Incubation of the pyrimidine [3'-2H]UDP with ribonucleotide reductase resulted in an isotope effect on the conversion to dUDP which varied as a function of pH and allosteric effectors (pH, kH/kF, effector); 5.6, 4.7, ATP; 7.6, 3.3, ATP; 7.6, 2.6, dATP; 7.6, 2.0, TTP; 8.4, 2.8, ATP. During this reaction H2O was also released. The lower the pH of the reaction, the larger the isotope effect, and the smaller the amount of H2O produced. At 50% conversion of UDP to dUDP and at pH 7.6, ~0.5% of total H present in solution was volatilized, while at pH 8.4, ~0.9% was volatilized. Similar experiments in which the purine [3'-2H]ADP was incubated with ribonucleotide reductase also resulted in an isotope effect on its conversion to dATP which varied as a function of pH (pH, kH/kF with dGTP as an effector); 6.6, 1.9; 7.6, 1.7; 8.6, 1.4. Furthermore, H2O was also released as a function of the extent of the reaction. At 50% turnover and pH 7.6, ~0.6% of H2O was volatilized, while at pH 8.6 ~1.25% was released. Two control experiments in which either the B1 subunit of ribonucleotide reductase was inactive with 2'-chloro-2'-deoxuryridine 5'-diphosphate or the B2 subunit of ribonucleotide reductase was inactive with 2'-azido-2'-deoxuryridine 5'-diphosphate and then the enzyme incubated with [3'-2H]UDP or [3'-2H]UDP indicated that in neither case was 2H released. Both B1 and B2 subunits are required for cleavage of the 3'-C—H bond. Incubation of [3'-2H]UDP with ribonucleotide reductase produced no measurable release of 2H. These data clearly indicate that conversion of a purine or pyrimidine diphosphate to a deoxynucleotide diphosphate by Escherichia coli ribonucleotide reductase requires the presence of the 3'-C—H bond of the substrate. The fate of the 3'-H of the substrate was also determined. Incubation of [3'-2H]UDP with ribonucleotide reductase resulted in the production of [3'-2H]dUDP.

Ribonucleoside diphosphate reductase from Escherichia coli catalyzes the conversion of nucleoside diphosphates, both purines and pyrimidines, to deoxynucleoside diphosphates. The enzyme has been extensively characterized by Thelander and Reichard (1). The enzyme is composed of two subunits: B1 is a dimer of M, = 160,000 and binds both the NDP substrates and the positive and negative allosteric effectors, and contains thiols which undergo oxidation upon substrate reduction. B2 is a dimer of M, = 78,000 and contains 2 Fe3+ and 1 organic tyrosylphenoxy radical. Both subunits are required for activity and Thelander and Reichard have proposed that the active site is a cleft between these two subunits (1).

Although this enzyme was purified to homogeneity several years ago, very little is known about the details of this unusual reduction and the structure of the unique cofactor. Recently, Thelander et al. (2) and Stubbe and Kozarich (3) studying the interaction of substrate analog 2'-chloro-2'-deoxyuridine 5'-diphosphate with ribonucleotide reductase, made some observations which are important in understanding the normal mechanism of reduction of NDPs. They observed that incubation of [3'-2H]CldUDP1 with ribonucleotide reductase resulted in production of uracil, chloride ion, inorganic pyrophosphate, and H2O and was accompanied by inactivation of the B1 subunit. Stubbe and Kozarich proposed the following working hypothesis to account for this data.

CldUDP is converted in an enzyme-catalyzed reaction to 3'-keto-2'-deoxyxynucleoside diphosphate II. This intermediate II has 2' and 4' Hs which are now acidic allowing elimination of uracil and inorganic pyrophosphate and the generation of the highly reactive sugar residue III. Nucleophilic attack by an amino acid side chain at the active site of B1 would result in B1 inactivation. In order to convert CldUDP to 3'-keto-2'-deoxyuridine diphosphate II, the 3'-C—H bond of substrate analog must be cleaved. We have recently observed that time-dependent inactivation of B1 is accompanied by time-dependent H2O release from [3'-2H]CldUDP.3

