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Title: Vaccinia Virus Ribonucleotide Reductase: Regulation of the Gene Products and Characterization of the Recombinant Small Subunit Protein

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Ribonucleotide reductase is a remarkable enzyme that catalyzes the rate-limiting step in the synthesis of the 2'-deoxynucleoside triphosphates. The intent of this project was to characterize the ribonucleotide reductase encoded by the orthopoxvirus, vaccinia. The first objective was to study the structural and functional features of the viral small subunit protein of ribonucleotide reductase. The viral reductase gene was engineered into an expression vector and expressed in *Escherichia coli*. The purified recombinant protein was then characterized and compared with other ribonucleotide reductase small subunits from different organisms. The physical characteristics of the vaccinia virus enzyme showed a strong similarity to the features of the mammalian counterpart.

A second aim of this project was to establish the transcriptional and translational kinetics of ribonucleotide reductase gene expression during the time course of viral infection in cultured mammalian cells. In addition, the activity and stability of the enzyme in the viral system was measured and the accumulation of ribonucleotide reductase protein was quantitated. By also quantitating the accumulation of viral DNA

synthesis, a direct comparison can be made between the the synthesis and utilization of deoxynucleotide precursors.

A third objective of this work was to detail the mechanism by which hydroxyurea inactivates the vaccinia virus ribonucleotide reductase. Visible spectroscopy and electron paramagnetic resonance spectroscopy clearly demonstrated that the inhibitor destroys the free radical moiety in the viral small subunit protein. In addition, *in vivo* studies revealed that inhibition by hydroxyurea can be circumvented during viral infection. The exogenous addition of deoxyadenosine reversed the block to viral growth that was imposed by hydroxyurea, and stabilized hydroxyurea induced deoxynucleotide pool imbalances. These inhibition studies suggest that there may be a differential sensitivity of the enzyme towards hydroxyurea in the presence of various substrates.

Vaccinia Virus Ribonucleotide Reductase: Regulation of the Gene Products and Characterization of the Recombinant Small Subunit Protein

by

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I. Introduction

One of the more significant scientific developments in the last thirty years has been the continued research towards an understanding of DNA replication. This process of reproducing one's own genetic code is essential to all living organisms, and malfunctioning of this event can result in gross cellular aberrations or cell death. Every organism evokes a distinctive mode of replication; from the simple strand displacements of characteristic prokaryotic viruses to the complicated schemes by which humans and other higher eukaryotes duplicate their DNA, each replication method reflects a different evolutionary path.

The synthesis of DNA requires a balanced supply of four precursor compounds called deoxynucleoside triphosphates (dNTP's). The supply of each dNTP needed at the replication fork for a given cell type depends not only on the metabolic state of the cell, but also on the specific deoxynucleotide base composition of the organism's DNA. The store of deoxynucleotides in a cell can be derived from either salvage processes, or from *de novo* biosynthesis. The salvage pathways function by utilizing material obtained from the degradation of RNA and DNA. Salvage enzymes, including kinases and phosphohydrolases, help regulate the flux of intracellular levels of nucleotides and deoxynucleotides through a process known as 'substrate cycling' (for a review, see Reichard, 1988). The *de novo* biosynthetic pathway produces deoxynucleotides by synthesis of nucleotides from small molecules and then subsequently by reduction of the nucleotides. This biosynthetic path is regulated by feedback inhibition and by the

allostery of two enzymes in the pathway, dCMP deaminase and ribonucleotide reductase. The combination of these two pathways must not only provide a balanced pool of each deoxynucleotide, but must regulate the overall levels of deoxyribonucleotide precursors to meet varying needs of the cell cycle-dependent replication process. In non-dividing eukaryotic cells, the basal levels of deoxynucleotides are extremely low (0.1-0.3 mM), well below the optimal level necessary for DNA replication. However, just prior to DNA synthesis, the dNTP pools increase markedly. These pool expansions are an essential requirement for DNA synthesis during the S-phase of the cell cycle (Reichard, 1988). Most organisms coordinately regulate deoxynucleotide production and initiation of DNA replication. However, the means by which this feat is accomplished is not yet fully understood.

Imbalances in deoxynucleotide pools can have drastic effects on cellular processes (reviewed by Weinberg et al., 1981). Pool biases can be caused by malfunctioning or mutation of enzymes involved in maintaining balanced pools or may be caused by severe metabolic changes within a cell. These imbalances not only lower the fidelity of DNA synthesis, but also impair the important role of DNA repair in preventing DNA strand breakage and base mispairing (Meuth, 1989; Kunz and Kohalmi, 1991).

The rate-limiting enzyme involved in *de novo* biosynthesis of deoxynucleotides is ribonucleotide reductase (Thelander and Reichard, 1979). Figure I.1 details the central role of ribonucleotide reductase in the deoxynucleotide metabolism of *E. coli* and higher eukaryotes. Reduction by ribonucleotide reductase is the first committed step in deoxynucleoside triphosphate synthesis. This complex enzyme catalyzes the reduction of the 2' hydroxyl group on the ribose moiety of precursor nucleoside diphosphates (or triphosphates) to form the corresponding 2'-deoxynucleoside 5'-diphosphate (or triphosphate) products. The enzyme irreversibly reduces the four

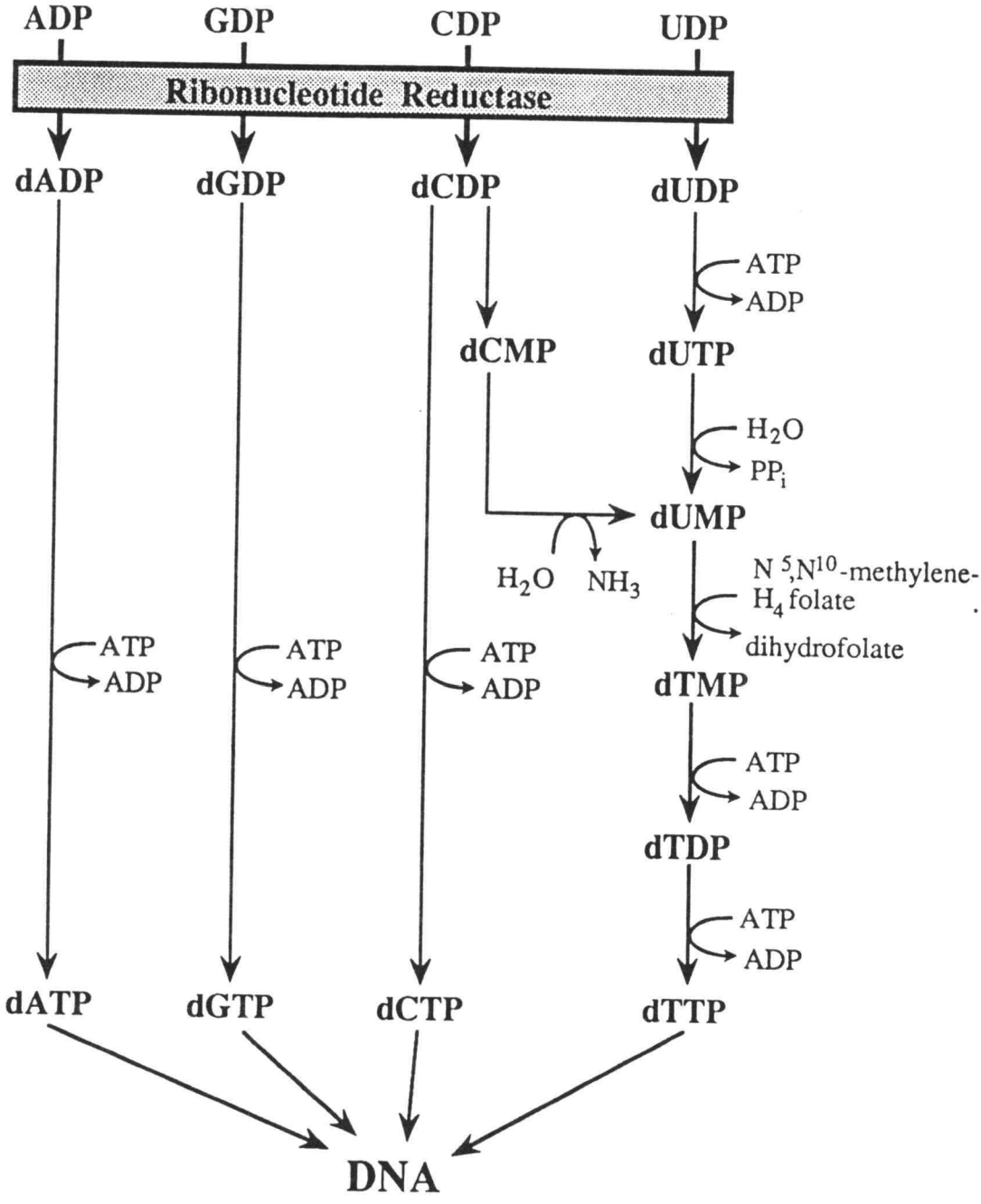

Figure I.1 Deoxynucleotide Biosynthesis

common nucleotides: adenosine diphosphate, guanosine diphosphate, uridine diphosphate and cytidine diphosphate. Because of the critical role of this enzyme reaction, this enzyme and its regulation has been the center of much important and onging research. The complex regulation of this metabolic step has been well studied in terms of both the amounts of enzyme activity during the cell life cycle (reviewed by Wright, 1989) and the allosteric effectors for the enzyme catalyzing the reaction (reviewed by Eriksson and Sjöberg, 1987). Furthermore, the central role of ribonucleotide reductase has made it an attractive target for the design of antineoplastic and antiviral agents (Moore and Hurlbert., 1985; Spector *et al.*, 1987). Selection of mutant forms of ribonucleotide reductase has led to a better understanding of deoxynucleotide pool imbalances and how these imbalances affect DNA synthesis. Studies examining the structural organization of ribonucleotide reductase and other enzymes involved in precursor synthesis and replication have led to a better understanding of how these two events function in concert (recently reviewed by Mathews, 1993).

This dissertation is an attempt to further characterize the ribonucleotide reductase enzyme from a large eukaryotic DNA virus. Vaccinia virus encodes many of the enzymes necessary for replication (including ribonucleotide reductase) and synthesizes its own DNA entirely within the host's cytoplasm. In addition, the virus utilizes *de novo* DNA synthesis for virion particle formation as host cell DNA synthesis is inhibited during infection (Jungwirth and Launer, 1968), and host cell nuclear DNA is made unavailable to the virus (Parkhurst *et al.*, 1973). For these reasons, and because the virus is well defined genetically and is easily manipulated, vaccinia is an ideal system with which to study the reductase enzyme and viral replication.

Since the virus infects mammalian hosts and viral enzymes share much sequence homolgy with their mammalian enzyme counterparts, it seemed plausible that

the vaccinia ribonucleotide reductase would share many biochemical characteristics with its host. Work described in this dissertation along with initial studies of the viral enzyme confirm this hypothesis. The earliest studies with the vaccinia virus ribonucleotide reductase characterized the requirements necessary for enzyme activity in virus-infected cells extracts and demonstrated that the viral enzyme is allosterically regulated in a manner similar to the mammalian and *E. coli* enzyme (Slabaugh *et al.*, 1984). In further studies, the locations of the two genes which encode the viral reductase were identified, and the genes were sequenced (Slabaugh *et al.*, 1988; Schmitt and Stunnenberg, 1988; Tengelson *et al.*, 1988). Northern blot analysis revealed that both genes are actively transcribed during a viral infection (Roseman and Slabaugh, 1990; Tengelson *et al.*, 1988). Concurrent with the work described herein, Slabaugh has pursued the purification and characterization of the recombinant large subunit protein of ribonucleotide reductase[1].

One incentive of this work is to provide a level of understanding and information about the viral enzyme which will readily lend itself to further structural and functional studies. To this end, this dissertation describes cloning of the small subunit gene of vaccinia virus ribonucleotide reductase into a bacterial expression system and characterization of the recombinant gene product. Because both subunits of vaccinia virus ribonucleotide reductase are now available in large quantities and in purified form, many interesting kinetic and physical association studies are now possible. Potential experiments for further study of this, and other work, are discussed in the conclusion chapter of this work. Another major goal of this dissertation is to compare the supply of deoxynucleotide precursors made available through the activity of the viral ribonucleotide reductase with the biosynthesis and accumulation of viral DNA. To accomplish this, the kinetics of ribonucleotide reductase expression are

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[1] M. Slabaugh, manuscript in preparation.

examined and the amounts of viral enzyme and viral genomic DNA accumulation are quantitatively determined. The third motivation for this work is to aid in the study of the mechanism of action of the ribonucleotide reductase inhibitor, hydroxyurea. To this end, we use purified vaccinia virus small subunit to spectrophotometrically examine enzyme inhibition by hydroxyurea. We also describe conditions (additions to the tissue culture medium) that allow for circumvention of hydroxyurea inhibition in the viral system. *In vivo* experiments demonstrate that deoxynucleotide pool imbalances are induced by hydroxyurea treatment and a proposal is put forth for the biochemical mechanism of hydroxyurea circumvention under these conditions.

The first section of this dissertation summarizes initial studies and recent work with the enzyme ribonucleotide reductase from various organisms. Special attention is given to current hypotheses on enzyme activation and formation of the tyrosyl radical in the small subunit protein. The literature review also describes the vaccinia virus system in terms of the viral lifecycle, expression of viral genes, and early work to characterize viral replication. The experimental results are described in three manuscripts. The first manuscript characterizes the recombinant small subunit protein of ribonucleotide reductase. A second manuscript compares regulation of ribonucleotide reductase with viral DNA synthesis. The third manuscript details circumvention of hydroxyurea inhibition during viral infection. Because of this author's more limited involvement in publication of the third work, the manuscript describing the hydroxyurea studies is presented in the appendix. Finally, the relevance of these studies is discussed, and specific conclusions are put forth.

II. Review of Literature

The intention of this literature review is to acquaint the reader with the current emphases in the study of ribonucleotide reductase. The elaborate and thorough investigations of this enzyme and its metabolic role convey its importance in biochemistry. Special attention is given to studies which characterize the small subunit polypeptide of the enzyme, because these specifically pertain to the work in this dissertation. Many exciting discoveries have recently been made in the area of cell cycle coordination. Ribonucleotide reductase is one of several eukaryotic enzymes that is regulated by these cellular events. The second section of this literature review is a survey of recent work describing ribonucleotide reductase gene and gene product expression and ribonucleotide reductase protein synthesis. Finally, this review describes the organism of study, vaccinia virus. The lifecycle of the virus is described and viral gene expression and replication are summarized. This review also outlines preliminary characterizations of ribonucleotide reductase in this viral organism.

II.1 Ribonucleotide Reductase

II.1.1 Different Classes of the Enzyme

Work in the ribonucleotide reductase field has recently accelerated to a vigorous rate. In the last ten years, two new forms of the enzyme have been described. This brings the total of distinct classes of ribonucleotide reductase to four. Because of the variability in the specific requirements for deoxynucleotide synthesis among different organisms, it is not suprising that several different types of enzyme have evolved to catalyze this critical reaction. All classes of reductase catalyze a reaction which involves

an unpaired electron or free radical species. The various classes of the enzyme can be distinguished by how the source of radical species is generated (Table II.1). The radical species is absolutely required for catalysis and plays a crucial role in the first step of the reaction mechanism.

Much of the early work characterized the first class of enzyme from organisms as diverse as *E. coli*, T4 bacteriophage, herpes simplex virus, vaccinia virus, yeast, clam, and mammalian and higher plant species. This first class of ribonucleotide reductase employs a conserved tyrosyl residue and a binuclear ferric center to generate a stable free radical species. The stability of the radical allows for its detection by electron paramagnetic resonance (EPR) spectroscopy and UV/visible spectroscopy. In this class of ribonucleotide reductase, the holoenzyme is composed of two nonidentical homodimer polypeptides. The smaller of these subunits contains the tyrosyl residue and the iron center. The larger subunit contains binding sites for the nucleoside diphosphate substrates and for allosteric effectors.

A second class of ribonucleotide reductase, characterized by several Gram positive bacteria, substitutes manganese for iron in forming a binuclear manganese center (Willing *et al.*, 1988a). This group of enzymes also generate a stable radical species at the conserved tyrosyl residue in the smaller subunit, but are insensitive to the negative allosteric effector of the class I reductases (Willing *et al.*, 1988b). In addition, these reductases are able to convert both nucleoside diphosphate and nucleoside triphosphate substrates to the corresponding deoxynucleotide product (Willing *et al.*, 1988a).

The ribonucleotide reductase from *L. leichmannii* is the prototype for the class of enzyme that uses adenosylcobalamin (AdoCbl) as a dissociable cofactor. This enzyme class includes both prokaryote and eukaryote species and the holoenzyme consists of only a single polypeptide. The reaction catalyzed by this enzyme is

Table II.1 Different Classes of Ribonucleotide Reductase

Class of Ribonucleotide Reductase	Source of Radical Species	Substrate
I. *Escherichia coli* mammal higher plant virus	Fe^{3+}, R2 subunit	NDP's
II. gram positive bacterium: *Brevibacterium ammoniagenes* *Micrococcus luteus* arthrobacter	Mn^{2+}, R2 subunit	NDP's, NTP's
III. *L. leichmannii* *Corynebacterium nephridii* *Thermus aquaticus* *Rhizobium leguminosarum* *Euglena gracilis*	Ado-Cbl, radical chain initiator	NTP's
IV. *Methanobacterium thermoautotrophicum* anaerobically grown *Escherichia coli*	S-AdoMet, ?? radical chain initiator ??	NTP's

restricted to the conversion of ribonucleoside triphosphates to deoxyribonucleoside triphosphates. In a reaction mechanism common to other AdoCbl-requiring enzymes, a homolytic cleavage of the carbon-cobalt bond results in formation of a 5'-deoxyadenosyl radical (Stubbe *et al.*, 1983). The radical species then performs the function of chain initiator to start catalysis (Ashley *et al.*, 1986).

The most recently characterized class of reductase includes the anaerobic methanogen, *Methanobacterium thermoautotrophicum*, and an *E. coli* form of the enzyme that is oxygen-sensitive and active only during anaerobic growth (Fontecave *et al.*, 1989; Hogenkamp *et al.*, 1987). This group of enzymes is thought to utilize S-adenosylmethionine (AdoMet) and a metal to generate a radical chain initiator (Eliasson *et al.*, 1990). The specific mechanism that generates this radical species is not yet understood. These enzymes are also restricted to the catalysis of the reduction of ribonucleoside triphosphates (Eliasson *et al.*, 1990).

II.1.2 Class I Ribonucleotide Reductase Holoenzyme

The best-characterized ribonucleotide reductase is the enzyme from *E. coli*. In 1990, the three-dimensional structure of the *E. coli* small subunit protein was published in detail to 2.2 Å (Nordlund *et al.*, 1990). Preliminary X-ray diffraction studies have resolved the large subunit dimer to 18-Å resolution (Ribi et al. 1987). This crystallographic work, as well as binding and kinetic studies and mutation analysis, have led to the proposed model of the *E. coli* holoenzyme shown in Figure II.1. This section describes that model and makes comparisons between the *E. coli* enzyme and other class I ribonucleotide reductases.

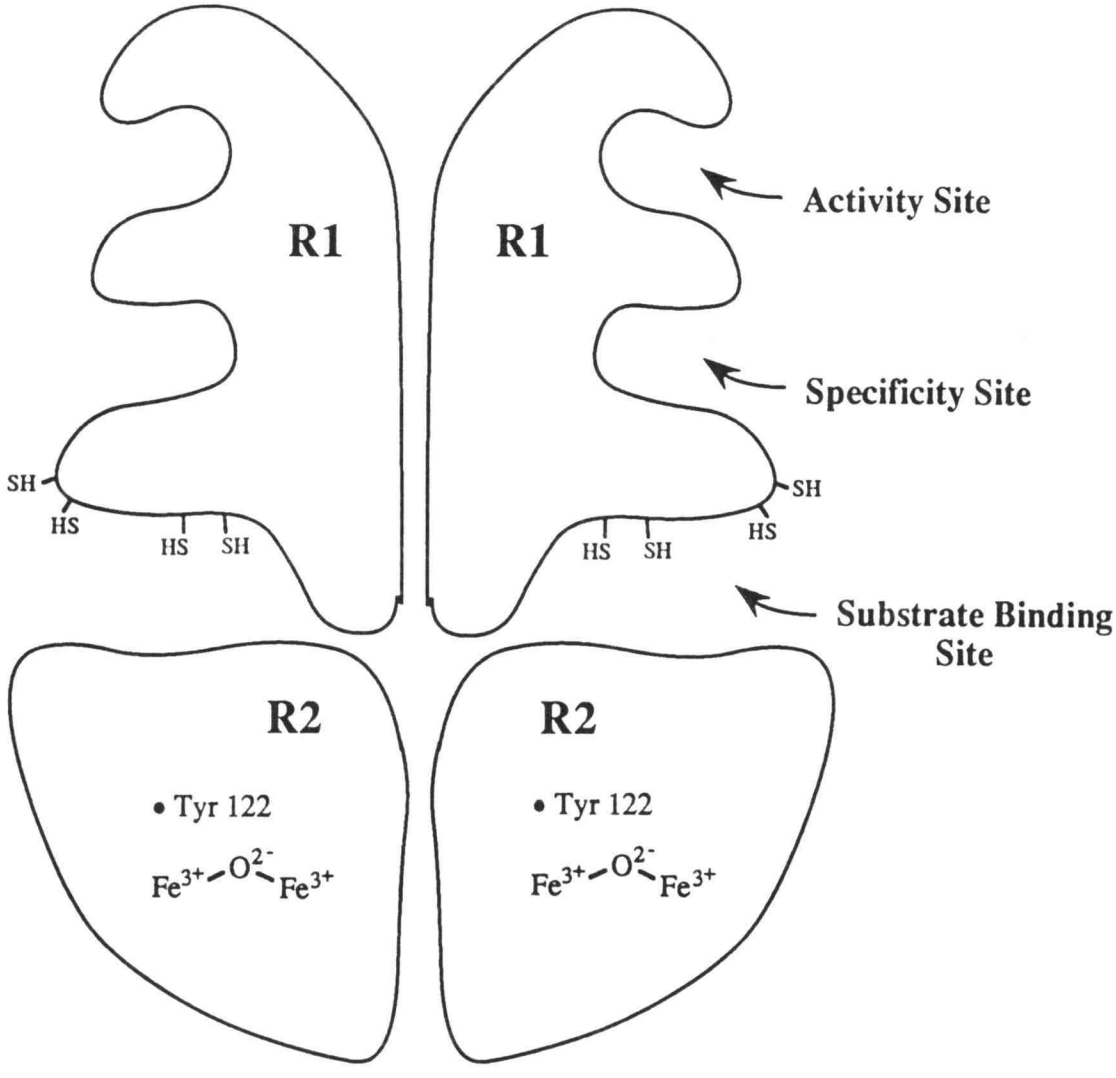

Figure II.1 Model of the Ribonucleotide Reductase Holoenzyme from *E. coli*

II.1.2.1 Large Subunits

As the model indicates, the larger subunit dimer contains three types of binding sites: activity sites, specificity sites, and substrate binding or 'active' sites. The physical model of the large subunit differs for mammalian reductase in that only one activity and specificity site was detected from Scatchard analysis of equilibrium dialysis studies for each large subunit dimer (Thelander *et al.*, 1980). The activity and specificity sites both confer allosteric regulation on the enzyme by binding positive and negative effectors. This control helps maintain a supply of precursor products commensurate to the needs for DNA synthesis. The complexities of the enzymes' allostery were uncovered and clarified by two Swedish laboratories under the direction of Reichard and Thelander. Initial studies by Reichard determined that in the absence of effector, the enzyme exhibits a very low turnover number (Larsson and Reichard, 1966a). Further studies demonstrated that the activity sites bind ATP and dATP and regulate the overall activity of the enzyme (Larsson and Reichard, 1966b). In the presence of dATP the enzyme is inactive. Only when ATP is bound at the activity site is the enzyme capable of substrate catalysis. Moreover, it was determined that binding and reduction of substrate requires the presence of additional effectors at the specificity sites (Larsson and Reichard, 1966a). The specificity sites bind ATP, dATP, dGTP, and dTTP. It was demonstrated that binding of a specific effector to these sites results in a conformational change at the active site which leads to preferential binding of a particular substrate (Brown and Reichard, 1969). It was subsequently established that the conformational shift results in a decrease of the K_M and an increase of the k_{cat} at the active site for the corresponding substrate (von Döbeln and Reichard, 1976).

Equilibrium dialysis studies by von Döbeln and Reichard (1976) also revealed that dATP binds to both specificity and activity sites but with different affinities. dATP binds with high affinity (K_d = 0.03 μM) to the specificity site to enhance the binding of

the substrates CDP and UDP. However, at higher concentrations of dATP, the low-affinity dATP activity sites, (K_d = 0.1-0.5 μM), become filled and this inhibits overall activity.

Determining the mechanism for dATP inhibition of enzyme activity was also the basis for a another series of studies which examined peptide interactions of the enzyme subunits (Brown and Reichard, 1969a; Thelander, 1980). Sucrose gradient sedimentation of the *E. coli* enzyme demonstrated that the holoenzyme changed into a heavier complex when inhibiting concentrations of dATP (above the dissociation constant for activity site binding) were added to the enzyme preparation (Brown and Reichard, 1969a). Isolation and analysis of these heavy complexes established that an inactive tetrameric form of the enzyme (two small subunit dimers and two large subunit dimers) was formed after dATP addition. Analytical ultracentrifugation of calf thymus reductase also demonstrated that the enzyme complexes into multimeric forms after addition of inhibiting concentrations of dATP (Thelander *et al.*, 1980). However, in this case, only the large subunit formed a tetrameric species. As competing amounts of ATP (positive effector) were added to the solution, the ratio of dimeric to tetrameric species changed accordingly. Taken together, these results suggest that dATP inactivates the enzyme by inducing conformational changes in the subunits that allow for these increased peptide interactions.

The conformational states of the *E. coli* ribonucleotide reductase in the presence of dATP and other effectors are outlined in Table II.2. The enzyme reduces both purine and pyrimidine substrates at the same rate in the presence of appropriate effectors. Substrate saturation curves show that the enzyme reaction displays hyperbolic kinetics in the presence or absence of effectors (Larsson and Reichard, 1966a). This demonstrates that there is no cooperativity for substrate binding. The system of allostery and feedback inhibition was first studied in 1966 by Larsson and

Table II.2 Allostery of Class I Ribonucleotide Reductases

Nucleotide Bound in			
Activity Site	Specificity Site	Activates Reduction of	Inhibits Reduction of
ATP	ATP , dATP	CDP , UDP	
ATP	dTTP	GDP	CDP , UDP
ATP	dGTP	ADP	CDP , UDP
dATP	any effector		ADP , GDP CDP , UDP

Reichard for the *E. coli* enzyme (Larsson and Reichard 1966a; Larsson and Reichard 1966b). Kinetic and photoaffinity studies with mammalian enzyme have shown an allosteric regulation pattern similar to the *E. coli* model (Eriksson *et al.*, 1979; Thelander *et al.*, 1980; Eriksson *et al.*, 1982; Caras *et al.*, 1982). In an intriguing discovery, the enzyme encoded by herpes simplex virus, which is also a class I-type enzyme, was found to contain neither type of effector binding site (Averett *et al.*, 1983). The activity of the herpes virus enzyme is constitutive.

The third type of binding site on the large subunit is at the site of catalysis. The four nucleoside diphosphate substrates compete for two substrate binding sites located on the large subunit (von Döbeln and Reichard, 1972; Thelander *et al.*, 1980). In addition to substrate binding, the active site also contains two sets of redox-active sulfhydryl groups, at or near the active sites. One set of thiol groups is directly involved in catalysis and stoichiometrically reduces substrate (Thelander, 1974). The second set of thiols, which is located near the active site, transfers the reducing equivalents to the active site from an electron transfer system on the outside of the enzyme. Evidence for the dual role of the thiol groups and the location of both thiol groups came from site-directed mutagenesis studies in which successive conserved cysteinyl residues were mutated (Lin *et al.*, 1987; Åberg *et al.*, 1989). These studies established that Cys-462 and Cys-225 were the thiols that participate at the active site and that two carboxyl cysteines, Cys-754 and Cys-759 are involved in transfer of reducing equivalents from the hydrogen donor systems.

II.1.2.2 Electron Transfer Systems

Concomitant with each reduction of NDP to dNDP is the net oxidation of one pair of redox active thiols. Reduction of the cystine disulfides to regenerate cysteine thiol groups is accomplished by either one of two electron transfer systems outlined in

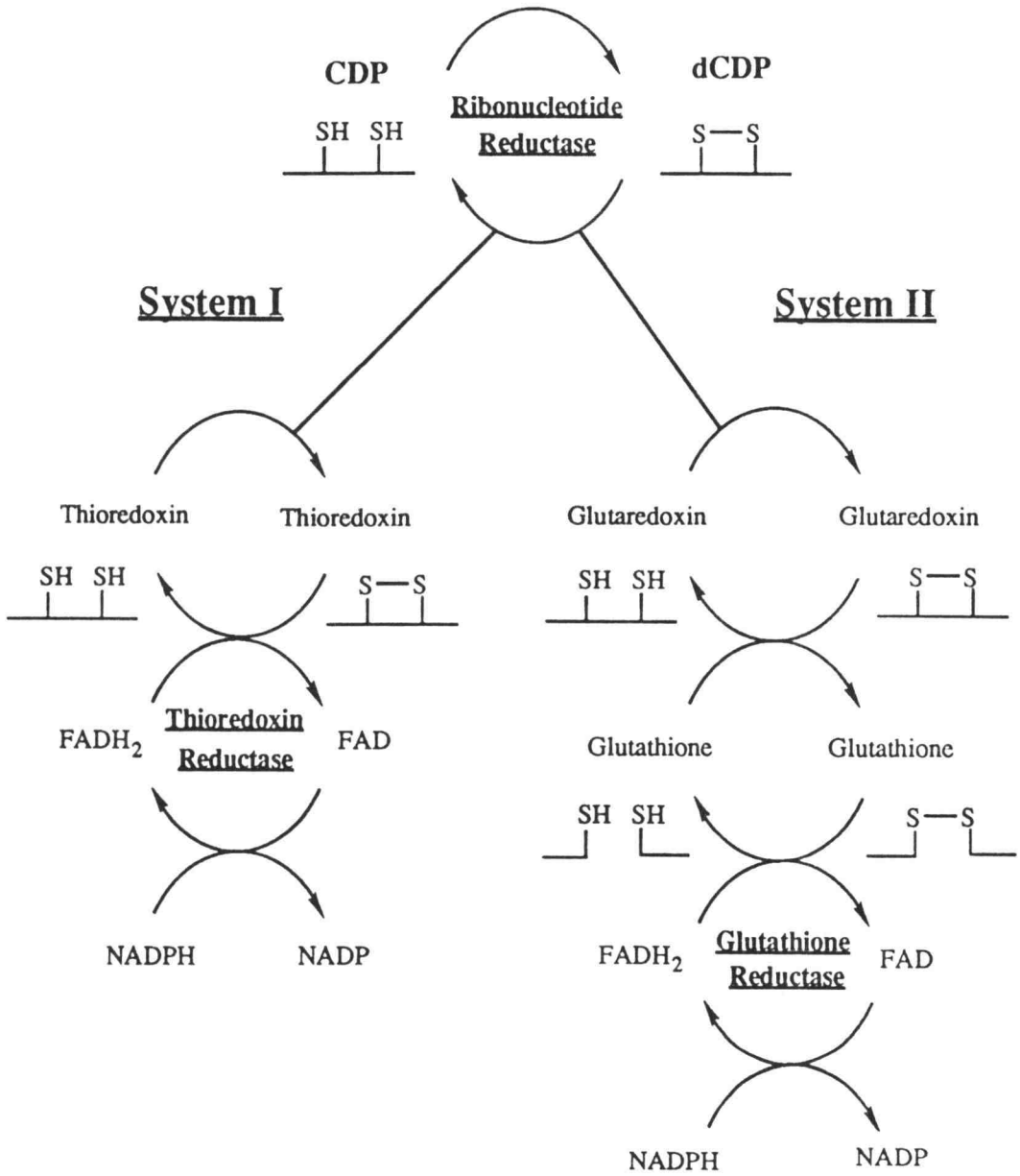

Figure II.2 Biological Reduction of Large Subunit Disulfides

Figure II.2. In the first system, reviewed by Holmgren (1985), a reduced form of the small protein thioredoxin, which itself contains a set of redox active thiols, serves as an efficient hydrogen donor for the disulfide groups on the outside of the enzyme. Thioredoxin is eventually reduced in the cell by NAD(P)H through the action of the flavoprotein, thioredoxin reductase. Thioredoxin operates by a ping-pong mechanism for both thioredoxin reductase and ribonucleotide reductase (Thelander, 1974). Thioredoxin was first characterized as the physiological electron donor for ribonucleotide reductase from *E. coli* (Laurent *et al.*, 1964). Analysis of thioredoxin mutants, however, led to the discovery of a second bacterial reducing system from the protein, glutaredoxin (Holmgren, 1976). The reduced form of glutaredoxin, another small protein which contains redox active thiols, is likewise able to reduce the ribonucleotide reductase disulfide. Glutathione, which is supplied via glutathione reductase and NADPH, then reduces the oxidized glutaredoxin. Glutaredoxin and thioredoxin have both been identified in mammalian cell extracts (Luthman and Holmgren, 1982). The principal hydrogen donor for the type-1 herpes simplex virus-encoded ribonucleotide reductase has been identified as the cellular thioredoxin (Darling, 1988). It has been demonstrated that bacteriophage T4 encodes and expresses its own thioredoxin (Berglund and Sjöberg, 1970), and that vaccinia virus contains an open reading frame with homology (40%) to the eukaryotic glutaredoxin (Goebel *et al.*, 1990). In fact, after overexpression of the putative vaccinia virus glutaredoxin, a 30-kDa protein was purified and it has been demonstrated that this viral protein contains the reducing activities of a *bona fide* glutaredoxin[2].

The overlapping functions of thioredoxin and glutaredoxin have made it very difficult to determine which system is primarily used *in vivo* for reduction of ribonucleotide reductase in *E. coli* and mammalian cells (Luthman *et al.*, 1979). *In*

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[2] W. Thresher, manuscript in preparation.

vitro studies with *E. coli* extracts have demonstrated that glutaredoxin has a ten-fold higher turnover number for ribonucleotide reductase than thioredoxin (Holmgren, 1979). However, thioredoxin is typically 50-100 times more abundant in logarithmically dividing bacterial cells than glutaredoxin (Holmgren, 1979). Deletion analysis of components from either reducing system was unable to establish the primary hydrogen donor (Kren *et al.*, 1988; Russell and Holmgren, 1988). Interestingly, after deletion of components from both systems at the same time, deoxynucleotide synthesis was still active (Russell and Holmgren, 1988). This new evidence suggests there may be a third unknown hydrogen donor for ribonucleotide reductase. The genes for the bacterial thioredoxin and glutaredoxin have been cloned and the recombinant proteins have been characterized (Holmgren, 1985; Wallace and Kushner, 1984). Further deletion analysis and kinetic studies will, one hopes, distinguish the roles of these proteins towards ribonucleotide reductase.

For *in vitro* studies of ribonucleotide reductase, the glutaredoxin and thioredoxin reducing systems are often replaced by the chemical reductants, dithiothreitol or ß-mercaptoethanol. The purified reductase enzyme, however, requires a dithiol reductant.

II.1.2.3 Small subunits

The small subunit dimers of class I ribonucleotide reductases contain two covalently bound binuclear centers which act as cofactors. Biophysical methods were used to elucidate the nature and location of these iron centers. X-ray diffraction studies on a radical-free diferric form of R2 established that the iron centers are buried 10 Å from the surface of the polypeptide and 5.3 Å away from the tyrosyl radical residue (Nordlund *et al.*, 1990). As shown in Figure II.3, the iron centers are composed of two high spin iron atoms in the 3+ oxidation state that are antiferromagnetically coupled

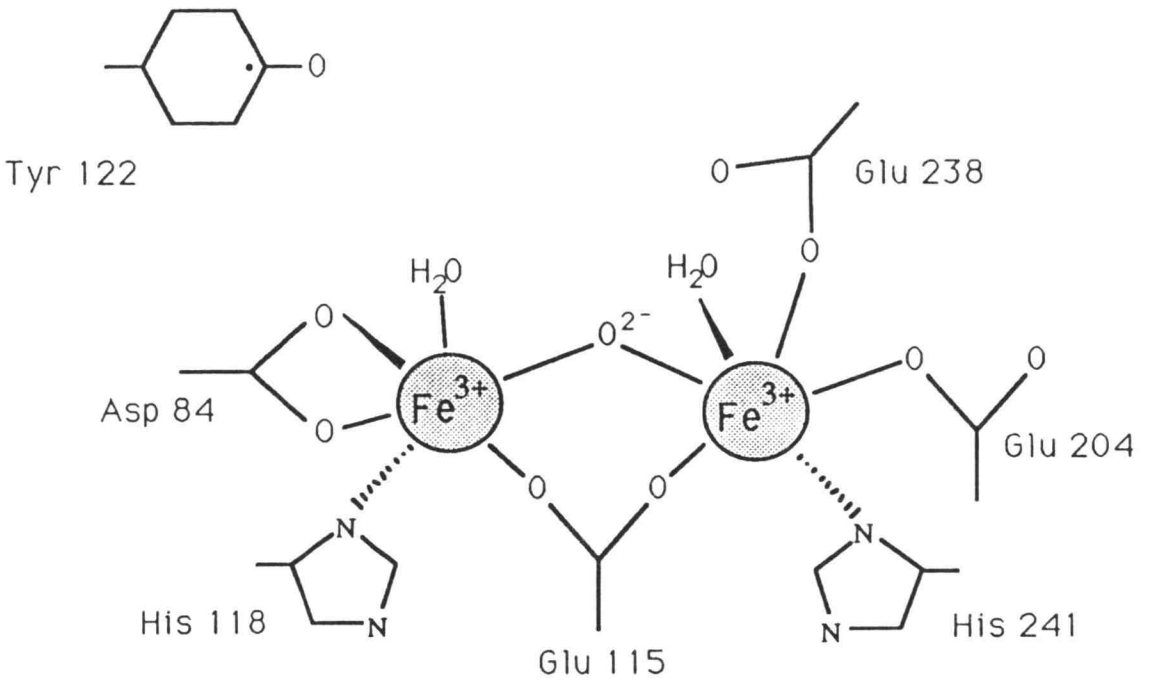


Figure II.3 Spatial Arrangement of the Binuclear Iron Center in the *E. coli* Small Subunit Protein

through a μ-oxo bridge. The iron cluster is covalently stabilized by histidine, glutamine, and aspartic acid residues of the R2 protein in addition to H_2O molecules. Early Mössbauer and magnetic susceptibility measurements on the *E. coli* protein revealed that there was a great deal of similarity between the iron center from ribonucleotide reductase, and the iron center from the well-characterized oxygen-transporting protein, hemerythrin (Atkins *et al.*, 1973; Peterson *et al.*, 1980). These techniques also established the oxidation and spin states of the iron atoms and the degree of antiferromagnetic coupling in the iron centers. The structure of the μ-oxo bridge was identified by resonance Raman spectroscopy (Sjöberg *et al.*, 1982). In addition, Raman studies were used to compare the Fe-O-Fe symmetric stretching vibrations of the small subunit protein with a model compound which also contained a μ-oxo bridge (Backes *et al.*, 1989). After addition of $H_2{}^{18}O$ to the reductase preparation, a characteristic shift in the Raman spectrum (due to exchange of ^{18}O) was seen that was consistent with the assignment of the bridge structure. Finally, X-ray absorption field spectroscopy (EXAFS), resonance Raman, and X-ray diffraction studies revealed the length and angle of the Fe-O-Fe bridge and identified ligands surrounding the iron center (Scarrow *et al.*, 1986; Backes *et al.*, 1989; Bunker *et al.*, 1987).

Involvement of a free radical species in the enzyme reaction was confirmed in 1972 by detection of an electronic signal using EPR spectroscopy (Ehrenberg and Reichard, 1972). The radical was unequivocally localized to the conserved Tyr-122 residue of the *E. coli* small subunit protein by analyzing a site-directed mutant in which the tyrosine residue was converted to a phenylalanine (Larsson and Sjöberg, 1986). The mutant Phe-122 enzyme lacked both tyrosyl radical and enzyme activity. Electron nuclear double resonance (ENDOR) and resonance Raman studies established that the tyrosyl residue forms a phenoxy radical and that the radical species is not protonated as

was earlier believed (Bender *et al.*, 1989; Backes *et al.*, 1989). Amino acid sequence homology of various small subunit proteins revealed that the location of the tyrosyl residue that forms the radical is strictly conserved in class I reductases.

Figure II.4 shows the EPR spectra of small subunit proteins from *E. coli*, mouse and herpes simplex virus. The radical EPR signal is a doublet that is centered at g = 2.0047. As the figure details, the hyperfine splitting of the radicals differ among the protein preparations. Differences also exist among these various radical proteins in the power dependence for microwave saturation of the radicals. These differences reflect variations in orientation of the ß-CH_2 group with respect to the tyrosine ring (Gräslund *et al.*, 1982) and the degree of magnetic interaction between the tyrosyl radical and the binuclear iron center (Sahlin *et al.*, 1987). The radical from the mouse enzyme appears, from these results, to be in closer association with the iron center than the radical from *E. coli*. The stoichiometry of the iron/radical cluster and the mechanism for its activation will be discussed in a subsequent section.

