but probably has an effect on the overall regulation of enzyme activity. It is evident from the data that Mg^{++}, in the absence of effectors, is a strong inhibitor of reductase activity especially at high concentrations. In L. leichmannii Mg^{++} can reverse the role of dTTP from that of an inhibitor to that of prime stimulator of GTP reduction. Preliminary data from the R. meliloti system indicates that the inhibitory effects of Mg^{++} can be reversed by dTTP. It is interesting that DNA polymerase requires Mg^{++} for activity (36, 81) while ribonucleotide reductase is inhibited by Mg^{++}. Mg^{++} may function in vivo as part of the regulatory process for ribonucleotide reduction in coordination with DNA synthesis.

In attempts to explain kinetic data in terms of protein structure various models based on subunit interaction have been proposed. The two most popular are the concerted or symmetry model of Monod and the sequential or induced-fit model of Kosland. According to Monod (71) the enzyme exists in an equilibrium between an active (R_0) and an inactive (T_0) state. Transition between these states involves concerted conformational changes among enzyme subunits with retention of symmetry. Kosland (60, 61) on the other hand has proposed a model in which binding of substrate at one subunit site can alter in a sequential manner the substrate affinity at

the other subunits without retention of symmetry.

In R. meliloti (30), as well as in E. coli (24), the in vitro substrate saturation curves are hyperbolic in the presence and absence of prime effectors and therefore the enzymes do not exhibit homotrophic co-operativity for substrate binding (24). According to both of these theories sigmoid saturation curves can only be explained in terms of multiple substrate sites and a higher affinity of substrate for the R_0 form of the enzyme as compared to the T_0 form. In a situation in which the enzyme exists predominantly in the R_0 state there can not be homotrophic co-operativity and thus no sigmoid substrate saturation curves. In the case of E. coli which is thought to contain only one catalytic site (66, 65) sigmoid curves would not be expected. For R. meliloti, in which the enzyme structure is unknown, the lack of sigmoid curves could be due to either a lack of multiple substrate sites or the existence of the enzyme in the R_0 state. Monod (71), however, has cautioned that a lack of co-operativity can also result from purification procedures. In general, due to the unique enzyme structures and complex patterns of allosteric regulation, ribonucleotide reductases do not exactly fit either the model proposed by Monod or Kosland.

An additional point along the line of enzyme structure is that reductase activity in R. meliloti could be stimulated only 2- to 3-fold by positive effectors. This lack of stimulation could also be due to the occurrence of the enzyme in

the R_O state or to an *in vitro* assay system containing too high a B_{12} concentration which would negate stimulatory capability (103).

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