Hydroxyurea inhibits DNA synthesis by destroying the catalytically essential free radical of class I ribonucleoside diphosphate (rNDP) reductase, thereby blocking the de novo synthesis of deoxyribonucleotides. In mammalian cells, including those infected by vaccinia virus, hydroxyurea treatment causes a differential depletion of the four deoxyribonucleoside triphosphate pools, suggesting that the activities of rNDP reductase are differentially sensitive to hydroxyurea. In the presence of different substrates and allosteric modifiers, we measured rates of free radical destruction in the vaccinia virus-coded rNDP reductase, by following absorbance at 417 nm as a function of time after hydroxyurea addition. Also, we followed enzyme activity directly, by using a recently developed assay that allows simultaneous monitoring of the four activities, in the presence of substrates and effectors at concentrations that approximate the intracellular environment. We found the primary determinant of radical loss to be not the ensemble of allosteric ligands bound but the activity of the enzyme. Nucleoside triphosphate effectors accelerated radical decay, compared with rates seen with the free enzyme. Adding substrate to the holoenzyme, under conditions where the enzymatic reaction is proceeding, further accelerated radical decay. Alternative models are discussed, to account for selective depletion of purine nucleotide pools by hydroxyurea.

Hydroxyurea (HU) inhibits DNA replication in cells that use the class I form of ribonucleotide reductase (RNR) by inactivating the tyrosyl radical required for enzyme activity (1). This inhibition is also observed in viruses such as vaccinia, which encodes a class I RNR that is closely related in structure and regulation to the mammalian cell RNRs. HU inhibits the de novo synthesis of deoxyribonucleotides, thereby starving the host cell and viral replication complexes for precursors. One might expect the inhibition of RNR by a drug like HU to result in an equivalent decrease in the rates of formation of all four products of the enzyme. However, when our laboratory measured the triphosphate forms of these products, the 2’-deoxyribonucleoside 5’-triphosphates (dNTPs) in vaccinia virus-infected cells, we found that hydroxyurea treatment had different effects upon the four dNTP pools; dATP was the most severely depleted, dCTP and dGTP were depleted to intermediate levels, and dTTP actually accumulated to some 2-fold over control values (2). Similar results had earlier been noted with uninfected mammalian cells (3–5). In vaccinia virus-infected cells, addition of deoxyadenosine to the culture medium, along with an adenosine deaminase inhibitor, reversed the inhibition of virus growth by HU (2). Also, labeling studies that estimated RNR flux rates in vivo confirmed that ADP reduction was the most severely affected of the four RNR activities. These observations suggested that the reduction of ADP is a specific biological target of hydroxyurea in vivo. This finding, in turn, has spurred attempts to use HU in conjunction with dideoxynucleosides to deplete white blood cells of dNTPs, and hence, to interfere with reverse transcription in cells infected with human immunodeficiency virus (6). In accord with the evident in vivo specificity of HU action, the RNR inhibitor is more effective when applied along with dideoxyinosine than with pyrimidine dideoxynucleosides.

In studies of the Escherichia coli class I RNR, Karlsson et al. (7) reported variations in the lifetime of the free radical in vitro, depending upon the presence of specific ligands bound at the regulatory sites. The mammalian and E. coli RNRs have similar regulatory features—two identical "activity sites" on each R1 (large) subunit, which bind either ATP or dATP at low affinity and control catalytic activity, and two "specificity sites," also on R1, which bind ATP, dATP, dGTP, or dTTP at higher affinity and control the specificity of the enzyme, with each ligand stimulating reduction of one or more substrates and inhibiting one or more activities. Because the vaccinia virus RNR is closely related to the mammalian enzymes (8, 9) and because it responds almost identically to allosteric effectors (10), the viral enzyme is almost certainly controlled by similar mechanisms. As with the E. coli enzyme, the mammalian and vaccinia RNRs both carry the catalytically essential tyrosyl radical on the R2 (small) subunit.

The findings of Karlsson et al. (7) suggested a hypothesis to account for the differential effects of HU upon dNTP pools, namely, that the binding of ligands at the allosteric sites somehow influences HU sensitivity. Specifically, because dGTP bound at the specificity site is a prime activator of the reduction of ADP to dADP (10), one could speculate that this form of the enzyme allows preferential access of HU to the tyrosyl radical, thereby specifically inhibiting that form of the enzyme poised to reduce ADP in vivo. This in turn could specifically inhibit dADP formation, and hence, diminish the steady-state pool of dATP.