II.1.3 Proposed Enzyme Mechanism

Through the use of chemical modeling studies, a hypothetical mechanism has been proposed for the 2' reduction of nucleotides by ribonucleotide reductase (reviewed by Stubbe, 1990). The proposed mechanism is shown in Figure II.5. The first step in the reaction is a protein-mediated hydrogen abstraction from the 3' carbon of the ribose moiety, which produces a 3' nucleotide radical (Panels A and B). Next, a H_2O molecule is lost through the facilitating action of a general acid catalysis (Panel C). The acid catalysis in this step is postulated to involve one of the redox active thiol groups on the large subunit. Cleavage of the H_2O molecule, therefore, results in the formation of a thiolate and a thiol within the active site. H_2O loss also results in the formation of a formyl methyl radical intermediate (Panel D) which displays an equilibrium between

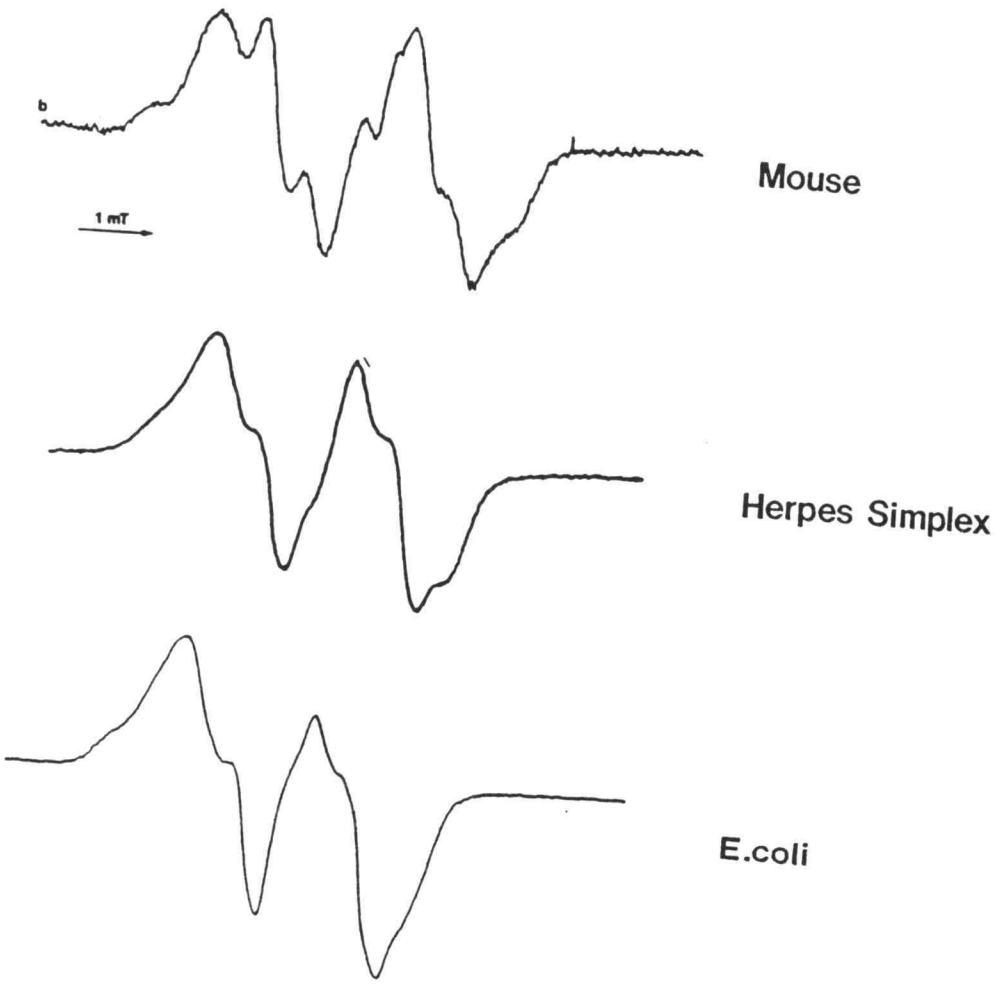


Figure II.4 Electron Paramagnetic Resonance Spectra of Various R2 Proteins

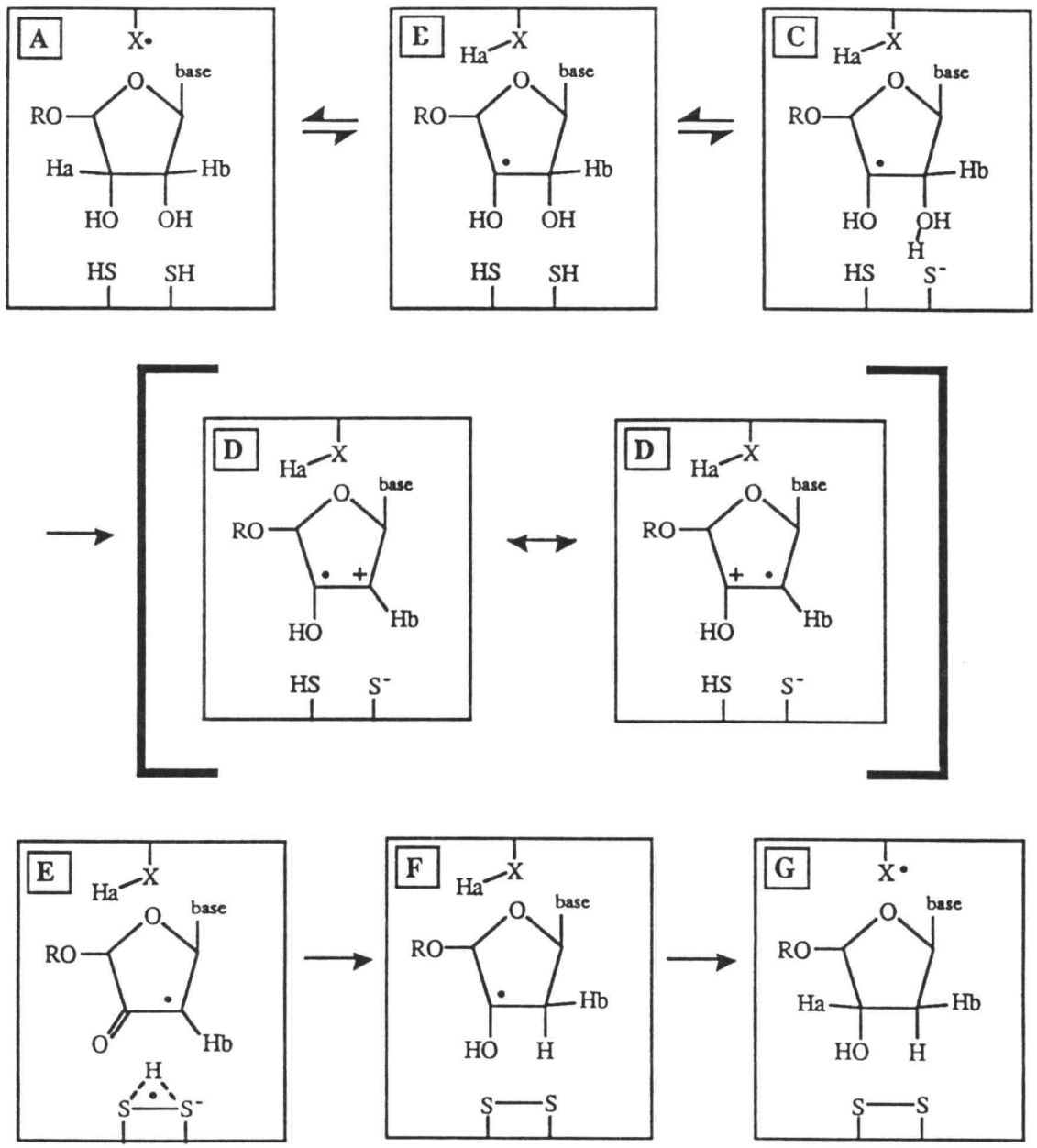


Figure II.5 Proposed Reaction Mechanism of Ribonucleotide Reductase

radical formation at the 2' and 3' ribose carbons. In the next step of the reaction (Panel E), reduction of the formyl methyl radical through an electron transfer from the thiolate produces a 3' ketone deoxynucleotide and a disulfide radical anion . This latter species mediates a one electron reduction of the 3' ketone to produce a 3-deoxynucleotide radical (Panel F). This intermediate then removes a hydrogen atom from the protein residue to regenerate the protein radical (Panel G).

Through the efforts of several laboratories utilizing isotopically-labeled substrates, substrate analogs, and site-directed mutants, support for Stubbe's model and the direct involvement of radical intermediates has been made. Studies using [3' -^{3}H or -^{2}H] nucleoside diphosphate substrates established that the hydrogen abstracted from the 3' carbon of the ribose moiety is returned to the same position during the final step of product formation (Stubbe *et al.*, 1983). Using the mechanism-based inhibitor, 2'-chloro-2'-deoxyuridine 5'-diphosphate (Cl-UDP), researchers demonstrated the formation of a 3'-keto-dUDP intermediate (Harris *et al.*, 1984; Ator and Stubbe, 1985; Salowe *et al.*, 1987). (The intermediate was subsequently shown to produce a highly reactive furanone which led to enzyme inactivation.) Another substrate analog, 2'-azido-2' deoxyuridine 5'-diphosphate (N_3UDP), was used in further studies and revealed the ability of the enzyme to mediate a radical-dependent reaction (Salowe *et al.*, 1987; Sjöberg *et al.*, 1983). In this reaction, one equivalent of N_3UDP inactivated the enzyme, producing one equivalent each of N_2, PP_i, and sugar. More than 90% of the tyrosyl radical was destroyed, and a new nitrogen-centered radical was formed from the N_3 moiety of N_3UDP.

Although the tyrosyl radical is absolutely required for activity, there had been much debate about whether the radical acted directly during catalysis or through some form of electron transfer. The crystallographic studies established that the iron center and radical are buried in the small subunit, and therefore excluded mechanisms

involving direct transfer of electrons (Nordlund *et al.*, 1990). The current theory about how electrons are transfered from the radical to the catalytic site is through an electron tunneling mechanism. Electron tunneling is the phenomenon of long distance (>5 Å) electron transfer through a protein, by interacting networks of chemical bonds that form a physical tunnel for electrons (Beratan *et al.*, 1991). In order to identify target residues for electron transfer (the end of the tunnel), site-directed mutagenesis studies have been undertaken (Mao *et al.*, 1989; Åberg *et al.*, 1989).

II.1.4 Iron Center/ Radical Activation

Another intriguing aspect of this complicated enzyme is the mechanism of assembly of the iron center and generation of the radical species. Although several other binuclear iron cluster proteins have been described (Que *et al.*, 1990; Sanders-Loehr, 1988; Fox *et al.*, 1988), only ribonucleotide reductase uses the iron center for a one-electron oxidation that generates a stabilized free radical. This section describes the stoichiometry of this reaction and the components that are necessary *in vivo* and *in vitro* to generate the iron/radical cluster. Models for the oxidative states of the cluster and the mechanism of activation are also discussed.

Recent evidence from plasma emission spectroscopy and X-ray diffraction has demonstrated that one binuclear iron center is generated per protomer and that one tyrosyl radical could potentially be formed per R2 peptide (Lynch *et al.*, 1989; Nordlund *et al.*, 1990). Once the tyrosyl radical is formed, however, the concentration of radical can fluctuate in the cell in response to the cell's growth cycle (Barlow *et al.*, 1983). This finding first suggested that cellular mechanisms are available *in vivo* to reduce and regenerate the radical. By fractionating *E. coli* extract with column chromatography, it was established that three protein components, Fe^{2+}, and O_2 participate *in vivo* to generate the tyrosyl radical (Fontecave *et al.*, 1987a). The enzyme

NAD(P)H:flavin oxidoreductase was identified as one of the protein components, and was shown to reduce both ferric ions to ferrous. These ferrous ions then self-assembled into the active center in the presence of O_2 and reductant. In addition, superoxide dismutase was found to be a required component during radical regeneration, and was shown to prevent the formation of superoxide anion (O_2^-) from reduced flavin oxidation (Fontecave *et al.*, 1987b). The third peptide component of the *E. coli* extract that was necessary for generation of the active center has not yet been identified (Eliasson *et al.*, 1986).

For *in vitro* systems, addition of Fe^{2+}, in the presence of O_2, to the reduced form of the enzyme (without the metal) led spontaneously to the reassembly of the active binuclear iron/radical cluster (Atkin *et al.*, 1974). The stoichiometry of this reaction was recently resolved for both the bacterial and mammalian enzymes (Elgren *et al.*, 1991; Ochiai *et al.*, 1990). Elgren and co-workers determined the balanced equation for O_2 activation of the bacterial R2 protein by simultaneously using an oxygraph to quantify oxygen consumption, EPR spectroscopy to measure tyrosyl radical content, and Mössbauer spectroscopy to determine the extent of Fe oxidation (Elgren *et al.*, 1991). Figure II.6 shows the chemical equation that was derived from these experiments. Analogous experiments on the mouse R2 protein revealed a similar stoichiometry (Ochiai *et al.*, 1990). In the equation, four reducing equivalents are necessary to form active protein; two of these electrons are provided by reduction of the diferrous center to a diferric center, and a third electron is donated by the Tyr-122. The source of the fourth electron *in vivo* has been intensely debated. One possible source, derived from the R2 protein itself, is discussed below.

Other than the inactive R2, which lacks both metal and radical, and the fully activated R2, two additional oxidative states for the R2 protein have been identified. Characterization of these states has led to a proposal for how the iron/radical center is

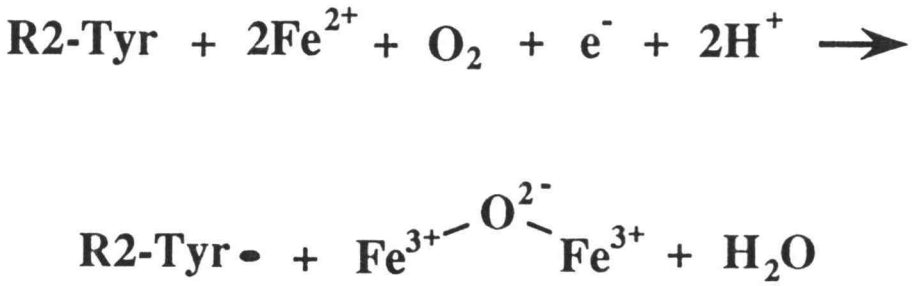


Figure II.6 Stoichiometry of Iron Center Activation

generated (Figure II.7). The first of the depicted subunit states, the inactive apo- form, lacks an iron center and contains a nonradical tyrosyl residue. Addition of two equimolar equivalents of ferrous ion under anaerobic conditions converts the apo- form of the enzyme into the $R2_{red}$ state. The $R2_{red}$ subunit contains a unique binuclear ferrous species, but no tyrosyl radical. In mammalian cells, admission of air spontaneously converts $R2_{red}$ into the active $R2_{ox}$; $R2_{ox}$ contains both the binuclear ferric center and the tyrosyl radical. For in vitro bacterial extracts, O_2, and dithiothreitol are able to convert $R2_{red}$ to $R2_{ox}$ only if the pH is maintained between 8.0-9.5 (Fontecave *et al.*, 1989). Presumably, at the increased pH, the iron center binds less tightly to the *E. coli* protein, and is more easily replaced with a reduced iron center. Because iron binding for the mammalian enzyme is weaker than for the *E. coli* enzyme, the higher pH is not needed to release iron. The final state of the subunit is formed by one-electron reduction of $R2_{ox}$. The product, $R2_{met}$, contains the intact ferric center, but is without tyrosyl radical. Hydroxyurea, a radical scavenger and potent inhibitor of ribonucleotide reductase, is capable of the one-electron reduction of $R2_{ox}$ that leads to its conversion to $R2_{met}$ (Sahlin *et al.*, 1989). $R2_{met}$ can be converted back to active $R2_{ox}$ by two different means. In an indirect route, the ferric center of $R2_{met}$ can be reduced back to the $R2_{red}$ ferrous center, and then oxidation by O_2 reforms the ferric species and simultaneously forms the tyrosyl radical. In an alternate route, treatment of $R2_{met}$ with hydrogen peroxide directly generates the free radical (Sahlin et al., 1990). There is evidence that conversion of $R2_{met}$ to $R2_{ox}$ proceeds through a ferryl (FeO^{2+}) intermediate (Sahlin *et al.*, 1990; Yamazaki and Piette, 1990).

Detection of transitory intermediates during the iron-mediated oxidation reaction has added additional insights to the understanding of this mechanism of radical generation. Two reactive intermediates were detected by Bollinger *et al.* (1991a), through stopped-flow absorption spectroscopy and rapid freeze-quench EPR

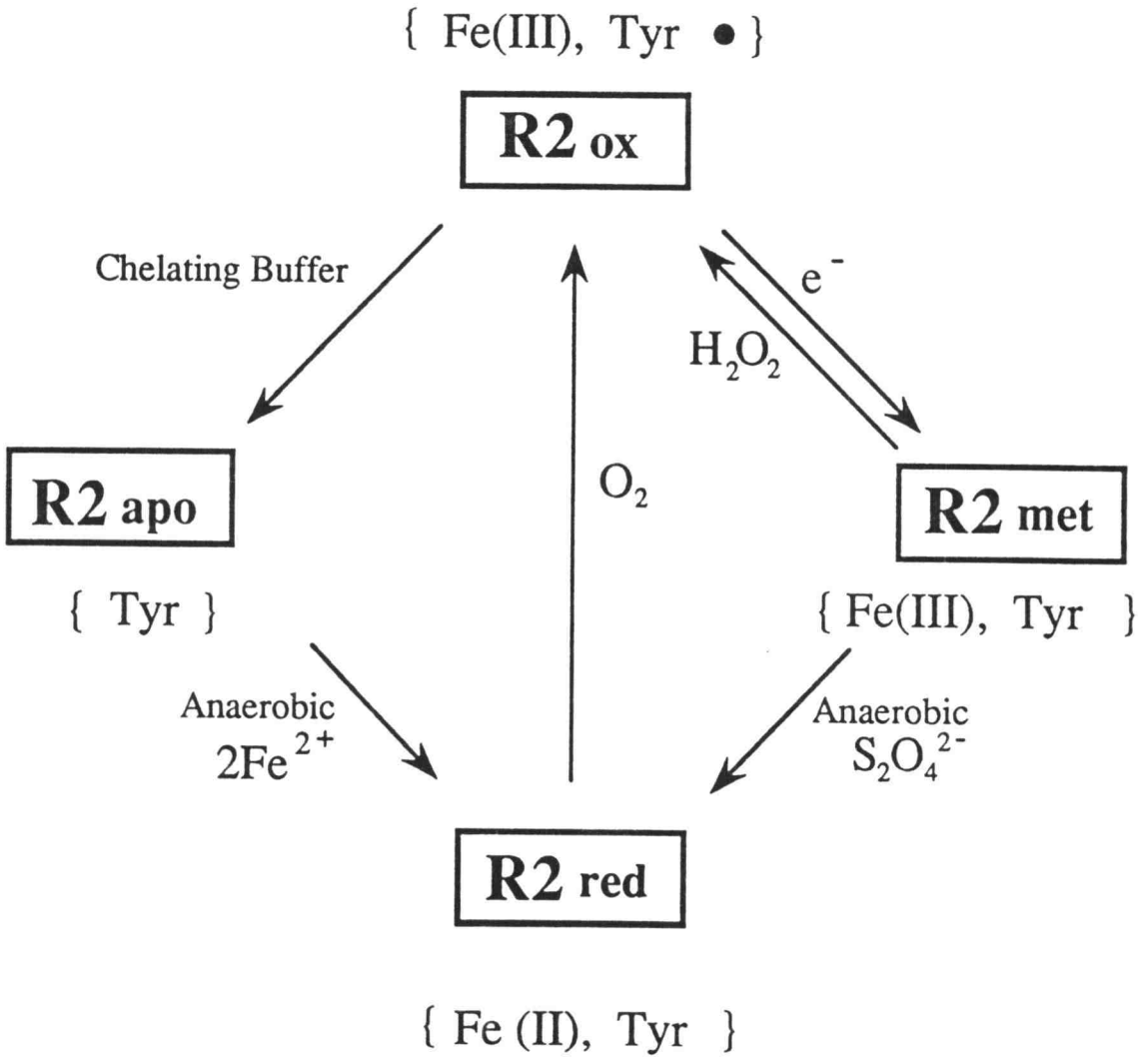


Figure II.7 Oxidation States of the R2 Protein

spectroscopy. They proposed that one intermediate is a μ-peroxodiferric complex and the other, an iron-coupled radical (Bollinger *et al.*, 1991a). Kinetic data showed that both new species were able to oxidize Tyr-122 to the stable tyrosyl radical. The proposal by these authors, for the oxidation reaction of $R2_{apo}$ to form $R2_{ox}$, is shown in Figure II.8. In the model, $R2_{apo}$ reacts with O_2 and two equivalents of ferrous ion to form the μ-peroxodiferric intermediate. In the presence of excess reductant (Fe^{2+}), the μ-peroxodiferric species is rapidly converted to the radical intermediate. This intermediate then undergoes one-electron reduction by Tyr-122 to generate the final products. One unique feature of this model is that it does not predict high-valence iron-oxo intermediates; the apparent intrinsic reactivity towards the one-electron reduction comes from the putative μ-peroxodiferric intermediate. Although the structure of the new radical intermediate has not been determined, Mössbauer spectroscopy indicates that the species contains two spin-coupled ferric ions (Bollinger *et al.*, 1991b).

An additional mixed valence form of the iron cluster Fe(II)Fe(III) was recently identified and characterized by two independent laboratories (Hendrich *et al.*, 1991; Gerez *et al.*, 1991). In a novel approach to EPR spectroscopy, Que's group submersed R2 subunit in liquid nitrogen, and then it was slowly heated in the presence of ionizing radiation from an X-ray source. The X-irradiation and slow heating stabilized the mixed valence intermediate, and allowed for its detection. Latour identified the same partially reduced iron species by treating R2 with a diimide (at high pH), and monitoring the disappearance of the characteristic EPR radical signal and the appearance of an intermediate signal. The intermediate signal was shifted to a lower microwave frequency and was identified as the mixed valence Fe(II)Fe(III) species. The existence of a mixed valence state does have precedence in other binuclear iron proteins (Fox *et al.*, 1988). Although the presence of the mixed valence state in ribonucleotide reductase seems to contradict the activation model proposed by Stubbe, Que describes a

unique role for the species. Que proposes that the fourth electron required to generate active protein comes from intra- or intermolecular electron transfers from neighboring iron clusters. Oxidation of one of the neighboring ferrous ions in a diferrous cluster, would then form the mixed valence species. The newly formed mixed valence center would then be incapable of oxidizing its own Tyr-122 to the radical state. This model not only provides a role for the mixed valent intermediate, but proposes a protein source for the elusive fourth electron.

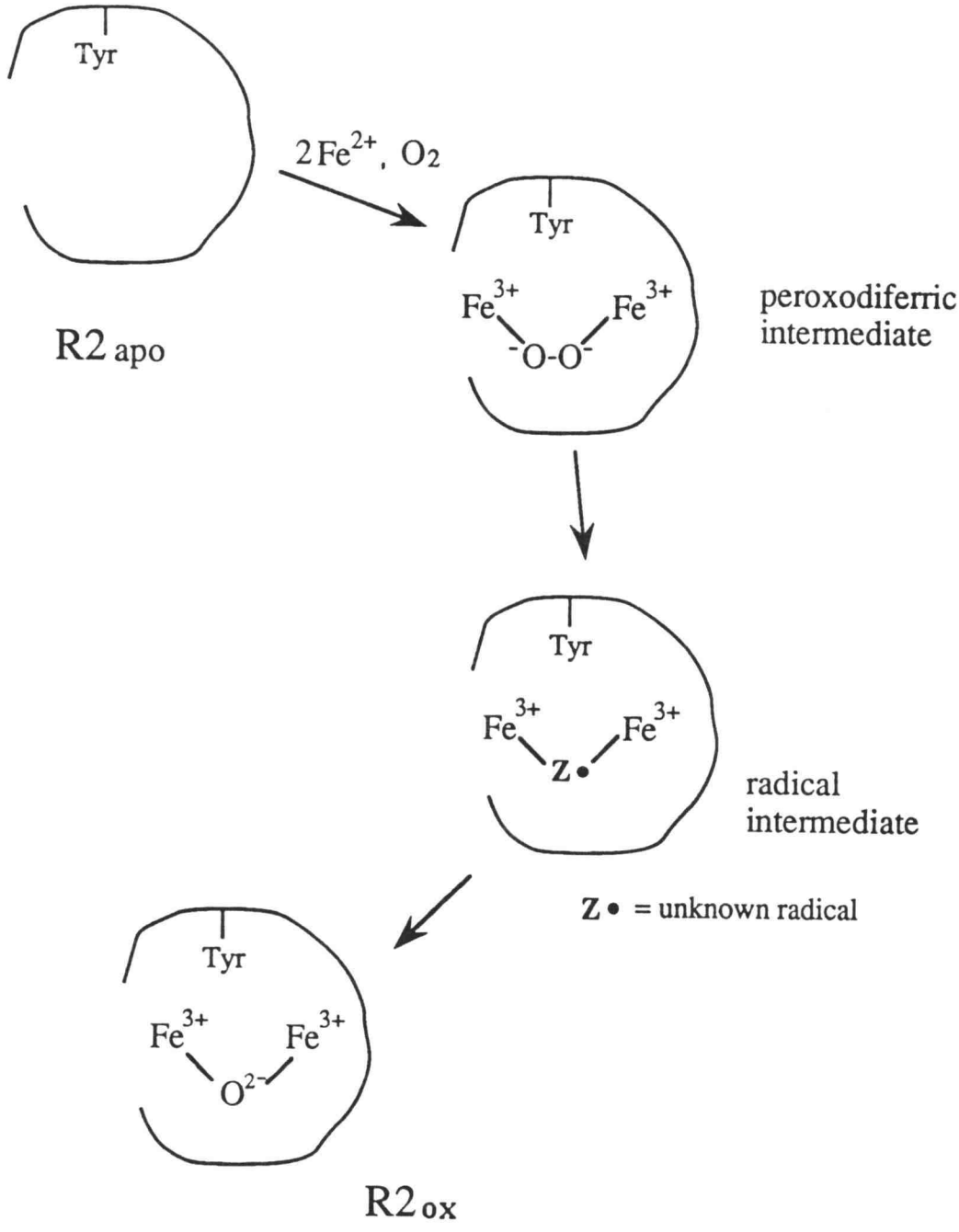
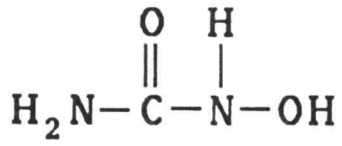


Figure II.8 One-Electron Oxidation of the Small Subunit

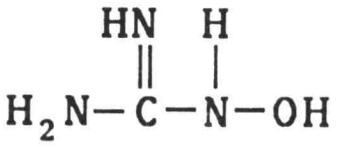
Although the maximum theoretical yield of radical is one per protomer, this amount of radical has never been detected; the largest number of radicals reported experimentally was 1.6 per R2 dimer for the mouse enzyme (Mann *et al.*, 1991). A theory presented to explain the discrepancy between the values for theoretical and experimental radical concentration involves the concept of "half-site reactivity" (Sjöberg *et al.*, 1987; Larsson *et al.*, 1988). In this model, only one of the Tyr-122 residues is activated and utilized for catalysis; it is suggested that the other Tyr-122 residue could assist in iron/radical center generation (Sjöberg *et al.*, 1987; Larsson *et al.*, 1988). The recent work by Que, describing the mixed valent intermediate, has given added credence to this model. Formation of the mixed valent species in an iron cluster, predicts less than complete occupancy of radical at that particular Tyr-122 (Elgren *et al.*, 1991).

II.1.5 Hydroxyurea Inhibition Studies

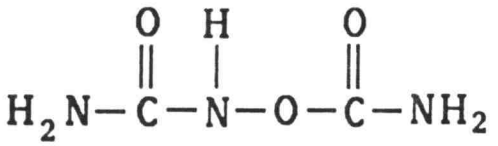
Hydroxyurea (HU) is a hydroxamic acid that is frequently used as an inhibitor of DNA synthesis, an agent for synchronizing cells, and as an antineoplastic chemotherapy drug (reviewed by Moore and Hurlbert, 1985). The structure of HU and other hydroxamic acids that inhibit ribonucleotide reductase are shown in Figure II.9. Treatment of cultured cells with HU leads to deoxynucleotide pool imbalances, reversible inhibition of DNA synthesis, reversible arrest of the cell cycle in early S phase, double-stranded breaks in DNA, chromosomal aberrations, and ultimately can cause cell death (reviewed by Timson, 1975). The primary target of HU was identified in 1964 as ribonucleotide reductase (Frenkel *et al.*, 1964). This section describes the mechanism of ribonucleotide reductase inhibition by HU, and examines how the structural and regulatory state of the enzyme affects HU inhibition. Molecular mechanisms for resistance to HU are also discussed.



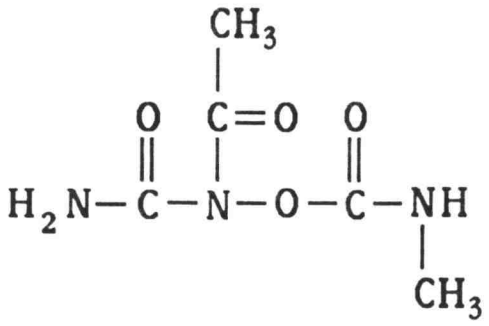
Hydroxyurea



Hydroxyguanidine



Carbamoyloxyurea



Caracemide

Figure II.9 Structure of Hydroxyurea and Related Compounds

II.1.5.1 Mechanism of Inhibition

Early mechanistic studies with hydroxyurea, hydroxamic acids, and related analogs established that the activity of the inhibitors towards ribonucleotide reductase could be correlated with the ability of these compounds to react with the model free-radical salt, potassium nitrodisulfonate (Kjöller-Larsen *et al.*, 1982). Since it had previously been established that the R2 protein of ribonucleotide reductase also contained a free radical species (Ehrenberg and Reichard, 1972), it was not long before experiments determined that the R2 radical was the direct site of inactivation by HU (Atkin *et al.*, 1973). Studies with the unusual ribonucleotide reductases (those that used AdoCbl and AdoMet as cofactors), revealed that these enzymes were insensitive to the effects of hydroxyurea (Lammers and Follman, 1983; Hogenkamp *et al.*, 1987). These results were expected, as these enzymes do not contain stabilized free radical species.

Subsequent experiments with the T4 and *E. coli* enzymes demonstrated that the tyrosyl radical exhibited a ten-fold higher susceptibility towards inactivation by HU during catalysis (Sahlin *et al.*, 1982; Kjöller-Larsen *et al.*, 1982). The rate of *E. coli* holoenzyme inactivation was recently examined in the presence of positive and negative effectors and substrate and product .[3] Formation of the R1/R2 holoenzyme was found to change the susceptibility of the radical towards HU in a manner than reflected the regulatory state of the enzyme. For example, addition of the positive effector ATP increased the sensitivity of the holoenzyme radical towards HU by 30%. Addition of inhibitory concentrations of dATP decreased radical susceptibility, two-fold, and addition of either CDP or dCDP increased the sensitivity of the radical. These results suggest that a conformational change in R2 is induced by binding of effectors and

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[3] M. Karlsson, M. Sahlin, B.-M.Sjoberg, submitted to *J. Biol. Chem.*

substrate. In addition, this conformational change appears to directly alter the ability of HU to reduce the radical species.

Comparative studies of ribonucleotide reductase from different organisms showed that the sensitivity to HU and structural impediments towards inactivation differed for the various enzymes. Enzyme activity from mammalian cells (Sahlin et al., 1982), T4 bacteriophage (Yeh and Tessman, 1978), herpes simplex virus (Langelier and Buttin, 1981) and pseudorabies virus (Lankinen *et al.*, 1982) were all found to be much more sensitive to HU than the *E. coli* enzyme (Yeh and Tessman, 1978). The mammalian and viral enzymes were also effectively inhibited by a wide size range of HU analogs. The *E. coli* enzyme, on the other hand, was insensitive to analogs with bulky side groups larger than 4 x 6 Å (Kjöller-Larsen *et al.*, 1982). Access of HU towards the *E. coli* radical is also shielded from negatively charged molecules (Sahlin *et al.*, 1989). These studies suggested that the mammalian and viral iron centers are more accessible to HU than is the *E. coli* center.

Other studies with the HU-inactivated mammalian enzyme demonstrated that HU inhibition of ribonucleotide reductase was reversible. After removal of HU, the mammalian enzyme was able to regenerate spontaneously under normal assay conditions (dithiol, O_2, Fe^{2+}), (Gräslund *et al.*, 1982). On the other hand, studies with purified *E. coli* ribonucleotide reductase showed that the HU-inactivated enzyme could be regenerated only by removing and replacing the iron-oxo bridge, or enzymatically, through the addition of Fe^{2+}, O_2, NAD(P)H:flavin oxidoreductase and superoxide dismutase (Petersson *et al.*, 1980; Eliasson *et al.*, 1986) As discussed in the previous section, the iron redox studies demonstrated that HU acted through a one electron reduction to convert $R2_{ox}$ to $R2_{met}$ (Sahlin *et al.*, 1989). It has since been suggested that treatment of mammalian enzyme with HU causes the loss of iron from the protein, while the *E. coli* iron center remains intact. In fact, a recent paper describes

destabilization of the mammalian iron center after HU treatment (McClarty *et al.*, 1990). Current mechanistic studies are aimed at confirming this prediction by kinetically measuring the dissociation of iron from both the *E. coli* and mammalian enzyme.

II.1.5.2 Deoxynucleotide Pool Effects

The functional role of ribonucleotide reductase is to supply balanced levels of deoxynucleotide precursors for DNA synthesis and repair. Inhibition of the enzyme would, therefore, be expected to decrease the flux of deoxynucleotides from the *de novo* source. Without the regulatory control from the allosteric enzyme, distortion of the relative concentrations of the individual pools might also be anticipated. Researchers have examined the effect of the inhibitor, HU, on deoxynucleotide pools and metabolism (Reviewed by Fox, 1985). The effect of inhibiting concentrations of HU on deoxynucleotide pool levels in various cultured cell lines is reviewed in Table II.3. Rather than the predicted reduction in synthesis and pool size of all deoxynucleotides, these studies consistently showed that HU addition appeared to differentially deplete just the purine pools. In most cases, the dATP pool was most drastically depleted, while the dGTP pool was also significantly diminished. The pyrimidine pools actually showed increases in dTTP and dCTP levels after HU treatment. Through the elegant work of Reichard and co-workers, the mechanism behind the pool size variations in the presence of this inhibitor was revealed (Bianchi *et al.*, 1986a: Bianchi *et al.*, 1986b). These researchers showed that an additional mechanism for regulating pyrimidine deoxyribonucleotides existed. This regulation occurred through 'substrate cycling' by directing the flow of pyrimidine deoxynucleotide across the cell membrane. In the presence of HU, the intracellular pyrimidine pools are fed by a net influx and phosphorylation of deoxyuridine from the

Table II.3 Effect of HU on Deoxynucleotide Pool Levels

Cell Type	Reference	Hydroxyurea Concentration	Fold change from Control, (where control = 1) dATP	dGTP	dCTP	dTTP
Mouse embryo cells	Skoog and Nordenskjold, 1971	1 mM (1 hr)	0.18	0.13	1.2	2.1
		10 mM (1 hr)	0.18	0	1.2	2.4
Activated Human Lymphocytes	Tyrsted, 1982	0.1 mM (28 hr)	0.2	0.7	0.8	0.7
		1.0 mM (28 hr)	0.1	0.4	0.4	0.6
Mouse Fibroblasts	Bianchi *et al.*, 1986b	0.1 mM	0.36	0.23	0.61	1.1
		3.0 mM	0.37	0.25	0.71	1.2

medium and by deamination of intracellular dCTP. No such cycling system exists for the purine deoxynucleotides and, therefore, these pools are depleted (Reichard, 1988).

Many attempts have been made to circumvent the effects of HU on cellular pools by the addition of one or more deoxynucleotide(s) or deoxynucleoside(s) (Adams and Lindsay, 1967; Gale, 1967; Plageman and Erbe, 1974; Lagergren and Reichard, 1987; Eriksson *et al.*, 1987; Walker *et al.*, 1977; Yarbro, 1968; Young *et al.*, 1967). Unexpectedly, the results of these investigations were preponderantly negative.

II.1.5.3 Mechanisms of Resistance

Drug resistance can occur through many different mechanisms. The cytotoxic effect of hydroxyurea makes it a very good selective agent for isolating drug-resistant cell lines with specific alterations in ribonucleotide reductase (reviewed by Wright *et al.*, 1990). Characterization of cultured cell lines grown in the presence of low to moderate amounts of HU has revealed that amplification of the small subunit R2 gene encoding ribonucleotide reductase is a common mechanism responsible for resistance (Wright *et al.*, 1987; Choy *et al.*, 1988; Tagger and Wright, 1988). At these concentrations of HU, R2 gene amplification leads to elevated R2 message and protein levels, without any changes in R1 gene expression. At higher concentrations of HU, R2 amplification is accompanied by large subunit R1 gene amplification and expression (McClarty *et al.*, 1987; Choy *et al.*, 1988; Hurta and Wright 1990). Molecular mechanisms for HU resistance involving ribonucleotide reductase alterations have also been studied for the yeast (Rittberg and Wright, 1989) and vaccinia virus (Slabaugh and Mathews, 1986; Slabaugh *et al.*, 1988) reductase genes. In both cases, the ability to overcome sensitivity to HU was dependent on small subunit gene amplification.

II.1.6 Regulation of Gene Products

Like many other enzymes involved in deoxynucleotide metabolism, the activity of ribonucleotide reductase increases when cells prepare for DNA synthesis (Reichard, 1988). This increase in activity does not depend on DNA synthesis but is regulated (in cultured and eukaryotic cells) in connection with the cell's mitotic cycle. Figure II.10 is a diagram depicting the four stages of the cell cycle. Experiments have been used to correlate increases in enzyme activities during the cell cyle, with increases in content of enzyme proteins and mRNAs. This section describes experiments that determined the modes of regulation for ribonucleotide reductase during *E. coli* growth and during the mammalian cell cycle.

II.1.6.1 *E. coli* Subunits

Synthesis of the two subunits of *E. coli* ribonucleotide reductase is coordinately regulated and occurs in parallel with DNA synthesis (Filpula and Fuchs, 1977). However, inhibition of DNA synthesis by chemical agents results in an increase in ribonucleotide reductase activity (Hanke and Fuchs, 1983). It was determined that the increased activity was due to an increased rate of reductase gene transcription (Hanke and Fuchs, 1983). Molecular analyses of the genes and promoter sequences around the bacterial ***nrd*** genes have led to a model for transcriptional regulation of *E. coli* ribonucleotide reductase (Tuggle and Fuchs, 1986, 1990). The two genes that encode the large and small subunits, are located on a single operon (Carlson *et al.*, 1984). Deletion analysis identified two negative regulatory regions flanking the reductase promoter (Tuggle and Fuchs, 1986). A positive regulatory site was also mapped upstream from the large subunit gene (Tuggle and Fuchs, 1986). The presence of both positive and negative regulatory elements allows the bacterial cell to communicate the need for DNA synthesis to the operon. A DNA/protein binding assay has been

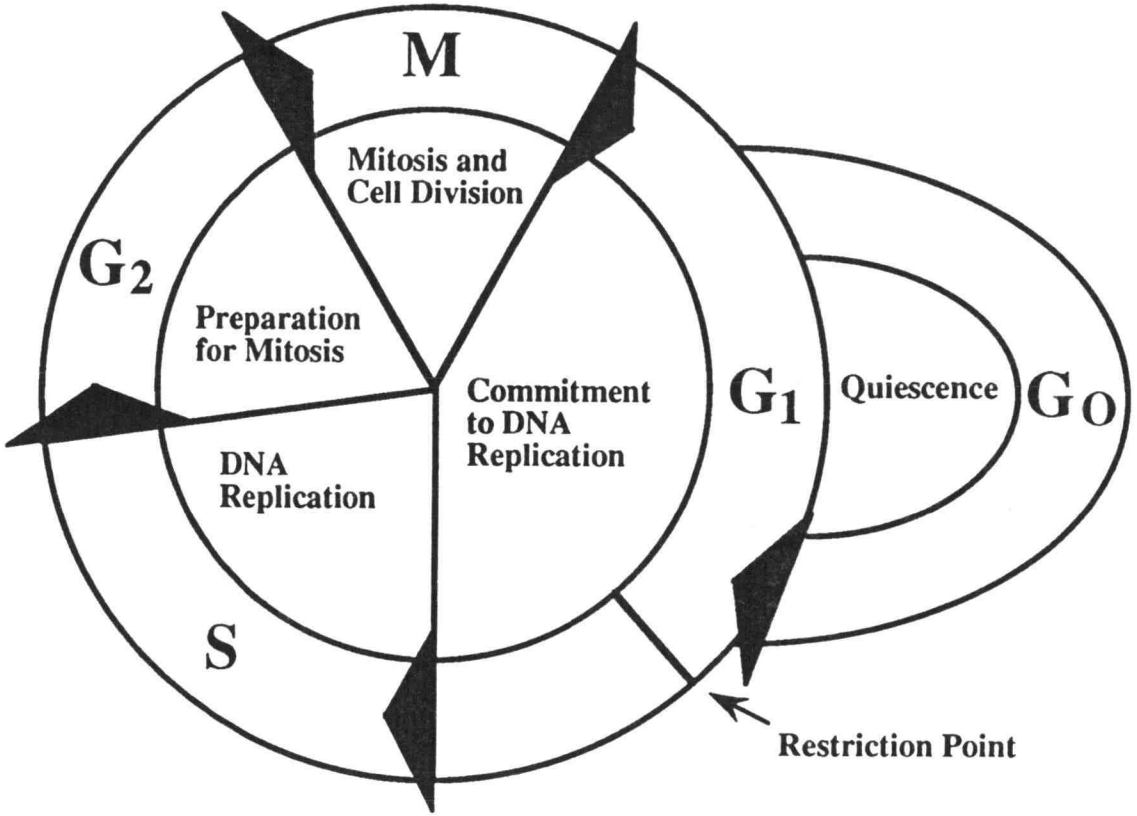

Figure II.10 Eukaryotic Cell Cycle

developed and is currently being used to identify the proteins responsible for regulation of these genes (Tuggle and Fuchs, 1990).