The abbreviations used are: CldUDP, 2'-chloro-2'-deoxyuridine 5'-diphosphate; NdUDP, 2'-azido-2'-deoxyuridine 5'-diphosphate; HPLC, high performance liquid chromatography; AdoCbl, adenosylcobalamin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

1 The abbreviations used are: CldUDP, 2'-chloro-2'-deoxyuridine 5'-diphosphate; NdUDP, 2'-azido-2'-deoxyuridine 5'-diphosphate; HPLC, high performance liquid chromatography; AdoCbl, adenosylcobalamin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 J. Stubbe, J. W. Kozarich, and M. Ator, unpublished observations.

3 M. Ator, unpublished results.

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These observations provided the impetus for these studies on the normal mechanism of reduction. The working hypothesis was that in order to cleave the 2'-C—OH bond of the NDP substrate, the 3'-C—H bond had to be cleaved. To test this hypothesis, Stubbe and Ackers synthesized [3'-'H]UDP and studied its interaction with ribonucleotide reductase (4). If cleavage of the 3'-C—H bond of the NDP is rate-determining or partially rate-determining, an isotope effect on this reduction ought to be observed. In a preliminary communication we reported that incubation of [3'-'H]UDP with ribonucleotide reductase resulted in an isotope effect of 3.3 on the reduction reaction and that this reduction was accompanied by a small amount of 3'H2O release. We concluded that ribonucleotide reductase catalyzed the cleavage of the 3'-C—H bond of NDP during turnover (4). Because these results are extremely important in understanding the reduction reaction and because the observed 'H selection effect is small, we undertook the studies reported in this paper which confirm and extend our earlier observations.

Specifically, both the isotope effects and 3'H2O release have been examined using [3'-'H]UDP and its interaction with ribonucleotide reductase at different pH values in the presence of positive and negative allosteric effectors. Furthermore, we undertook the studies reported in this paper which confirm and extend our earlier observations.

**Materials and Methods**

Uridine phosphorylase (EC 2.4.2.3) and purine nucleoside phosphorylase (EC 2.4.2.1) were purified from E. coli as previously described (28) except that the last ECTEOLA-cellulose chromatography step was replaced by a Sephadex G-100 chromatography step. This was performed in 4 M guanidine·HCl, pH 7.0, containing 1 mM EDTA and 5% glycerol. Fractions having the highest specific activities were combined and brought to 70% saturation with ammonium sulfate and stored as a suspension at 3°C. The specific activity of the uridine phosphorylase preparation was 9.3 nmol/min/mg of protein. This value for purine nucleoside phosphorylase was 40.

Ribonucleoside diphosphate reductase was isolated (specific activity of 30 nmol/min/mg) using a dATP Sepharose affinity column synthesized by extensive modifications of the procedure of Bergland and Eckstein (5) as described by Krore et al. (6). Thioredoxin and thioredoxin reductase were isolated as previously described (7-9). Protein concentrations were determined by the procedure of Bradford (10) or by the Lowry procedure.

[14C]NaBH4 and [U-14C]uridine were purchased from New England Nuclear. [U-1-'H]ADP was purchased from Amersham. NaB3H4 was purchased from Aldrich. [3'-1-'H]UDP was purchased from P-L Biochemicals. NADPH, ATP, dithiothreitol, dATP, TTP, and dTTP were purchased from Sigma. dTTPs were purified directly before use by DEAE-Sephadex A-25 column chromatography using a 0 to 0.8 M triethylammonium bicarbonate gradient. All other materials were purchased in the highest purity available.

Isolation and separation of uridine, deoxyuridine, adenine, and deoxyadenosine were accomplished using an Altex HPLC system equipped with a microprocessor and a Whatman ODS-1 reverse phase column. All solutions were analyzed for radioactivity using Aquasol mixture and a Packard 310 scintillation counter. NMR spectra were recorded on a Bruker 270 MHz spectrometer.