This paper describes two independent in vitro techniques
developed to test this hypothesis—first, a physical method that monitors the HU-induced decay of the radical directly, and second, an enzymatic assay that monitors the four activities of the enzyme as a function of time after hydroxyurea addition. Both approaches use the vaccinia virus-coded RNR, which is an excellent model for the closely related mammalian enzymes. The physical method monitors loss of the free radical directly with time after HU addition, through analyses of a light absorption maximum (417 nm) associated with the radical and a nearby iron-bridged oxygen atom (11, 12). By these means, we followed the HU-induced loss of the radical in different allosteric conformations, and in addition, we monitored radical loss in the presence of substrate, under conditions where the enzyme is catalytically active. The activity assay (13) simultaneously monitors all four activities of rNDR reductase in one reaction mixture, thereby allowing the differential effects of HU to be examined in a single reaction mixture, under conditions approximating the intracellular environment.

EXPERIMENTAL PROCEDURES

Overexpression and Purification of Recombinant Subunits—Both subunits of vaccinia ribonucleotide reductase were previously cloned into IPTG-inducible pET expression vectors and were overexpressed as described (12, 14).

The purification procedure for the R2 subunit was essentially as reported (12), except for the incorporation of an additional radical reactivation step. In this step, recombinant apoR2 was activated to form the tyrosyl radical and iron-oxo center by addition of ferrous iron under anaerobic conditions. The reactivation was accomplished during gel exclusion chromatography by the addition of ferrous ammonium sulfate to extensively degassed column buffer. Once the size exclusion column had been equilibrated with this solution, partially purified R2 was applied, allowed to bind iron, and then eluted as described (12). The tyrosyl radical was formed when the protein eluted from the anaerobic environment of the column and was exposed to air in the fraction collector.

Purification of the R1 protein was based on the affinity of the protein for dATP. A dATP-Sepharose affinity resin was synthesized by coupling 2'-deoxyadenosine-5'-[γ-4-aminophenyl]-triphosphate (United States Biochemical), via the aminophenyl moiety, to cyanogen bromide-activated Sepharose, using minor modifications of the coupling procedure specified by the supplier (Amersham Pharmacia Biotech). Routinely, 3–5 g of cells containing overexpressed vaccinia R1 was lysed in a French press and centrifuged at 12,000 rpm for 20 min. The clarified lysate was filtered and applied to a column packed with 1.0 ml of dATP-Sepharose resin, and the column was washed extensively with column buffer containing 50 mM HEPES, 100 mM KCl, 1 mM dithiothreitol, pH 8.2. After washing the column back to baseline, a 7.0-ml wash of column buffer containing 5 mM ATP was used to elute any E. coli R1 or other bound proteins resulting from the expression system. Finally, the recombinant vaccinia R1 protein was eluted with column buffer containing 75 mM ATP, Centronic-30 centrifugation devices (model 4209; Amicon Inc., Beverly, MA) were used to concentrate the purified R1 and remove the ATP used in the elution step. Both R1 and R2 proteins were analyzed by SDS-polyacrylamide gel electrophoresis and found to contain no detectable contaminating proteins, as reported previously (12, 14).

Hydroxyurea-mediated Radical Decay Assays—Purified holozyme, or R2 alone, plus the appropriate effectors and substrates, was mixed with HU at zero time, and the time-dependent change in absorbance at 602 nm was recorded on a Beckman DU-64 spectrophotometer. Typically, assays were done in 50 mM HEPES, pH 8.2, containing 15 μM R1, 20 μM R2, 800 μM dNTP effector, 1 mM rNDR substrate, and 20 mM dithiothreitol in a final volume of 75 μl. R1 was added in excess to assure that most of the R2 would be bound in the holozyme form. HU was added at the concentrations specified in the relevant tables and figures.

Ribonucleotide Reductase Four-substrate Assay—The assay was performed essentially as described by Hendricks and Mathews (13). In the first of two steps, boronate affinity chromatography was used to separate the RNR products (dNDPs) and allosteric effectors (dNTPs) from the substrates (rNDPs) and the effector ATP. In the second step, the dNDPs were resolved into individual nucleotides by high performance liquid chromatography. Vaccinia R2 was added to the reaction in a 29520-fold molar excess over vaccinia R1. In all reactions, vaccinia R1 was present at 1.0 μM final concentration. ATP and dithiothreitol were present in all reaction mixtures at 2.0 and 50 mM, respectively. Except as indicated, all four rNDR substrates were added to the reaction mixtures at equimolar concentrations, usually 0.15 mM each. In the inhibitor-treated samples, the final HU concentration ranged from 0.2 to 2.5 mM.