II.1.6.2 Mammalian subunits

Like the *E. coli* reductase genes, the genes that encode the mammalian enzyme are coordinately regulated. The genes encoding both the human (Yang-Feng *et al.*, 1987) and mouse (Brissenden *et al.*, 1988) ribonucleotide reductase have been characterized and located to separate chromosomes. Using antisense RNA probes, it was established that the levels of both genes increased when cultured mouse cells progressed from either G_0 or G_1 into the S-phase of the cell cycle (Björklund *et al.*, 1990). The decline in message levels of both subunit mRNA's was also found to be coordinate (Björklund *et al.*, 1990). Dispite these findings, posttranslational regulation of the two proteins was found to be quite disparate. Expression of the small subunit protein in proliferating cells correlates strictly with the S-phase of the cell cycle (Eriksson *et al.*, 1984). The levels of R1 protein, on the other hand, are constant during the cell cycle (Engström *et al.*, 1984). This regulatory paradox of coordinate mRNA expression and uncoordinate protein level accumulation was resolved when the stability of the two proteins was measured. Breakdown of the R2 protein occurs readily, with a protein half-life of 3 h (Eriksson *et al.*, 1984). However, the R1 protein is very stable (half life of 20 h), and therefore remains intact throughout the cell cycle (Mann *et al.*, 1988). Indeed when R1 protein was measured in quiescent or terminally differentiated cells, the levels were almost undetectible (Engström *et al.*, 1984; Mann *et al.*, 1988). It has been demonstated that eukaryotic ribonucleotide reductase activity is cell cycle-dependent and that activity correlates with cellular DNA content (Eriksson *et al.*, 1984). This finding first suggested that the R2 protein was limiting at times other

than S-phase and that enzyme activity was regulated by the levels of small subunit protein.

II.2 Vaccinia Virus

Vaccinia is a member of the Poxviridae family of viruses, which infect both vertebrate and invertebrate hosts. Within the genus Orthopoxvirus is the notorious variola virus, the causative agent of smallpox. Nonetheless, the prototype for this genus is the well-characterized vaccinia virus. Vaccinia virus was originally identified as an immunizing agent against cowpox in 1798 by Edward Jenner (Jenner, 1959). Because of the serological relatedness of the vaccinia and variola viruses, vaccinia was administered as a live vaccine in the campaign by the World Health Organization to eradicate smallpox (World Health Organization, 1988). Vaccinia continues to be used as a vector for developing recombinant viruses for use as live vaccines (reviewed by Fenner, 1988; Moss, 1991). The virus is particularly well-suited for use as a vaccine because it has a broad mammalian host range and the genome has a large capacity to accept foreign DNA (up to 25 kilobases). Because of these advantages, vaccinia has also become extensively used as a eukaryotic cloning and expression vector (Fuerst *et al.,* 1987).

The vaccinia virus particle is a large brick-shaped structure composed of an outer lipoprotein envelope, a single duplex DNA genome, and two lateral protein bodies with unknown function. With the advent of molecular biology, it was discovered that the poxviruses exhibit the unusual ability to replicate within the cytoplasm, rather than the nucleus, of the infected host cell (reviewed by Moyer and Graves, 1981). Because of the cytoplasmic site of replication, viral nucleotide and deoxynucleotide metabolism are controlled entirely by viral gene products. In fact,

vaccinia encodes functions necessary for control of viral transcription, mRNA processing, DNA replication, and DNA recombination.

II.2.1 Genome Organization and Replication

The vaccinia virus genome is a covalently-closed linear duplex DNA molecule that contains two 10-kilobase regions of AT-rich inverted terminal repeats (reviewed by Traktman 1990). A Copenhagen strain of the vaccinia genome has been completely sequenced and it was determined that the 190 kilobases of viral DNA encode 198 open reading frames (ORF's) of at least 60 amino acids (Goebel *et al.*, 1990). The ORF's are bidirectional, tightly spaced, and contain no intervening sequences. Each viral gene contains its own transcriptional promoter. Computer analysis has demonstrated a high degree of sequence homology within the central region of the genome among different members of the poxvirus family. Among the viral genes of known function that have been sequenced, there is also much sequence homology with mammalian protein counterparts (reviewed by Moss, 1990). Sequence variability is found at the ends and within the terminal repeats of the genomes. Through the use of temperature sensitive mutants and genetic analyses, many essential genes have been physically mapped within the genome (Condit and Motyczka, 1981; Condit *et al.*, 1983).

Although the host cell nucleus is required for maturation of virus particles, vaccinia virus is able to replicate its DNA within enucleated cells (Prescott *et al.*, 1971). Accordingly, the virus must encode all the functions necessary for viral DNA replication. The locations of the genes encoding many of the enzymes involved in replication and deoxynucleotide metabolism have been determined and are shown in Figure II.11. It should be noted that the genes that encode the viral ribonucleotide reductase proteins are separated by 35 kilobases.

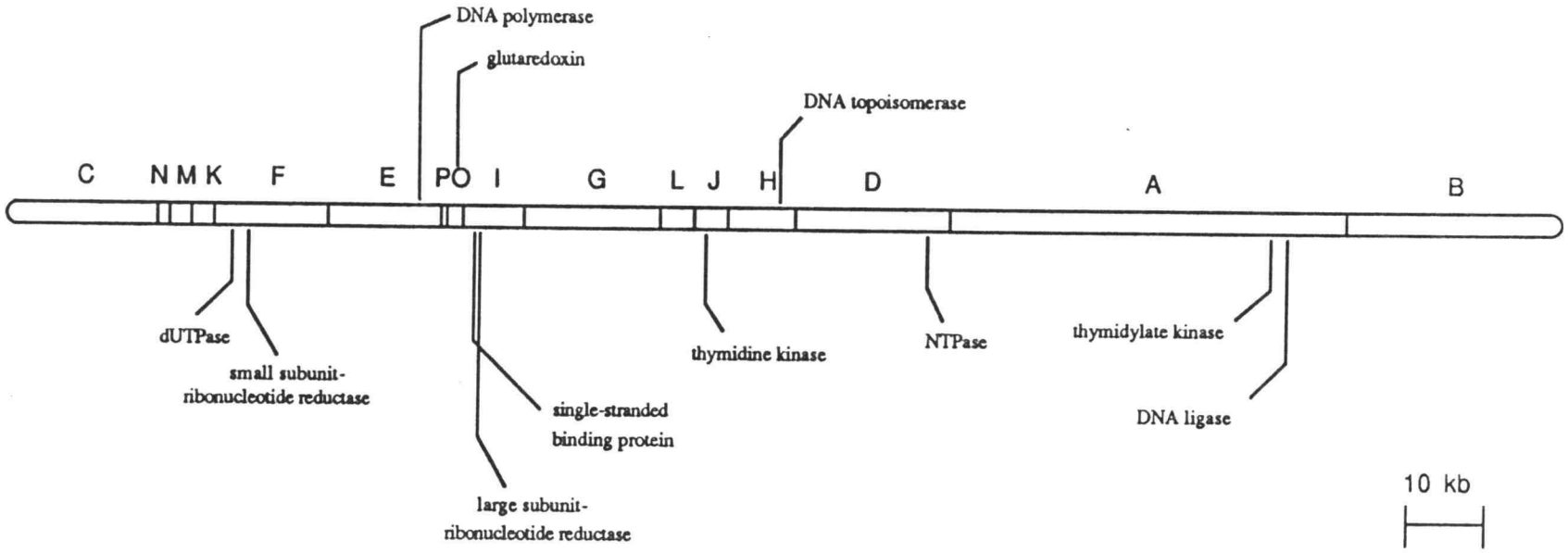

Figure II.11 Location of Genes Encoding Replication and Nucleotide Metabolism Proteins

Viral DNA replication takes place in cytoplasmic inclusion bodies called virosomes (Dahl and Kates, 1970). Virosomes are composed of viral nucleoprotein and immunolocalization studies have revealed the presence of ribonucleotide reductase (Davis, 1992) and other replication enzymes at these viral DNA factories. Early studies demonstrated that the rate of viral DNA replication is several times that of dividing eukaryotic cells and that a full 30% of all virally-replicated genomes are packaged into virions by 24 h post infection (Joklik and Becker).

Experiments which measure [^{3}H]-thymidine incorporation into acid-precipitable material have been used to estimate the time course of viral DNA replication. These studies concluded that DNA synthesis begins approximately 1.5 h after viral entry into a cell, reaches a maximal rate at 2.5 to 3 hours post infection, and then declines rapidly (Joklik and Becker, 1964). However, recent quantitative filter hybridization experiments to measure accumulation of viral DNA have shown contradictory results. The hybridization experiments demonstrate that increases in intracellular vaccinia DNA are detected by 3 h post infection and then accumulate at a constant rate until approximately 10-12 h post infection (Ensinger, 1987; Rempel *et al.*, 1990). This experimental discrepancy towards the viral DNA replication kinetics probably reflects the inherent innaccuracies in measurements of deoxynucleotide pools (thymidine levels) in an active metabolic system. A basic assumption of the [^{3}H]-thymidine incorporation experiments is that the intracellular concentration of thymine nucleotides is constant. However, this assumption has not been tested in virus-infected cells. Moreover, deoxynucleotide pools in other eukaryote systems have been shown to fluctuate extensively during replication (Reichard, 1988; Bianchi *et al.*, 1986). The hybridization experiments, therefore represent a more accurate measure of viral DNA synthesis.

The mechanism of viral replication has been well characterized (reviewed by Moss *et al.*, 1983; Moyer and Graves, 1981). Briefly, DNA nicks are introduced near either end of the parental genome soon after viral infection and replication begins at these sites. Viral DNA is apparently self primed and synthesis occurs in a semi-discontinuous fashion. During replication, hairpen loop structures form at the inverted terminal genome repeats. Replication eventually generates viral concatamers in which genomes are joined head-to-head and tail-to-tail. Resolution of each concatameric junction results in the formation of two full length genomes.

II.2.2 Viral Infectivity and Inhibition ofCellular Macromolecular Synthesis

Infection of a single eukaryotic cell with vaccinia virus can lead to the production of several hundred to thousands of infectious virus particles, depending on the type of cell infected, and the multiplicity of virus used for the infection (Joklik and Becker, 1964). This section discusses the viral mode of infection and the effect of the virus on host macromolecular processes.

Poxvirus entry into the host cell occurs naturally through the cellular process of membrane pinocytosis (Dales, 1963). Because entry of virus is not restricted, the host range of the poxviruses is determined not by cell entry, but through the action of a virally-encoded protein protein. In vaccinia virus, an *hr* (host range) gene has been sequenced (Gillard *et al.*, 1986) and a viral host range protein has been physically characterized (Gilard *et al.*, 1989). Mutant deletion studies suggest that the host range protein acts as an antagonist towards host factors which inhibit viral protein synthesis (Drillien *et al.*, 1981)

In order to replicate and package so many virions in a relatively short period of time (20-30 h), the poxviruses suppress the metabolic activities of their hosts. Vaccinia virus inhibits host cell DNA synthesis (Jungwirth and Launer, 1969), cell protein

synthesis (Moss, 1968), transfer of nuclear RNA into the cytoplasm, and eventually RNA synthesis (Becker and Joklik, 1964). These degradative events allow the virus to control, and in some cases, to utilize the metabolic potential of the cell. In the case of host cell DNA synthesis, it is believed that upon infection, a host specific deoxynuclease is released directly from the virus core. Host cell DNA synthesis inhibition begins immediately after infection and cellular DNA is degraded into large DNA fragments of discrete sizes (Parkhurst *et al.*, 1973). The degraded cellular DNA is not made available to the virus for utilization under normal conditions (Kit and Dubbs, 1962).

II.2.3 Viral Lifecycle and Regulation of Viral Gene Transcription

Within the viral life cycle, there are three distinct classes of genes and gene transcription; early, intermediate, and late. These stages form a cascade of temporal gene expression; each class of gene is expressed at a specific period in the viral lifecycle and only for a discrete length of time. For a complete review of viral transcription regulation, see Moss, (1990). Depending on the cell type and multiplicity of infection, the entire viral life cycle is completed within approximately 8-12 h. All of the enzymes necessary for early gene expression are packaged within the virion core of the infectious particle (Nevins and Joklik, 1977). Upon infection, the virion enters the host cell through direct penetration or membrane fusion (Dales, 1963). Two uncoating processes then release the intact viral core from various membrane proteins and glycoproteins. Transcription of early genes begins immediately after infection, continues for about 3-4 h, and then ceases. Early genes include those encoding replication functions, virion proteins, and intermediate transcription factors. Coincident with the start of DNA replication (approximately 2 h post infection) is the activation of intermediate genes by the intermediate transcription factors. The intermediate genes

primarily encode late transcription factors. Late transcription factors activate synthesis of structural coat proteins, other virion proteins, early transcription factors, and the early transcription enzymes destined for encapsidation. Early, intermediate, and late genes can be distinguished by differences in promoter consensus sequences. Also, because early genes contain termination signal sequences, early messages are of a discrete size. Intermediate and late genes do not contain termination signals, however, and synthesize run-on messages. In addition, there are differences in 5'-capping and 3'-polyadenylylation of the various temporal messages.

A complex series of morphogenetic events are responsible for virus particle formation. Along with DNA replication, immature virion formation takes place within and surrounding the virosomes. Crescent shaped structures derived from host cell lipids form around a portion of the viroplasm, and then through a series of morphologic steps (which are clearly identifiable with an electron microscope) the infectious particle takes form. Many of the viral core and membrane polypeptides contain posttranslational modifications, including proteolytic cleavage, phosphorylation, glycosylation, and ADP-ribosylation (VanSlyke and Hruby, 1990). These modifications help to regulate the events in viral morphogenesis.

II.3 Vaccinia Virus Ribonucleotide Reductase

Preliminary characterization of the viral ribonucleotide reductase was accomplished in large part by the work of both Slabaugh and Roseman. As mentioned previously, the genes that encode the viral ribonucleotide reductase have been sequenced and localized within the HindIII F (R2) and HindIII I (R1) genomic restriction fragments (Slabaugh *et al.*, 1988; Tengelson *et al.*, 1988; Schmitt and Stunnenberg, 1988). (Figure II.12). Primer extension and S1 nuclease mapping

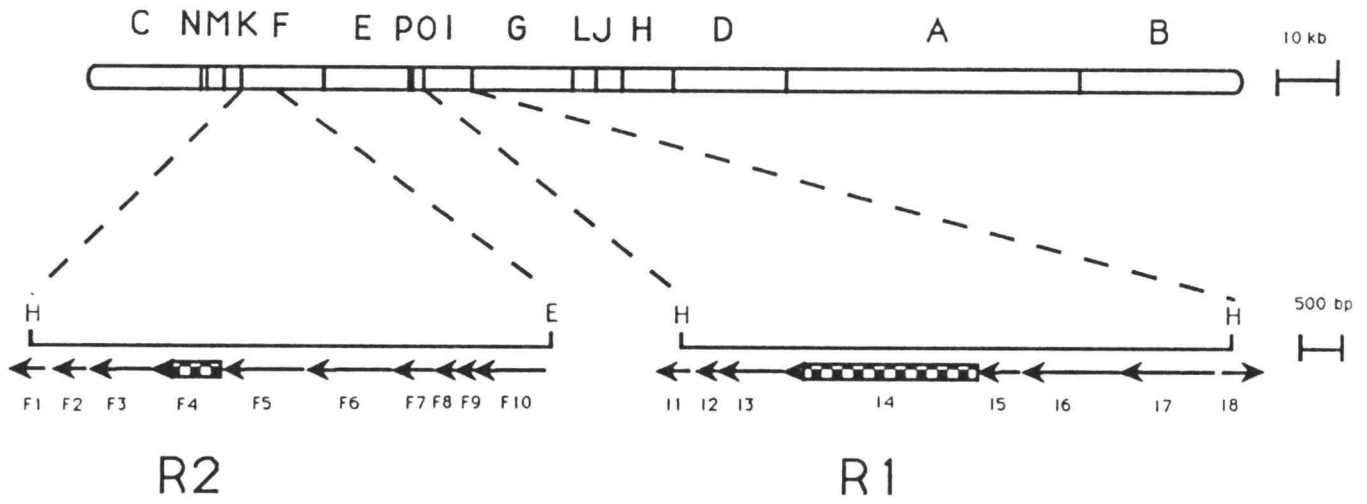

Figure II.12 Position of ORF's Encoding Ribonucleotide Reductase Subunits

experiments were subsequently used to define the reductase transcriptional units. In addition, Northern blot analysis has demonstrated that both genes are transcribed early in the viral life cycle (Tengelson *et al.*, 1988; Roseman and Slabaugh, 1990).

Comparison of the amino acid sequences of ribonucleotide reductases from several species reveals a high degree of homology between the mammalian reductase and vaccinia virus polypeptides (Slabaugh *et al.*, 1988; Tengelson *et al.*, 1988). Figure II.13 is an amino acid comparison of nine different R2 proteins. The mouse and vaccinia small and large subunit amino acid sequences share 80% and 72% amino acid identity, respectively. In contrast, there is only 20% homology between the vaccinia virus and *E. coli* or T4 bacteriophage R2 amino acid sequences. The three herpes simplex viral polypeptide sequences share only 30% homology with the vaccinia R2 sequence. Slabaugh proposes that the strong sequence homology between the mouse and vaccinia enzymes suggests that the viral enzymes are of eukaryotic origin. Figure II.14 is a phylogenetic tree that was generated by computer analysis of the R2 amino acid sequences. The tree also suggests a large degree of evolutionary relatedness between the vaccinia and mouse R2 proteins.

Initial characterization of the vaccinia virus ribonucleotide reductase also demonstrated allosteric similarities of the enzyme to its mammalian counterpart (Slabaugh and Mathews, 1984a). Unlike the T4 bacteriophage and herpes simplex virus enzymes, which are insensitive to many of the regulatory signals for the cellular reductases (Averett *et al.*, 1983; Berglund, 1972), vaccinia virus R2 is allosterically inhibited by both dATP and dTTP and shows a strict requirement for a positive activator. Further characterization of the vaccinia virus enzyme proved that there were also distinctions between the mammalian and viral enzymes (Slabaugh and Mathews, 1984b). The viral K_M for CDP is three-fold lower than that for the mammalian

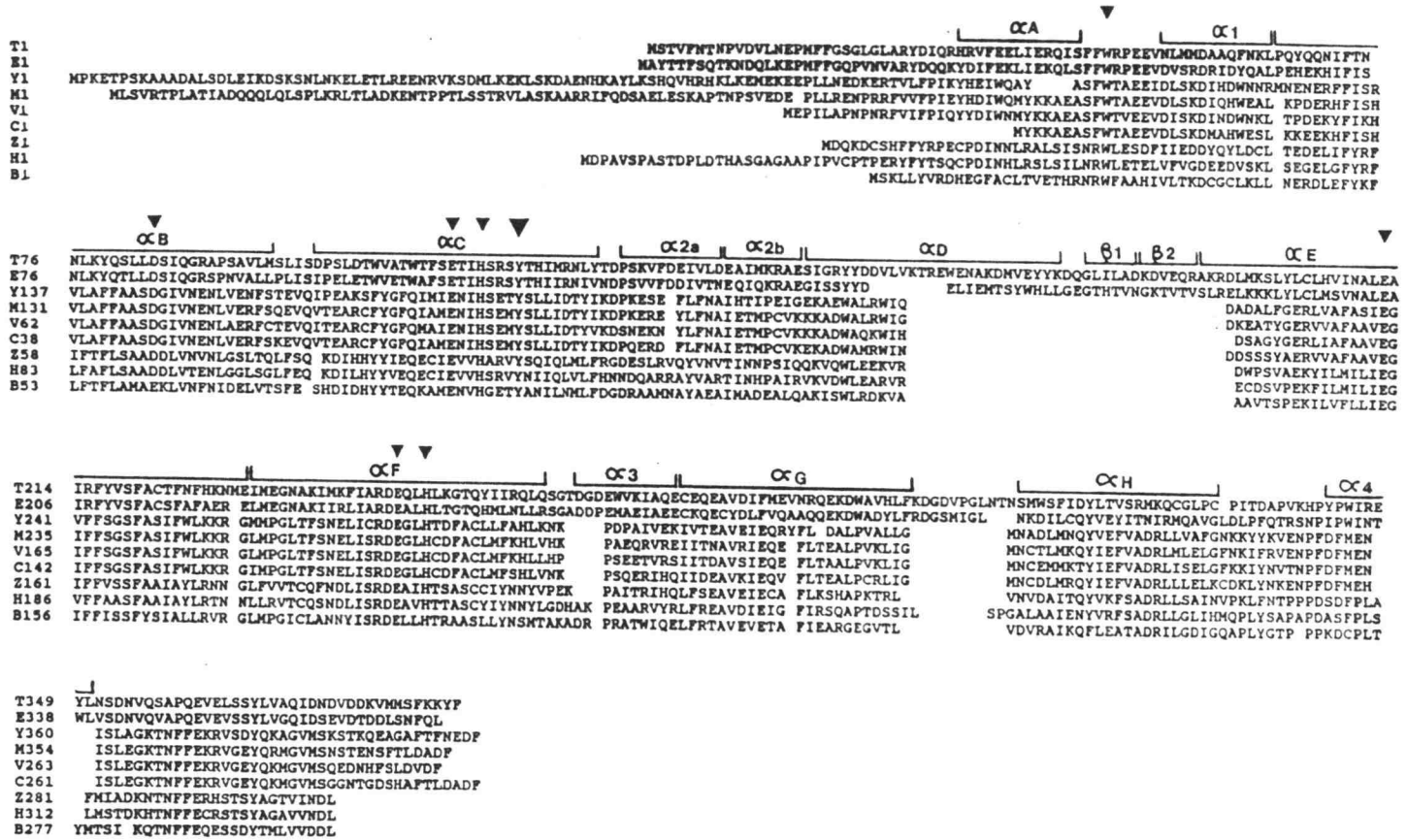

Figure II.13 Amino Acid Comparison of Nine Different Small Subunit Proteins. T, T4 bacteriophage; E, *E. coli*; Y, yeast; M, mouse; V, vaccinia; C, clam; Z, Varicella Zoster virus; H, herpes simplex virus; B, Epstein-Barr virus

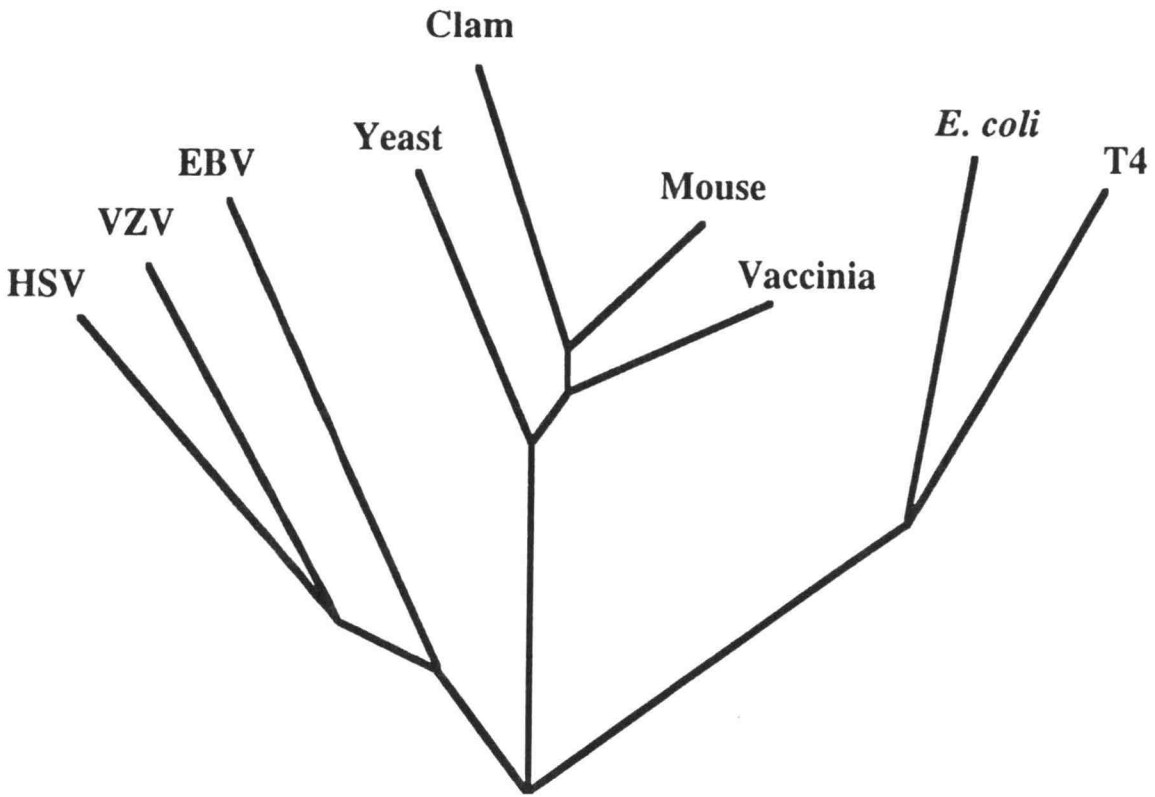

Figure II.14 Phylogenetic Tree of R2 Amino Acid Sequences

enzyme. Additional differences in Mg^{2+} requirements and substrate affinities were also uncovered. To more carefully compare the kinetic and allosteric properties of the viral and mammalian enzymes, large quantities of purified active enzyme from both organisms would be required. The importance of further characterizing the vaccinia virus ribonucleotide reductase were motivating factors towards the subsequent work to produce and overexpress recombinant viral enzyme.

Although viral insertion and deletion mutants had been isolated in regions spanning the entire viral genome, it was indeed surprising when it was revealed that viable viral mutants could be isolated with mutations harbored in both the R1 and R2 genes (Child et al., 1990; Perkus et al., 1986). In all cases, the viral mutants showed reduced virulence, but were capable of sustaining viral growth after infection of either resting or dividing cells. These data firmly establish that neither reductase subunit is essential for viral replication. How then, is the supply of deoxynucleotide precursors supplied for viral replication in these mutants? The answer to this question may rest with the cellular host reductase. Immunolocalization of cellular R1 and R2 indicates that the subunits are both cytoplasmic (Engstrom et al., 1984; Engstrom et al., 1988). It is possible that the mammalian host enzyme substitutes for the viral enzyme in these insertion and deletion mutants. An informative experiment would be to compare the expression kinetics of the host ribonucleotide reductase during a viral infection using wild-type vaccinia, with that from a viral infection using the ribonucleotide reductase minus mutants. It would also be very interesting to determine if the host ribonucleotide reductase remained cell-cycle dependant after infection with these mutant viruses. Another untested explanation for the viability of the vaccinia reductase mutants is that one of the host range subunits (large or small) may complement and activate the viral reductase with the missing subunit component. In this case, the reductase activity

would not come from the holoenzyme, but from a hybrid combination of viral subunits and host subunits.

III. Cloning of the Vaccinia Virus Ribonucleotide Reductase Small Subunit Gene. Characterization of the Gene Product Expressed in *Escherichia coli*.

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III.1 Abstract

During its infectious cycle, vaccinia virus expresses a virus-encoded ribonucleotide reductase which is distinct from the host cellular enzyme (Slabaugh *et al* 1984a, 1984b). We have cloned the gene for the small subunit of vaccinia virus ribonucleotide reductase (designated VVR2) into *Escherichia coli*, and expressed the protein using a T7 RNA polymerase plasmid expression system. Following isopropyl ß-D-thiogalatopyranoside induction, accumulation of a 37-kDa peptide was detected by SDS-polyacrylamide gel electrophoresis, and this peptide reacted with polyclonal antiserum raised against a TrpE-VVR2 fusion protein. The 37-kDa protein was purified to homogeneity, and gel filtration of the purified protein revealed that the recombinant protein existed as a dimer in solution. Purified recombinant VVR2 protein was shown to complement the activity of purified recombinant ribonucleotide reductase large subunit, with a specific activity that was similar to native VVR2 from a virus-infected cell extraxt. A CD spectrum of the recombinant viral protein showed that like the mouse protein, the vaccinia virus protein has 50% α-helical structure. Like other iron-containing ribonucleotide reductase small subunits, recombinant VVR2 protein contained a stable organic free radical that was detectable by EPR spectroscopy. The EPR spectrum of purified recombinant VVR2 was identical to that of vaccinia virus-infected mammalian cells. Both the hyperfine splitting character and microwave saturation behavior of VVR2 were similar to those of mouse R2 and distinct from *E. coli* R2. By using amino acid analysis to determine the concentration of VVR2, we determined that ≈ 0.6 radicals were present per R2 dimer. Our results indicate that vaccinia virus R2 is similar to mammalian ribonucleotide reductases.

III.2 Introduction

Ribonucleotide reductase is an essential enzyme in DNA replication, providing the only source of de novo-synthesized deoxyribonucleotides. In both *Escherichia coli* and eukaryotic organisms, the holoenzyme is composed of two homodimers: an allosteric/catalytic subunit, designated R1[2] , whose protomers have molecular weights of 83,000 to 87,000, and a smaller iron-containing subunit, designated R2, with protomer molecular weights of 37,000 - 45,000 (Sjöberg and Gräslund, 1983). Although several types of ribonucleotide reductase have been described, accumulating evidence suggests that all forms of the enzyme utilize a free radical mechanism to initiate reduction of the four ribonucleotide substrates (reviewed in Stubbe, 1990). In the iron-containing reductases, the source of the radical is a tyrosine side chain (Larsson and Sjöberg, 1986) that has recently been shown by x-ray crystallography to reside within a hydrophobic pocket in each R2 protomer, adjacent to a diferric iron center (Nordlund *et al.*, 1990).

The mechanism by which the diferric tyrosyl radical center in R2 is formed and maintained is currently the subject of intense research (Sahlin *et al.*, 1989; Ochiai *et al.*, 1990; Fontecave *et al.*, 1990; Sahlin *et al.*, 1990; Stubbe, 1990). Iron binding to mouse R2 apoprotein appears to be highly cooperative (Ochiai *et al.*, 1990), and evidence from the *E. coli* and mouse proteins indicates that generation of the radical is concomitant with oxidation of the diferrous protein to the oxo-bridged diferric state. However, tyrosyl radical quantitation by EPR of R2 proteins from various sources has yielded variable results, usually around 0.5 radical/polypeptide chain, rather than the

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[2] The abbreviations used are: *E. coli*, *Escherichia coli*; R2 and R1, small and large subunit of ribonucleotide reductase, respectively; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonylfluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; mW, milliwatts.

theoretical maximum of 1.0/R2 monomer (Lynch *et al.*, 1989; Sahlin *et al.*, 1989). Additionally, the mechanistic role of the iron center and its associated radical remains obscure since changes in the redox state of either entity have never been detected under substrate turnover conditions (reviewed in Stubbe, 1990).

We have previously shown that ribonucleotide reductase is encoded by the eukaryotic orthopoxvirus, vaccinia, and is actively expressed during viral infection. The genes for the large and small subunits, which are separated by 35 kilobases on the vaccinia genome, are transcribed early in the infective cycle (Roseman and Slabaugh, 1990; Tengelsen *et al.*, 1988). Because of the difficulties involved in obtaining large amounts of enzyme from vaccinia-infected cell extracts, we chose to overexpress the genes for both subunits of ribonucleotide reductase in a bacterial expression system. Comparison of the amino acid sequences of the vaccinia gene products to other ribonucleotide reductase subunits revealed that the vaccinia virus enzyme has far greater similarity to the mammalian and yeast reductases than it does to the corresponding enzyme from *E. coli*, T4 bacteriophage, or the herpes viruses (Slabaugh *et al.*, 1988). In fact, the vaccinia proteins share 70-80% amino acid identity with the respective mouse subunits. In contrast to the herpes virus ribonucleotide reductase, which is unregulated (Averett *et al.*, 1983), the vaccinia virus-induced enzyme exhibits the same pattern of allosteric regulation as the mammalian enzyme (Slabaugh *et al.*, 1984). The regulatory and structural similarity of the VVR2 protein to the mammalian R2 subunits as well as the genetic advantages offered by the readily manipulated nature of the vaccinia virus genome (Traktman, 1990) have led us to pursue study of the vaccinia virus ribonucleotide reductase.

In this report, we describe the cloning and overexpression of the vaccinia small subunit ribonucleotide reductase gene in *E. coli*. We describe purification of VVR2 and compare the specific activities and migration on an SDS-polyacrylamide gel, of the

native and recombinant proteins. We show that the recombinant protein is susceptible to hydroxyurea inactivation, as evidenced by a decrease in the light absorption spectrum of the R2 free radical and iron center. The radical content of the small subunit is quantitated, and the EPR and CD spectra of recombinant protein are compared to spectra of small subunit proteins from other sources.

III.3 Materials and Methods

Buffers. Buffers used in this work were as follows. Buffer A: 50 mM HEPES (pH 7.6), 2 mM dithiothreitol, 1 mM PMSF, 5% glycerol, 2 mM $MgCl_2$. Buffer B: 100 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol, 5% glycerol, 1 mM PMSF. Buffer C: 50 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 5% glycerol, 1 mM PMSF. Buffer D: 50 mM Tris-HCl (pH 9.5), 150 mM NaCl, 2 mM EDTA, 0.1% polyoxyethylene sorbitan monolaurate. Dithiothreitol and PMSF were added immediately before use of the buffer.

Bacterial Strains and Media. The *E. coli* strain BL21(DE3) is a lambda lysogen that carries the gene for T7 RNA polymerase under the control of the inducible p_L promoter (Rosenberg *et al.*, 1987). The plasmids pET11d (Studier *et al.*, 1990) and pETlld-R2 were transformed into BL21(DE3) by the method of Chung *et al.* (1990). pET plasmids and suitable bacterial hosts were generously supplied by William Studier, Department of Biology, Brookhaven National Laboratory. Bacteria containing plasmids were routinely grown at 30°C in Luria broth medium (per liter; 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH) containing 100 μg/ml ampicillin.

The *E. coli* strain N6405/pSPS2, which overproduces the R2 subunit of *E. coli* ribonucleotide reductase, was a gift of JoAnne Stubbe (Salowe and Stubbe, 1986).

Cloning and Overexpression. The gene for the 37-kDa VVR2 protein was previously localized to the left end of the *Hin*dIII F fragment of the vaccinia virus genome (Slabaugh *et al.*, 1988). A 2.3-kilobase *Xba*I fragment containing the gene was cloned into the *Xba*I site in the polylinker of the phagemid vector pIBI30 (International Biotechnologies, Inc., New Haven, CT). An *Nco*I site was engineered at the initiating ATG start codon of the VVR2 gene by site-directed mutagenesis (Kunkel *et al.*, 1987) using the oligonucleotide 5'-GGATGGGTTCCATGGCTAAATTAACAATAA- 3'. This generated a two base pair change (AA to GG; underlined in the oligonucleotide sequence) directly upstream of the start codon. The manipulation did not change the coding sequence of the VVR2 gene. The new construct, pXR2a-*Nco*I, was digested with *Nco*I and *Hin*dIII and the fragment containing the VVR2 gene was ligated into the expression vector pET11d (Rosenberg *et al.*, 1987). Routinely, a 1-liter culture of BL21(DE3) cells harboring the plasmid pET11d-R2 was grown overnight at 30°C. The saturated culture was diluted 1:1 with Luria broth and incubated at 30°C for 1 h before induction for 4 h with 0.4 mM isopropyl ß-D-thiogalactopuranoside.

Large-Scale Purification of Recombinant VVR2 Protein. The purification scheme used to isolate the recombinant VVR2 protein involved streptomycin sulfate and ammonium sulfate precipitations, gel filtration, and anion-exchange chromatography. Precipitation steps were performed at 0-4°C, and column chromatography was done at ambient temperature. Beyond the ammonium sulfate step, we found it advantageous to include glycerol (5%, v/v) in purification buffers to avoid precipitation of vvR2 protein. Cells were harvested by centrifugation at 3,500 x *g*, and the cell pellet was resuspended in 5 volumes of Buffer A and lysed in a French press. Debris was pelleted by centrifugation at 10,000 x *g* for 20 min. To remove nucleic acids, solid streptomycin sulfate was added to the clarified extract to 0.5% (w/v). After

stirring on ice for 30 min, the solution was centrifuged at 8,500 x *g* for 20 min to remove insoluble material. To concentrate the protein extract, solid ammonium sulfate was added to 40% saturation with stirring for 30 min on ice, and the following suspension was centrifuged at 8,500 x *g* for 20 min. The supernatant was discarded and the precipitate was resuspended in Buffer A and applied directly to a Superose 6 HR16/50 fast protein liquid chromatography gel filtration column (16 x 50 mm, Pharmacia LKB Biotechnology Inc.) that had been pre-equilibrated with Buffer B. Following elution in Buffer B, fractions were analyzed for the presence of VVR2 by SDS-polyacrylamide gel electrophoresis. The recombinant protein eluted as a single peak. For the final step in purification, anion-exchange chromatography, pooled eluate from the Superose 6 column was loaded onto a Mono Q HR5/5 fast protein liquid chromatography column and eluted with a triphasic salt gradient of 0-0.3 M NaCl in Buffer C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blots. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels as described by Laemmli (1970). Peptide bands were visualized by staining with Coomassie Brilliant Blue. For immunoblots, proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) for 30 min at 200 mA by using a semidry electroblotter (Idea Scientific, Minneapolis, MN) as described by Towbin *et al.* (1979). The membrane was blocked in Buffer D with 1% gelatin, incubated in Buffer D containing polyclonal antiserum to the vaccinia R2 protein at a dilution of 1:2,000, and then incubated in Buffer D containing goat anti-rabbit antibody conjugated to alkaline phosphatase at a dilution of 1:5,000 (Boehringer Mannheim).

Polyclonal Antisera. An EcoRV-XbaI DNA fragment encoding sequences for 30 kDa of the vaccinia virus R2 gene product was cloned into a pATH bacterial expression vector (Dieckmann and Tzagoloff, 1985). TrpE-VVR2 fusion protein was

purified by SDS-polyacrylamide gel electrophoresis and injected into rabbits by using established procedures. Antiserum to VVR2 was demonstrated to be specific for vaccinia virus R2 by Western blot and immunoprecipitation[3].

Enzyme Activity Measurements and Preparation of Native VVR2 Extracts. The assay for ribonucleotide reductase measured conversion of [^{3}H]-CDP to [^{3}H]-dCDP and was performed as described previously (Slabaugh *et al.*, 1984). Bovine serum albumin was added to reaction mixtures to normalize the protein concentration. Assay reactions were run in triplicate, and the results were averaged.

A unit of enzyme activity is defined as the amount of VVR2 protein which catalyzes the formation of 1 nmol of dCDP in 1 min in the presence of an excess of complementary vaccinia virus ribonucleotide reductase large subunit (VVR1) protein at 30°C. Specific activity is defined as units of enzyme activity per mg of protein. The most reliable measurements of enzyme activity were made when VVR2 protein was present at 0.01-0.5 units/20 µl reaction, and the complementary VVR1 protein was present in a 5-fold molar excess. In preliminary experiments, we incubated preparations of recombinant VVR1 and VVR2 together before initiation of the reaction to determine whether preassociation of the two subunits affected enzyme activity. Pre-incubation periods of 1, 10, or 40 min at 30 or 37°C did not affect the activity detected in a 10-min period immediately after the preincubation.

Virus-infected and uninfected cell extracts were prepared as described previously (Slabaugh and Mathews, 1984). VVR1 and host cell R1 protein were specifically removed from virus-infected and uninfected extracts by using an affinity matrix, dATP-Sepharose. Crude extracts were incubated at 4°C for 30 min with dATP-Sepharose resin, and separated from the resin by brief centrifugation. This resulted in extracts with no endogenous ribonucleotide reductase activity and no immunoreactive

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[3] Howell *et al.*, manuscript in preparation.

VVR1. For specific activity measurements, VVR2 protein was complemented with purified recombinant vaccinia virus large subunit[4] . We choose to use recombinant VVR1 in our complementary enzyme assay because although native VVR1 can be recovered from the dATP-Sepharose affinity resin, it is contaminated with host R1. For the activity assay measurements in Figure III.4, 0.27 ug of VVR1 was included in each reaction.

To add equivalent amounts of recombinant and native VVR2 to the reactions we estimated the concentration of native VVR2 in the cellular extract by quantitative Western blot analysis. Incremental amounts of purified recombinant VVR2 (0.07-0.70 μg) were electrophoresed on an SDS-polyacrylamide gel alongside aliquots of extract from virus-infected cells. This procedure allowed us to estimate the VVR2 concentration in the extract to within 10%.

Protein Concentration Determination. Protein concentrations were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard. The relative amount of VVR2 protein in extracts was determined by scanning laser densitometry of Coomassie-stained extracts in SDS-polyacrylamide gels using a Zeineh model SL-504-XL instrument (Biomed Instrument Inc., Fullerton, CA). The extinction coefficient of purified VVR2 was determined by amino acid analysis. This procedure was performed as described by Malencik *et al.*, (1990). Briefly, vacuum-dried VVR2 protein (approximately 5 μg) was subjected to acid hydrolysis with 6 M HCl in the vapor phase for 24 h at 160°C. After dabsyl chloride derivatization, approximately 100 total nmoles of amino acid were chromatographed on a reverse-phase Ultracarb 20 HPLC column. Absorbance was monitored at 460 nm and peak areas were determined by using Dynamic Solutions' Baseline data acquisition

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[4] Slabaugh *et al.*, manuscript in preparation.

system (version 3.0). Based on parallel analysis of a known amount of chicken lysozyme, recovery of VVR2 was quantitative.

Amino acid analysis yielded a molar extinction coefficient for active VVR2 of 119,000 M^{-1} cm^{-1} at 280 nm.

Circular Dichroism. The circular dichroism spectrum for VVR2 was recorded on a Jasco J-41A spectropolarimeter that was calibrated by using (+)-10-camphorsulfonic acid, $\Delta\Sigma \approx 2.42$ at 290.5 nm. The measurement was made using a 200 μm pathlength cell and the protein solution was 5.7 μM in 50 mM sodium phosphate buffer (pH 7.8). The secondary structure prediction was made using the variable selection method computer program outlined by Compton and Johnson (1986), with a basis set of 26 proteins.