**Experimental**

Preparation of [3'-1-'H]Adenosine—A reaction mixture of final volume 1.8 ml contained [3'-1-'H]uridine (specific activity, 1 x 106 cpm/μmol), 50 mM adenine, 25 mM potassium phosphate (pH 7.4), 1 mM NaN3, 0.02%, uridine phosphorylase (5 μl, 3.8 units) and purine-nucleoside phosphorylase (5 μl, 2 units) were centrifuged in an Eppendorf tube for 1 min. The supernatant was removed with a paper tissue and 50 μl of 1 M potassium phosphate (pH 7.4) were added to the pellet. The resulting solution was added to the reaction mixture and was incubated at 37°C for 28 h. At this time HPLC analysis using an ODS-1 reverse phase column (flow 1.7 ml/min; compound, retention time) uridine, 5.9 min; adenosine, 17.2 min; adenine, 19 min) indicated that the reaction had reached equilibrium. The reaction mixture was diluted with 2 ml of H2O and the pH adjusted to 10.5 with 5 drops of concentrated NH2OH. The solution was chromatographed on a Dowex 1-formate column (0.5 X 5 cm). [3'-1-'H]Adenosine was eluted by washing the column with 45 ml of 0.1 M NH2OH, giving 31.7% yield based on uridine. Adenosine was purified by preparative thin-layer chromatography (TLC) using POCi3 and PO(OEt)3, (26) and converted to the diprophosphate by the carbonyldimidazole and Ph method described by Kuzar et al. (27).

Isotope Effect Studies and Release of H2O

[3'-1-'H]ADP—A typical reaction mixture contained HEPES (pH 7.6), 50 mM Mg(OAc)2, 15 mM EDTA, 1 mM GTP, 0.1 M NAPDPH, 1 mM [3'-1-'H]ADP, 0.11 mCi (specific activity, 1.3 × 106 cpm/micromol); [14C]ADP, 0.38 mCi (specific activity, 5.8 × 105 cpm/micromol); thioredoxin, 0.064 mg; thioredoxin reductase, 0.021 mg; ribonucleoside diphosphate reductase, 0.03 mg in a total volume of 0.75 ml. In studies done at pH 5.6, 2-[N-morpholino]ethanesulfonic acid, 50 mM, replaced HEPES, while tris[hydroxymethyl]methyl amino propanesulfonic acid, 50 mM, was used at pH 8.6. At fixed time intervals, 120-μl aliquots were taken and incubated in a boiling H2O bath for 1 min to stop the reaction.

Sixty μl of 0.12 M ethanolamine (pH 9.7) and 2.3 units of bovine liver alkaline phosphatase were then added to the sample and the mixture was incubated at 37°C for 1 h. The samples were boiled for 1 min and the protein removed by centrifugation. Following the addition of 320 μl of H2O to each sample, the supernatant was transferred to a 3-m1 pear-shaped flask. H2O was removed by bulb distillation and the amount of H2O release determined by scintillation counting.

The residue was dissolved in 105 μl of H2O and injected onto an Altex Partisil ODS reverse phase HPLC column (flow rate: 1.7 ml/min, eluant: 15% methanol) and 0.5-min fractions were collected. Compounds and retention times: adenosine, 8 min; deoxyadenosine, 14 min. These fractions were counted and the adenosine and deoxyadenosine peaks each pooled. Each peak was recounted with 20,000 cpm present in each peak.