RESULTS

Hydroxyurea-mediated Chromophore Decay—Fig. 1 shows visible and near-UV spectra of the vaccinia R2 protein taken at approximately 90-s intervals following the addition of HU. The spectrum of the untreated R2 protein, as reported previously (12), is nearly indistinguishable from that of the closely related mouse R2 protein (the two proteins share 80% sequence identity). As shown for the mouse enzyme, the 417-nm absorption peak and the 395-nm shoulder result primarily from the tyrosyl radical, and the 370-nm peak arises chiefly from the iron-bridged oxygen moiety, which participates in forming the tyrosyl radical. Because of the sequence relatedness of the mouse and vaccinia R2 proteins, the near-identity of their absorption spectra, and also the near-identity of their electron paramagnetic resonance spectra (12, 15), we assume that the spectral features of the two proteins derive from the same chromophores. Therefore, we conclude from Fig. 1 that hydroxyurea causes near-simultaneous destruction of both the tyrosyl radical and the iron-oxo chromophores in the vaccinia R2 protein.

In the experiment shown in Fig. 2, we followed the decline in absorbance at 417 nm as a function of time after HU addition at 60 mM. Comparison of the decay rates of R2 alone with R2 plus R1 indicates that the presence of R1 protects the R2 tyrosyl radical and iron-oxo center from HU. This protection was lost once allosteric effectors were added to the holozyme, because the addition of either dGTP or dTTP accelerated the decay in A417 beyond what was seen with R2 alone.

A further acceleration was seen when substrates were provided to the R1-R2 holozyme in addition to allosteric effectors. In the experiment shown in Fig. 3, we compared the decay rates in the presence of dGTP—the prime allosteric activator for ADP reduction—with dGTP plus the substrate ADP. Similarly, we compared the decay rate in the presence of dTTP—the
prime activator for GDP reduction—with dTTP plus GDP. These experiments had to be run in the absence of ATP, an allosteric activator that stimulates all four activities of the enzyme by binding to the activity site on the R1 protein. However, ATP binds also to the specificity site, thereby stimulating the reduction of CDP and UDP. Because we wanted to compare enzyme-ligand complexes designed to reduce ADP and GDP, respectively, we omitted ATP in this experiment. Under these conditions, where the enzyme is at least partially active, we noted that the presence of either substrate significantly accelerated loss of the radical, when compared with the enzyme in the presence of effector alone. Equally important, perhaps, we saw no significant difference in the decay rate when the ADP-reducing enzyme was compared with the GDP-reducing enzyme.

The acceleration of radical decay by substrate was seen also in experiments (not shown) that involved pyrimidine reduction. When holoenzyme plus ATP was compared with holoenzyme plus ATP plus CDP, the addition of the substrate CDP evidently destabilized the radical, just as seen for ADP and GDP in Fig. 3. Although variations were seen in individual experiments, the results shown in Figs. 2 and 3 were seen reproducibly; i.e., 1) adding R1 to R2 appeared to protect the radical and iron-oxo center when compared with the decay rate seen with R2 protein alone; 2) the addition of effectors increased sensitivity to hydroxyurea; and 3) the addition of substrate further increased the rate of chromophore decay.

**Measurement of HU Inhibition Using the Four-substrate Assay**—The experiments described above were carried out to test the hypothesis that different allosteric forms of ribonucleotide reductase vary in their sensitivity to hydroxyurea and that these variations could explain why hydroxyurea specifically targets ADP reduction in vivo. However, these experiments did not support the hypothesis, because we did not observe significant differences in rate of chromophore loss after HU addition, among the different enzyme-effector combinations tested. However, what we did see reproducibly was sensitization of the enzyme to HU when the enzyme was acting on a substrate.

As another approach to the initial question—how to explain the evident specificity of HU against ADP reduction in vivo—we turned to an assay system, recently developed in our laboratory (13), which permits the simultaneous monitoring of the four RNR activities under conditions approximating the intracellular environment. Our earlier report (13) showed that for T4 phage-coded aerobic ribonucleotide reductase, the rates of the four reactions, measured together, are in close proportion to the representation of the four deoxyribonucleotides in the phage genome, but only under conditions where the four ribonucleoside diphosphate substrates and the four allosteric effectors (ATP, dATP, dGTP, and dTTP) are all provided at their intracellular concentrations, as estimated from pool measurements. In the accompanying paper (16), we show the same to be true for the vaccinia virus RNR.