Electron Paramagnetic Resonance Measurements. EPR spectra were obtained on a Varian E-109 spectrometer equipped with an E-102 microwave bridge and an Air Products liquid helium cryostat and appropriate cavity for measurements at 30 K. For quantitation of the tyrosyl radical content, measurements were performed at 108 K (by use of a Varian variable temperature accessory that flows chilled nitrogen gas over the sample) at a power of 1 mW and a modulation amplitude of 5 gauss. The area of the double integral was compared with that obtained from a 1.0 mM $Cu(ClO_4)_2$ standard in 2 M $NaClO_4$, 10 mM HCl (pH 2) under similar instrumental conditions.

III.4 Results

III.4.1 Overexpression of the VVR2 Subunit in E. coli

As shown in Figure III.1, induction of BL21(DE3) cells containing pET11d-R2 with isopropyl ß-D-thiogalactopyranoside resulted in high-level expression of a 37-kDa peptide (*lanes 2-5*). This peptide was not detected in BL21(DE3) cells containing the control plasmid, pET11d (*lane 1*). After 4 h of induction, VVR2 accumulated to approximately 15% of the total soluble protein as determined by densitometric scanning of Coomassie-stained electrophoretic bands. Approximately 40 mg of recombinant VVR2 was produced per liter of bacterial culture.

pET11d-R2 was induced at several different temperatures in an attempt to increase the ratio of soluble to insoluble VVR2 protein in the cell extracts. When cultures were induced at 37°C, only 50% of the total amount of expressed VVR2 protein was soluble. However, when the cultures were induced at 30°C, more than 70% of the recombinant protein was expressed in soluble form. Induction at temperatures below 30°C under the same growth conditions decreased the level of total protein expression.

III.4.2 Summary of VVR2 Purification

A typical purification of recombinant VVR2 is summarized in Table III.1. Two liters of bacterial culture yielded 18.8 mg of purified VVR2 protein with a specific activity of 48 units/mg. The final step in purification, chromatography on a strong anion-exchange matrix, separated full-length VVR2 monomers from partially degraded VVR2 polypeptides. As shown in Figure III.2*A*, VVR2 eluted in two peaks. The first peak, eluting at 0.20 M NaCl, contained full-length 37-kDa VVR2 as well as several peptides of discrete sizes (35.5, 34.5 and 33 kDa) whose electrophoretic mobilities

Figure III.1 Expression of Recombinant VVR2 protein in *E. coli* at 30°C. Isopropyl β-D-thiogalactopyranoside was added to cultures to 0.4 mM and 1-ml aliquots were removed from cultures of pET11d-R2 at 0, 1, 2, and 4 h post induction (*lanes 2-5*) and from pET11d at 4 h (*lane 1*). Aliquots were centrifuged, and each cell pellet was resuspended in 200 µl of SDS-containing sample buffer. Extracts were sonicated, heat denatured at 100°C for 2 min, and 20 µl was loaded onto an SDS-polyacrylamide gel.

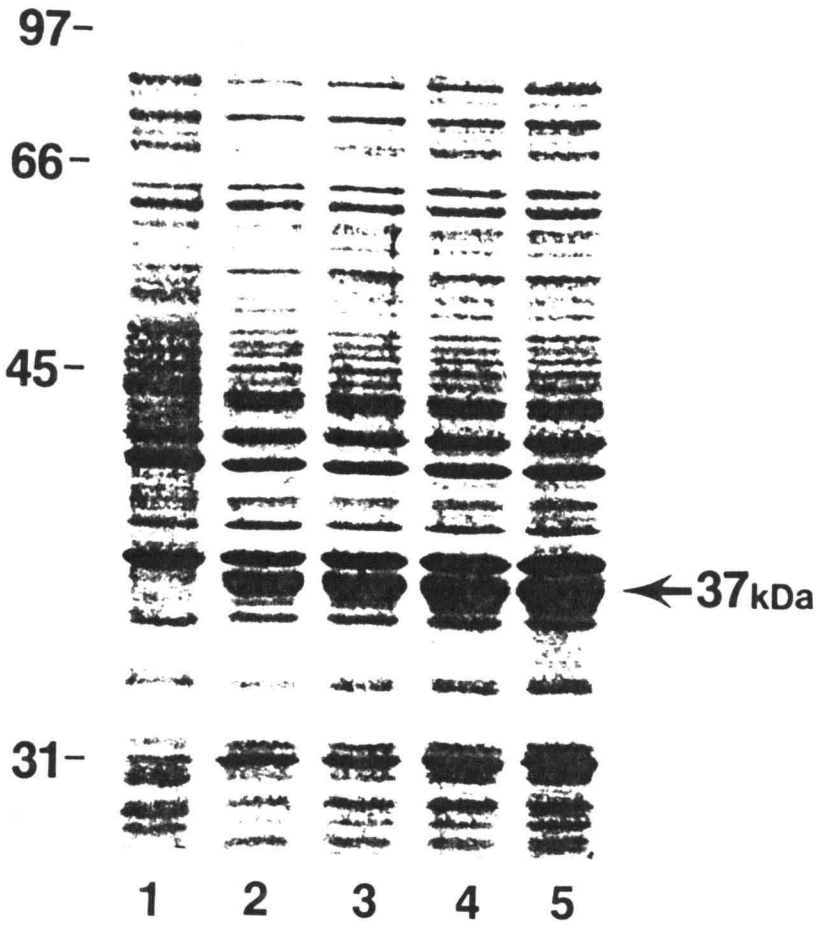

Figure III.1

Figure III.2 Anion-Exchange Chromatography of Recombinant VVR2 Small Subunit. Partially-purified VVR2 (~100 μg) was applied to a Mono Q HR5/5 column and eluted using a triphasic salt gradient of 0-0.3 M NaCl in Buffer B. *Panel A*, NaCl gradient and absorbance at 280 nm of the Mono Q eluate. *Panel B*, 50 μl of each 0.5-ml fraction analyzed on an SDS-polyacrylamide gel. The fraction numbers for the sample are indicated below the elution profile and below the corresponding *lanes* of the gel.

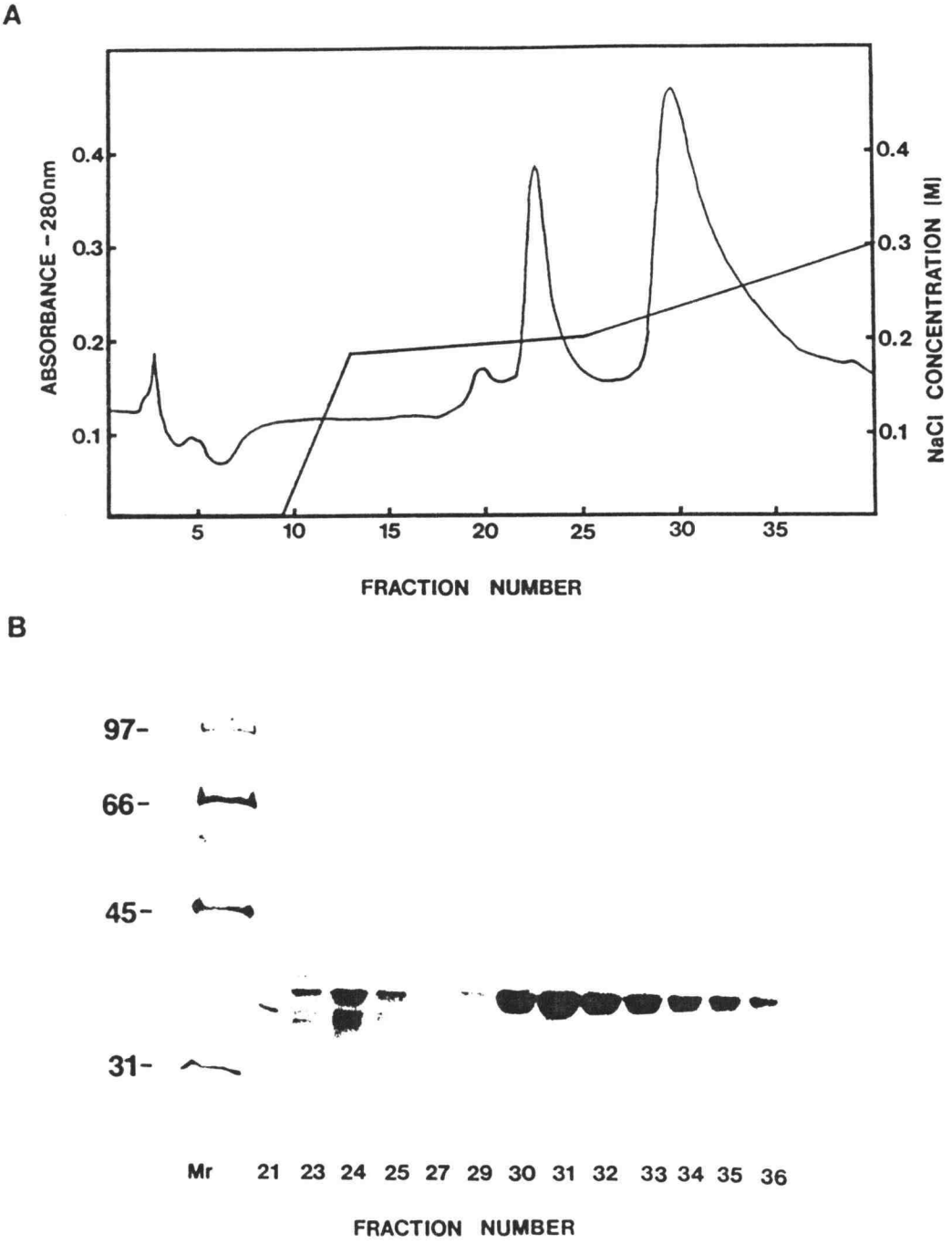
A
ABSORBANCE - 280nm
0.4
0.3
0.2
0.1
NaCl CONCENTRATION (M)
5
10
15
20
25
30
35
FRACTION NUMBER
B
97-
66-
45-
31-
Mr
21
23
24
25
27
29
30
31
32
33
34
35
36
FRACTION NUMBER

Figure III.2

Table III.1 Purification of Recombinant R2

Purification Step	Total Volume (ml)	Total Protein (mg)	Enzyme Activity (units)	Specific Activity (units/mg)	Yield %	Purification -fold
crude extract [a]	30	630	5090	8.1	100	1.0
amm. sulfate	5	151	1330	8.8	26	1.1
gel filtration	12	73	1190	16.3	23	2.0
anion-exchange	6	18.8	900	48	17.6	6.0

[a]Crude extract denotes the supernatant fraction obtained after the removal of cell debris

were slightly greater than full-length VVR2 (Figure III.2*B*, Fractions 23-25). All peptides in these fractions reacted positively to anti-VVR2 antiserum in Western blot analysis, suggesting that the shorter fragments were derived from full-length VVR2. The second peak, which eluted at 0.25 M NaCl, contained predominantly 37-kDa monomer.

Sjöberg *et al.* (1987), described fractionation on a Mono Q HPLC anion-exchange column as a method for separating *E. coli* R2 homodimers from heterodimers consisting of one full-length and one R2 polypeptide of smaller molecular mass. The truncated *E. coli* polypeptide was shown to be a carboxy-terminal proteolytic digestion product that was present when PMSF was omitted from purification buffers. Mann et al. (1991) reported that recombinant mouse R2 is also susceptible to partial degradation during purification even in the presence of protease inhibitors, but that in this case, degradation was from the amino-terminal end of the protein. Similarily, addition of the protease inhibitors PMSF, pepstatin, leupeptin, and aprotinin to purification buffers did not prevent formation of the degradation products that we observed. Analogous to the formation of *E. coli* heterodimers, we hypothesize that the first fraction eluted from the Mono Q column represents various VVR2 heterodimers of different molecular masses.

The mobility of purified recombinant VVR2 was compared to that of native VVR2 from a vaccinia infected-cell extract by migration of the two proteins in SDS-PAGE (Figure III.3). Staining of a blot of the gel with antiserum against a TrpE-VVR2 fusion protein revealed a single 37-kDa band in the lanes containing recombinant and native VVR2, but no band in the lane containing extract from uninfected cells. Native VVR2 degradation fragments were not detected in the lane containing VVR2. Additional Western blot analysis of recombinant VVR2 during purification revealed that the protein is not proteolytically degraded until after lysis of the bacterial cells.

Figure III.3 Protein Gel and Western Blot Analysis of Recombinant and Native VVR2. *Lane 1*, Purified recombinant VVR2 (0.35 μg). *Lane 2*, 8 μl of a vaccinia virus-infected cell extract (1.2 mg/ml total protein). *Lane 3*, 8 μl of an uninfected cell extract (1.2 mg/ml total protein).

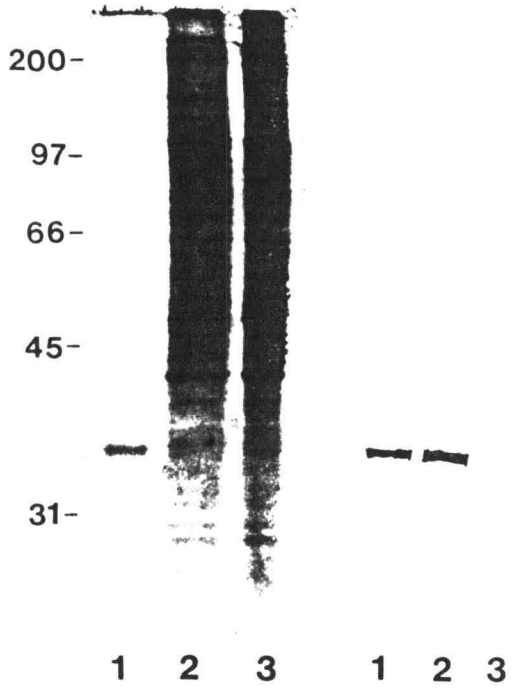

Figure III.3

To determine whether the recombinant vaccinia ribonucleotide reductase small subunit was biologically active, VVR2 was combined with purified recombinant VVR1. Neither VVR2 nor VVR1 preparations exhibited enzyme activity when assayed alone. However, as shown in Figure III.4, recombinant VVR2 did complement the recombinant VVR1 preparation. To compare directly the specific activities of recombinant and native VVR2 proteins, equivalent amounts of VVR2 were assayed for enzyme activity in the same experiment. (Native VVR2 concentration was estimated in crude extracts from infected cells as described under "Materials and Methods"). The saturation curve obtained by assaying increasing amounts of R2 with a constant amount of R1 showed that the specific activity of the purified recombinant VVR2 was equivalent to that of VVR2 produced in an infected eukaryotic cell.

III.4.3 Determination of the Apparent Molecular Mass for Native VVR2 Protein

A Superose 12 HR10/30 fast protein liquid chromatography gel filtration column (10 x 30 mm) was used to estimate the molecular mass of native R2 protein in solution. The molecular mass of VVR2 monomer calculated from the deduced amino acid sequence is 36,975 Da and the recombinant protein has an apparent size of 37 kDa as compared to standard proteins on an SDS-polyacrylamide gel. Purified VVR2 eluted as a single peak on a Superose 12 gel filtration column at a retention time of 67.4 min (Figure III.5), corresponding to an estimated molecular weight of 64,000. We conclude that the VVR2 protein exists in solution as a dimer. These results agree with experiments on corresponding proteins from *E. coli* (Thelander, 1973) and mouse (Thelander *et al.*, 1985), which also describe the native R2 unit as a dimer.

Figure III.4 Complementation of Recombinant and Native VVR2 with Purified Recombinant VVR1. Vaccinia and host R1 were removed from the cell extracts by adsorption with dATP-Sepharose. Assay parameters and the method for determining equivalent amounts of recombinant and native VVR2 are described under "Materials and Methods". *Solid line/open circle,* Purified recombinant VVR2; *Dashed line/solid circle*, Vaccinia virus-infected cell extract; *Dashed line/open triangle*, uninfected cell extract.

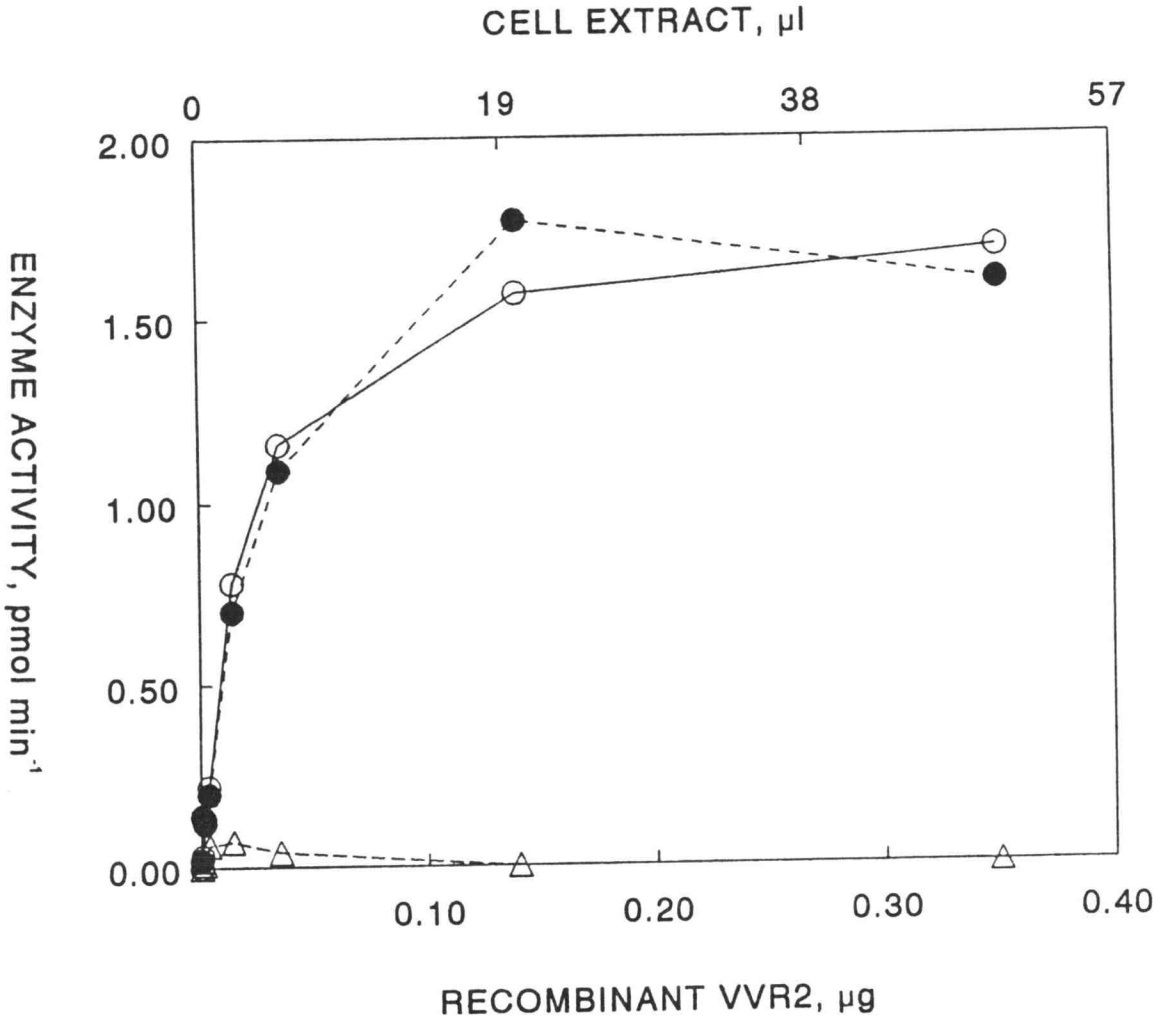
CELL EXTRACT, µl
0
19
38
57
2.00
1.50
1.00
0.50
0.00
ENZYME ACTIVITY, pmol min-1
0.10
0.20
0.30
0.40
RECOMBINANT VVR2, µg

Figure III.4

Figure III.5 Gel Filtration Column Chromatography of VVR2. Purified VVR2 protein was chromatographed on a Superose 6 molecular sieve column in 50 mM Tris-HCl (pH 7.6), 5% glycerol and 2 mM dithiothreitol. The flow rate was 1.0 ml/min and 1-ml fractions were collected. The *inset panel* depicts the correlation between the migration of molecular weight standards and the retention time from the column. *Letters* indicate elution of standards: *d*, aldolase at 148,000 Da; *c*, BSA at 68,000 Da; *b*, ovalbumin at 45,000 Da; *a*, cytochrome C at 12,500 Da. *e*, Migration of recombinant VVR2 relative to standards.

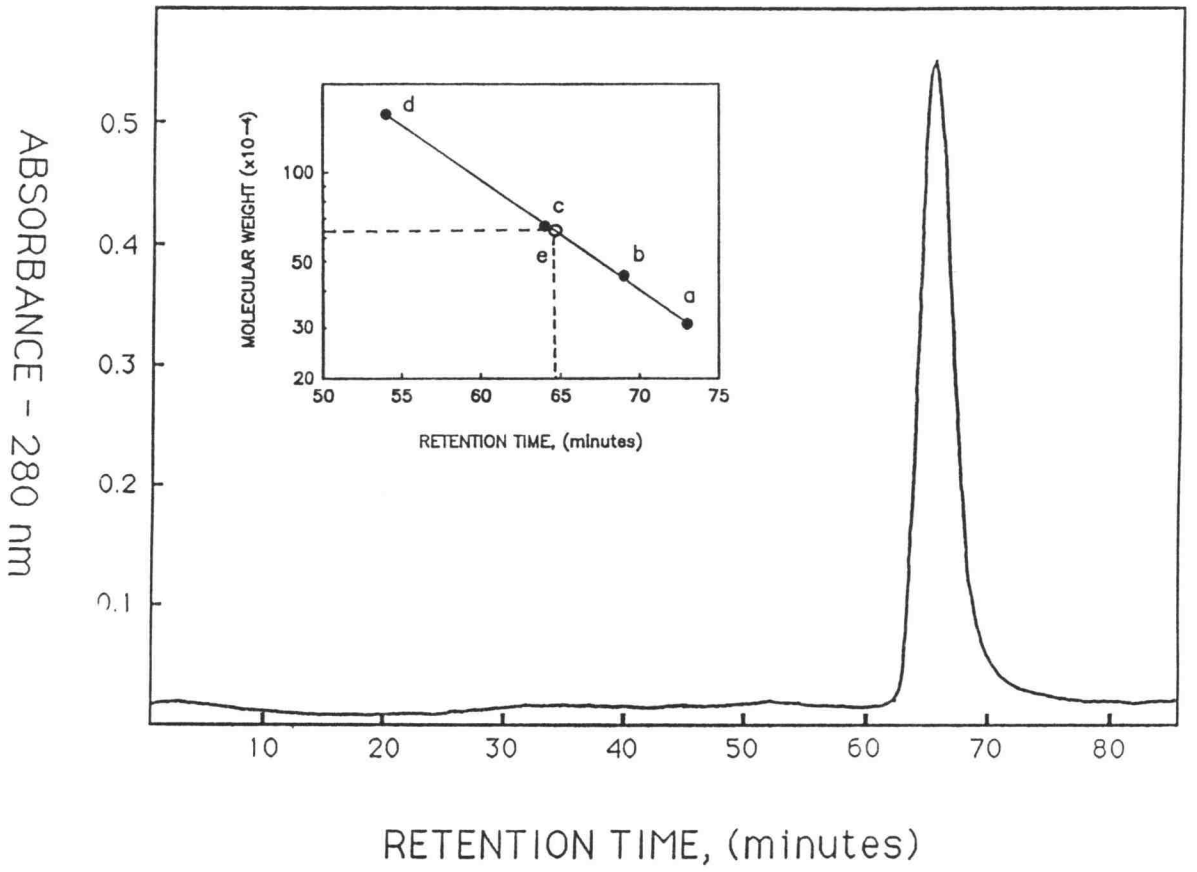
ABSORBANCE - 280 nm
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
RETENTION TIME, (minutes)
MOLECULAR WEIGHT (x10⁻⁴)
100
50
30
20
50
55
60
65
70
75
RETENTION TIME, (minutes)
d
c
e
b
a

Figure III.5

III.4.4 Circular Dichroism

The circular dichroism spectrum recorded for VVR2 is shown in Figure III.6. Analysis of the spectrum revealed the secondary structure composition of VVR2 to be 47% α-helix, 6% ß-sheet, 21% ß-turn, and 29% other structures. Recent CD spectra of mouse and *E. coli* R2 and three-dimensional structure analysis of *E. coli* R2, have distinguished the two proteins on the basis of secondary structure (Mann *et al.*, 1991; Nordlund *et al.*, 1990). It was demonstrated that *E. coli* R2 has a high α-helical content (70%) corresponding to the helix-turn-helix structural motif. Mouse R2 exhibited a lower α-helical content (50%), and had a significant secondary structure contribution from ß-sheet (17%).

III.4.5 Effect of Hydroxyurea on the Absorption Spectrum of VVR2 Protein

The near UV absorption spectra of the iron-containing ribonucleotide reductases exhibit characteristic bands that have been ascribed to the dinuclear iron center (325 and 370 nm) and the tyrosyl radical (390 and 410/416 nm) (Atkin *et al.*, 1973; Thelander *et al.*, 1985). The mammalian radical peak at 416 nm (Mann *et al.*, 1991) is slightly shifted from the sharp *E. coli* radical peak at 410 nm (Petersson *et al.*, 1980). The absorption spectrum of purified VVR2 protein from 300 to 500 nm is shown in Figure III.7*A*, *curve a*. In addition to absorption bands at 325, 370, and 390 nm, and similar to the published spectrum for mouse R2, a VVR2 peak is also detected at 416 nm.

Vaccinia virus ribonucleotide reductase activity is inhibited by the radical scavenger, hydroxyurea (Slabaugh and Mathews, 1986). Inactivation of mouse ribonucleotide reductase by hydroxyurea results in a decrease in intensity of all the near-UV absorbance bands (Thelander *et al.*, 1985). In contrast, hydroxyurea inhibition of *E. coli* R2 is characterized by the marked decrease in intensity of the maxima at 390 and 410 nm but only slight changes in the ferric iron center

Figure III.6 Circular Dichroism Spectrum of Recombinant VVR2. The spectrum of recombinant protein (0.42 mg/ml) in 50 mM sodium phosphate buffer, pH 7.6, was recorded with a 200 μm pathlength at 25°C.

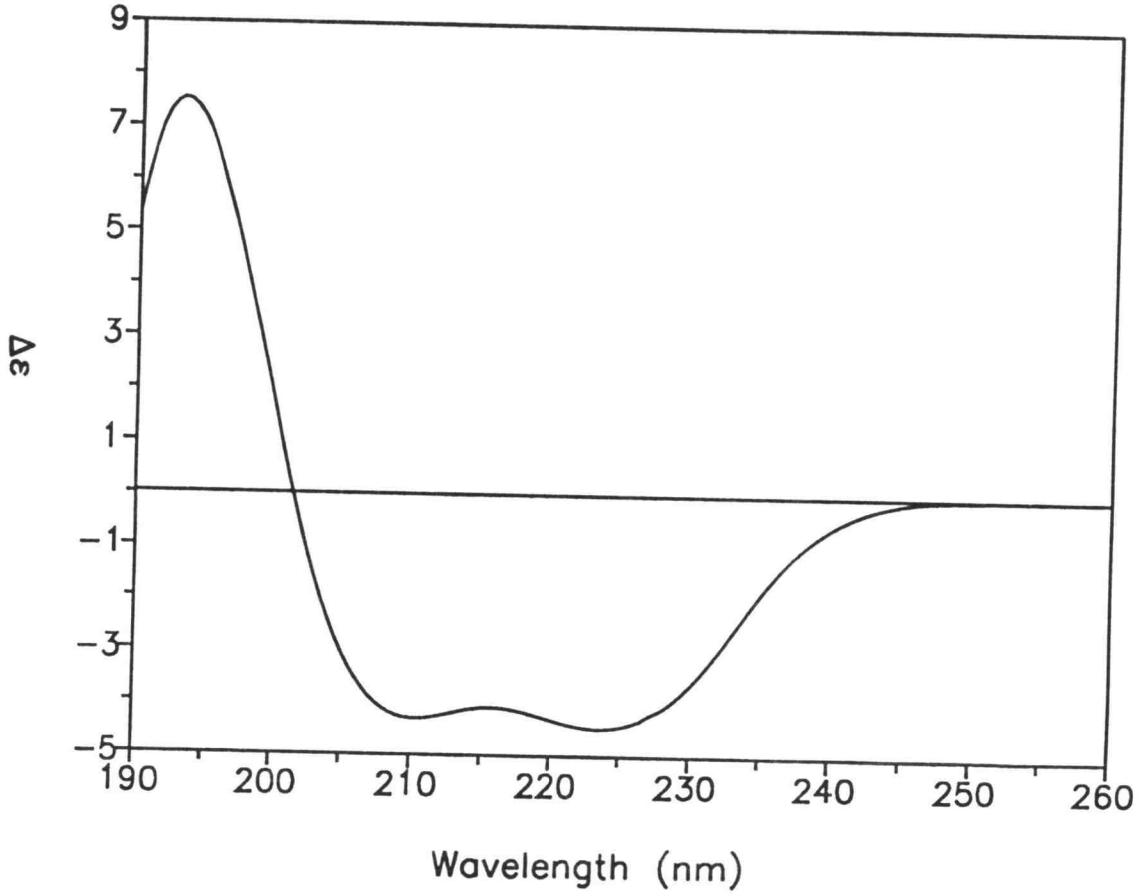

Figure III.6

Figure III.7 Light Absorption Spectra Above 300 nm for the vvR2 Subunit of Ribonucleotide Reductase. *Panel A*, The electronic spectra of *a*, protein VVR2 (19 μM) in Buffer C and *b*, an identical sample after incubation with 10 mM hydroxyurea for 20 min at 25°C. The absorbance peaks exhibited by mouse R2 protein are indicated by *arrows*. Spectral features are assigned to iron center (*I*) or radical (*R*). *Panel B*, difference spectrum derived from data in *A*.

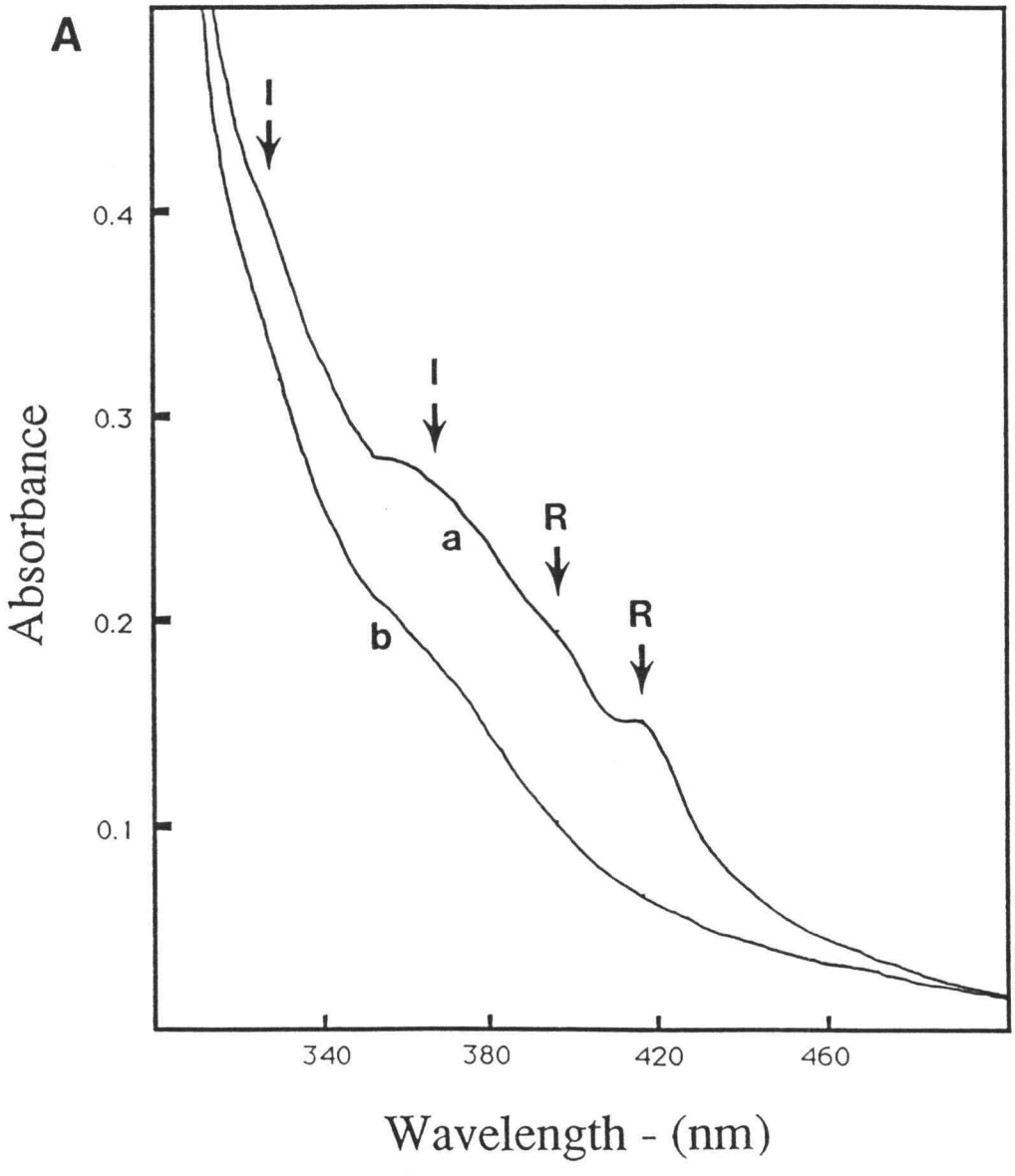

Figure III.7 Panel *A*

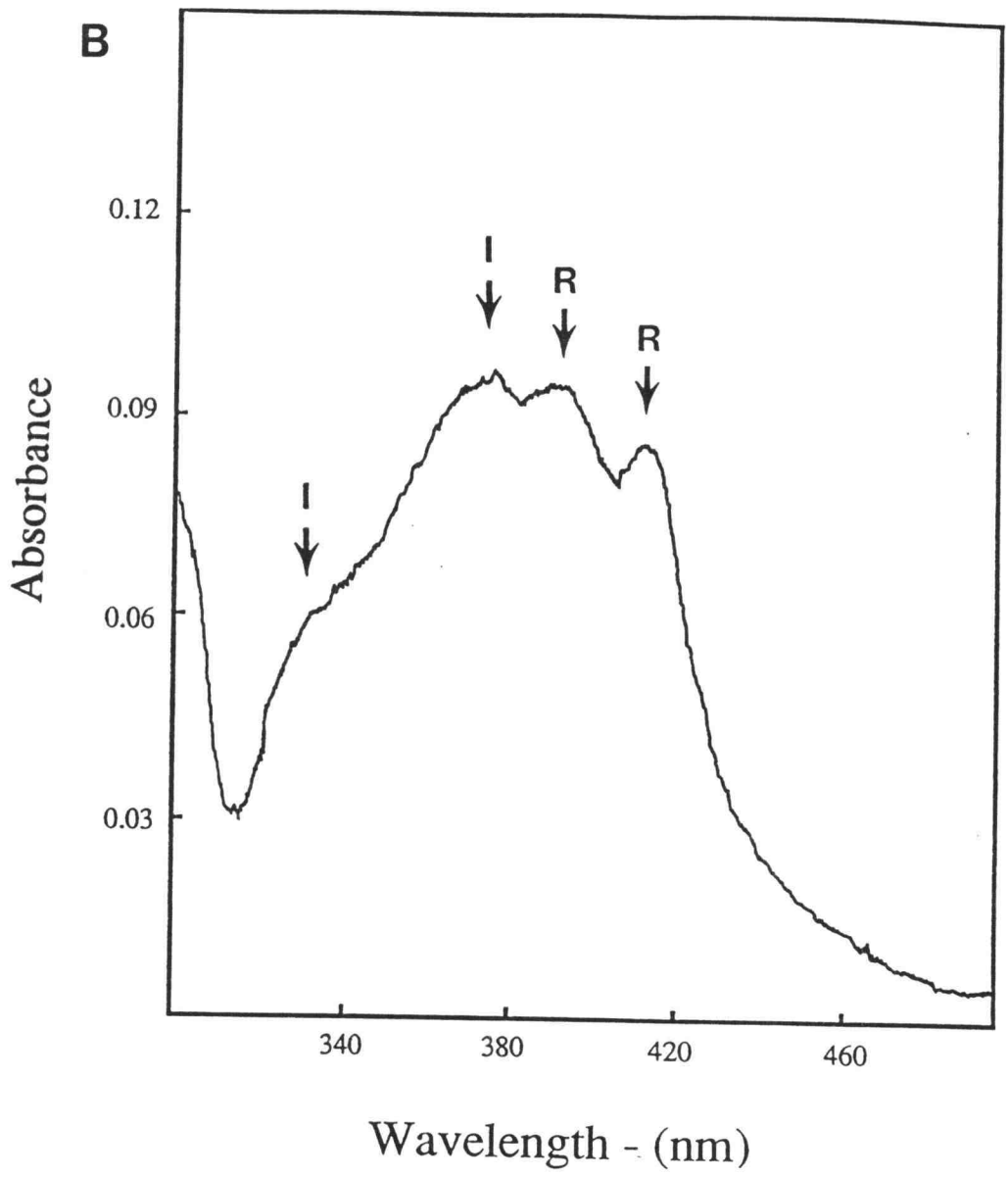

Figure III.7 Panel *B*

absorbances. (The 360-390 nm region contains overlapping contributions from both the free radical and the iron center and this has made quantitative analysis of the absorption signals somewhat ambiguous.)

To monitor the effects of hydroxyurea on the VVR2 protein, the electronic signals of the free radical and the iron species were measured after treatment of purified VVR2 with the inhibitor (Figure III.7*A*, *Curve b*). Hydroxyurea was added to the purified VVR2 sample to 10 mM in the presence of 2 mM dithiothreitol, and the preparation was incubated at 25°C for 30 min. A difference spectrum (Figure III.7*B*), derived by subtracting *curve b* from *curve a* , showed that absorbance maxima from the tyrosyl radical (390 and 416 nm) were decreased by 45 and 40% respectively, and that the iron center absorbance maxima (325 and 370 nm) were also significantly decreased (20 and 35%, respectively).

III.4.6 Characterization of Tyrosyl Radical by EPR Spectroscopy

Purified vaccinia virus R2 protein exhibited a g = 2.0 EPR signal (Figure III.8*B*) that is characteristic of a tyrosyl radical (Sjöberg and Gräslund, 1983). The EPR spectrum showed a hyperfine splitting pattern that was identical to that of native vaccinia protein expressed in virus-infected cells (Figure III.8*C*). No such signal was present in uninfected cells (Figure III.8*D*). Treatment of recombinant VVR2 with hydroxyurea, which destroys the enzymatic activity and the 416-nm absorption band associated with the tyrosyl radical (see above), also caused the disappearance of the tyrosyl radical EPR signal. (The spectrum of VVR2 treated with 10 mM hydroxyurea for 30 min, looked the same as the spectrum in Figure III.8*D*).

The hyperfine splitting of the tyrosyl radical signal appears to be extremely sensitive to the orientation of the ß-CH_2 group with respect to the tyrosine ring (Gräslund *et al.*, 1982). The close similarity of the EPR spectra in Figures III.8*B* and

Figure III.8 EPR Spectra at 30 K of Tyrosyl Radicals in R2 Proteins. *A*, *E. coli* R2 protein from strain N6405/pSPS2 (0.1-mW power, 1.6 gauss modulation amplitude). *B*, Purified recombinant VVR2 protein, 10 μM in R2 dimer (3 mW, 1.6 gauss). *C*, VVR2 protein from virus-infected BSC_{40} cells (3 mW, 4 gauss). *D*, Protein from uninfected BSC_{40} cells (3 mW, 4 gauss). *E*, Hydroxyurea-resistant, R2-overproducing mouse fibroblast 3T6 cells [data from Lankinen *et al.* (1982)]. Samples in *A*, *C*, and *D* were partially purified by ammonium sulfate fractionation. *mT*, millitesla.