[3'-1-'H]UDP—A typical reaction mixture contained 2-[N-morpholino]ethanesulfonic acid, 50 mM (pH 6.6); Mg(OAc)2, 15 mM; EDTA, 1 mM; ATP, 1.5 mM; NADPH, 1.5 mM; [3'-1-'H]UDP, 0.55 mM (specific activity, 1.7 × 106 cpm/micromol); [14C]UDP, 0.75 mM (specific activity, 8.8 × 105 cpm/micromol); thioredoxin, 0.045 mg; thioredoxin reductase, 0.021 mg; ribonucleoside diphosphate reductase, 0.86 mg in a total volume of 0.55 ml. The Protocol was identical to that for [3'-1-'H]ADP, except: HPLC analysis was performed on an Altex Ultrasil ODS reverse phase column (flow rate: 1.7 ml/min; eluant: H2O). Compounds and retention times: uridine, 8.5 min; deoxyuridine, 13.5 min. NADPUD-inactivated Ribonucleotide Reductase [3'-1-'H]ADP and [3'-1-'H]UDP (pH 7.6)—Dithiothreitol was removed from ribonucleotide reductase by centrifugation through a 1-ml column of Sephadex G-25 medium (13). The protein (0.475 mg) was added to an inactivation mixture which contained: HEPES, 50 mM (pH 7.6); Mg(OAc)2, 15 mM; EDTA, 1 mM; ATP, 1 mM; NADPH, 1 mM; 2'-NUDP, 0.57 mM; thioredoxin, 25 μg; thioredoxin reductase, 3 μg, in a volume of 160 μl. After incubation at room temperature for 20 min, the inactivation mixture was divided and subjected to centrifugation through 1-ml columns of Sephadex G-25. The recovery of protein after gel filtration was at least 80% in all cases. One of these aliquots was used to determine the amount of ribonucleotide reductase activity remaining after incubation with NADPUD. The amount of ribonucleotide reductase activity remaining after incubation with NADPUD was determined by addition of 40 μl of Sephadex inactivation mixture to an assay mixture containing HEPES, 50 mM (pH 7.6); Mg(OAc)2, 15 mM; EDTA, 1 mM; ATP, 1.0 mM; NADPH, 1.0 mM; [14C]UDP, 0.8 mM (specific activity, 1.0 × 105 cpm/micromol); thioredoxin, 32 μg; thioredoxin reductase, 3 μg, in a final volume of 100 μl. After 30 min at room temperature, the sample was incubated for 1 min in a boiling H2O bath. The sample was then treated with 0.5 M Tris (pH 8.5), 50 μl, and bovine intestinal alkaline phosphatase, 1.5 units, for 1 h at 37°C. Cytidine and deoxyguanosine were separated on a Dowex 1-borate column (0.5 X 5 cm). The deoxyguanosine in the H2O effluent was quantitated by scintillation counting.

The release of H2O from [3'-1-'H]UDP was measured by addition of 96 μl of the Sephadex inactivation mixture to the following reaction mixture: HEPES, 50 mM (pH 7.6); Mg(OAc)2, 15 mM; EDTA, 1 mM; retention time) uridine, 5.9 min; adenosine, 17.2 min; adenine, 19 min).
dGTP, 0.1 mM; [3'-3H]ADP, 0.5 mM (specific activity, 5.3 × 10^6 cpm/μmol); NADPH, 1 mM; thioredoxin, 16 μg; thioredoxin reductase, 3 μg; in a final volume of 300 μl. At 0 and 60 min after addition of ribonucleotide reductase, 140-μl aliquots were taken and incubated in a boiling water bath for 1 min. H2O, 360 μl, was added to each aliquot, the solvent was removed by bulb to bulb distillation, and the amount of H2O released determined by scintillation counting.

A similar experiment was performed using [3'-3H]JUDP. The inactivation mixture was identical with that described above, but the assay solution contained HEPEs, 50 mM (pH 7.6); Mg(OAc)2, 15 mM; EDTA, 1 mM; ATP, 1.5 mM; [3'-3H]UDP, 1 mM (specific activity, 4.70 × 10^6 cpm/μmol); NADPH, 1 mM; thioredoxin, 32 μg; thioredoxin reductase, 3 μg; in a total volume of 250 μl; aliquots (110 μl) were withdrawn at 0 and 45 min after addition of inactivation mixture and were treated as described above.

Two control experiments were performed in parallel with the experiments described above for both [3'-3H]JADP and [3'-3H]JUDP. In one, N3dUDP was omitted from the inactivation mixture, and in the second ribonucleotide reductase was omitted. All other aspects of these experiments were identical with those described above.

**CldUDP-inactivated Ribonucleotide Reductase with [3'-3H]JADP and [3'-3H]JUDP (pH 7.5).—**The protocol followed was identical with that described for N3dUDP inactivated ribonucleotide reductase, except 3.3 mM CldUDP replaced N3dUDP in the inactivation mixture.