Fig. 4 shows the results of a typical four-substrate assay. In
this experiment, the four rNDP substrates were provided at 0.15 mM each, and the allosteric effectors ATP and dGTP were added at 2.0 mM and 10 μM, respectively. These conditions should direct the enzyme toward ADP reduction; if hydroxyurea, in fact, specifically inhibits this reaction, these conditions should help us to observe this effect. In this experiment, which was run in duplicate, HU was added to one of the samples at time zero, and the reaction mixtures were incubated at 37 °C. After a 6-min incubation, the reactions were terminated, and the products were analyzed by high performance liquid chromatography as described (13). The difference between the peak areas for each dNDP product in the treated and untreated samples gives the percentage of inhibition of product formation by HU. In the experiment shown in Fig. 4, those percentage of inhibition figures were as follows: ADP, 42%; CDP, 30%; GDP, 38%; and UDP, 26%.

The results of this experiment showed slightly preferential inhibition of ADP reduction under the conditions tested. This result led to a series of experiments, summarized in Tables I–III, in which we carried out this assay with a series of effector combinations and at different hydroxyurea concentrations. For these experiments, we measured only the activities of the enzyme on ADP, CDP, and GDP, because UDP reductase activities were almost too low to measure. As discussed elsewhere (17, 18), much or most of the dTTP pool and the DNA-dTMP in T4 and mammalian DNAs comes from deoxycytidine nucleotides, via dCMP deaminase, and the same appears to be true for vaccinia virus DNA synthesis, in light of the extremely low activities of the viral enzyme with UDP as substrate.

Because the experimental error in the four-substrate assay is approximately 5% (13), a large number of assays were needed to determine whether the different RNR activities varied in their sensitivity to hydroxyurea. The assay reported in Table I involved reactions run with 2 mM ATP as the sole effector. The assays reported in Table II were run in the presence of ATP plus one dNTP effector (dGTP to stimulate ADP reduction, dATP to stimulate CDP reduction, and dTTP to stimulate GDP reduction). Finally, the assays reported in Table III were run in the presence of a “physiological” mix of effectors (13, 16). In none of these experiments were we able to establish that the reduction of ADP is more sensitive to hydroxyurea than are the other activities of the enzyme. In fact, all three activities mentioned were indistinguishable in their responses to HU, a result that we might have expected from the fact that the tyrosyl radical is essential for catalysis of all four reactions of the enzyme.

**DISCUSSION**

There are both conceptual and practical reasons for our interest in the problem posed in this study. At the conceptual level, understanding how an inhibitor that should have identical effects on all four activities of RNR reductase achieves apparent selective inhibition of just one of those activities in vivo relates to our long-standing interest in using in vitro enzymology to learn how enzymes behave within their natural environment, the living cell. At the practical level, hydroxyurea has been used for some time as an anticancer agent, which targets ribonucleotide reductase, and HU has shown activity as an antiviral agent in HIV-infected cells (6, 19, 20). In these more recent studies, HU displayed a synergistic effect when applied together with 2',3'-dideoxynosine, but only additive effects in combination with dideoxyctydine or dideoxyuridine. These observations are readily explained if HU specifically targets purine dNTP synthesis in HIV-infected cells, for dideoxyinosine must compete with endogenous purine nucleotide pools in order to be anabolized to the biologically active dideoxynosine triphosphate forms.

Our initial experiments measuring rates of decay of the tyrosyl radical were based upon comparable experiments with the *E. coli* type I RNR (7). In those experiments, Karlsson et al. (7) monitored radical decay indirectly, by following loss of enzyme activity as a function of time after HU addition. Our direct measurements of radical decay showed that the vaccinia RNR, like the comparable *E. coli* enzyme, is strongly affected in its sensitivity by the ensemble of nucleotide substrates and effectors bound. However, we were not able to confirm our initial hypothesis, namely, that the enzyme poised to act as an ADP reductase is most sensitive to hydroxyurea. We reached the same conclusion from our simultaneous measurements of enzyme activities in the presence of hydroxyurea.

How, then, can we explain why hydroxyurea preferentially depletes dATP pools in vaccinia virus-infected cells (and probably also in uninfected cells (3–5))? Two factors seem particularly relevant. First, as shown by Reichard and colleagues (21, 22), dNTP pools in mammalian cells are in a continual state of synthesis and degradation. Reichard (23) has proposed that “substate cycles,” operating between deoxyribonucleosides and deoxyribonucleoside monophosphates, contribute significantly toward the regulation of dNTP pool sizes. Large proportions of the deoxyribonucleosides produced from dNTP turnover are excreted from the cell, with far more pyrimidine nucleosides being excreted than purines (21, 22). Similar results for vaccinia virus-infected cells have been seen in our

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**Table I**

| % inhibition of reduction of | mm HU |
|-----------------------------|-------|
| rCDP | 0.5 | 15 | 24 | 37 | 46 |
| rADP | 14 | 21 | 27 | 36 | 44 |
| rGDP | 5 | 18 | 36 | 41 | 49 |