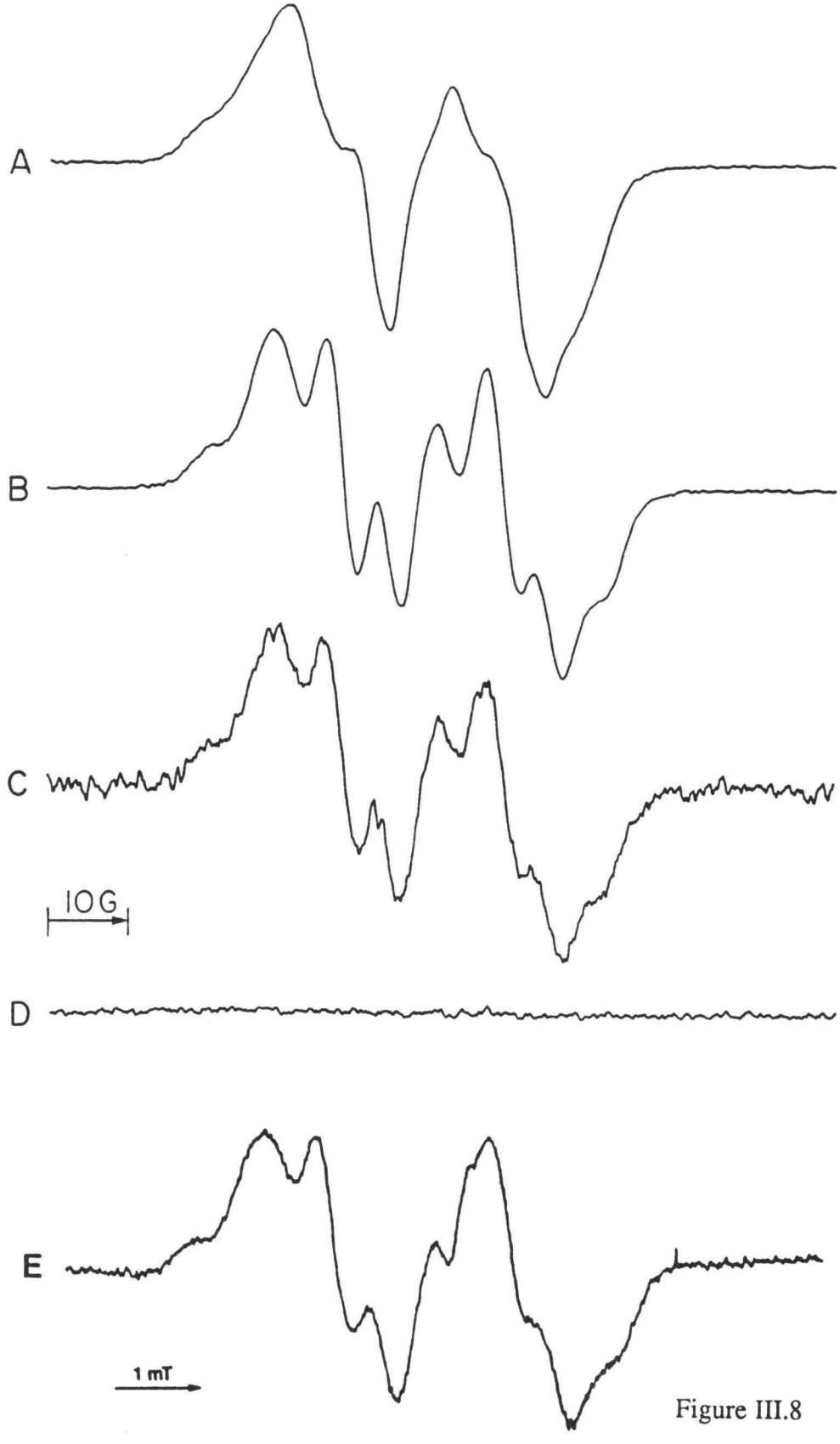

Figure III.8

8*C* indicates that expression of the vaccinia virus R2 gene in *E. coli* yields a protein whose metal center is indistinguishable from that of the native virus. The hyperfine splitting pattern of vaccinia virus R2 is readily distinguished from that of *E. coli* (Figure III.8*A*). In fact, the EPR spectrum of vaccinia virus R2 closely matches the spectra of ribonucleotide reductases from eukaryotic sources such as mouse (Figure III.8*E*, Gräslund *et al.*, 1982) and yeast (Harder and Follmann, 1990). The spectra of the R2 radicals from the pseudorabies virus (Lankinen *et al.*, 1982), herpes simplex virus (Mann *et al.*, 1991), and bacteriophage T4 (Sahlin *et al.*, 1982) are all distinctively different. These findings suggest that the vaccinia R2 gene is closely related to and presumably derived from a mammalian gene, in agreement with amino acid sequence comparisons (Slabaugh *et al.*, 1988).

Another point of similarity between vaccinia and mouse R2 proteins is in the power dependence for microwave saturation. As in the case of mouse R2 (Mann *et al.*, 1991), recombinant VVR2 begins to show saturation of its EPR signal (i.e., >10% reduction in $S/P^{1/2}$) when the microwave power exceeds 1 mW at 30 K. In contrast, the EPR signal of *E. coli* R2 begins to saturate already at 0.1 mW at 30 K, whereas that of herpes simplex R2 saturates only above 10 mW at 30 K (Mann *et al.*, 1991). The differences in saturation behavior are believed to be related to the degree of magnetic interaction between the tyrosyl radical and the dinuclear iron center (Sahlin *et al.*, 1987). Such an interaction enhances the spin-lattice relaxation rate, thereby making the signal less susceptible to saturation. Thus, it would appear that the tyrosyl radical is more closely associated with the dinuclear iron center in vaccinia and mouse R2 than it is in *E. coli* R2.

The spin concentrations in two different preparations of purified recombinant VVR2 were calculated by comparison to a copper standard. The protein concentration was determined by amino acid analysis. These measurements indicated that at the

completion of the purification procedure, 0.3 tyrosyl radicals were present per VVR2 polypeptide chain.

III.4.7 Attempted Reconstitution of Vaccinia Virus R2

In an attempt to increase the iron and tyrosyl radical content of recombinant VVR2 protein, purified protein was subjected to aerobic (Thelander *et al.*, 1985) and anaerobic (Mann *et al.*, 1991) reconstitution procedures. Briefly, this involved exposing the protein to excess ferrous iron in the presence of dithiothreitol (aerobic reactivation) or sodium ascorbate (anaerobic reactivation). To determine what effect reactivation had on the VVR2 protein, we compared the enzymatic activities of reconstituted and unreconstituted protein samples and measured the radical content of the protein before and after reactivation procedures. Enzyme assay results reflected no increase in activity resulting from either aerobic or anaerobic treatment of VVR2 compared to untreated protein. Additionally, inclusion of $FeCl_3$ or $Fe(NH_4)_2(SO_4)_2$ in the enzyme assay had no effect on the activity detected in treated or untreated VVR2 protein. As measured by EPR, neither reactivation procedure significantly increased the radical content of the vaccinia virus protein.

We also asked whether the activity (tyrosyl radical content) of recombinant VVR2 could be increased by supplementing the bacterial growth medium with exogenous iron or adding ferrous iron to lysis buffers. Neither of these approaches increased activity above the value of approximately 50 units/mg reported in Table III.1.

III.5 Discussion

In these experiments, we have characterized the 37-kDa vaccinia virus ribonucleotide reductase small subunit protein that has been overexpressed in *E. coli*.

By several criteria, we have shown that the recombinant VVR2 subunit is functionally and physically equivalent to the R2 subunit expressed in vaccinia virus-infected mammalian cells. Polyclonal antiserum made to a fusion protein containing a major portion of the vaccinia virus R2 protein reacted with the recombinant protein expressed in *E. coli*. When native and recombinant VVR2 were complemented with purified recombinant VVR1, ribonucleotide reductase activity was readily detected, and the specific activity of both proteins was the same. In addition, the EPR spectrum of recombinant protein matched that of a partially purified preparation of VVR2 protein produced by viral infection of BSC_{40} cells.

The circular dichroism spectrum of VVR2 revealed the viral protein to be similar to mouse R2 in α-helical content (~50%). Thus, the eukaryotic R2 molecule appears to diifer significantly in secondary structure from *E. coli* R2 which has been shown by CD and crystallography (Mann *et al.*, 1991; Nordlund *et al.*, 1990) to be exceptionally rich in α−helical content (70%). ß-sheet contributions to the CD spectrum in the case of VVR2 (6%), were intermediate between the reported values for *E. coli* (3%) and mouse R2 (17%).

From our spectroscopic studies we conclude that the protein environment of the tyrosyl radical in VVR2 is similar to that of the previously characterized mouse and calf thymus proteins and exhibits certain distinctions from the *E. coli* R2. The location of the radical absorbance peak at 416 nm in the electronic spectrum of the recombinant VVR2 protein differs from the 410 nm maximum for the *E. coli* protein but closely matches the 416 nm absorption band of the mouse protein (Mann *et al.*, 1991). Hydroxyurea inhibition of VVR2 protein revealed that like analogously treated mouse R2, vaccinia virus R2 exhibits an absorption spectrum in which all four near UV electronic signals are attenuated in the presence of inhibitor. This contrasts with the effect of hydroxyurea on *E. coli* R2, in which the ferric iron center electronic signals

are only slightly decreased. One interpretation of these results is that the mammalian ferric iron center is more susceptible to reduction by hydoxyurea than is the *E. coli* iron center. Either reduction of ferric iron to the ferrous form or dissociation of iron from the protein could cause the decreased intensity of the iron center electronic signal. We are continuing inhibitor studies on the VVR2 protein to examine more closely the mechanism of hydroxyurea inhibition.

The EPR spectrum of the recombinant VVR2 more closely resembles the EPR spectrum for mouse protein than *E. coli* R2, both in the hyperfine splitting pattern and in the susceptibility to microwave power saturation at low temperature. The former is indicative of a conserved conformation for the tyrosyl radical side chain in the mouse and vaccinia proteins. The latter suggests increased interaction between the tyrosyl radical and the dinuclear iron center in mouse and vaccinia virus R2 relative to *E. coli* protein.

Various preparations of recombinant VVR2 yielded protein with 25-30% of the theoretical maximum of one tyrosyl radical per R2 protomer. For comparison, calf thymus, mouse, and herpes virus R2 proteins purified from natural or recombinant sources exhibit even lower levels of radical content (0-14%) and corresponding low iron occupancies (0-6%) (Thelander *et al.*, 1983; Mann *et al.*, 1991). However, whereas mouse and *E. coli* R2 can be reactivated to 70-100% iron occupancy (3.2-4.0 Fe/dimer), and up to 80% radical content (1.6 radical/dimer) (Bollinger *et al.*, 1991; Mann *et al.*, 1991), identical reactivation procedures applied to the vaccinia virus enzyme did not increase radical/R2 monomer ratios above 30%. Iron/radical centers in herpes virus R2 were also reported to be resistant to full reactivation (Mann *et al.*, 1991). Additionally, inclusion of ferrous or ferric iron in the enzyme assay had no effect on the activity measured in "unreactivated" or "reactivated" VVR2 preparations.

This contrasts with the apparent lability of the iron center in calf thymus R2, which may require continual regeneration during enzyme turnover (Thelander *et al.*, 1983).

The lower specific activities of vaccinia and herpes virus R2 proteins (approximately one-sixth that of mouse R2) may be caused by the lower steady-state radical contents exhibited by these viral enzymes *in vitro*. Our results, taken together with studies of R2 from other species, emphasize the variable nature of the stability of the iron/radical center in this metalloprotein.

III.6 Acknowledgements and Author Contributions

We thank Dr. Dean Malencik for performing the amino acid analysis, Jeannine Lawrence for help recording and analyzing CD spectra, and Drs. Helmut Beinert and Claire Kennedy for advice on the quantitation of the EPR spectra.

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The contributions of the authors are as follows. C. K. Mathews provided insightful discussion during this work and provided constructive and critical review of this manuscript. M. B. Slabaugh assisted in the design of many of the experiments and also provided constructive and critical review of this manuscript. N. A. Roseman designed preliminary engineering schemes for cloning the R2 gene into expression vectors, and provided helpful assistance in the actual cloning manipulations. N. A. Roseman also provided VVR2 antisera for use in this study and assisted in the design of R2 purification. J. Sanders-Loehr and T. M. Loehr directed the electron paramagnetic resonance experiments and contributed valuble discussion and critic to the manuscript. M. L. Howell performed all other experiments.

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IV. Vaccinia Virus Ribonucleotide Reductase: Correlation Between Deoxynucleoside Triphosphate Supply and Demand

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IV.1 Abstract

Ribonucleotide reductase is considered a rate-limiting enzyme in DNA synthesis. In the past, we have characterized the virus-encoded small subunit protein of ribonucleotide reductase from the orthopoxvirus, vaccinia [Howell, M. L., Sanders-Loehr, J., Loehr, T. M., Roseman, N. A., Mathews, C. K., and Slabaugh, M. B. (1992) *J. Biol. Chem.* 267, 1705-1711] and in an accompanying manuscript we describe characterization of the large ribonucleotide reductase subunit protein (Slabaugh *et al.*, this issue). In this report, we use quantitative analysis of ribonucleotide reductase protein levels and DNA accumulation in vaccinia virus-infected cell extracts to correlate the supply of deoxyribonucleotides with the demand for these precursors in viral DNA synthesis. To facilitate our quantitation, polyclonal antisera were generated to TrpE fusion proteins constructed from the carboxyl termini of both subunits of viral ribonucleotide reductase. S1 nuclease and immunoprecipitation analysis were used to determine the transcriptional and translational kinetics of vaccinia virus ribonucleotide reductase expression. Enzyme activity and ribonucleotide reductase protein stability were also assayed during the viral time course of infection. Enzyme-linked immunoassays were used to quantitate protein levels, and dot blot filter hybridizations were used to measure the accumulation of viral DNA. Our results demonstrate that the rate of catalysis of ribonucleotide reduction is sufficiently high in vaccinia virus-infected cells to synthesize substrates at rates needed to sustain viral DNA synthesis.

IV.2 Introduction

Ribonucleotide reductase catalyzes the direct reduction of ribonucleotides to deoxyribonucleotides and occupies the first committed step in the *de novo* synthesis of

DNA (Thelander and Reichard, 1979). As such the enzyme has been extensively studied for its role in, or response to, cell divison and cell cycle control (Hurta and Wright, 1992; Hurta *et al.*, 1991; Elledge *et al.*, 1992). Biochemical studies with the mammalian enzyme have demonstrated that *in vivo* ribonucleotide reductase activity is at a high level during S-phase, coincident with cellular DNA synthesis and metabolic requirements for DNA precursors (Engström *et al.*, 1985; Reichard, 1988). Further work elucidated that expression of the mRNA's encoding the two subunits of mouse ribonucleotide reductase occurs coordinately during S-phase (Björklund *et al.*, 1990; Engström and Rozell, 1988; Mann *et al.*, 1988), and that variation in the stability of the two polypeptide subunits is the specific factor that regulates the level of enzyme activity (Björklund *et al.*, 1990; Mann *et al.*, 1988).

Vaccinia virus, the prototypic poxvirus, contains a large duplex DNA genome (187 kb) and is a member of the only known family of DNA viruses that replicate in the cytoplasm of infected cells (Moss, 1990; Goebel *et al.*, 1990). Considering the requirement for a ready supply of precursors for DNA synthesis by vaccinia virus, it is not surprising that the virus encodes and expresses many of the enzymatic activities required for DNA metabolism and synthesis, including ribonucleotide reductase (Slabaugh *et al.*, 1984). The genes that encode the viral ribonucleotide reductase subunits have been sequenced and localized to positions on the vaccinia genome separated by 35 kilobases (Slabaugh *et al.*, 1988; Tengelson *et al.*, 1988; Schmitt and Stunnenberg, 1988). Like the mammalian enzyme, the vaccinia virus holoenzyme consists of two dissimilar homodimers, both of which are essential for activity. The viral large subunit (designated R1)[1] is a homodimer comprising two 86-kDa polypeptides and contains binding sites for nucleotide substrates and allosteric effectors

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[1]The abbreviations used are: R1 and R2, large and small subunit of ribonucleotide reductase, respectively; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

(Slabaugh *et al.*, 1984; Slabaugh and Mathews, 1984). The small subunit (designated R2) is a homodimer comprising two 37-kDa polypeptides and contains an iron-stabilized free radical that is required for catalysis (Howell *et al.*, 1992). Amino acid sequence comparisons and physical characterization of the viral R2 ribonucleotide reductase subunit have revealed several similarities with the mammalian counterpart (Slabaugh *et al.*, 1988; Howell *et al.*, 1992).

The events of vaccinia virus replication have been well characterized. Early studies, which measured viral DNA synthesis through incorporation of [^{3}H]-thymidine into acid precipitable material, demonstrated that during a vaccinia virus infection, DNA replication begins at approximately 1-2 h post infection (Joklik and Becker, 1964; Slabaugh and Mathews, 1986; Jungwirth and Launer, 1968). Synthesis of DNA was shown through these measurements to remain high for 2-3 h and then sharply decline to background levels by 8 h post infection. These studies also concluded that the maximal rate of viral DNA replication is several times that of DNA replication in uninfected cells (Joklik and Becker, 1964), and coincident with the burst of viral DNA synthesis is a rapid inhibition of host-cell DNA synthesis (Jungwirth and Launer, 1968). After inhibition, the host cell DNA is subsequently cleaved by a viral endonuclease to discrete-sized fragments (Parkhurst *et al.*, 1973) and these are not made available to the virus for reutilization under normal conditions (Kits and Dubbs, 1962; Oki et al., 1971). Viral DNA replication, therefore, relies on the synthesis of deoxyribonucleotide precursors. Measurements of deoxyribonucleotide pools show that, as in other organisms, free deoxyribonucleotides do not accumulate in the host cell, but are found in very small amounts (4-20 pmol/10^6 cells at 4 h post infection; Slabaugh *et al.*, 1991). These results imply that following ribonucleotide reduction, deoxyribonucleotides are immediately incorporated into DNA. If indeed the reduction of ribonucleotides is limiting towards DNA replication, establishing the rate of synthesis and accumulation

of ribonucleotide reductase is crucial to an understanding of the flux of precursors into DNA.

In this study we determine the kinetics of expression of both vaccinia virus ribonucleotide reductase subunits by an analysis of the products of transcription and translation. The generation of polyclonal antisera to R1 and R2 of VV has enabled us to monitor the synthesis and stability of ribonucleotide reductase proteins. Additionally, by immunochemical quantitation of ribonucleotide reductase we have determined the stoichiometry of R1 and R2 polypeptide levels during a viral infection. To our knowledge, this is the first report to directly correlate the accumulation of ribonucleotide reductase in any viral system with the rate of DNA replication and the amount of accumulated viral DNA.

IV.3 Materials and Methods

Cells and Virus. Vaccinia virus, strain WR, and BSC_{40} monkey kidney cells were maintained as previously described (Earl et al., 1988). Monolayer cultures were grown in Eagle minimum essential medium supplemented with 5% (v/v) heat-inactivated bovine calf serum (Hyclone Laboratories Inc. Logan, UT)

Transcription analysis. Viral mRNA was isolated as described (Weinrich *et al.*, 1985). S1 nuclease mapping was performed as described (Roseman and Hruby, 1987).

Antibodies and antisera production. Overexpression of sequences in or portions of viral R1 and R2 proteins was accomplished by fusion of viral sequences downstream of the bacterial *trpE* gene in pATH expression vectors (Dieckmann and Tzagoloff, 1985). Initially, the vaccinia virus *Hin*dIII F and I fragments were digested with the restriction enzyme *Xba*I, and DNA fragments containing the entire coding

sequences for R1 and R2 were isolated and cloned into pUC18 vectors. Plasmids containing the full length reductase genes were then used in all further manipulations. In both cases the *Hin*dIII site described below is derived from the vector. The construct for the 2316-nucleotide R1 gene consisted of a *Bgl*II-*Hin*dIII fragment which contained 733 nucleotides of the carboxyl terminus of R1 coding sequence ligated to pATH 1 digested with *Bam*HI-*Hin*dIII. For the 956-nucleotide R2 gene, an *Eco*RV-*Hin*dIII fragment containing 818 nucleotides of the carboxyl terminus of R2 coding sequence was ligated to the *Sma*I-*Hin*dIII site of pATH 2.

Synthesis of the fusion proteins was induced in JM83 strains containing these plasmids by the addition of indoleacrylic acid. The insoluble fusion protein was then purified as described (Miner and Hruby, 1989). The insoluble pellet was applied to a preparative SDS-polyacrylamide gel and electrophoresed. The insoluble fusion protein band was excised and the protein eluted in an Elutrap device (Schleicher and Schuell Inc., Keene, NH). Rabbits were initially injected intradermally with 100-200 μg of purified protein in Freund's complete adjuvant. Subsequent intramuscular injections occurred at 1 week intervals with approximately 100 μg of protein in Freund's incomplete adjuvant. Rabbits were bled two weeks following the third injection of protein.

The mouse monoclonal antibody AD 203, which recognizes mammalian ribonucleotide reductase R1, was purchased from InRo Biomedtek (Umeå, Sweden). The rat α-tubulin monoclonal antibody YL1/2, which cross reacts with mammalian ribonucleotide reductase R2, was purchased from Serotec (Station Field, IN).

Western Blots. For immunoblots, BSC_{40} cells were virus-infected (10 plaque forming units/cell) or mock-infected and harvested at 6 h post infection. Cells were pelleted, resuspended in Laemmli buffer (Laemmli, 1970) and lysed by sonication. Proteins were separated by electrophoresis on 11% SDS-polyacrylamide

gels and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) for 60 min at 200 mA by using a semidry electroblotter (Idea Scientific, Minneapolis, MN). The membranes were blocked in buffer (50 mM Tris [pH 9.5], 150 mM NaCl, 2 mM EDTA, 0.1% polyoxyethylene sorbitan monolaurate) with 1% gelatin, incubated in buffer containing primary antiserum, and then incubated with secondary antiserum conjugated to alkaline phosphatase. Anti-VVR1 and VVR2 sera were used at dilutions of 1:500 and 1:2000, respectively, and AD 203 and YL-1/2 antisera were diluted 1:250. Goat anti-rabbit alkaline phosphatase conjugate at a dilution of 1:5000 was used for VVR1 and VVR2 antisera, and anti-rat and anti-mouse conjugate were used for anti-YL-1/2 and anti-AD 203 sera, respectively, at dilutions of 1:2500.

Enzyme assays. Ribonucleotide reductase activity in the viral extracts was determined by measuring conversion of [^{3}H]-CDP to [^{3}H]-dCDP as previously described (Slabaugh *et al.*, 1984). For the time course experiment, the 20-µl reaction volume contained 10 µl viral extract and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.2), 10 mM DTT, 20 µM $Fe(NH_4)_2SO_4$, 2 mM $MgCl_2$, 2 mM 5'-adenylylimido diphosphate, 100 µM CDP and [^{3}H]-CDP at 100 cpm/pmol. In the experiment to compare ribonucleotide reductase activity in the presence of different reducing agents, dithiothreitol was substituted with either glutathione (1 mM), purified virally-encoded glutaredoxin (0.1 mM), or both. The purified glutaredoxin was a generous gift of Dr. Wayne Thresher. Enzyme activity was expressed as pmol of dCDP produced/min/10^6 cell equivalents. Each extract was assayed in triplicate and the error bars in Figure 5 represents the standard deviation of the three determinations.

Immunoprecipitations. BSC_{40} monolayers at 95% confluence were infected at a multiplicity of 10 plaque-forming units/cell and polypeptides were labeled by the addition of [^{35}S]-methionine (25 or 50 µCi/ml). For pulse labeling experiments,

cells were labeled for sequential 1 h periods up to 8 h post infection and then harvested. For pulse-chase experiments, cells were labeled from 2.5 to 3 h post infection, washed twice with phosphate buffered saline, and incubated in medium containing a one hundredfold excess of unlabeled methionine. Radioactively labeled cells were harvested with a rubber policeman, centrifuged, washed with phosphate buffered saline, and resuspended in 1 ml of lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% [v/v] Nonidet P-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] sodium dodecyl sulfate) and sonicated. Extracts derived from 1.5×10^6 cells were incubated with 5 µl of antisera for 3 h on a rotating platform at 4°C. 100 µl of a 10% (v/v) suspension of Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) beads were added to each of two 500 µl aliquots and incubated at 4°C for a minimum of 4 hours. The beads were pelleted, the supernatant removed, and the beads were washed six times with lysis buffer. Immune complexes were released by boiling the pellet in 50 µl Laemmli buffer (Laemmli, 1970). Following electrophoresis on 11% SDS-polyacrylamide gels, proteins were visualized by fluorography.

Polyclonal antibody-ELISA for R1 and R2. The competitive inhibition immunoassay was performed essentially as described by Arkoosh and Kaattari (1990). Briefly, in all cases, purified R1 or R2 was used as coating antigen to coat the microtiter plate wells at concentrations of 0.8 and 0.2 µg/ml, respectively. Plates were then extensively washed in TBST buffer (50 mM Tris [pH 8.0], 100 µM EDTA, 300 mM NaCl, 1% [v/v] polyoxyethylenesorbitan monolaurate), and blocked to further protein binding by incubation with a solution containing 1% (w/v) bovine serum albumin, fraction *V*, in TBST buffer. Standard curves were prepared as follows. First, purified R1 or R2 protein, in a solution of 1% (w/v) bovine serum albumin in TBST, was added to the plate wells in serial dilutions. Next, primary (anti-VVR1 or anti-VVR2) serum was also added to the plate wells at dilutions of 1:500. The purified

R1 and R2 proteins in solution act as antigen inhibitor to compete with the R1 and R2 coating antigen for the primary antisera. After incubation, immune complexes that are not attached to the wells (antigen inhibitor complexed to primary antibody) are washed away using TBST/bovine serum albumin buffer. The remaining immune complexes (coating antigen complexed to primary antibody) are then further incubated with an excess of secondary mouse anti-rabbit IgG conjugated to horseradish peroxidase antiserum (Sigma, St Louis, MO). Immunoreactive antibody conjugate was then quantitated by colorimetric detection on a Titertek multiscan plus plate reader (Flow Laboratories Inc., Emeryville, CA). To quantitate R1 and R2 in virus-infected extracts, aliquots of serial dilutions of the extracts were added to the plate wells (after the addition of coating antigen) in TBST/bovine serum albumin buffer to be used as antigen inhibitor. The viral R1 and R2 proteins in the extracts (antigen inhibitor) compete with the purified R1 and R2 coating antigen for the primary antiserum. After incubation, immunocomplexes in solution were washed away, and bound immunocomplexes were incubated with an excess of secondary mouse anti-rabbit IgG conjugate and quantitated as described above. The amount of the ribonucleotide reductase subunits in the extracts was established through a kinetic-based analysis of the immunoassay (Arkoosh and Kaattari, 1990). The concentrations of R1 and R2 in the extracts were determined by comparison of the immunoassay data with the standard curve of purified viral ribonucleotide reductase proteins. Each data point in Fig. IV6 represents the average concentration of R1 or R2 protein, determined in duplicate, from each of three separate extracts.

In these ELISA experiments, the VVR2 antiserum described in this manuscript was used for R2 quantitation. Polyclonal antiserum prepared against full length R1 protein (Davis, 1992) was used for R1 quantitation because this was demonstrated to be more specific (than antiserum against partial R1) for the vaccinia virus R1 protein.

The full length VVR1 antiserum was a generous gift from Dr. Ralph Davis of this laboratory.

Quantitation of viral DNA by dot blot hybridization. Confluent 100-mm dishes of BSC_{40} cells were infected at a multiplicity of 10 plaque-forming units/cell. After 1 h of adsorption, the inoculum was removed, and cultures were rinsed three times with phosphate buffered saline and then incubated, again, in medium. At the indicated times, cells were harvested with a rubber policeman, collected by centrifugation, and resuspended in 0.3 ml of buffer (50 mM Tris pH 8.0, 100 mM NaCl). To make extracts, the infected cells were frozen and thawed three times and sonicated. Extracts were treated with 10 µg/ml deoxyribonuclease-free ribonuclease A (Sigma) for 60 min at room temperature to remove RNA. 20-µl aliquots of each extract were then heat denatured and 3 µl was applied directly to Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, CA) and bound to the membrane by UV crosslinking for 3 min with a UV Stratalinker 1800 transilluminator (Stratagene, La Jolla, CA). Hybridization probe was synthesized from a 2.3-kb *Xba*I fragment containing the *VVR2* coding sequence by random hexamer priming and digoxigenin-dUTP labeling using the Genius nonisotopic nucleic acid labeling and detection kit from Boehringer Mannheim Co. (Indianapolis, IN). Extracts were hybridized overnight according to the standard hybridization protocol included in the kit. The hybridized filters were detected by reaction with an immunochemiluminescent formulation (Martin et al., 1990) and examined by autoradiography. Hybridization signal was quantitated by scanning laser densitometry of the autoradiographs using a Zeineh model SL-504-XL instrument (Biomed Instrument Inc., Fullerton, CA). The concentration of DNA in the viral extracts was determined by comparison with a standard curve. The standard curve was derived from increasing amounts of purified *Hin*dIII F fragment DNA hybridized to the labeled probe and detected as described

above. The *Hin*dIII F fragment DNA was quantitated by A_{260} and the purity of the DNA was confirmed by agarose gel electrophoresis.

IV.4 Results

IV.4.1 Transcriptional Kinetics of Vaccinia Virus Ribonucleotide Reductase

The precise locations of the 5' ends of the vaccinia virus ribonucleotide reductase transcriptional units have been previously determined (Slabaugh *et al.*, 1988; Tengelson *et al.*, 1988). To determine when in the replication cycle viral mRNAs encoding R1 and R2 are present, viral RNA was isolated at various times post-infection and the presence of R1 and R2 transcripts were determined by S1 nuclease analysis (Fig. IV.1). Appropriate 5' single-ended labeled probes (Fig. IV.1, *Panel C*) were hybridized with viral mRNA isolated from infected cells in the presence of cycloheximide or at the indicated times post infection. Following S1 nuclease digestion, protected hybrids were fractionated by SDS-polyacrylamide gel electrophoresis. The protected hybrids for R1 and R2 migrate at the expected sizes of 540 and 490 nucleotides, respectively. Both genes are transcribed in the presence of cycloheximide and both are maximally transcribed between 1 and 3 h post infection. This analysis demonstrates that R1 and R2 are members of the immediate early gene class. While it appears that mRNAs from both genes are present up to 9 h post infection, we do not know whether this is due to continued transcription or mRNA stability.

An 820-nucleotide protected band in Fig. IV.1, *Panel A*, present late in infection is consistent with a transcriptional start just upstream of the start of the I5 open reading frame, thus defining ORF I5 as a late gene. The 860-nucleotide band in

Figure IV.1. S1 Nuclease Analysis of the Transcriptional Kinetics of the VVR1 (*A*) and VVR2 (*B*) Genes. The 5' single end-labeled probes (*) are diagrammed relative to restriction sites and open reading frames (*arrows*) found in the region of the genome (*Panel C*). Probes were hybridized to RNA isolated from cells infected in the presence of cycloheximide (*Lane C*); or at 1, 3, 5, and 9 h post infection in the absence of drug; or to tRNA (*Lane* -) as a control. P, probe; A *Acc*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; RV, *Eco*RV; X, *Xba*I. Markers sizes (expressed in nucleotides) are shown on the left; sizes of protected fragments (expressed in nucleotides) are shown on the right.

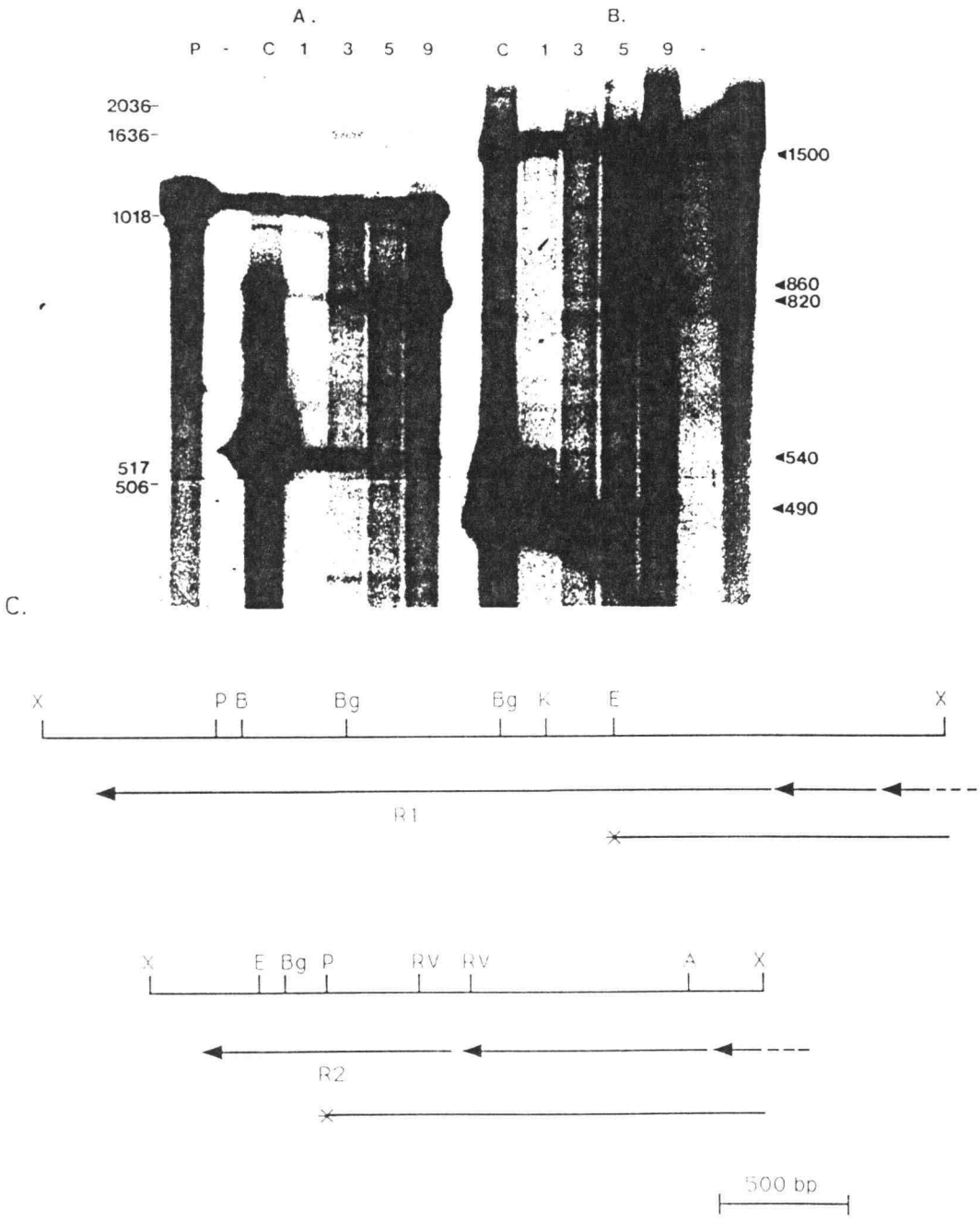
A.
B.
P - C 1 3 5 9
C 1 3 5 9 -
2036-
1636-
1018-
517
506-
1500
860
820
540
490
C.
X
P B
Bg
Bg K
E
X
R1
X
E Bg P
RV RV
A
X
R2
500 bp

Figure IV.1

Fig. IV.1, *Panel B* maps a 5' end approximately 330 nucleotides into the coding sequence of ORF F5. Analysis of the sequence in this region does not resemble any proposed late promoter signals (Davidson and Moss, 1989). The 1500-nucleotide protected fragment in Fig. IV.1, *Panel B* (*lane C*), maps initiation at ORF F5 and is consistent with previous work (Slabaugh *et al.*, 1988; Roseman and Slabaugh, 1990). ORF F5 appears to be expressed at a low level during infection since initiation from this open reading frame in the absence of cycloheximide gives a weak signal.

IV.4.2 Characterization of Vaccinia Virus Ribonucleotide Reductase Antisera

Upon induction with indoleacrylic acid, bacteria containing plasmids bearing the carboxyl termini of either the R1 or R2 gene fused to the amino terminus of the *trpE* gene both expressed a fusion protein of the predicted molecular weight. The fusion proteins were purified by elutriation after SDS-polyacrylamide gel electrophoresis and injected into rabbits. Sera were collected as described in "Materials and Methods". To determine whether an immune response was elicited by the highly conserved ribonucleotide reductase proteins, VVR1 and VVR2 antisera were used for Western blot analysis of virus-infected and mock-infected cell extracts (Fig. IV.2). No cross reactivity with host or viral proteins was seen with pre-immune sera (data not shown). Immunoblots of vaccinia virus-infected cells with anti-VVR1 (*lane* 1) and anti-VVR2 (*lane* 3) sera demonstrated that each is specific for a protein of the expected molecular weight of 86 and 37 kDa respectively.

IV.4.3 Translational Kinetics of Vaccinia Virus Ribonucleotide Reductase

To determine whether the transcriptional kinetics of the viral reductase genes is consistent with the time course of ribonucleotide reductase protein synthesis, immunoprecipitations of pulse-labeled infected cells were done. Mock-infected or

Figure IV.2. Characterization of VVR1 and VVR2 Antisera by Western Blot Analysis. Extracts made from BSC_{40} cells which were virus-infected (*Lanes 1* and *3*) or mock-infected (*Lanes 2* and *4*) were separated on an 11 % SDS-polyacrylamide gel and incubated with antisera to either VVR1 (*Lanes 1* and *2*) or VVR2 (*Lanes 3* and *4*). Molecular weight standards are shown on the left; the molecular weights of immunoreactive proteins are shown on the right.

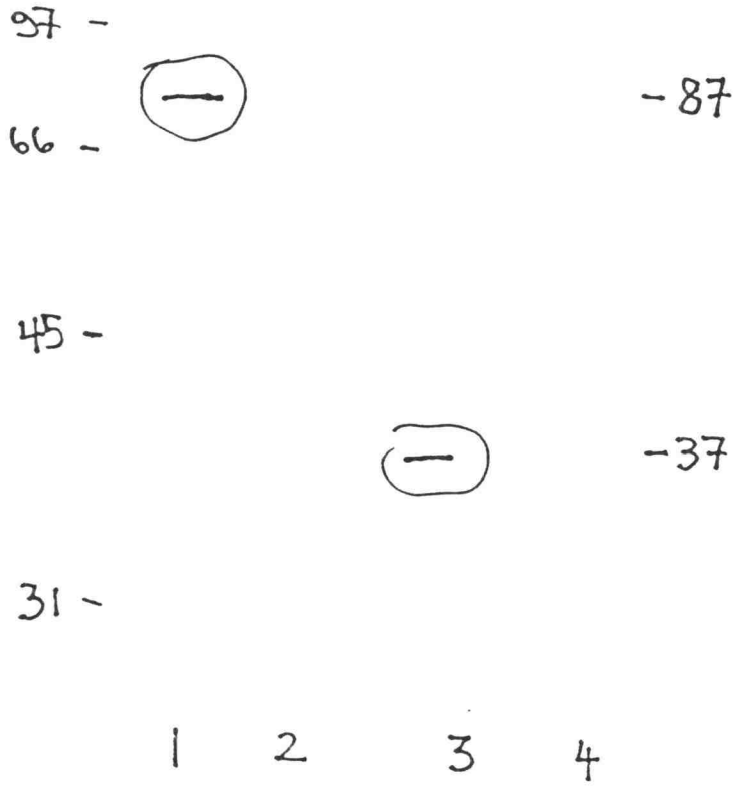

Figure IV.2

virus-infected cells were pulse-labeled at the indicated times for one hour and then harvested. Cell extracts were incubated with either VVR1 (Fig. IV.3, *Panel A*) or VVR2 (Fig. IV.3, *Panel B*) antisera, followed by immunoprecipitation with protein-A Sepharose. Both R1 and R2 proteins are present in virus-infected cells by 1 h post infection and the peak of protein synthesis for both subunits occurs between 3 and 4 h post infection. Translation of these proteins continues up to 9 h post infection. These results are consistent with the transcriptional data. Since the relative quantity of each protein in the infected cell cannot be derived by this analysis, competitive ELISA was used to quantitate the amounts of R1 and R2 proteins.

The VVR2 antiserum cross-reacted with a host protein of 42 kDa while the VVR1 antiserum faintly cross-reacted with a host protein of 92 kDa. Infection by vaccinia virus appears to result in the cessat ion of synthesis and turnover of both of the immunoreactive host proteins. The following results demonstrate that the cross-reacting 92-kDa band is the host large subunit of ribonucleotide reductase. Immunoblot analysis of the infected and mock-infected extracts using AD 203 monoclonal antiserum, which reacts to mammalian R1 (Engström, 1982; Engström *et al.*, 1984), revealed cross reactivity with the 92-kDa host protein, but no immunoreactivity was detected with the vaccinia 87-kDa R1 protein (data not shown). Immunoblot analysis using monoclonal antiserum to rat α-tubulin, which has been demonstrated to cross-react with mouse and calf ribonucleotide reductase R2 protein (Kilmartin et al., 1982; Thelander et al., 1985), revealed no cross reactivity with the 42-kDa host monkey protein, and only faint reaction with the viral 37-kDa R2 protein after incubation of the antibody conjugate overnight with alkaline phosphatase substrate. We were therefore unable to identify the 42-kDa band on the immunoblot as the corresponding host ribonucleotide reductase protein.

Figure IV.3. Immunoprecipitation Analysis of the Time Course of Vaccinia Virus Ribonucleotide Reductase Protein Synthesis. Pulse-labeled cell extracts were immunoprecipitated with antisera to either VVR1 (*Panel A*) or VVR2 (*Panel B*). Mock-infected (*M*) or virus-infected cells (10 plaque forming units/cell) were pulse-labeled with 50 μCi/ml [^{35}S]-methionine for 1 h at the indicated times post-infection. Molecular weight standards are shown on the left; the molecular weights of immunoprecipitated proteins are shown on the right.