### Measurement of the Initial Rate of Reduction of ADP as a Function of pH

The rate of ADP reduction by ribonucleotide reductase was measured as a function of pH using the following reaction mixture in a total volume of 100 μl: MES (pH 6.6), 50 mM; HEPES (pH 7.6), or TAPS (pH 8.6), 50 mM; Mg(OAc)2, 15 mM; EDTA, 1 mM; ATP, 1.6 mM; NADPH, 0.1 mM; [3'-3H]UDP, 0.064 μM (specific activity, 1.9 × 10^6 cpm/μmol); thioredoxin, 8 μg; thioredoxin reductase, 2 μg; ribonucleotide reductase, 20 μg. At 1 and 2 min after addition of ribonucleotide reductase, 45-μl aliquots of the reaction mixture were removed and placed in a boiling H2O bath for 1 min. After treatment with 25 μl of 0.5 M Tris (pH 8.5) and 1 unit of bovine intestinal alkaline phosphatase (37 °C, 1 h), adenosine and deoxyadenosine were separated on a column (0.1 × 5 cm) of Dowex 1-borate (14). The deoxyadenosine-containing eluate was evaporated to dryness, dissolved in 1 ml of H2O, and the amount of radioactivity determined by scintillation counting.

### Measurement of the Initial Rate of Reduction of UDP as a Function of pH

The rate of UDP reduction by ribonucleotide reductase was measured as a function of pH using a reaction mixture which contained MES (pH 7.6), HEPES (pH 7.6), or TAPS (pH 8.5), 50 mM; Mg(OAc)2, 15 mM; EDTA, 1 mM; ATP, 1.6 mM; NADPH, 1 mM; UDP, 1 mM (specific activity, 1.9 × 10^6 cpm/μmol); thioredoxin, 8 μg; thioredoxin reductase, 2 μg; ribonucleotide reductase, 50 μg, in a volume of 110 μl. At 10 and 20 min after addition of ribonucleotide reductase, 50-μl aliquots were withdrawn and placed in a boiling H2O bath for 1 min. Each aliquot was treated with 25 μl of 0.5 M Tris (pH 8.5) and 2 units of bovine intestinal alkaline phosphatase for 1 h at 37 °C. Uridine and deoxyuridine were separated by reverse phase HPLC and the amount of product quantitated by scintillation counting of the deoxyuridine-containing fraction.

### Conversion of [3'-3H]JUDP to [3'-3H]JUDP

Conversion of [3'-3H]JUDP to [3'-3H]JUDP was carried out in two reaction mixtures which each contained HEPES (pH 7.6), 50 mM; Mg(OAc)2, 15 mM; EDTA, 1 mM; diethiothreitol, 0.5 mM; ATP, 1.6 mM; NADPH, 3 mM; [3'-3H]UDP, 2 mM; thioredoxin, 0.064 μg; thioredoxin reductase, 0.021 mg; ribonucleotide reductase, 0.03 mg in a total volume of 1.0 ml. After 140 min at room temperature, an additional 0.93 μg of ribonucleotide reductase, 0.064 μg of thioredoxin, and 0.021 mg of thioredoxin reductase were added.

After an additional 2 h, the reaction mixtures were pooled. To the combined solution was added bovine intestinal alkaline phosphatase, 68 U/ml; 0.12 M ethanolamine (pH 9.7). The sample was incubated at 37 °C for 1.5 h and placed in a boiling H2O bath for 1 min to denature the protein. The protein was removed by centrifugation and the supernatant was evaporated to dryness. The resulting residue was redisolved in 1 ml of H2O and injected onto a Whatman ODS-1 reverse-phase HPLC column. [3'-3H]Deoxyuridine was isolated (flow rate, 1.7 ml/min; eluant, H2O; retention time, 7.5 min) and evaporated to dryness. The sample was exchanged into D2O and sodium 2,2-dimethyl-2-silapentane-5-sulfonate was added as an internal chemical shift standard.