**Table II**

| % inhibition of reduction of | mm HU |
|-----------------------------|-------|
| rCDP | 1.0 | 35 | 17 | 44 | 5 |
| rGDP | 1.5 | 38 | 22 | 38 | 4 |
| rGDP | 2.0 | 41 | 16 | 37 | 2 |

**Table III**

| % inhibition of reduction of | mm HU |
|-----------------------------|-------|
| rCDP | 1.0 | 32 | 14 | 7 |
| rADP | 2.5 | 28 | 9 | 10 |
| rGDP | 0.5 | 24 | 11 | 4 |
laboratory (2). It seems likely that high intracellular levels of adenosine deaminase and purine nucleoside phosphorylase degrade purine nucleosides to nucleobases, which do not readily exit the cell. Evidence supporting this idea comes from our observation that adenosine addition to the medium allows bypass of an HU-mediated block to vaccinia virus replication, but only when an adenosine deaminase inhibitor is added simultaneously (2).

When a hydroxyurea block is imposed, making the cell dependent upon salvage pathways for deoxyribonucleotide synthesis, pyrimidine deoxyribonucleosides are readily taken back into the cell and converted to dNTPs (23). It seems likely that this is the reason for dTTP accumulation in hydroxyurea-treated cells, both uninfected (3, 17) and vaccinia virus-infected (2). However, if purine nucleosides have been catabolized to nucleobases, anabolic pathways would convert those bases to ribonucleotide pools, which would have to flow through ribonucleotide reductase in order to replenish purine dNTP pools. Thus, these pathways cannot significantly overcome a hydroxyurea block.

The above considerations can explain how hydroxyurea treatment selectively depletes purine deoxyribonucleotide pools; indeed, these arguments largely restate what was shown by Bianchi et al. (3, 17) for uninfected mammalian cells and is shown in our work for virus-infected cells (2). However, these considerations do not explain how dATP is selectively depleted when compared with dGTP. A possible explanation relates to the fact that dGTP pools are underrepresented in mammalian cells, usually comprising just 5–10 mol % of the entire dNTP pool (24). The same is true for vaccinia virus-infected cells (2). When hydroxyurea is added and further dNTP synthesis is blocked, then continued DNA synthesis, drawing upon the preexisting pools and those that can be re-created from salvage pathways, can be expected to deplete the least abundant dNTP pool most rapidly. That pool is dGTP.

dGTP is the prime allosteric activator for reduction of ADP by mammalian RNRs (10, 23) and also for the vaccinia virus enzyme (16). Therefore, in either infected or uninfected cells, a partial dGTP depletion could limit ADP reduction in vivo, by depriving the enzyme of the effector it needs to efficiently reduce ADP to dADP. We have estimated the intranuclear concentration of dGTP in S-phase-enriched HeLa cells to be about 10 μM (25), and dNTP pool measurements suggest that the levels in vaccinia virus-infected cells are comparable. Minimum optimal dNTP concentrations for regulating vaccinia RNR are also about 10 μM (16). These semiquantitative considerations suggest that a partial dGTP depletion could have a significant effect on flux through ADP reduction and thereby cause a specific dATP pool depletion.

Another possible explanation comes from our recent studies of regulation of vaccinia virus rNDP reductase. As detailed in the accompanying paper (16), we have found that ADP at concentrations within the physiological range specifically inhibits ADP and GDP reduction, with ADP reduction being the more sensitive target. Perhaps hydroxyurea inhibition causes the ribonucleoside diphosphate substrates of rNDP reductase to accumulate, with consequent inhibition by ADP of the synthesis of dADP and hence of dATP. Both models advanced to explain selective dATP depletion in response to HU are amenable to experimental test.

Finally, we can speculate on the reasons why ribonucleotide reductase is more sensitive to HU inhibition when it is catalytically active. Crystallographic analysis of both the E. coli and mouse R2 proteins (26, 27) shows the tyrosyl radical to be buried within the interior of the protein. Sjoberg and co-workers (28) have obtained evidence for an electron transfer pathway in the E. coli R1-R2 holoenzyme, which moves the radical character from Tyr-122 through specifically identified residues, some of them on the protein surface, finally reaching a particular cysteine residue, Cys-439, in the catalytic site on the R1 protein; in the process, an unpaired electron is moved from Cys-439 in R1 to Tyr-122 in R2. Because access to the tyrosyl residue is apt to be hindered, even for small molecules like hydroxyurea, one might speculate that hydroxyurea can react not only with the tyrosyl radical within the R2 protein but with a surface residue that is transiently radicalized, as an intermediate in creating the active-site radical that is essential for catalysis.

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