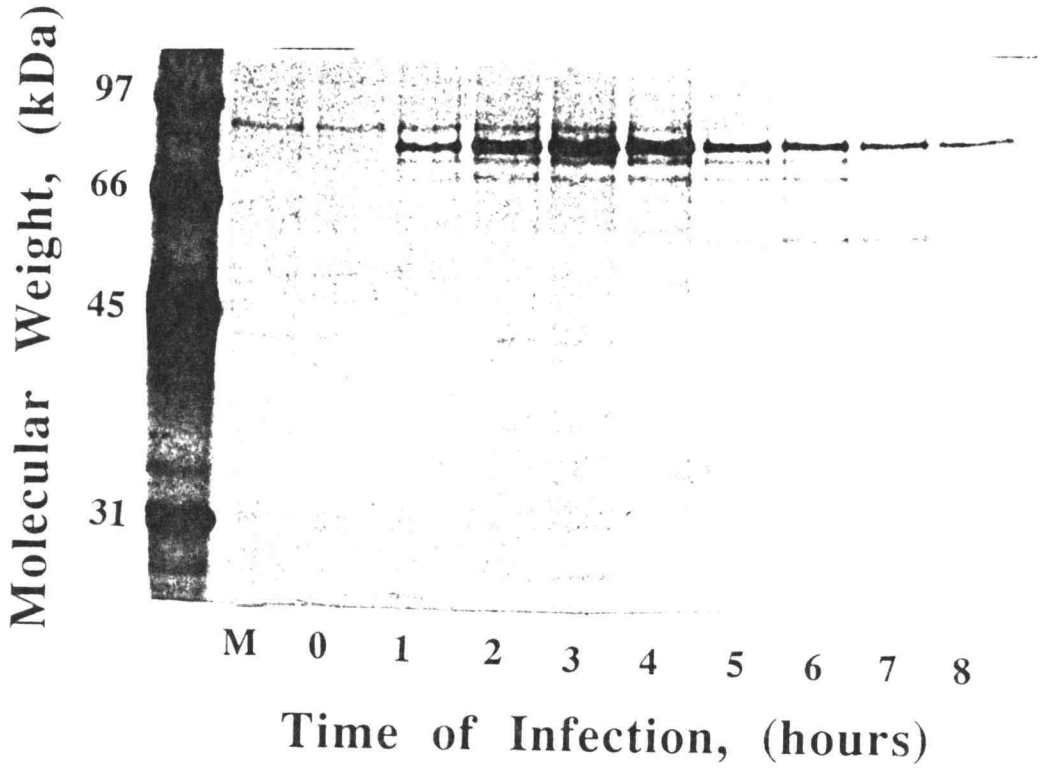

Figure IV.3 Panel *A*

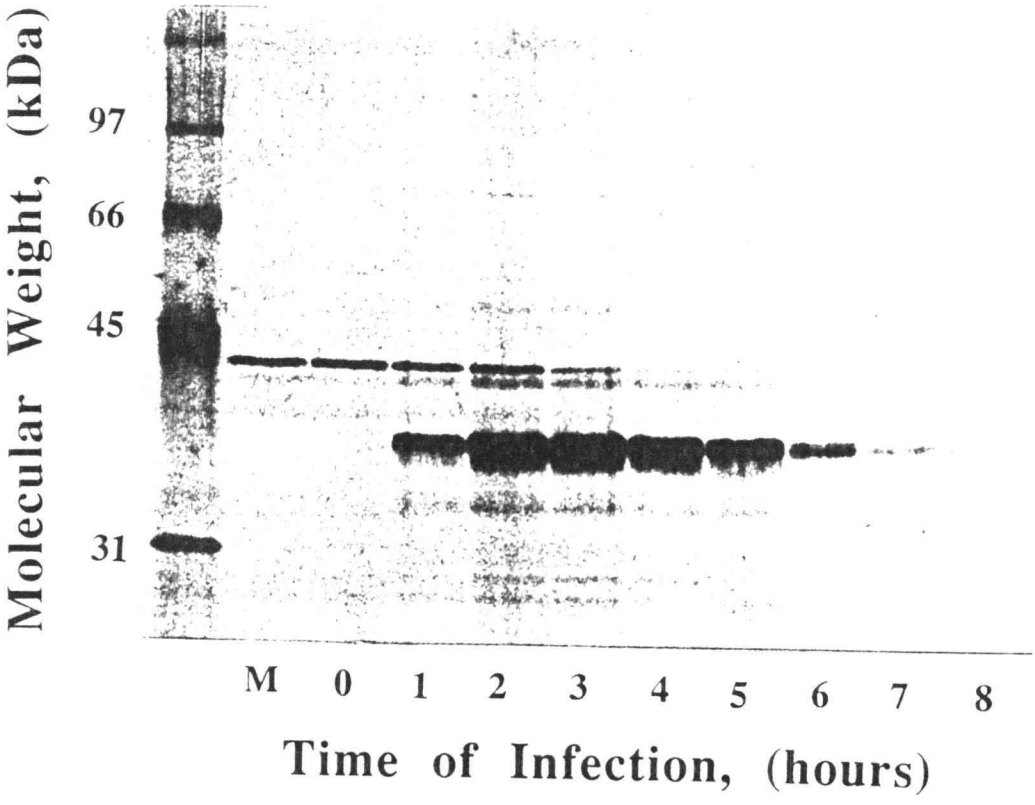

Figure IV.3 Panel *B*

IV.4.4 Protein Stability of Vaccinia Virus Ribonucleotide Reductase

To determine the stability of the R1 and R2 proteins during the time course of viral infection, immunoprecipitations of pulse-labeled cell extracts were performed. Mock-infected or virus-infected cells were labeled from 2.5 to 3 h post infection with [^{35}S]-methionine and then chased with an excess of unlabeled methionine for the indicated times and then harvested. Cell extracts were incubated with a combination of VVR1 and VVR2 antisera (Fig. IV.4), followed by immunoprecipitation with protein-A Sepharose. The levels of both R1 and R2 proteins were relatively constant over the 9 h of incubation in unlabeled methionine, indicating that the turnover numbers of these proteins are low.

IV.4.5 Ribonucleotide Reductase Activity in Vaccinia Virus-Infected Extracts

Ribonucleotide reductase assays were performed with mock-infected and virus-infected cell extracts to determine the time course of enzyme activity. To prevent degradation of substrate in the reaction mixture (presumedly due to catalysis by nucleoside diphosphate kinase) ATP was substituted with the uncleavable analog 5'-adenylylimido diphosphate. The kinetics of ribonucleotide reductase activity in the infected cell extracts is shown in Figure IV.5. Activity increased from 1 to 6 h post infection and the maximal activity of virus-infected extracts (689 *f*mol/min/10^6cells) was greater than ten times that of mock-infected extracts (61 *f*mol/min/10^6 cells). The specific activity of ribonucleotide reductase (approximately 5 nmol/min/mg R1) was established by using the quantitative ELISA results to determine the amount of R1 (limiting) protein in the crude extracts.

The recent expression and purification of a virally encoded glutaredoxin[2] enabled us to examine the effect of the glutaredoxin reducing system on ribonucleotide

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[2] W. Thresher, manuscript in preparation.

Figure IV.4. Immunoprecipitation Analysis of the Stability of Vaccinia Virus Ribonucleotide Reductase Protein. Pulse-chased cell extracts were immunoprecipitated with antisera to both VVR1 and VVR2. Virus-infected (*VV*) or mock-infected (*M*) cells were pulsed with 25 μCi/ml [^{35}S]-methionine between 2.5 and 3.0 h post infection and harvested at the indicated times post infection. *Panel A*, Stability of VVR1 protein. *Panel B*, Stability of VVR2 protein.

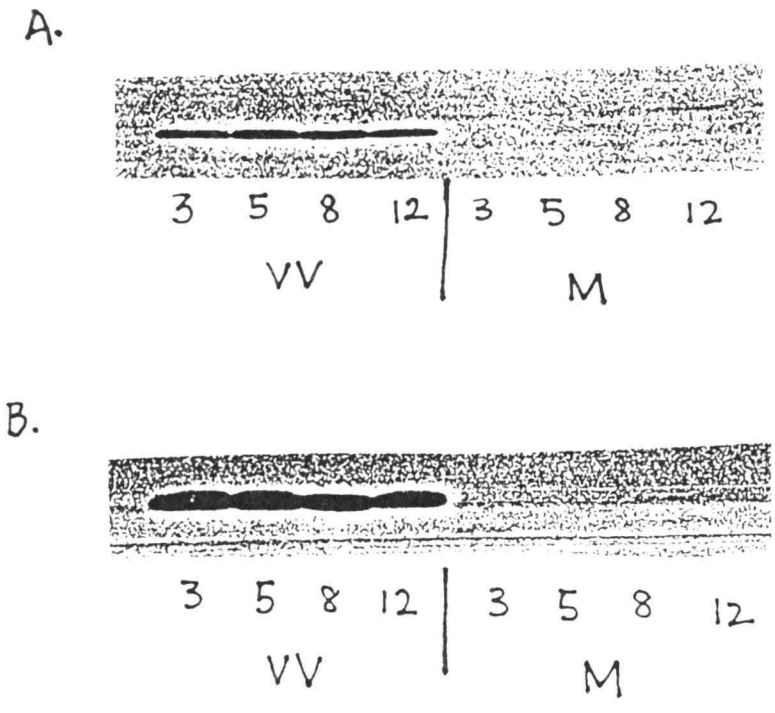


Figure IV.4

Figure IV.5. Time Course of Induction of Vaccinia Virus Ribonucleotide Reductase Activity. BSC_{40} cells were infected with 10 plaque forming units/cell and harvested at the indicated times post infection. Extracts were assayed for enzyme activity using the assay parameters described in the text. Each bar represents the average of triplicate determinations.

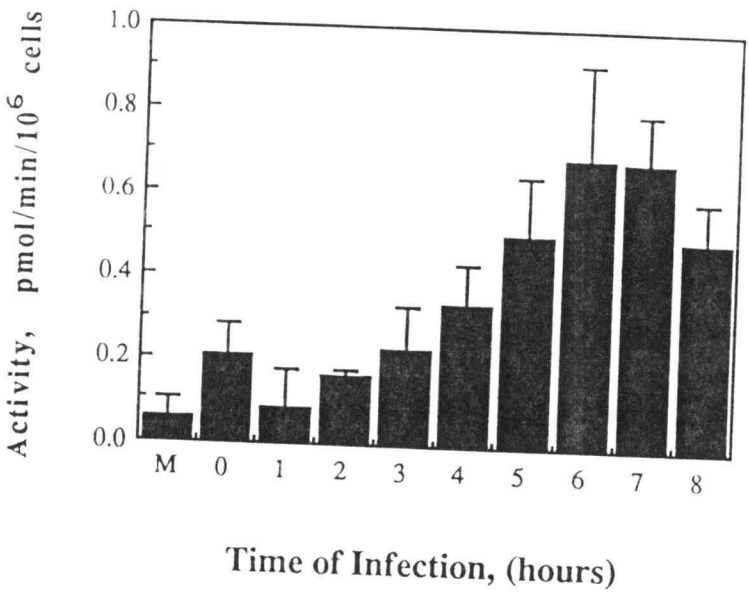

Activity, pmol/min/10^6 cells
1.0
0.8
0.6
0.4
0.2
0.0
M
0
1
2
3
4
5
6
7
8
Time of Infection, (hours)

Figure IV.5

reductase activity in the crude extracts. Substitution of dithiothreitol with either glutathione, glutaredoxin, or both (at concentrations tenfold higher than the K_M values for mammalian glutathione and glutarexon), maintained but did not increase the level of enzyme activity (data not shown).

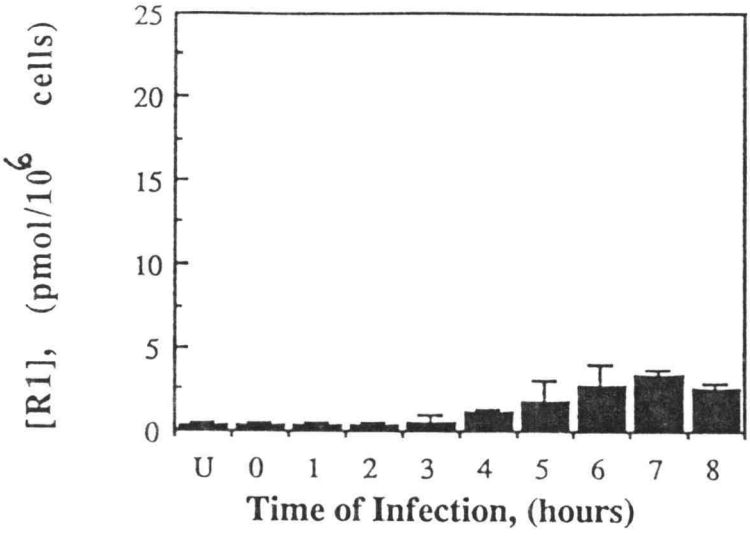
IV.4.6 ELISA of R1 and R2 Proteins in Vaccinia Virus-Infected Extracts

The kinetics of ribonucleotide reductase protein accumulation was quantitated by using a competitive inhibition enzyme-linked immunoassay. Extracts made from cells infected at the indicated times were used to inhibit the reaction of purified viral ribonucleotide reductase with anti-VVR1 or anti-VVR2 sera. Antibody complexes were measured by reaction of the antisera with a horseradish peroxidase anti-rabbit conjugate and colorimetric detection of the enzyme reaction by an ELISA plate reader. The time course of accumulation of both viral proteins is shown in Figure IV.6, *Panels A* and *B*. Both vaccinia proteins accumulated through 6 h of infection to maximal levels of 4.2 x 10^6 R1 molecules/cell and 23 x 10^6 R2 molecules/cell. The kinetics of protein accumulation, shown in Figure IV.6, duplicated that of ribonucleotide reductase activity. This direct correlation between protein accumulation and enzyme activity suggests that enzyme activity is regulated solely by subunit protein levels.

The immunoassay results also define the molar stoichiometry of the two reductase subunits during infection. The R2 subunit (20.3 pmol monomer/10^6 cells) accumulated in the extracts to a level that is six times higher than the concentration of the R1 subunit (3.4 pmol monomer/10^6 cells). These results establish that the R1 subunit is limiting in vaccinia virus-infected cell extracts. These data are supported by recent kinetic analysis of the purified recombinant enzyme. In those experiments, maximal enzyme activity was obtained only when the R2 subunit was present in a fourfold excess

Figure IV.6. Quantitative ELISA Analysis of the Accumulation of Vaccinia Virus Ribonucleotide Reductase Protein. BSC_{40} cells were infected with 10 plaque forming units/cell and harvested at the indicated times post infection. VVR1 and VVR2 protein levels in the extracts were measured by competitive inhibition assay and quantitated by comparison with purified recombinant VVR1 and VVR2 protein standards as described in the text. *Panel A*, time course of VVR1 accumulation. *Panel B*, time course of VVR2 accumulation.

A.



B.

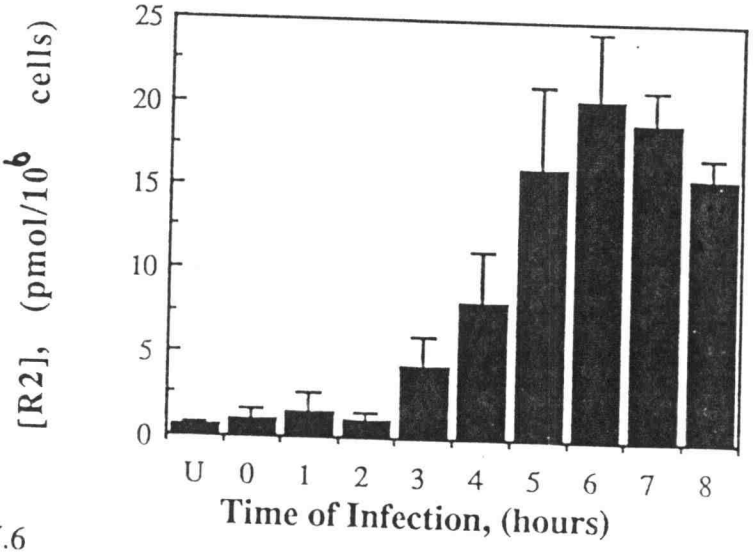


Figure IV.6

IV.4.7 Viral DNA Accumulation in Vaccinia Virus-Infected Cells

Vaccinia virus DNA replication takes place in cytoplasmic foci, termed virosomes (Cairns, 1960). The rate of DNA synthesis at the virosomes has been estimated by incorporation of [^{3}H]-thymidine into acid-precipitable material (Joklik and Becker, 1962; Condit and Motyczka, 1981; Slabaugh and Mathews, 1986) These measurements, however, assume that there is a constant flow of thymine nucleotides into DNA and that the thymidine triphosphate pool size remains constant and, therefore, so does the specific activity of [^{3}H]-thymidine. To the contrary, deoxynucleotide pool measurements in eukaryotic cells demonstrate extensive pool fluctuations during DNA synthesis (Bianchi *et al.*, 1986a; Bianchi *et al.*, 1986b). To circumvent these problems, we used a dot blot filter technique to more directly measure the accumulation of viral DNA. Cell extracts infected with virus at a multiplicity of 10 plaque forming units/cell were hybridized to an excess of random-primed vaccinia virus-specific probe. Hybridization was quantitated by immunochemiluminescent detection (Martin *et al.*, 1990) and the DNA concentration in the extracts was determined by comparison with a standard curve of hybridized purified vaccinia virus DNA. Control experiments were performed to establish that the hybridization probe was in excess and that hybridization was due to DNA and not RNA in the extracts. The kinetics of accumulation of virus-specific DNA sequences in infected cell extracts is shown in Figure IV.7. Viral DNA accumulated throughout the measured time course and the rate of accumulation was constant from 3-8 h post infection. Quantitatively, more than 1200 viral genomes equivalents are replicated in each cell by 8 h post infection under the experimental conditions. The kinetics established in this experiment are consistent with previous work which also measured vaccinia DNA accumulation by the hybridization technique (Ensinger 1987; Hooda-Dhingra *et al.*, 1989; Rempel *et al.*, 1990). These groups all found that DNA accumulation is constant until 10-12 h post infection. The quantitative

Figure IV.7. Quantitative Dot Blot Hybridization Analysis of the Accumulation of Vaccinia Virus DNA. BSC_{40} cells were infected with 10 plaque forming units/cell and harvested at the indicated times post infection. Cells were disrupted and a portion of each sample was bound to a filter and probed with a nonisotopically-labeled *Hin*dIII F fragment of vaccinia virus DNA. Hybridization was measured by an immunochemiluminescent formulation and quantitated by comparison with purified *Hin*dIII F fragment DNA.

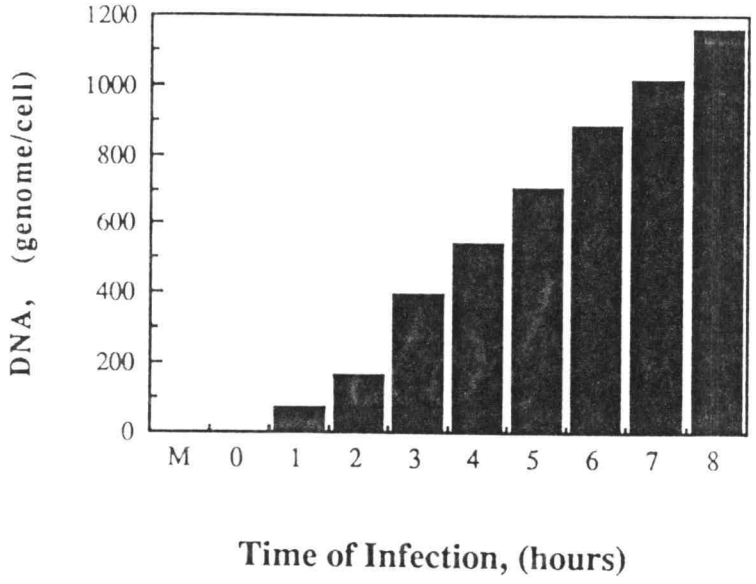

1200
1000
800
600
400
200
0
DNA, (genome/cell)
M 0 1 2 3 4 5 6 7 8
Time of Infection, (hours)

Figure IV.7

amounts of virally-synthesized DNA determined in this study for BSC_{40} cell infections also agree with early studies that quantitated infectious particle formation of vaccinia virus in other host cell types (Joklik and Becker, 1964; Jungwirth and Launer, 1968). These early studies also demonstrated that 30% of viral DNA (by weight) is packaged into virions during an infection within a 24 h period.

IV.4.8 Comparison of the Accumulation of Viral DNA with Viral Ribonucleotide Reductase Levels During Vaccinia Infection

Since the kinetics of viral DNA accumulation and ribonucleotide reductase accumulation have both been measured quantitatively, a direct comparison can be made between the supply of deoxynucleotide precursors and their utilization. This comparison (Table IV.1) demonstrates that the levels of ribonucleotide reductase in virus-infected cells are indeed sufficient to supply adequate levels of deoxyribonucleotides for the accumulation of viral DNA. From the DNA hybridization experiment, it was established that viral DNA replication results in the synthesis of more than twelve hundred viral genomes/cell by 8 h of infection (*Column 2*). To synthesize this amount of DNA by 8 h, the number of limiting ribonucleotide reductase subunits (R1) in the infected cell must be at least 2.3×10^6 molecules (*Column 3*) or 94,000 molecules (*Column 4*), depending on which value for enzyme specific activity is used for the calculations. ELISA analysis to determine ribonucleotide reductase protein levels demonstrate that there is indeed enough enzyme at 8 h (3.1×10^6 molecules R1 and 1.9×10^7 molecules R2) to synthesize appropriate amounts of DNA (*Column 5*). Only during the early hours of viral DNA synthesis (1-3 h post infection, where quantitation of the diminutive amounts of DNA and enzyme is difficult and inaccurate) is the level of ribonucleotide reductase insufficient to account for the rate of

Table IV.1 Comparison of Deoxyribonucleotide Supply and Demand

Hours of Infection (h)	**DNA accumulation** [a] (genomes/cell)	**Requirement for R1** [b] (molecules/cell)	**Requirement for R1** [c] (molecules/cell)	**Accumulation of R1** [d] (molecules/cell)
0	-	-	-	-
1	72	1,000,000	43,000	320,000
2	162	1,200,000	48,000	320,000
3	397	1,900,000	79,000	560,000
4	545	1,900,000	82,000	1,400,000
5	708	2,000,000	84,000	2,200,000
6	885	2,100,000	88,000	3,300,000
7	1010	2,100,000	87,000	4,200,000
8	1260	2,300,000	94,000	3,100,000

[a] Values determined from dot blot filter hybridization experiment as described in text

[b] Values calculated as described in text using specific activity of 5 nmol/min/mg R1 for virus-infected extracts

[c] Values calculated as described in text using specific activity of 120 nmol/min/mg R1 for purified holoenzyme

[d] Values determined from ELISA experiment as described in text

accumulation of viral DNA. This inadequacy is also only found when the lower specific activity value is considered.

The numbers in *Column 2* were determined by first calculating the weight of genome equivalents in the infected cells from the DNA hybridization data: for the 8 h calculation, (1263 genomes/cell) x (187,000 bp/genome) x (660 g bp/mol) ÷ (6.02 x 10^{23} molecules/mol) = 2.6 x 10^{-13}pg bp/cell; then by determining the molar concentration of nucleotides needed as substrate for DNA synthesis in the infected cells, 1 pg bp = 1 pg nucleotide, (2.6 x 10-13 pg nucleotide/cell) ÷ (330 g/mol nucleotide) = 7.9 x 10^{-16} mol nucleotide/cell; then by establishing the rate at which the nucleotide substrates must be provided by dividing by the length of time in which the DNA accumulation measurements were made, 7.9 x (10^{-16} mol nucleotide/cell) ÷ (8 h) x (1 h/60 min) = 1.6 x 10^{-18} mol nucleotide/min/cell; and finally by dividing this sum by the specific activity of viral ribonucleotide reductase and the molecular weight of the R1 (limiting) subunit, (1.6 x 10^{-18} mol nucleotide/min/cell) ÷ (5 x 10^{-9} mol nucleotide/min/mg R1) x (6.02 x 10^{23} molecules R1/mol R1) x (1 mol R1/87,000 g R1) = 2.3 x 10^{6} molecules R1/cell.

To ensure that the calculations in Table 1 are valid, the values used for the specific activity of ribonucleotide reductase (5 nmol/min/mg R1) must be considered. Slabaugh and coworkers have recently demonstrated that the specific activity of ribonucleotide reductase can be dramatically increased by optimizing reaction conditions for the purified enzyme[3]. The value obtained for the purified enzyme under the optimized reaction conditions (50 mM dithiothreitol instead of 10 mM and ATP instead of 5'-adenylylimido diphosphate) was 120 nmol/min/mg of R1. However, we felt that because the specific activity value used in the quantitation calculations should approximate the physiological conditions of virus-infected cells, it might be inaccurate

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[3] M. Slabaugh, manuscript in preparation.

to use the value for purified enzyme. Accurate specific activity determinations on virus-infected cell extracts, however, have been elusive. Activity measurements on virus-infected cell extracts in the presence of 10 mM dithiothreitol and 2 mM 5'-adenylylimido diphosphate have given specific activity values between 3-8 nmol/min/mg R1 (this study). Unfortunately, the optimal reaction conditions for the purified enzyme are severely inhibitory ($\geq 90\%$) to the activity found in virus-infected crude extracts. Much of this inhibition is almost certainly due to phosphorylation of CDP to CTP by nucleoside diphosphate kinase and the effects of other ATP utilizing enzymes, which render the adenosine nucleoside products unable to activate ribonucleotide reductase. Moreover, enzyme assays on purified ribonucleotide reductase have demonstrated that ATP is tenfold more effective as an activator than is 5'-adenylylimido diphosphate. Therefore, specific activity measurements in infected cell extracts in the presence of 5'-adenylylimido diphosphate would be expected to give estimates that are much lower than the actual value. For these reasons, we have used the specific activity values determined for both purified ribonucleotide reductase (120 nmol/min/mg R1) and that determined in virus-infected cell extracts (5 nmol/min/mg of R1) in our calculations.

IV.5 Discussion

The availability of deoxynucleotides is a crucial factor in determining the rate at which a cell or organism is able to replicate its DNA. The *de novo* synthesis of these precursors was thought to be limited by the rate of catalysis of the enzyme ribonucleotide reductase (Thelander and Reichard, 1979). In this report we have measured both the accumulation of DNA and the accumulation and activity of ribonucleotide reductase in BSC_{40} cells infected with vaccinia virus. Quantitation of

these processes has allowed us to directly relate the synthesis (supply) of deoxynucleotide precursors with their demand in viral DNA replication. Vaccinia expresses the enzymes necessary for viral DNA synthesis and directs its own replication in the cytoplasm of the host cell. Moreover, vaccinia expresses all of the functions necessary for its own cytoplasmic DNA replication and is not dependent on host factors for this process. Because host DNA synthesis is inhibited by viral infection and cellular DNA is not used by the virus in its own DNA synthesis, we reasoned that vaccinia virus provided an ideal system for this study. Since host DNA synthesis is inhibited and not available for viral DNA synthesis, these parameters did not have to be considered in the quantitations.

This work is of key significance in that through quantitative experimentation we can answer the central question of whether the rate of *de novo* deoxynucleotide synthesis is sufficiently high to account for the accumulation of DNA in this viral system. This question of deoxyribonucleotide supply and demand has been approached in the past by measuring the ribonucleotide reductase activities in extracts from different cell types and comparing these values with estimates of the cellular requirements for deoxyribonucleotides (Eriksson *et al.*, 1977; Feller *et al.*, 1980, Larsson, 1969). These early investigations however, reported enzyme activities that were too low to account for the cellular demand for deoxynucleotides. As in our work, the standard reaction conditions used to measure enzyme activity in these reports (5'-adenylylimido diphosphate), gave much lower values than measurements with purified enzyme which use physiological enzyme activator (ATP) (Mann *et al.*, 1991; and Slabaugh[4]). Our approximation of specific activity from virus-infected cells is most certainly an underestimate, while the conditions used to determine the higher specific activity value for purified vaccinia virus ribonucleotide were not physiological. We feel

[4] M. Slabaugh, manuscript in preparation.

confident however, that the bona fide value for viral ribonucleotide reductase specific activity falls between these two determinations.

S1 nuclease and immunoprecipitation analysis revealed that the transcriptional and translational expression of the genes encoding ribonucleotide reductase occur coordinately and early during viral infection. The time course of expression and the stability of the ribonucleotide reductase proteins parallel other virus-induced enzymes involved in DNA metabolism. Similar studies with viral DNA polymerase (Traktman *et al.*, 1984), thymidylate kinase (Smith *et al.*, 1989), and thymidine kinase (Hruby and Ball, 1981) all demonstrate similar expression kinetics. These gene products all belong to the 'immediate early class' of viral gene expression (Moss, 1990). Like ribonucleotide reductase, these replication proteins are also quite stable and are resistant to post-translational degradation processes.

The finding that R1 was limiting with respect to R2 during a viral infection was somewhat suprising since it was previously demonstrated that in mammalian cells, enzyme activity is limited during the cell cycle by R2 protein levels (Engström *et al.*, 1985; Mann *et al.*, 1988; Eriksson *et al.*, 1984) In other studied viral systems (herpes simplex virus and T4 bacteriophage), both subunits of ribonucleotide reductase form tight $a_2ß_2$ subunit complexes and are present in stoichiometric amounts in infected cell extracts (Ingemarson and Lankinen, 1987; Berglund, 1975). It appears that this enzyme has recruited quite a range of regulatory schemes. That vaccinia virus expresses an excess of the R2 protein is seemingly advantageous in light of recent experiments that demonstrated that vaccinia virus R2 preparations contain a low tyrosyl radical content (0.6 radicals/R2 dimer) (Howell *et al.*, 1992). The excess R2 protein in vaccinia-infected extracts may compensate for the low radical content detected in vaccinia virus R2 preparations.

We used competitive inhibition ELISA to quantitate viral ribonucleotide reductase levels and dot blot filter hybridization experiments to measure viral DNA accumulation. To relate these values we determined the specific activity of ribonucleotide reductase in virus-infected extracts. Our specific activity determinations revealed values that were significantly lower than that for purified enzyme (Slabaugh *et al.*, this issue). Significant differences in specific activities of purified enzyme and enzyme from crude extracts have also been demonstrated for ribonucleotide reductases from herpes simplex (references), bacterial (references), and mammalian (references) sources. This discrepancy is not understood but may in part be due to naturally occurring enzyme inhibitors in the extracts (Schimpff *et al.*, 1978; Lewis *et al.*, 1977). Because we did not want to overestimate ribonucleotide reductase activity in the viral extracts we used the lower value for vaccinia virus specific activity. Even with this lower estimate our calculations still reveal an adequate supply of enzyme.

IV.6 Acknowledgments and Author Contributions

We thank Dr. Stephen Kaattari for advice on the ELISA quantitation and Dr. Ralph Davis for insightful discussion.

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The contributions of the authors are as follows. C. K. Mathews provided insightful discussion during this work and provided constructive and critical review of this manuscript. M. B. Slabaugh performed preliminary immunoprecipitation and Western blot experiments and provided insightful discussion as to the design of many of the experiments. N. A. Roseman performed the S1 nuclease experiments that characterized the transcriptional kinetis of R1 and R2 expression (including Fig. IV.1) and also performed the protein stability immunoprecipitation experiments (including Fig. IV.4) In addition, N. A. Roseman provided constructive and critical review of this manuscript. M. L. Howell performed all other experiments.

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V. CONCLUSIONS

The work presented in this dissertation adds to the wealth of knowledge about a very complex and fascinating enzyme, ribonucleotide reductase. This work characterizes the small subunit of the enzyme encoded by the orthopoxvirus, vaccinia, and demonstrates that the enzyme shares many physical similarities with the well-characterized mammalian ribonucleotide reductase. In addition, the accumulation of viral ribonucleotide reductase and viral DNA have been quantitated and a direct relationship has been made between these two processes. Finally, the effects of the inhibitory drug, hydroxyurea, have been examined by deoxynucleotide pool measurements of virus-infected cells, and a mechanism for circumvention of hydroxyurea inhibition has been detailed. The relevance of each of these projects and further experiments that would additionally enhance these studies are presented below.

V.1 Small Subunit Characterization

The manuscript describing the physical characteristics of the small subunit protein demonstrated that the circular dichroism spectrum, visible spectrum and EPR spectrum of the viral protein and recombinant viral protein were nearly identical (in many respects) to those from the mammalian R2 protein (Howell *et al.*, 1992). Purification of VVR2 from a recombinant source has allowed us to prepare large amounts of protein for further study. Taken together, the biochemical results in this manuscript and amino acid sequence comparisons with other R2 proteins, provide strong evidence that the genes encoding the vaccinia ribonucleotide reductase are derived from a mammalian host. Because of these strong physical and genetic

similarities between the two proteins, and because of the ease of genetic manipulations in the virus, vaccinia represents an ideal model system within which to continue study of the mammalian ribonucleotide reductase.

However, one important difference was uncovered between the viral and mammalian R2 subunits in our experiments, and this deserves further study. Not only did the vaccinia R2 protein contain a much lower radical content (one-third that of the mammalian enzyme), but the radical content of purified viral R2 protein could not be increased by anaerobic reconstitution procedures. [These reconstitution procedures significantly increased the radical content of purified mouse R2 protein (Mann *et al.*, 1991).] To begin to understand why the radical content of the vaccinia virus R2 protein is significantly low and well below the theoretical maximum, two central questions must be answered.

First, it is important to determine whether the iron binding capability of the viral R2 protein can account for the lower levels of radical formation. A hypothesis can be put forth that radical formation is limited in the vaccinia R2 protein by the amount of iron that the viral dimer is able to bind. Initial steps have been made to examine this question by performing iron assays on the vaccinia R2 protein and by examining conditions which regenerate iron center/radical after treatment with hydroxyurea. The preliminary experiment to measure R2 iron content (presented in Appendix B) demonstrated that iron is present in purified recombinant R2 preparations and that the iron can be removed by treatment with a chelating agent (EDTA) or removed (to a lesser degree) by treatment with hydroxyurea. The results also revealed that iron was present in substoichiometric amounts; only one iron atom was bound /R2 monomer. Unfortunately, this study did not examine the iron content of ribonucleotide reductase from virus-infected cells, and therefore, conclusions about the iron center stoichiometry of the native viral enzyme can not be made. Additional preliminary studies were

performed to examine the requirements for iron center and radical regeneration after hydroxyurea treatment . These data are present in Appendix C. The results of these experiments show that radical formation (as detected by UV/visible spectroscopy) is regenerated after hydroxyurea treatment only by the addition of both iron and dithiothreitol. These experiments, although revealing, were not detailed studies. To precisely determine the amount of iron bound to VVR2 protein and examine the oxidation state of iron bound in the vaccinia virus R2 protein, atomic absorption and further quantitative iron and radical analyses are necessary.

The second question of interest is whether important differences in the stoichiometry of iron/radical center formation or activation of the vaccinia R2 protein can account for the lower radical concentrations. Because the vaccinia radical EPR spectrum was so similar to the mouse spectrum, it is improbable that there are large differences in the radical environment or in the oxidation state of the stabilizing iron center (reviewed in Chapter II.). It seems more likely that the source of the fourth (extra) electron which is necessary for radical formation may be derived from an alternate source in the vaccinia protein (Figure II.6). Therefore, this second question would best be approached by studies similar to those that determined the stoichiometry of bacterial and mammalian R2 protein activation. Quantitative oxygen consumption and Mössbauer studies could be used to reveal the source of electrons used in formation of the viral iron/radical center. Perhaps a half-sites reactivity model (which predicts less than full radical occupancy) would best fit the activation mechanism for the viral protein.

We now know (from the quantitative ELISA experiments) that R2 protein is synthesized in excessive amounts (sixfold higher) compared to the R1 protein . Northern blot analyses of the mRNA's encoding the R1 and R2 subunits reveal that the strength of the R2 promoter is much greater than that of the R1 promoter (Roseman and

Slabaugh, 1990; Tengelson *et al.*, 1988). It can be postulated that R2 overexpression is an evolutionary mechanism that the virus has developed to compensate for the intrinsically low radical content of the protein.

V.2 Holoenzyme Interactions

The work to characterize the small subunit protein of vaccinia virus ribonucleotide reductase has been augmented by complementary studies by Dr. Mary Slabaugh (previously in this laboratory) with the large subunit of the enzyme[1]. In addition to purifying and physically characterizing recombinant viral large subunit protein, Dr. Slabaugh has optimized reaction conditions for holoenzyme activity in studies by using both viral recombinant reductase subunits in purified form. Still, there are many more interesting aspects of holoenzyme kinetics and other interaction studies that remain to be examined. Preliminary studies by Dr. Slabaugh suggest that viral enzyme activity is maximal when the R2 protein is present in a fourfold excess over R1 protein levels. In addition, a preliminary K_D value has been determined for R2 binding to the R1 protein. Notwithstanding these experiments, none of the binding kinetics studies with substrate and/or effectors have been performed with the purified viral enzyme. It is currently not known, for instance, whether the viral enzyme reduces the various substrates with the same efficiency in the presence of the appropriate effector. The importance of this question and the need for performing these binding studies becomes clear when the hydroxyurea studies (Appendix A of this dissertation) and the work by Karlsson and co-workers[2] are considered. These studies both suggest that

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[1] M. Slabaugh, manuscript in preparation.

[2] M. Karlsson, M. Sahlin, and B.-M. Sjoberg, submitted: Journal of Biological Chemistry.

hydroxyurea may display a differential effect on reduction of the various substrates. In the case of the vaccinia virus system, the work in this dissertation has demonstated that the phenotype of differential substrate reduction is not due to the phenomena of substrate cycling. Additionally, whether the action of hydroxyurea indirectly affects the specificity or substrate binding site is unknown. The premise of differential substrate reduction should be examined in the viral system by substrate and effector binding studies with the purified holoenzyme in the presence and absence of drug.

Another aspect of the ribonucleotide reductase that has not been examined is the native structure of the holoenzyme. Chapter III. demonstrates that the VVR2 protein exists in solution as a dimer. Analogous studies to determine the native structure of the VVR1 protein have not yet been performed. An additionally compelling binding study that would further characterize the viral holoenzyme is to examine the effect of dATP on the physical structure of the holoenzyme. Dr. Ralph Davis (previously in this laboratory) has performed preliminary glycerol gradient analyses and demonstrated that the viral ribonucleotide reductase subunits do not co-migrate[3]. This experiment suggests that the interaction of the two holoenzyme subunits is not sufficiently strong to stabilize higher-order or multimeric species. Addition of dATP to holoenzyme preparations of mammalian, bacterial, or T4 bacteriophage ribonucleotide reductase caused the formation of tetrameric or higher-order reductase species consisting of both subunits in one case. The effect of dATP (and perhaps other effectors or substrates) on the native structure of vaccinia reductase should be examined by gel filtration chromatography, glycerol gradient sedimentation, and/or by analytical ultracentrifugation. Clearly, these studies would add to our understanding of the enzyme structure.

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[3]R. Davis, PhD Thesis, Oregon State University.

Other important interaction studies with vaccinia virus ribonucleotide reductase are currently being pursued in our laboratory. One unresolved aspect of viral ribonucleotide reductase is the source of biological reducing equivalents that are utilized by the enzyme. To this end, the vaccinia virus gene encoding glutaredoxin has recently been identified (Goebel *et al.*, 1990) and overexpressed in bacteria by Dr. Wayne Thresher in our laboratory[4]. Dr. Thresher has purified the putative glutaredoxin protein and has demonstrated biological glutaredoxin activity in purified preparations. In addition to the work on the glutaredoxin protein, ribonucleotide reductase assays were recently performed on virus-infected cell extracts in studies designed to determine the size of reducing factors needed *in vivo* for efficient reductase activity. This experiment is presented in Appendix D. The results of this experiment demonstrate that at least two reducing components are required for maximal reductase activity: one of very small size (may be replaced by dithiothreitol) and one of molecular mass between 5,000 to 30,000. Because no viral thioredoxin system has been detected, the glutaredoxin/glutathione/glutathione reductase system is the obvious candidate to serve as the biological reductant for ribonucleotide reductase in this viral system. We hope that further characterization of the viral glutaredoxin system will demonstate its catalytic role or interaction with the vaccinia virus ribonucleotide reductase.

Another important physical interaction that has recently been examined is that between the virally-encoded single-stranded DNA binding protein and the R2 subunit of vaccinia ribonucleotide reductase. Dr. Davis identified this interaction by preparing anti-idiotypic antiserum to VVR2 subunit, and then analyzing immunoprecipitations with virus-infected extracts[5]. The addition of a purified preparation of single-stranded binding protein was subsequently shown to stimulate the activity of ribonucleotide

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[4]W. Thresher, manuscript in preparation.

[5]R. Davis, PhD Thesis, Oregon State University.

reductase in preliminary studies. Affinity binding studies are currently being performed (by Dr. Thresher) to directly characterize this particular interaction. The physical interaction studies with both the viral glutaredoxin and single-stranded binding protein demonstrate the importance of considering the physiological environments and interactions of enzymes when studying enzyme structure or function.

V.3 Viral Ribonucleotide Reduction and Replication

In Chapter IV., we characterized the ribonucleotide reductase in virus-infected crude extracts. We demonstrated that the transcriptional and translational kinetics of reductase expression are consistent with those of other enzymes that are involved in DNA replication. We also demonstrated that there are adequate levels of ribonucleotide reductase activity to synthesize the required amounts of deoxyribonucleotides for viral replication (except at early hours in the viral life cycle and under certain conditions[6]). These results represent the first thorough quantitation of ribonucleotide reductase protein levels in any system and also represents the first quantitative comparison of deoxyribonucleotide supply and demand during replication.

Unfortunately, the basis of our calculations required a determination of the enzyme specific activity, and measurement of this value was complicated by the reaction conditions that are required to assay activity in crude extracts. The complexity of the *in vivo* system (enzymes that utilize ATP and CDP, the positive activator and substrate of our enzyme assay) made our activity measurements impossible with the activator, ATP. Additionally, the uncleavable analog 5'-adenylylimido diphosphate, that was used instead of ATP and remedied the interference from other enzymes, was a

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[6]Special conditions represent specific activity measurement of 5 nmol/min/mg R1 in which enzyme assays were performed with the inefficient activator, 2'-adenylylimido 5' diphosphate.

much worse activator than ATP in activity measurements with the purified ribonucleotide reductase. We remedied this problem by using both the specific activity value that we determined for crude viral extracts (in the presence of 5'-adenylylimido diphosphate) and the value that was recently determined for purified holoenzyme[7] (in the presence of ATP). Since 5'-adenylylimido diphosphate lowers measurements of enzyme activity, the correct specific activity value for ribonucleotide reductase is most assuredly in the range of our determinations. To improve the accuracy of our reductase activity determinations in crude extracts (and, therefore, our estimation of deoxyribonucleotide supply in the viral system), it would be very beneficial to find a fully active analog to substitute for ATP. Although several exist, one commercial (inexpensive) analog to test for its ability to activate ribonucleotide reductase would be γ-adenylylthio 5'-diphosphate.

Our comparison of deoxynucleotide supply and demand demonstrates that the activity of ribonucleotide reductase does not limit the rate of viral DNA replication under the conditions tested. This finding is contradictory to early studies of the bacterial enzyme that suggested, albeit indirectly, that ribonucleotide reductase was the limiting enzyme of DNA synthesis (reviewed by Thelander and Reichard, 1979). These studies evoked the presence of temperature-sensitive ribonucleotide reductase mutants (deficient in DNA synthesis) and insufficient enzyme activities in crude extracts as indications of the importance of the enzyme to DNA synthesis. With purified recombinant enzymes and immunological reagents, these researchers are now much better equipped to directly determine the role of ribonucleotide reductase in DNA synthesis. Whether the vaccinia virus replication system represents a suitable model for mammalian or bacterial replication is unclear. However, studies such as this one will most assuredly provide insights into the regulation of these replication systems.

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[7]M. Slabaugh, manuscript in preparation.