### RESULTS

Our initial observation of an isotope effect of (kH/kD) 3.3 on the ribonucleotide reductase catalyzed reduction of UDP, accompanied by release of H2O, indicated that the 3'-C—H bond of the substrate is cleaved during turnover (4). Because this value is small for a primary tritium isotope effect, however, additional studies have been performed in an attempt to maximize the isotope effect for the ribonucleotide reductase-catalyzed reaction. Three approaches have been taken. 1) Variation of the pH of the reaction. This manipulation often changes the relative rates of various steps in the mechanism, resulting in a change in the expression of the isotope effect. 2) Use of a purine nucleoside substrate, [3'-3H]JADP. Reduction of this substrate requires a different allosteric effector and probably a different active site conformation which might result in observation of a larger isotope effect. 3) Use of different effectors of ribonucleotide reductase, both positive and negative.

**Isotope Effects with [3'-3H]JUDP: Variation of pH and Allosteric Effector—**All isotope effects were measured using the double label technique described under "Materials and Methods." The results of a typical experiment are shown in Fig. 1. The ratio of the 3H/14C for uridine and deoxyuridine extrapolated to time zero is a measure of the isotope effect on the reaction. At pH 8.4, kH/kD is 2.8, while at pH 6.6, kH/kD is 4.7. Furthermore, at 100% reaction the 3H/14C ratio for deoxyuridine is the same as that of uridine at 0% reaction (data not shown). The results of similar studies are summarized in Table I. The observed isotope effects increase with decreasing pH. There is, however, no correlation between the observed isotope effects and the turnover of the enzyme due to altered

**FIG. 1.** Isotope effects on the reduction of [3'-3H]JUDP by *E. coli* ribonucleotide reductase at varying pH values.
pH (Table I). However, it should be pointed out that these turnover experiments are probably done under $V_{\text{max}}$ conditions. At present the trends of $V_{\text{max}}/K_m$ as a function of pH have not been established. Furthermore dATP, a negative effector of UDP turnover by ribonucleotide reductase gave an isotope effect of 2.6 (pH 7.6). In this case, the reaction was followed to less than 10% completion due to the low turnover rate. TTP and ATP, both positive effectors of UDP turnover, gave isotope effects of 2.0 and 3.3, respectively (pH 7.6). Thus, no obvious trend between turnover rate due to allosteric effector and magnitude of the observed isotope effect is apparent.

### TABLE I

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<th>Allosteric effector</th>
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Isotope Effects with $[3'-3^H]$ADP: Variation with pH—The double label method described under “Materials and Methods” was again used. The positive allosteric effector employed in all cases was dGTP. The result of a typical series of experiments is shown in Fig. 2. The data is summarized in Table II. As in the case of the pyrimidine UDP, the isotope effect increases with decreasing pH and has no correlation with turnover regulated by pH. The observed isotope effects with the purine ADP are much smaller than that for UDP. $^{3}$H$_2$O Release as a Function of pH—As indicated in the original report of Stubbe and Ackles, during ribonucleotide reductase catalyzed turnover of $[3'-3^H]$UDP a small, but significant, amount of $^{3}$H$_2$O was released as a function of the extent of reaction (4). These experiments have been extended to examine the amount of $^{3}$H$_2$O released as a function of pH and substrate, and to further substantiate that this $^{3}$H release is enzyme-catalyzed.