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APPENDICES

Appendix A. Deoxyadenosine Reverses Hydroxyurea Inhibition of Vaccinia Virus Growth

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A.1 Abstract

Hydroxyurea, an inhibitor of ribonucleotide reductase, blocks replication of vaccinia virus. However, when medium containing hydroxyurea and dialyzrd serum was supplemented with deoxyadenosine, the block to viral reproduction was circumvented, provided that an inhibitor of adenosine deaminase was also present. Deoxyguanosine, deoxycytidine and deoxythymidine were ineffective alone and did not augment the deoxyadenosine effect. In fact, increasing concentrations of deoxyguanosine and deoxythymidine, but not deoxycytidine, eliminated the deoxyadenosine rescue, an effect that was reversed by addition of low concentrations of deoxycytidine. These results suggested that the inhibition of viral replication by hydroxyurea was primarily due to a deficiency of dATP. Deoxyribonucleoside triphosphate pools in vaccinia virus-infected cells were measured at the height of viral DNA synthesis after a synchronous infection. With 0.5 mM hydroxyurea, the dATP pool was >90% depleted, the dCTP and dGTP pools were 40 to 50% reduced, and the dTTP pool was increased. Assay of ribonucleotide reductase activity in intact virus-infected cells suggested that HU may differentially affect reduction of the various substrates of the enzyme.

A.2 Introduction

The ability of hydroxyurea (HU) and related hydroxamic acids to preferentially inhibit DNA metabolism was initially described more than 25 years ago (Young and Hodas, 1964). HU-treated cells are reversibly arrested in the early S phase, but prolonged exposure results in cytotoxicity, leading to cell death (Bacchetti and Whitmore, 1969; Fallon and Cox, 1979; Walters et al., 1976). Although neither the

acute nor long-term effects are fully understood, HU continues to be used clinically as an antitumor agent (Anonymous, 1984).

The rate-limiting enzyme in dNTP biosynthesis, ribonucleotide reductase (EC 1.17.4.1), is the immediate target of HU (Skoog and Nordenskjold, 1971), and the site of HU action has been localized to the smaller of the two subunits of the enzyme (Krakoff et al., 1968). Inhibition is thought to result from the ability of the drug to destroy a stable organic free radical carried on a tyrosine residue that is invariant in small subunits from sources as diverse as *Escherichia coli*, yeast species, mice, and vaccinia virus (Sjöberg et al., 1985; Slabaugh et al., 1988). Although mechanistic details of the reaction catalyzed by ribonucleotide reductase remain obscure, the tyrosyl radical has been proposed to initiate reduction of the ribonucleotide substrates (Sjöberg et al., 1983; Stubbe, 1988).

If HU acts by starving the replication apparatus for precursors, then the block should be reversed if dNTPs are supplied as exogenous deoxyribonucleosides, just as thymidine can relieve inhibition of DNA synthesis by fluorodeoxyuridine, an inhibitor of thymidylate synthase. Nevertheless, numerous attempts to reverse HU inhibition have yielded preponderantly negative results (Adams et al., 1967; Cameron and Jeter, 1973; Eriksson et al., 1987; Gale, 1967; Lagergren and Reichard, 1987; Plageman et al., 1974; Walker et al., 1977; Yarbro, 1968; Young et al., 1967), and several of the reported restorations of DNA sythesis can be ascribed to changes in [^{3}H]dTTP specific activity (Scott and Forsdyke, 1980). These and other observations (Bachetti and Whitmore, 1969; Wawra and Wintersberger, 1983) have led to the suggestion that HU may have additional sites of action (Adams et al., 1971; Skoog and Nordenskjold, 1971; Yarbro, 1968), or that certain precursor pools derived by salvage pathways are excluded from replication forks (Mathews and Slabaugh, 1986; Scott and Forsdyke, 1980; Snyder, 1984).

Although no additional primary targets for HU have been identified, and HU-resistant cell and viral mutants all contain either an altered or amplified ribonucleotide reductase small subunit (for a review, see Moore and Hurlbert, 1985), the cytotoxic effects of the drug or its metabolites (e.g. chromosome breakage [Jacobs and Rosenkranz, 1970] and membrane damage [Lassmann and Liermann, 1989]) may preclude effective rescue of cell growth from HU. To circumvent possible complications due to secondary effects, we have re-examined the reversal of HU-inhibition using vaccinia virus, which encodes ribonucleotide reductase subunits that are highly homologous to their mammalian counterparts (Schmitt and Stunnenberg, 1988; Slabaugh et al., 1988; Tengelson et al., 1988). Vaccinia virus reproduction is inhibited by HU with an 50% effective concentration of 0.5 to 0.7 mM, and the virus-encoded small subunit is presumed to be a target for the drug, since resistant mutants with amplified genes for this protein have been isolated (Slabaugh et al., 1989).

We found that HU-mediated inhibition of vaccinia virus reproduction was reversible by exogenous deoxyribonucleosides, but only if an inhibitor of adenosine deaminase was present. Surprisingly, deoxyadenosine (dAdo) alone was as effective as a mixture of all four deoxyribonucleotides. The implications of these results for understanding the interaction of HU with ribonucleotide reductase are discussed.

A.3 Materials and Methods

Materials. [2,8-^{3}H]Adenosine ([^{3}H]Ado, 39 Ci/mmol) and [5-^{3}H]cytidine ([^{3}H]Cyd, 25 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). [8-^{3}H]Guanosine ([^{3}H]Guo, 11 Ci/mmol) was a gift from Dr. Buddy Ullman, Oregon Health Sciences University. [2,8-^{3}H]Adenosine 5'-diphosphate (25 Ci/mmol), [5-^{3}H]cytidine 5'-diphosphate (25.3 Ci/mmol), and [8-^{3}H]guanosine 5'-diphosphate (5

Ci/mmol) were obtained from DuPont (NEN Research Products, Boston, Mass.). Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) was donated by Burroughs Wellcome Co. (Reseach Triangle Park, N.C.). HU and calf intestinal phosphatase (CIP) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Deoxyribonucleoside triphosphates (dNTPs), poly(dA-dT), and poly(dI-dC) used in the dNTP assays were purchased from Pharmacia (Piscataway, N.J.). Pol 1 (Klenow fragment) was obtained from United States Biochemical (Cleveland, Ohio). All other biochemicals were purchased from Sigma (St. Louis, Mo.).

Cells and virus. BSC_{40} monkey kidney cells and the WR strain of vaccinia virus were maintained as described (Slabaugh et al., 1984a), except that virus stocks were subjected to two freeze-thaw cycles rather than trypsinization before infections were initiated. Periodic screening for mycoplasma with Hoechst 33258 dye revealed no evidence of infection. Confluent cell monolayers received fresh medium containing 5% dialyzed fetal calf serum (Gibco) 16 h before the beginning of an experiment and again at the time of infection. For plaque reduction assays, cells were infected with vaccinia virus at a dilution that yielded 100 to 300 plaques per dish in the absence of drugs or nucleoside. Plaques were visualized by staining monolayers with 0.5% (wt/vol) methylene blue in 50% methanol. In most experiments, triplicate dishes were used for each data point. Except where plaque numbers were low, the standard deviation was <15% of the mean value.

Analysis of dNTP pools. Confluent monolayers of BSC_{40} cells in 100-mm dishes were infected with vaccinia virus (multiplicity of infection, 10). Triplicate dishes were prepared for each data point. Treatments (HU and/or deoxyribonucleosides) were initiated at 3.5 h postinfection (hpi) and terminated at 4.5 hpi by aspirating medium and rapidly washing the monolayers twice with 2 ml of cold Tris-buffered saline (10mM Tris-HCl [pH8], 150 mM NaCl). Nucleotides were

extracted into 2 ml of 60% methanol at -20°C for 1 h. After insoluble material was pelleted, extracts were taken to dryness and suspended in 5% trichloroacetic acid (100 μl per 100-mm dish). After 30 min on ice, extracts were centrifuged to remove insoluble material and supernatants were extracted with an equal volume of 0.5 M tri-*n*-octylamine-78% Freon. dNTPs were quantitated by the enzymatic method, essentially as described (North et al., 1980).

Analysis of ribonucleotide pools. Portions of the extracts prepared for dNTP analysis were analyzed on a Partisil-10 SAX high-pressure liquid chromatography ion-exchange column (Whatman, Clifton, N.J.) exactly as described (McKeag and Brown, 1978). Peaks were detected by A_{254} and identified by their retention times as compared to standards.

Assay of ribonucleotide reductase activity. For *in vitro* assays, vaccinia virus ribonucleotide reductase was partially purified from virus-infected cells, and CDP reduction measured as described (Slabaugh et al., 1984a; Slabaugh and Mathews, 1986). For measurement of ADP and GDP reduction, reaction mixtures (20 μl) contained 100 mM 4-(-2-hydroxyethyl)-1-piperazine-*N'*-2-ethanesulfonic acid (pH 8), 10 mM dithiothreitol, 4 mM adenylylimido diphosphate, 2 mM magnesium acetate, 20 μM $FeCl_3$, 50 μM tritiated substrate (specific activity 100 cpm/pmol) and effector nucleotides at various concentrations. Reactions were stopped by heating at 100°C. Nucleotides were dephosphorylated by CIP (1 U per reaction, 37°C, 2 h), and reaction products were chromatographed as described (Schrecker et al., 1968), and quantitated by scintillation counting.

To measure *in situ* ribonucleotide reductase activity, we adapted a method previously used in mammalian cells to estimate CDP reduction after HU treatment (Bianchi et al., 1986). Infected cell monolayers (35-mm dishes; multiplicity of infection, 10 to 15) were treated with 0 to 5 mM HU at 3.5 hpi and then exposed from

4 to 5 hpi to a trace amount (0.3 μM) of radioactive ribonucleoside precursor ([^{3}H]Ado, [^{3}H]Guo, or [^{3}H]Cyd). Methanol-soluble molecules were extracted from the cells, and radioactivity in deoxyribonucleosides and deoxyribonucleotides was quantitated using the same chromatography systems utilized for *in vitro* assays. The methanol-insoluble fraction was digested with 0.3 N NaOH to hydrolyze RNA, and radioactivity incorporated into DNA (base-resistant, acid-precipitable material) was collected on GF/C filters. Radioactivity incorporated into DNA in the presence of 0.1 M cytosine ß-D-arabinoside was taken as background. This cytosine ß-D-arabinoside concentration is 50-fold greater than the level required to eliminate vaccinia virus plaque formation. To quantitate extracellular radioactivity in deoxyribonucleosides, a portion of the medium was concentrated and analyzed.

In preliminary experiments to characterize this method in the vaccinia virus-infected cell system, cultures were harvested 0, 30, 60, and 90 min after the addition of labeled compounds. The rate of uptake of [^{3}H]Ado and [^{3}H]Cyd into the methanol-soluble fraction was not affected by vaccinia virus infection. [^{3}H]Cyd reached isotopic equilibrium after 30 to 60 min, wheeras [^{3}H]Ado did not achieve equilibrium even after 90 min. This difference can be attributed to the ATP pool being much larger than the CTP pool. The acid-precipitable radioactivity present after base digestion was 3 to 5% of the total radioactivity present in the mehanol-insoluble fraction.

A.4 Results

A.4.1 Inhibition of Adenosine Deaminase Potentiates dAdo Toxicity.

Before attempting to circumvent HU effects on vaccinia virus growth, we addressed the problem of dAdo diversion resulting from the action of the salvage enzyme adenosine deaminase (Plageman and Erbe, 1974). Using dAdo toxicity to

virus growth as an assay (a phosphorylated metabolite of dAdo, dATP, inhibits the activity of ribonucleotide reductase toward all four of its substrates, leading to a depression of DNA synthesis as dATP accumulates), we asked whether the adenosine deaminase inhibitor, EHNA, was effective in countering diversion of dAdo (Henderson et al., 1977) in the vaccinia-infected cell. EHNA decreased the 50% effective concentration of dAdo from 360 μM (0 EHNA) to 60 μM (5 to 10 μM EHNA). In subsequent experiments we included EHNA at 5 μM whenever dAdo was added to the medium, unless otherwise noted.

A.4.2 HU-Mediated Inhibition of Plaque Formation Can be Reversed by Deoxyribonucleosides and EHNA.

To see whether exogenous deoxyribonucleosides could reverse HU inhibition of vaccinia virus growth, plaque assays were conducted under the various conditions indicated in Figure A.1. Only one combination of medium supplements tested -- 10 μM each dAdo, dGuo, dCyd, dThd, and 5 μM EHNA -- supported plaque formation in the presence of 2 mM HU. Under these conditions, the number of plaques per dish was 75% of the control value (no HU or deoxynucleoside additions). Without EHNA, neither a 10 μM nor a 50 μM deoxyribonucleoside supplement was effective. The higher concentration of precursors appeared to potentiate the inhibition by HU, whether EHNA was present or not. It was also apparent that in the absence of HU, the combination of EHNA plus 50 μM deoxyribonucleosides was inhibitory.

A.4.3 Reversal of HU Inhibition is a Function of dAdo Concentration.

To determine the concentration of each of the four deoxyribonucleosides which maximized reversal of HU inhibition, cells were infected with virus in the presence of HU (2 mM), EHNA (5 μM), and three of the four deoxyribonucleosides (10 μM). The

Figure A.1 Effect of Deoxyribonucleosides and EHNA on HU-Mediated Inhibition of Plaque Formation. Symbols; ○, control; ●, 5 μM EHNA; △, 10 μM dAdo, dGuo, dCyd, and dThd; ▲, 50 μM dAdo, dGuo, dCyd, and dThd; □, 5 μM EHNA, 10 μM dAdo, dGuo, dCyd, and dThd; ■, 5 μM EHNA, 50 μM dAdo, dGuo, dCyd, and dThd. Results from three dishes were averaged for each data point.

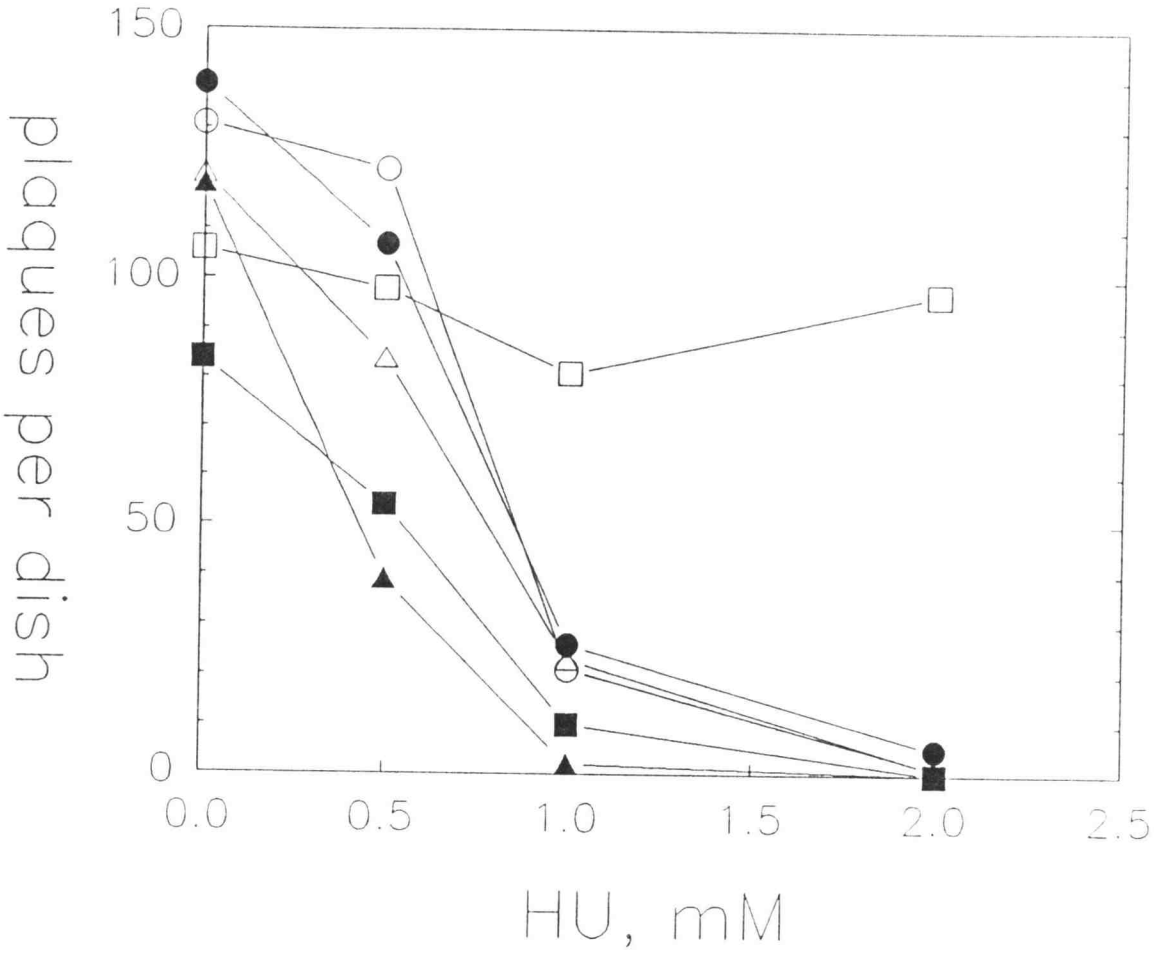

Figure A.1

fourth precursor was added at concentrations ranging from 0 to 50 μM. The unexpected result was that restoration of plaque formation was solely a function of the dAdo concentration (Figure A.2*A*). Furthermore, dAdo was effective only at concentrations below 30 μM. The finding that the efficiency of plaque restoration did not change as dCyd, dThd, and dGuo were varied suggested that their presence was not essential. This was confirmed by the experiment shown in Figure A.2*B*. Conditions were identical to those in *panel A*, except that only one deoxyribonucleoside precursor was added to each set of cultures. The results clearly demonstrared the potency of dAdo at 3 to 10 μM, but only in combination with EHNA. The maximum rescue efficiency achieved by dAdo plus EHNA was 73&.

A more sensitive measure of HU inhibition and its reversal is provided by a two-step growth assay --a protocol in which the agents are present during several cycles of viral reproduction in culture, followed by titration of total infectious virus by plating in the absence of the inhibitor. The ability of dAdo alone to support virus growth in the presence of HU was compared to the effect of all four precursors. As shown in Figure A.3, HU alone depressed virus reproduction by three orders of magnitude at 5 mM. Both treatments being tested supported a 13-fold increase in viral reproduction in the presence of 2 mM HU, and a 60- to 90-fold increase in the presence of 5 mM HU.

A.4.4 dAdo-Mediated Reversal of HU Inhibition Can be Eliminated by dThd and dGuo.

One explanation for the effectiveness of dAdo in counteracting HU is that the drug affects vaccinia replication primarily by inhibiting ADP reduction, leading to a critical depletion of the dATP pool. To investigate the activity of ribonucleotide reductase toward its pyrimidine-containing substrates under dAdo rescue conditions,

Figure A.2 (A) Effect of Varying the Concentration of Each Deoxyribonucleoside on HU-Mediated Inhibition of Plaque Formation. HU was present in all dishes at 2 mM, EHNA was present at 5 μM, and three of the four deoxyribonucleosides were present at 10 μM. The concentration of the fourth deoxyribonucleoside is indicated on the *x* axis. Symbols: ○, dAdo varied; ●, dCyd varied; Δ, dGuo varied; ▲, dThd varied. The control value (no HU) was 63 plaques per dish. Results from three dishes were averaged for each data point.

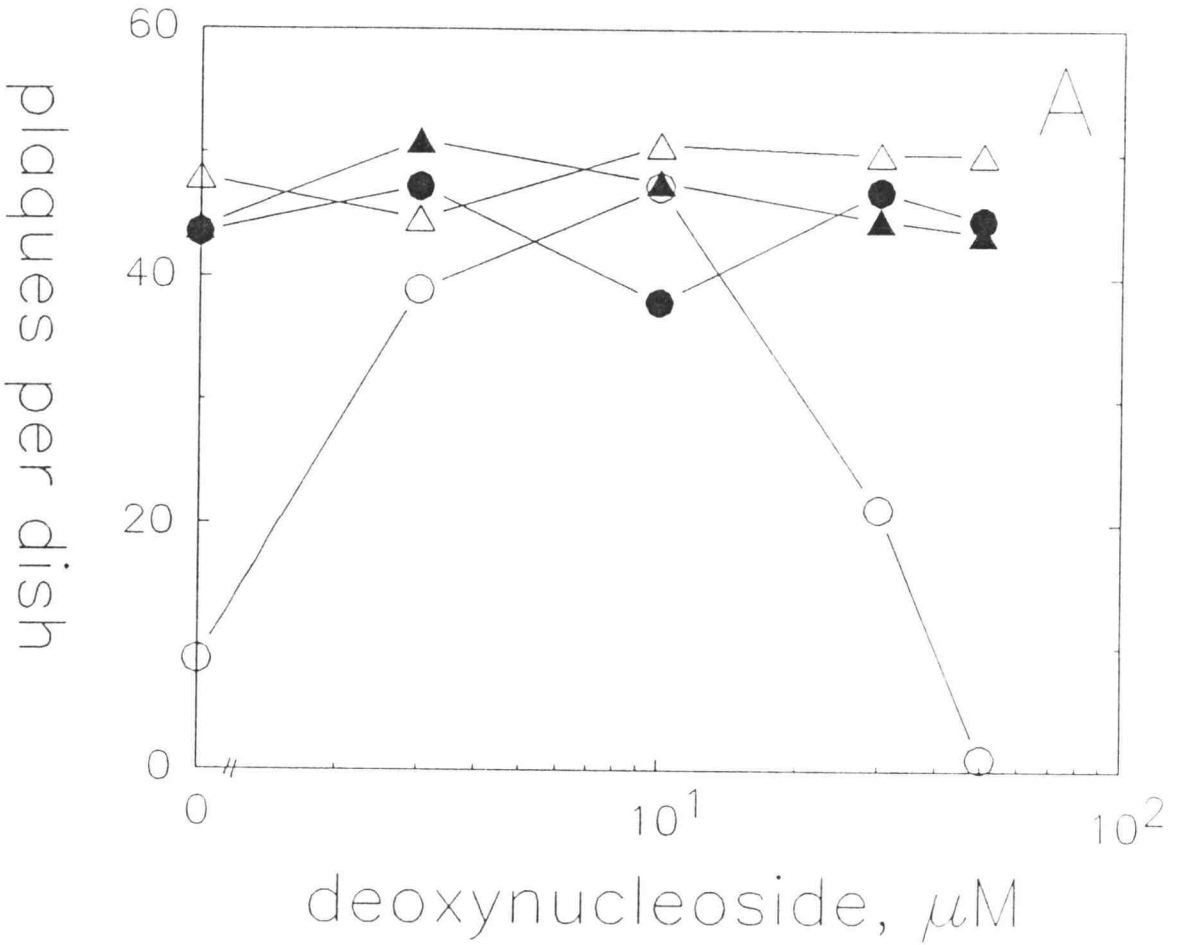

Figure A.2(A)

Figure A.2 (B) Effect of Single Deoxyribonucleosides on HU-Mediated Inhibition of Plaque Formation. HU was present in all dishes at 2 mM. Symbols: ○, dAdo plus EHNA (5 μM); ●, dAdo; △, dCyd plus EHNA; ▲, dGuo plus EHNA; □, dThd plus EHNA. Control value (no HU) was 141 plaques per dish. Each data point is the average of three determinations.

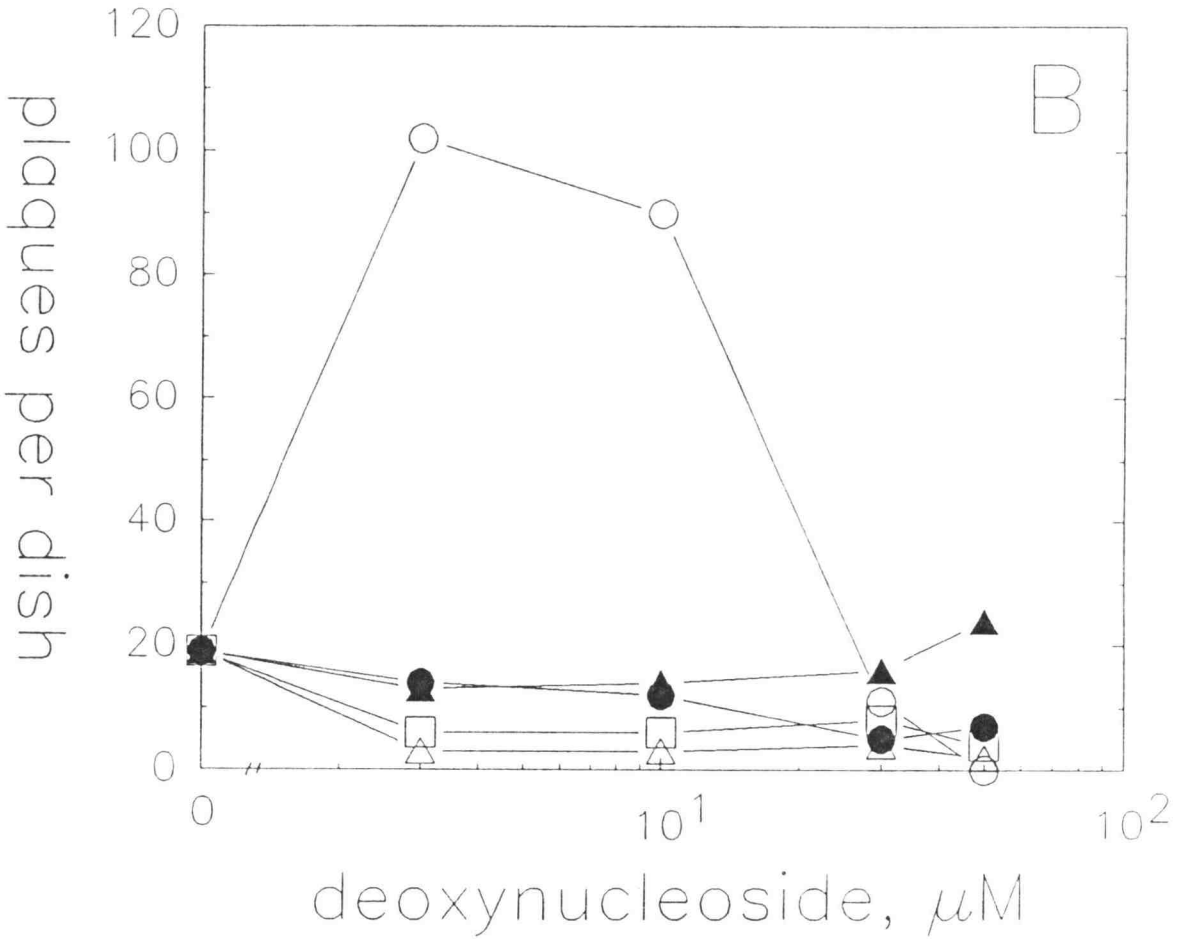
B
120
100
80
60
40
20
0
plaques per dish
0
10^1
10^2
deoxynucleoside, μM

Figure A.2(B)

Figure A.3 Effect of HU on Vaccinia Virus Growth .
Effect of HU on vaccinia virus growth in the absence (○) or presence (●) of dAdo (10 μM) plusEHNA (5 μM) or in the presence of all four deoxyribonucleosides (10 μM) plus EHNA (Δ). Monolayers of BSC_{40} cells were infected at a multiplicity of infection of 0.1 and harvested after 24 h; titers were determined by plaque assay.

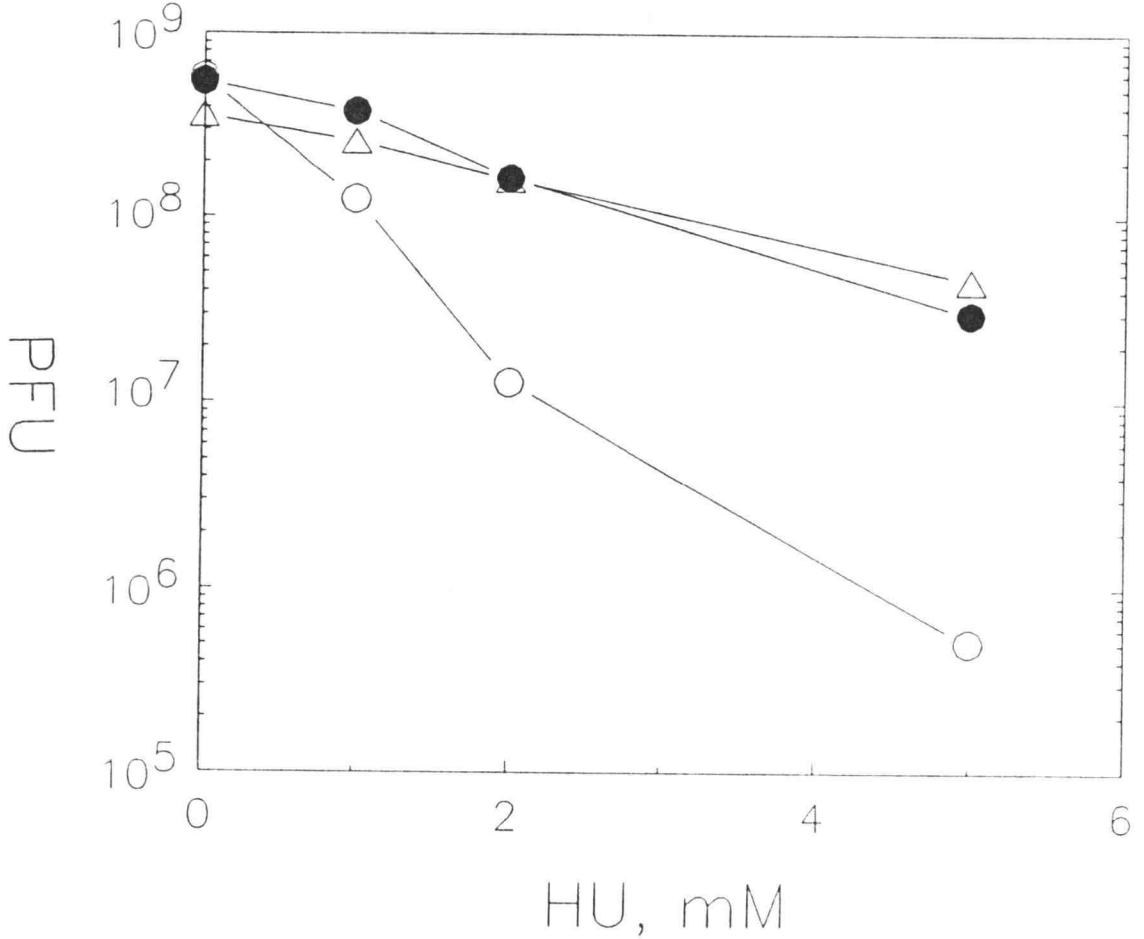

Figure A.3

we took advantage of the allosteric regulatory properties of the enzyme. As isolated from both *E. coli* and mammalian cells, the enzyme is inhibited toward reduction of CDP or UDP by either dTTP or dGTP, presumably because these effectors play positive allosteric roles in GDP and ADP reduction, respectively (Brown and Reichard, 1969; Eriksson et al., 1979). We first determined that vaccinia-encoded ribonucleotide reductase is regulated similar to the well-characterized E. coli and mammalian enzymesin that dTTP and dGTP inhibited CDP reduction, but stimulated GDP and ADP reduction, respectively (data not shown). Although these experiments were done using crude extracts, the activities measured almost certainly represent the virus-encoded enzyme, since the level of CDP reductase activity exceeded that in extracts from uninfected cells by 10-fold. Additionally, it has recently been shown that the increase in ribonucleotide reductase activity seen following vaccinia infection is dependent on an intact viral large subunit gene (Child et al., 1990). If the dAdo-mediated reversal of HU inhibition was dependent on continued reduction of pyrimidine substrates, therefore, the reversal should be sensitive to dThd or dGuo added to the culture medium. This was tested in the experiment shown in Figure A.4*A*, in which addition of either dThd or dGuo to the medium resulted in a dose-dependent elimination of the reversal. The dAdo-mediated reversal, however, was insensitive to exogenous dCyd; dCTP has no role in regulating ribonucleotide reductase (Eriksson et al., 1979). To obtain evidence that the negative effects depicted in panel A were, in fact, due to depletion of the dCTP and/or dTTP pools, we added a low concentration of dCyd to culture containing HU, dAdo, EHNA, and various concentrations of dGuo. Indeed, 10 μM dCyd restored circumvention of the HU block (Figure A.4*B*). The difference in mean plaque number in cultures with and without dCyd in the absence of any dGuo was not statistically significant (mean ± S.D. was 298 ± 39 vs. 250 ± 34, respectively).

Figure A.4(A) (A) Effects of dGuo (○), dThd (●) and dCyd (Δ) on Reversal of HU Inhibition by dAdo Plus EHNA. HU (2 mM), dAdo (10 μM), and EHNA (5 μM) were present in all dishes. The control value (no HU) was 54 plaques per dish. Each data point is the average of three determinations.

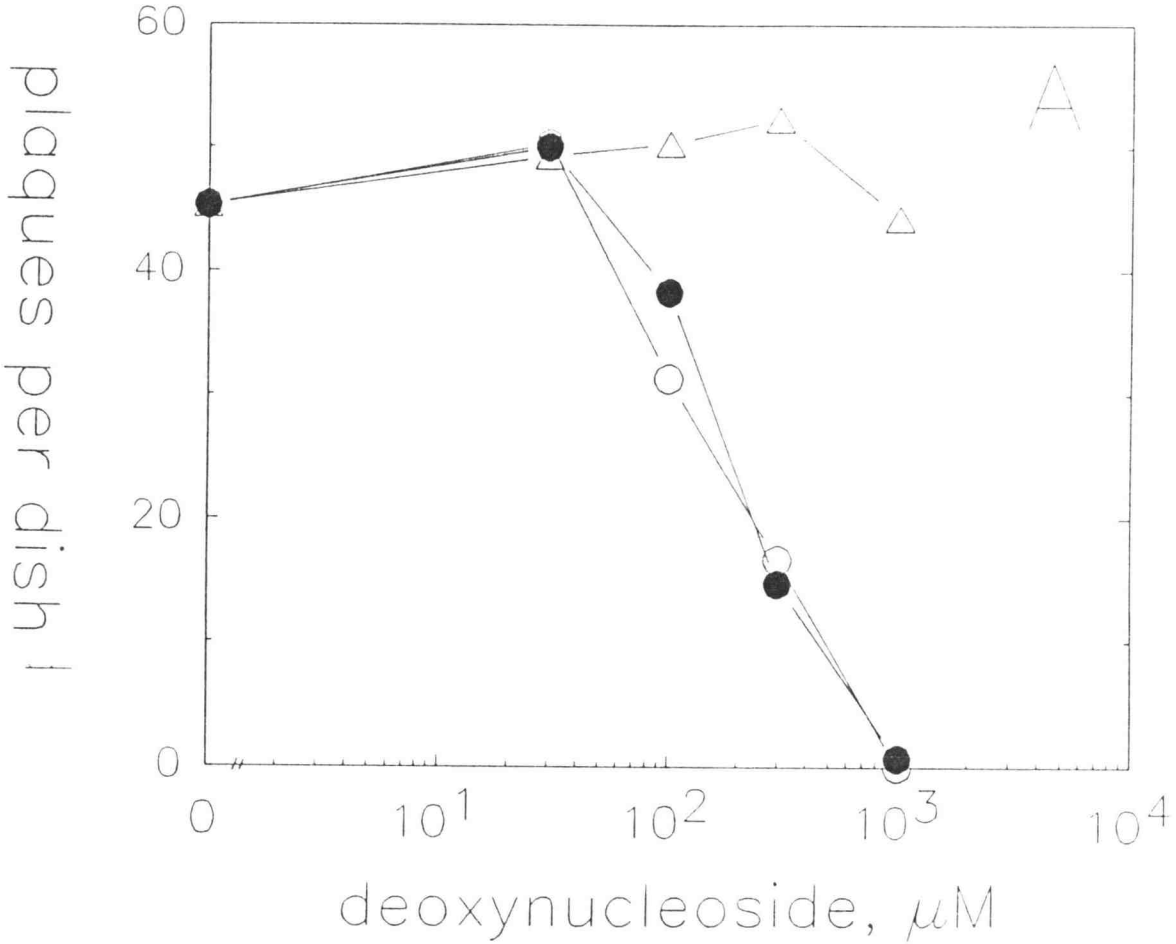
plaques per dish
60
40
20
0
0
10^1
10^2
10^3
10^4
deoxynucleoside, μM
A

Figure A.4(A)

Figure A.4 (B) Reinstatement by dCyd of Reversal of HU Inhibition. HU, dAdo, and EHNA at the concentrations indicated for *panel A* were present in all dishes. The effects of increasing concentrations of dGuo with (●) or without (○) dCyd (10 μM) are plotted.

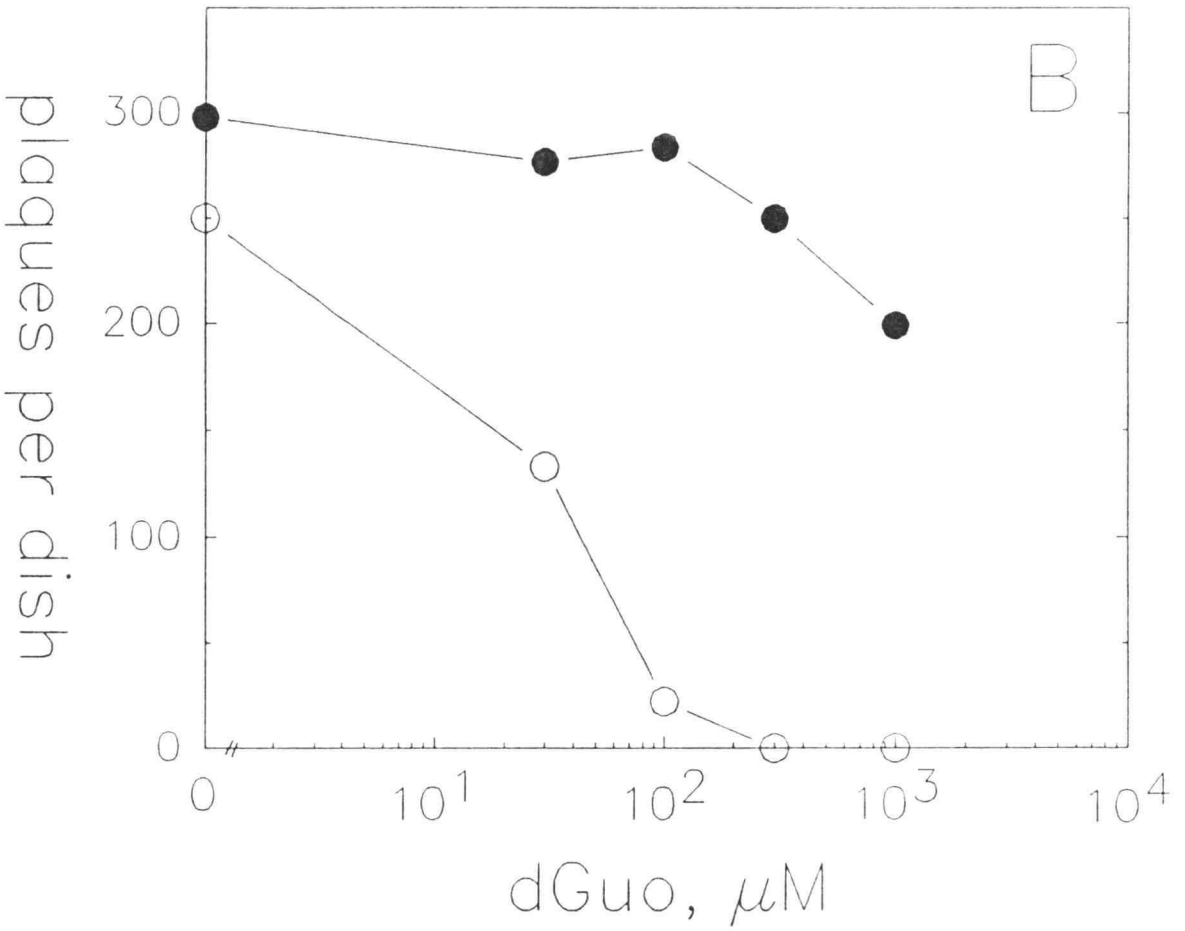

Figure A.4(B)

A.4.5 Effects of HU on dNTP Pools in Virus-Infected Cells.

To determine the effects of HU on DNA precursor pools during the period of active viral replication, we measured dNTP levels 1 h after addition of various concentrations of HU. The dATP pool was most dramatically affected by HU; it was depleted to 10% of the untreated level by 0.5 mM HU (Figure A.5). Changes in the dGTP, dCTP and dTTP pools were more moderate and were continuous over the range of HU concentrations tested. The dGTP pool, (the smallest in the absence of HU) and the dCTP pool were both reduced to about half of control values by 0.5 mM HU, and to about one-third of control values by 2 mM HU. The dTTP pool nearly doubled in the HU range from 0 to 5 mM.

A.4.6 Replenishment of the dATP Pool by Addition of dAdo and EHNA

To further explore the mechanism by which dAdo and EHNA rescued vaccinia from HU inhibition, we measured the effects of these compounds on dNTP pool sizes in the presence of various concentrations of HU. As anticipated, dAdo elevated the dATP pool and this pool remained elevated at all HU concentrations tested (Figure A.6). Aditionally, dAdo appeared to moderate the HU-induced increase in the dTTP pool and augment the drug-mediated decrease in the dGTP pool. However, since the conditions imposed in the Figure A.6 experiment are those that most effectively rescue virus reproduction, the observed changes in the dTTP and dGTP pools may not be a direct result of the dAdo supplementation but secondary to the demands of ongoing DNA synthesis.

Figure A.5 Effect of HU on dNTP pools in Vaccinia Virus-Infected Cells. Pool sizes were measured 1 h after the addition of HU to the concentrations indicated. HU was added 3.5 hpi; the multiplicity of infection was 10. Error bars indicate standard deviation for triplicate dishes.

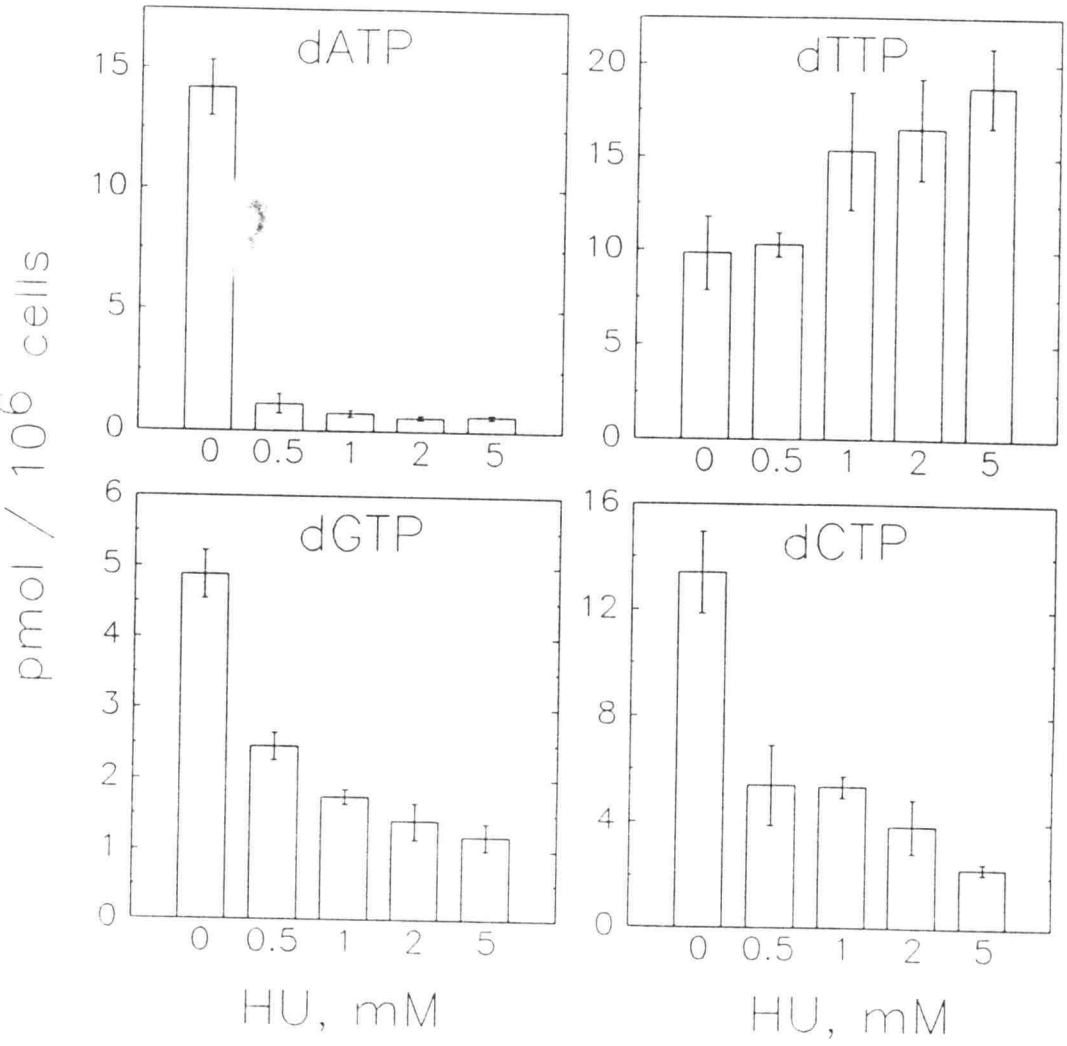

Figure A.5

Figure A.6 Effect of HU on dNTP Pools in the Presence of dAdo (10 μM) and EHNA (5 μM). Experimental details were as described in the legend to Fig. A.5.

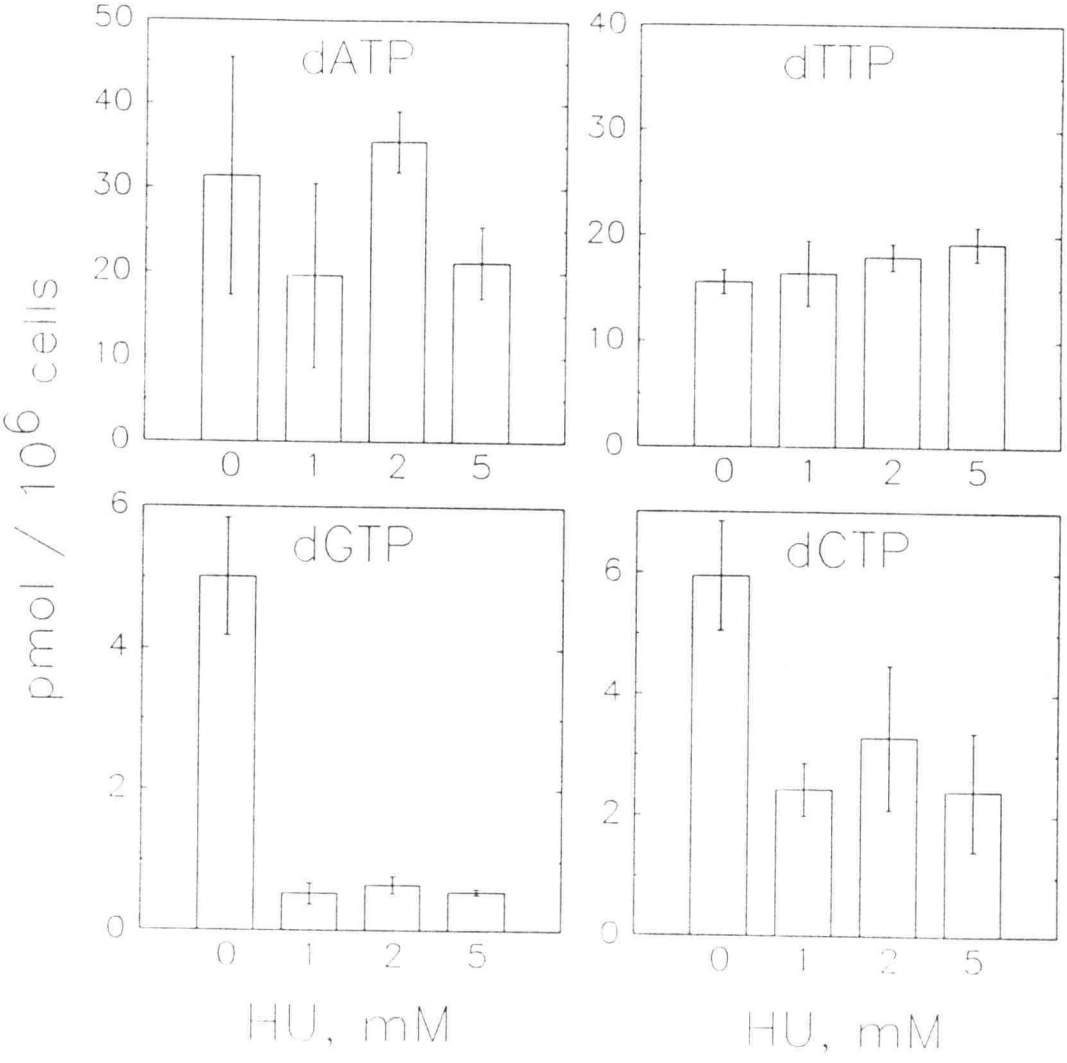
dATP
dTTP
dGTP
dCTP
pmol / 10^6 cells
HU, mM
HU, mM
0
1
2
5
10
20
30
40
50
4
6

Figure A.6

A.4.7 HU-Mediated Inhibition of the Flow of Tritiated Ribonucleosides into DNA and Deoxyrinonucleoside and Deoxyribonucleotide Pools

Since all four substrates of ribonucleotide reductase (ADP, GDP, CDP, and UDP) are thought to be reduced in the same catalytic site, by a mechanism involving the same HU-sensitive radical, differential inhibition of the four reactions catalyzed by this enzyme was unexpected. To more directly examine the effects of HU on ribonucleotide reductase in the vaccinia-infected cell, we measured the relative activity of the enzyme with respect to three of its four substrates by using a protocol based on measurement of the enzyme activity in uninfected cells (Bianchi et al., 1986). In the method, cells are exposed to a tracer amount of tritiated ribonucleoside ([^{3}H]Ado, [^{3}H]Guo, or [^{3}H]Cyd); after a period of time, intra- and extracellular fractions containing deoxyribonucleoside and deoxyribonucleotide derivatives of the precursors are analyzed. Summation of the radioactivity that has passed through ribonucleotide reductase during the labeling period yields an estimate of the relative activity of the enzyme under various conditions.

Incorporation of all three precursors into DNA was profoundly inhibited by 0.5 mM HU and was almost undetectable after treatment with 2 or 5 mM HU (Table A.1). In contrast to the effects of HU on the incorporation of ribonucleoside precursors into DNA, however, the drug exhibited an apparent differential effect on precursor incorporation into deoxyribonucleoside and deoxyribonucleotide pools. HU at 0.5 mM decreased radioactivity flowing into [^{3}H]Ado-, [^{3}H]Guo-, and [^{3}H]Cyd-derived deoxy pools by 96, 63, and 49%, respectively. Label derived from radioactive Cyd was present in both dCyd- and dUrd-containing nucleotides at about a 1:1 ratio whether HU was present or not (data not shown), suggesting that deamination of dCyd-containing compounds was not affected by HU. Cytosine ß-D-arabinoside (0.1 mM) had no effect on accumulation of radioactivity into any deoxyribonucleoside or

Table VII.1 **Relative Effects of HU on *In Situ* Ribonucleotide Reductase Activity.** Cultures infected with vaccinia virus (multiplicity of infection, 15) were labeled from 3.5 to 4.5 h post infection with the indicated compounds (0.3 μM). The radioactivity in DNA is methanol-insoluble, base-resistant, acid-precipitable radioactivity. Background values (subtracted) were determined in the presence of 0.1 mM cytosine ß-D-arabinoside (113 cpm for [^{3}H]Cyd, 260 cpm for [^{3}H]Ado, 13 cpm for [^{3}H]Guo). Values tabulated are the mean of determinations made on three independent 35-mm dishes of infected cells. ND, Not detected.

Table A

Precursor	HU (mM)	cpm per 35-mm dish ± SD			
		DNA	Intracellular deoxy pools	Extracellular deoxy pools	Total (%)
[^{3}H]Cyd	0	2,418 ± 178	3,147 ± 303	7,897 ± 1,389	13,462 (100)
	0.5	174 ± 45	1,997 ± 133	3,587 ± 756	5,758 (43)
	1.0	129 ± 51	1,570 ± 153	2,743 ± 309	4,442 (33)
	2.0	3 ± 24	1,547 ± 253	2,583 ± 476	4,133 (31)
	5.0	0	1,850 ± 273	2,243 ± 335	4,093 (30)
[^{3}H]Ado	0	5,261 ± 442	1,033 ± 180	ND	6,294 (100)
	0.5	204 ± 41	46 ± 29		250 (4)
	1.0	10 ± 36	51 ± 39		61 (1)
	2.0	0	29 ± 108		29 (<1)
	5.0	0	27 ± 68		27 (<1)
[^{3}H]Guo	0	546 ± 82	112 ± 26	ND	658 (100)
	0.5	76 ± 38	41 ± 12		117 (18)
	1.0	53 ± 21	20 ± 28		73 (11)
	2.0	17 ± 5	23 ± 24		40 (6)
	5.0	56 ± 34	24 ± 23		80 (12)

deoxyribonucleotide pool (data not shown), indicating that the observed effects were not simply secondary to cessation of DNA synthesis. Although excretion of purine deoxyribonucleosides was not detected, more than half the total radioactivity in the deoxy fractions derived from [^{3}H]Cyd was present as extracellular [^{3}H]dCyd-[^{3}H]dUrd. The excretion and re-utilization of pyrimidine deoxyribonucleosides in mammalian cells have been well characterized (Nicander and Reichard, 1985). Summing the radioactivity in the various fractions yielded the data shown in the right-hand column of Table A.1. The apparent relative sensitivity to HU at all concentrations of the drug tested was ADP reduction > GDP reduction >> CDP reduction.

Although HU is reported to have little or no effect on RNA metabolism (Yarbro, 1968), and our preliminary labeling studies indicated no significant effect of the drug on incorporation of precursors into RNA (data not shown), we wished to determine directly whether HU altered ribonucleotide pool sizes, since such effects could cause differential changes in the specific activities of ribonucleotide reductase substrates. Methanol-soluble compounds extracted from vaccinia virus-infected cells were resolved by chromatography on high pressue liquid chromatography anion-exchange column (McKeag and Brown, 1978), and the percentage of the total absorbance present in each peak was calculated. Comparison of the values obtained after a 1-h treatment with 0, 2 or 5 mM HU revealed that there was no significant effect of the drug on any NMP or NTP pool, as shown in the representative tracings from 0 mM and 5 mM HU treatments shown in Figure A.7. Additionally, the pyrimidine nucleoside diphosphate pools were not detectably altered. However, treatment with either 2 or 5 mM HU resulted in a twofold increase in the size of the peaks migrating at the positions of GDP and ADP. From this analysis, we concluded that the specific activity of [^{3}H]CDP was unaltered but that the specific activity of [^{3}H]ADP and [^{3}H]GDP may have decreased approximately twofold after treatment with 2 to 5 mM

Figure A.7 Nucleotide Pools in Vaccinia-Infected Cells at 4.5 h Post Infection Determined From High-Pressure Liquid Chromatography. *Upper tracing*, control (90,148 total arbitrary absorbance units); lower tracing, 1h of treatment with 5 mM HU (96,330 total arbitrary absorbance units).

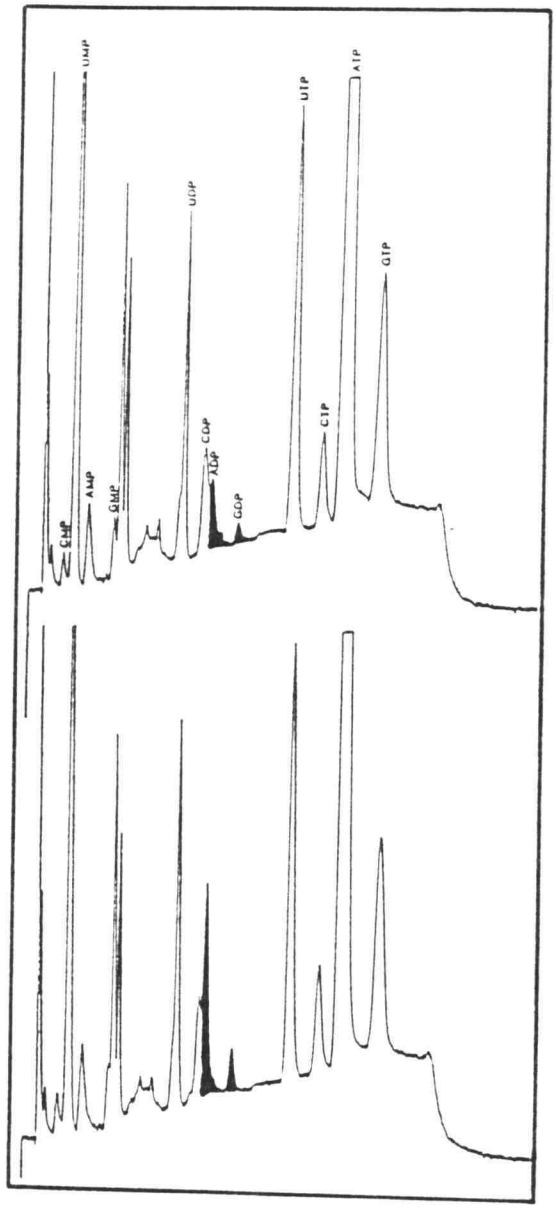

Figure A.7

HU. Changes of this magnitude, however, would not alter the conclusions we have drawn from the data in Table A.1. The apparent increase in ADP and GDP pools observed after 1 hr of HU treatment suggests that the drug block results in accumulation of ribonucleotide reductase substrates. Additional characterization of these peaks will be required before definite conclusions can be drawn.

A.5 Discussion

In the present study we report the novel and unexpected finding that HU inhibition of vaccinia virus growth was circumvented by addition of dAdo to the growth medium. Although partial reversal of HU-inhibited *Tetrahymena pyriformis* growth was achieved by adding the four deoxyribonucleoside precursors to the medium (Cameron and Jeter, 1973), reversal of growth inhibition in mammalian cells has not been reported. The conditional nature of the drug in the vaccinia virus system allowed us to explore several parameters of HU action, and the results lead us to suggest that HU inhibition of ribonucleotidereductase *in vivo* may be more complex than previously assumed.

We considered various hypotheses that might account for our finding that dAdo reversed HU inhibition of virus growth. Since resistance to HU can be mediated by elevated levels of target enzyme (resulting most commonly from gene amplification), we asked whether dAdo might act by increasing the amount of either subunit of ribonucleotide reductase produced during a vaccinia infection. As measured by immunoblotting and enzyme assays, there was no such effect (data not shown). We next considered the possibility that exogenously added dAdo provided a source of deoxyribose that was then recycled by purine nucleoside phosphorylase to supplement all four DNA precursor pools. Several results of our study, however, conflict with this

hypothesis. EHNA, a potent inhibitor of adenosine deaminase, counteracts the conversion of dAdo to deoxyinosine. Since deoxyinosine is a much better substrate than dAdo for purine nucleoside phosphorylase (Stoeckler et al., 1978), the recycling hypothesis predicts that EHNA would inhibit the dAdo rescue effect. Instead, we found that EHNA was required to achieve the rescue effect, suggesting a specificity for dAdo-containing compounds. Additionally, dGuo which is efficiently degraded and recycled by purine nucleoside phosphorylase, was ineffective as a rescue agent. The third hypothesis we considered is that HU inhibits preferentially the reduction of purine substrates, especially the reduction of ADP, and that dAdo relieves inhibition by replenishing directly the dATP pool and, perhaps indirectly, the dGTP pool. Three lines of evidence, discussed in the following paragraphs, are consistent with this hypothesis.

(i) Elimination of the dAdo rescue effect by dThd and dGuo. These latter two deoxyribonuclosides are precursors of compounds that serve as allosteric regulators of ribonucleotide reductases extracted from *E. coli* and mammalian cells (Brown and Reichard, 1969; Eriksson et al., 1979) and from vaccinia-infected cells (this study). The simplest explanation of the results shown in Figure A.4 is that the reduction of pyrimidine substrates is occuring at a rate sufficient to support viral replication, albeit at a reduced level, in the presence of HU and dAdo, and that this residual reduction can be eliminated by the allosteric effectors dTTP and dGTP. Of course, these results do not eliminate the possibility that dThd anf dGuo. or their metabolites, deplete the dCTP pool by some as-yet-undescribed mechanism.

(ii) dNTP pool studies. The differential pattern of dNTP pool changes we observed in HU-treated, vaccinia virus-infected cells was strikingly similar to that reported by a number of other investigators using a variety of cell types (Åkerblom and Reichard, 1985; Bianchi et al., 1986; Collins and Oates, 1987; Eriksson et al., 1987;

Lagergren and Reichard, 1987; Nicander and Reichard, 1985; Skoog and Nordenskjold, 1971; Snyder, 1984; Walters et al., 1973). We found the dATP pool to be depressed even more severly than what is usually reported, but this may be due to the fact that in our experiments essentially all of the cells were engaged in (viral) DNA synthesis, whereas most studies have been conducted on unsynchronized cell populations. However, the concentration of HU that caused a 90% depletion of the dATP pool (0.5 mM) effects only a 50% inhibition of virus growth. This anomaly is likely due to the different time scales of the two types of experiment. The biological response of vaccinia virus to agents that inhibit DNA synthesis is abnormally prolonged transcription and translation of early genes, a class that includes both ribonucleotide reductase genes. The presence of HU during 24 to 28 h of virus growth thus leads to overproduction of ribonucleotide reductase, which eventually confers partial drug resistance on viral replication (Slabaugh and Mathews, 1986).

(iii) Measurement of *in situ* ribonucleotide reductase activity. To determine more directly whether HU differentially affects reduction of purine versus pyrimidine substrates, we estimated relative ribonucleotide reductase activity in the presence of increasing concentrations of HU by summing radioactivity present in deoxyribose-containing molecules (DNA plus intra- and extracellular pools) labeled from ribonucleoside precursors added to the cell growth medium. The accumulation of radioactivity into total intracellular methanol-soluble and -insoluble pools was not significantly affected by HU; however, inhibition of the flow of label into dAdo-containing acid-soluble pools paralleled the decrease in DNA labeling we observed with each of the three precursors; the radioactivity flowing into dGuo- and dCyd-containing acid-soluble pools was less markedly affected. In these experiments, we attempted to account for all significant metabolic fractions into which products of ribonucleotide reductase might flow; however, we were unable to measure conversion of dUMP to

dTMP by thymidylate synthase by measuring tritiated water in the cell culture medium (Rode et al., 1980), because of a high background contributed by the [^{3}H]Cyd label. Without knowing what fraction of the total dTTP pool is derived from dCTP in vaccinia virus-infected cells, we cannot estimate how much of the total CDP reduction output was lost from our analysis by this omission. Nevertheless, since thymidylate synthase is insensitive to feedback inhibition by elevated dTTP levels (Jackson, 1978), this likely resulted in an underestimate of CDP reduction in the presence of HU. A metabolic fate of the dCTP pool unrelated to DNA synthesis, incorporation into liponucleotides, utilizes 10 to 20% of this pool in some cell types (Esko et al., 1981; Spyrou and Reichard, 1987; Spyrou and Reichard, 1989). Any [^{3}H]dCTP so incorporated in the present study would have been included in the thin-layer chromatographic quantitation of intracellular [^{3}H]dCMP after sample preparation by acid hydrolysis (see Materials and Methods).

A differential effect of HU on purine versus pyrimidine substrate reduction has not been demonstrated *in vitro*. Nevertheless, several authors have presented indirect evidence that reduction of purines, especially ADP, is more sensitive to HU than reduction of pyrimidines (reviewed by Moore and Hulbert, 1985). A partial reversal of HU-induced pool changes and DNA synthesis inhibition was recently reported in hamster lung fibroblasts after combined treatment with dAdo (plus EHNA) and dGuo (Lagergren and Reichard, 1987). Data obtained from isotope dilution analysis of rat thymus cells led to the suggestion that the increased dTTP pool observed after HU treatment resulted from residual function of ribonucleotide reductase (Scott and Forsdyke, 1980). Additionally, evidence of a differential effect of HU on ribonucleotide reduction was presented in a 1973 study of Chinese hamster cells (Walters et al., 1973). Synchronized by mitotic shake-off and then exposed to HU during the G1 phase of the cell cycle, these cells accumulated dTTP, dCTP, and dGTP

but not dATP as they approached the time they would normally have initiated DNA synthesis. Therefore, although we have studied a virus-infected cell system, it seems likely that our results are relevant to an understanding of the effects of HU on cellular replication.

That HU destroys the proteinaceous free radical of all known iron-containing ribonucleotide reductases has been widely documented. However, the concentrations of HU required *in vitro* to yield a certain inhibition, and the conditions required for regeneration of enzyme activity vary widely. Analyses that depend on assay of enzyme activity may be complicated by spontaneous regeneration of the radical under the usual reaction conditions employed (e.g. air, iron, dithiothreitol) (Thelander et al., 1983), and the recent demonstration of a strong pH dependence of the regeneration reaction *in vitro* may provide a partial explanation for the differing results reported previously (Fontecave et al., 1990).

Although we have interpreted the results of the present study as most consistent with a differential susceptibility of ribonucleotide reductase to HU, a mechanistic explanation in light of the accepted model of HU action is not readily apparent. Substrate specificity is governed by allosteric effector sites on the large subunit of the enzyme, whereas HU sensitivity is a property of the small subunit. The structure of the *E. coli* small subunit was recently determined and revealed that the tyrosine residue on which the HU-sensitive radical resides is buried within the protein some 1.0 nm from the nearest surface. The possibility of electron transfer between the iron-tyrosyl center and a surface residue at the interface between the large and small subunits was suggested (Nordlund et al., 1990). In light of this, the allosteric conformation of the large subunit might influence exposure of the radical in the holoenzyme. A thorough *in vitro* study of this enigma awaits the availability of purified enzyme subunits from cloned sources.

A.6 Acknowledgments and Author Contributions

We thank Jodi Bibler for assistance with plaque reduction assays and Buddy Ullman for helpful discussions.

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The contributions of the authors are as follows. C. K. Mathews provided insightful discussion during this work and provided constructive and critical review of this manuscript. Y. Wang performed preliminary tissue culture experiments. M. L. Howell confirmed the results of the experiments performed by Yu Wang and performed all of the tissue culture experiments that characterized the conditions which circuvent HU inhibition. M. L. Howell also performed the deoxynucleotide pool quantitation experiments, performed preliminary enzyme assays, and assisted in preparation of the manuscript. M. B. Slabaugh performed enzyme assays with the various substrates and quantitated the flow of precursors into ribonucleosides, deoxyribonucleoside and deoxyribonucleosides upon treatment with hydroxyurea (Table A.1). M. B. Slabaugh was also central to the preparation of this manuscript.

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APPENDIX B. Iron Analysis of Vaccinia Virus R2 Protein

B.1 Materials and Methods

Iron assays were performed as previously described (Atkins *et al.*, 1973). Briefly, purified preparations of R2 protein were incubated with 5% trichloroacetic acid for 10 min at room temperature (to remove iron from the protein), and then precipitated protein was pelleted and removed. 40 µl of the iron-containing supernatant was added to 36 µl H_2O, 15 µl 0.1% (w/v) sodium bathophenanthroline-sulfonate, and 5 µl 60 mM acetic acid. After a 30 min incubation, 4 µl of saturated ammonium acetate was added (to buffer the solution), and the color reaction (iron content) was monitored by measuring the A_{535} - A_{650}. Iron was quantitated by comparison with a standard curve (Figure B.1) derived from iron assays on serial dilutions of a reagent $Fe(NH_4)_2(SO_4)_2$ solution.

Iron was chelated from R2 preparations by two different treatment methods. One preparation (500 µg R2, treatment 2 in Fig. B.2) was incubated with 20 mM hydroxyurea for 30 min. The other aliquot (500 µg R2, treatment 3 in Fig. B.2) was dialyzed overnight against 100 mM EDTA. To determine how much iron remained in the treated samples, chelated iron was removed from both samples by gel exclusion using G-50 Sephadex desalting spin columns. Protein concentration were normalized before desalting. A control preparation containing unchelated R2 protein(500 µg R2, treatment 1 in Fig. B.2) was also desalted using the spin columns.

B.2 Results and Discussion

Iron assays were performed to determine the iron content of purified recombinant R2 protein and to determine the iron content of recombinant R2 protein treated with different chelating agents. The standard curve that was used to quantitate the supernatants from R2 preparations in shown in Figure B.1 and results of the iron assays are presented in Figure B.2. The quantitative values from these iron assays demonstrate that the stoichiometry of iron (0.0065 μmole) to recombinant R2 monomer (0.011 μmole) is approximately 1:2 in the purified R2 preparations. This result suggests that iron is bound to purified recombinant R2 preparations in substoichiometric amounts. In addition, the low iron content is not the result of iron deficiency in the bacterial growth media, as the addition of iron to bacterial media and to protein purification buffers did not increase the radical content of R2 preparations (data not shown). In a similar manner, the presence of iron in the enzyme reaction mixture did not increase the activity of R2 preparations as measured by enzyme assays (data not shown). One explanation for the low iron center content is that the conformational state of the recombinant R2 protein may limit the extent of iron binding. Because of the large amounts of protein required for this analysis, we were unable to measure the iron content of R2 protein from virus-infected cells. Therefore, it is not known whether the low iron content is a unique characteristic of the recombinant R2 protein.

Treatment with the chelating agents, hydroxyurea or EDTA, caused reduction in the iron content of the R2 preparation by 33 and 53%, respectively. This demonstrates that iron binding is reversible in the recombinant viral protein. The finding that hydroxyurea causes the dissociation of iron from the recombinant viral R2 protein is significant and consistent with the proposed role of hydroxyurea in iron dissociation within the mouse R2 protein (McClarty *et al.*, 1990).

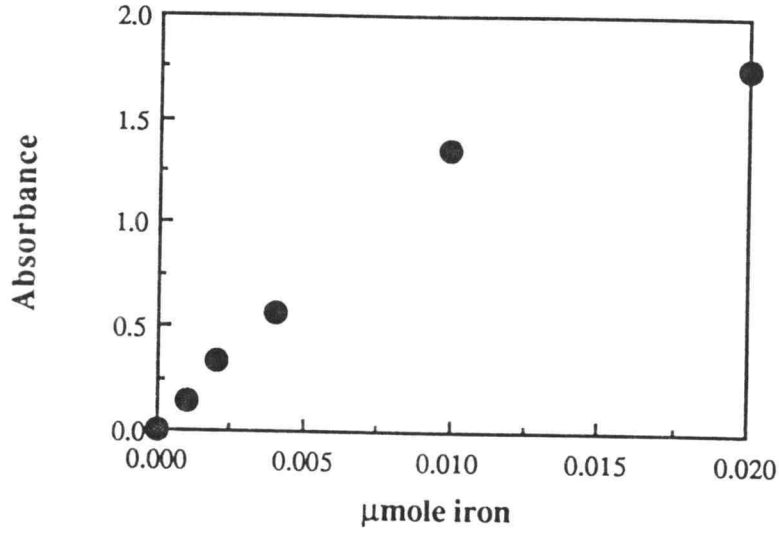

Figure VII.8 Standard Curve for Iron Analysis of R2 Protein

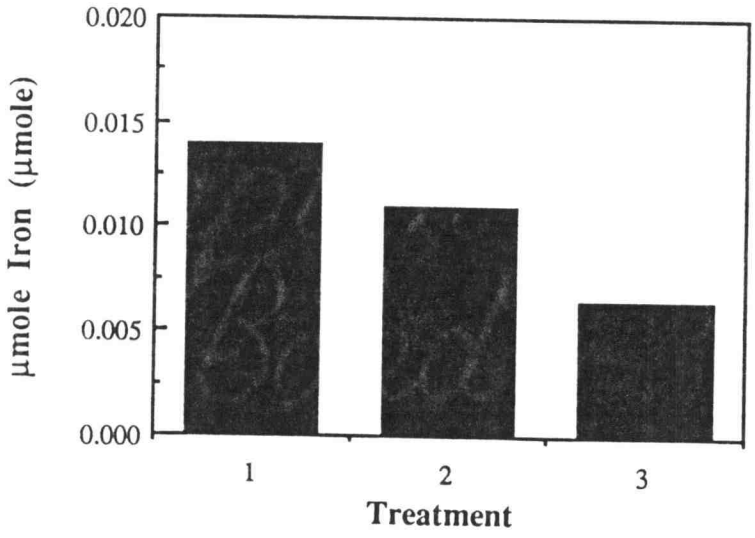

Figure VII.9 Iron Analysis of Treated R2 Preparations

APPENDIX C. Regeneration of Vaccinia Virus R2 Radical

C.1 Materials and Methods

The radical/iron center of a purified R2 preparation (5 mg) was inactivated by treatment with 50 mM hydroxyurea for 30 min at room temperature. Hydroxyurea was subsequently removed by gel exclusion using a G-50 Sephadex desalting column.The recovered preparation containing inactivated R2 was then incubated with the following solutions for 30 min. Solution 2 contained only Buffer X (100 mM Tris [pH 7.8], 150 mM NaCl). Solution 3 contained Buffer X and 10 mM dithiothreitol. Solution 4 contained Buffer X and 100 μM $Fe(NH_4)_2(SO_4)_2$. Solution 5 contained Buffer X, 10 mM dithiothreitol, 100 μM $Fe(NH_4)_2(SO_4)_2$. R2 preparations containing the various treatments were analyzed for iron center and radical content by UV/visible spectroscopy. Enzyme activity of the various R2 preparations was measured by the enzymatic assay described by Slabaugh *et al.*, (1984). The R2 preparations were complemented with purified recombinant R1 for activity measurements. For treatment 2 in figures C.1 and C.2 (corresponding to solution 2), hydroxyurea was added to the preparation before enzyme activity was measured. Treatment 1 in figures C.1 and C.2 represent R2 radical.iron center before inactivation by hydroxyurea. All protein concentrations of R2 preparations were normalized before analyses.

C.2 Results and Discussion

These experiments examine the requirements for regeneration of inactivated R2 preparations. Initially, R2 radical/iron center in the protein was inactivated by treatment with hydroxyurea and then hydroxyurea was removed from the preparations. Addition of iron and dithiothreitol to these preparations, partially restored the iron center/radical content of inactivated R2 protein (Fig. C.1, panels *B-D*) as measured by UV/visible spectroscopy. Solutions containing R2 and both iron and dithiothreitol restored the R2 radical content to the same level as untreated R2 (Fig. VII.10, *panels A and E*). These results suggest that only iron and dithiothreitol are required to regenerate the iron center/radical component of the viral R2 protein after hydroxyurea inactivation.

To examine the activity of the various R2 preparations, ribonucleotide reductase assays were performed on the treated preparations. The results of the assays are presented in Figure C.2 Although the iron/radical content of R2 protein was fully regenerated by the addition of iron and dithiothreitol, enzyme activity was only partially regenerated. Treatment of inactivated samples with dithiothreitol or dithiothreitol and iron, restored activity levels to only 55-60% of the untreated R2 (Fig. C.2, *column 1, 3, 4*). The highest activity was achieved after treatment with iron alone (75% of untreated R2). The lower activity measurement in the R2 sample with iron and dithiothreitol may result from the lack of additional components (besides dithiothreitol and iron) which may be necessary to restore full ribonucleotide reductase activity. The high concentrations of dithiothreitol and iron that were used in the assays may also have been inhibitory (in combinantion) to the activity measurement.

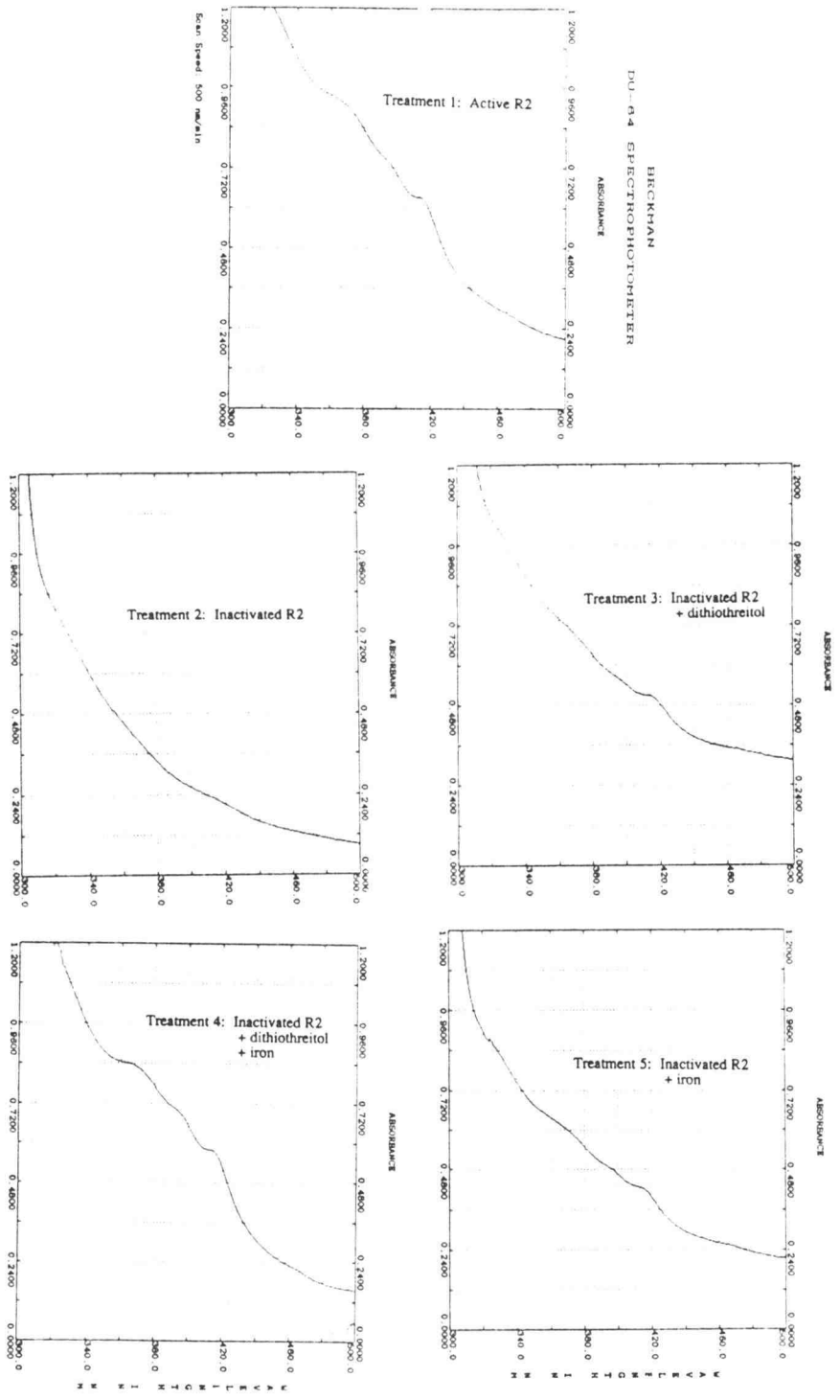

Figure VII.10 UV/visible Spectroscopy of Treated R2 Preparations

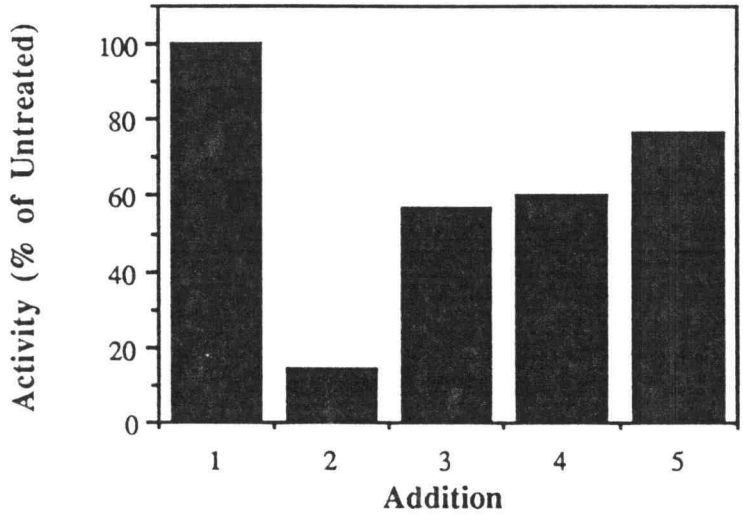

Figure VII.II Enzyme Assays on Treated R2 Preparations

APPENDIX D. Size Determination of Biological Reductants Necessary for *In Vivo* Ribonucleotide Reductase Activity

D.1 Materials and Methods

Virus-infected cell extracts were prepared as previously described (Chapter IV., this dissertation). To remove extract components, extracts were spun through either G-25 or G-50 Sephadex spin columns. The G-25 spin columns retained only very small molecules (less than 5 kDa) while the G-50 column retained molecules of molecular mass less than 30,000. A control experiment with purified R2 protein revealed that there was full recovery of small subunit protein from the flow through of the G-50 Sephadex spin column. Extracts were assayed for enzyme activity as described (Slabaugh *et al.*, 1984), except that dithiothreitol was omitted in the indicated samples. Background activity from host cell reductase was monitored by assaying mock-infected extracts, and was subtracted from the viral extracts. The activity of the host reductase was less than 10% of the ribonucleotide reductase activity from virus infected cells.

D.2 Results and Discussion

Enzyme activity measurements indicate that at least two different components are required to serve as biological reducing agents for the viral ribonucleotide reductase. The results of this experiment are shown in Figure D.1. Removal of very small molecules (< 5 kDa) decreased the reductase activity of virus-infected extracts by 60% (*column 2*). Removal of molecules of molecular mass less than 30,000, completely eliminated ribonucleotide reductase activity (*column 3*). However, the addition of

dithiothreitol to the assay reaction mixture was able to restore or partially restore the activity of both depleted extract preparations (*columns 4, 5*). The results with dithiothreitol demonstrate that at least one component of the *in vivo* reducing system may be replaced by this disulfide reductant.

Bacterial and mammalian systems encode genes for both thioredoxin and glutaredoxin and both proteins are capable participating in the reduction of the ribonucleotide reductase disulfide. Recently, a gene for glutaredoxin has been identified in the vaccinia genome (Goebel *et al.*, 1990). Because the glutaredoxin systems in *E. coli* and mouse require components of the sizes indicated as necessary for viral reductase activity, these experiments indirectly suggest that a viral glutaredoxin system may provide the necessary reducing equivalents to ribonucleotide reductase.

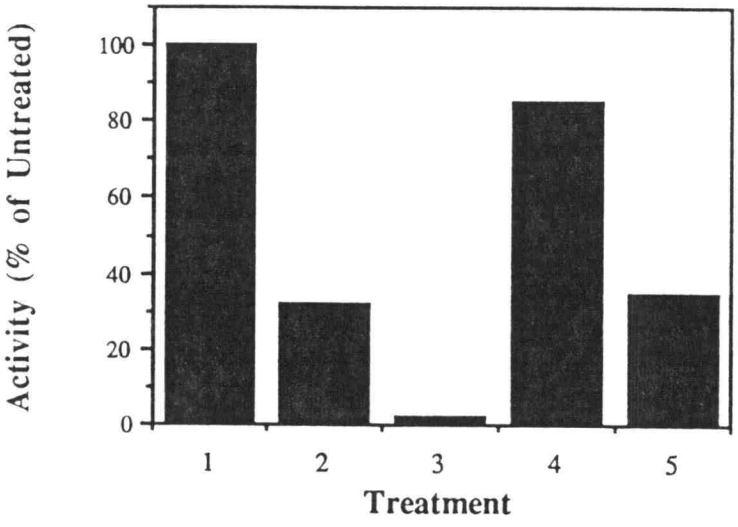

Figure VII.12 Enzyme Assays of Treated Virus-Infected Extracts