As indicated in Fig. 3, incubation of $[3'-3^H]$UDP resulted in $^{3}$H$_2$O release which increased with increasing pH. At 50% turnover and pH 7.6, 0.5% of the total radioactivity was volatilized, while at pH 8.4, 0.9% of the total radioactivity was volatilized. This trend is the opposite of the effect of pH on the isotope effect. Similar experiments using $[3'-3^H]$ADP gave the results shown in Fig. 4. Once again the trend is the same, increase in pH results in increased $^{3}$H$_2$O release. At 50% reaction and pH 7.6, 0.6% of total radioactivity was volatilized. At pH 8.6, this value was 1.25%. The conditions giving rise to the smallest isotope effect result in the largest amount of $^{3}$H$_2$O volatilized (Tables I and II). Again no correlation is observed between amount of $^{3}$H volatilized and rate of turnover of ADP as a function of pH.
Control Experiments—Due to the small amount of $^3$H volatilized during ribonucleotide reductase incubation with $[3'-^3H]UDP$ and $[3'-^3H]ADP$, it was important to establish that this release was ribonucleotide reductase-dependent. Thelander et al. (2) reported that CldUDP inactivated the $B_1$ subunit of ribonucleotide reductase, while N$_3$dNDP inactivated the $B_2$ subunit of ribonucleotide reductase. We have utilized ribonucleotide reductase inactivated by both compounds to examine the relationship of $^3$H$_2$O release to enzyme activity. In one control experiment CldUDP-inactivated ribonucleotide reductase was incubated with $[^3H]UDP$ or $[^3H]ADP$. When ribonucleotide reductase is less than 1% active, only 0.05% of the total radioactivity was volatilized compared with 0.7% in the experimental reaction. The second control experiment involved N$_3$dUDP-inactivated ribonucleotide reductase. Again, with 6% of ribonucleotide reductase remaining less than 0.05% $^3$H was volatilized. The results of these experiments are shown in Table III and clearly indicate that both the $B_1$ and $B_2$ subunits are required for $^3$H release from $[3'-^3H]UDP$ and $[3'-^3H]ADP$. Furthermore, the volatilization of $^3$H in the presence of active ribonucleotide reductase and $[^3H]NDP$ provides unequivocal evidence for cleavage of the $3'$ C—H bond of an NDP.

The final control experiment involves incubation of $[3'-^3H]dADP$ or $[3'-^3H]dUDP$ with ribonucleotide reductase to rule out the possibility that $^3$H is released from product rather than substrate. The results indicate that <0.01% of the total radioactivity is volatilized.

Incubation of $[3'-^3H]UDP$ with Ribonucleotide Reductase: Fate of the $^3$H in Reisolated dUDP—The isotope effect data indicate that the $3'$ C—H bond of NDP is cleaved during turnover, and the $^3$H$_2$O release indicates that a small percentage of $^3$H is released to the media. Mechanistically, it is important to determine the location of the remaining $^3$H in the dUDP produced. Ribonucleotide reductase was incubated with $[3'-^3H]UDP$ so that the results would be analyzed by NMR spectroscopy and the reaction monitored by HPLC to insure total conversion to dUDP. The phosphates were removed with bovine intestinal alkaline phosphatase the deoxyuridine isolated by HPLC and analyzed by NMR. Fig. 5 illustrates the entire spectrum of deoxyuridine isolated from the experiment and a control with $[3'-^3H]deoxyuridine$. In the deoxyuridine isolated from the experiment, a $3'$ proton is missing at 4.45 ppm and the splitting pattern of the $4'$-H ($6 = 4.1$ ppm) and $2'$ region ($6 = 2.32$ ppm) have been notably simplified. The peaks in the experiment which are absent in the control are due to oxidized dithiothreitol which comigrates with deoxyuridine on reverse phase HPLC. An expanded spectrum of the $4'$-H and $2'$-H region of deoxyuridine is shown in Fig. 6. These spectra are compared with a specimen of authentic deoxyuridine which has been irradiated in the $3'$-H region. These spectra are identical and indicate that $^3$H in position $3'$ in the substrate is returned to position $3'$ in the product.

![Fig. 5. NMR spectrum of deoxyuridine from incubation mixture of ribonucleotide reductase with (A) $[3'-^3H]UDP$ and (B) $[3'-^3H]UDP$.](http://www.jbc.org/)

![Fig. 6. Expanded NMR spectrum of the $2'$ and $4'$ region of deoxyuridine.](http://www.jbc.org/)
Radical mechanisms may be important in all ribonucleotide reductase-catalyzed reactions. Recent work by Sjoberg et al. (15-17) implicated an organic tyrosine radical and 2 Fe³⁺ as a cofactor in the reduction reaction catalyzed by *E. coli* ribonucleotide reductase. This radical is observable by EPR spectroscopy as well as visible spectroscopy (410 nm). Removal of the radical by using radical scavengers such as NH₂OH or hydroxyurea or by chelation of the Fe³⁺ using Li₂8-hydroxyquinoline-sulfamate results in total loss of enzyme activity. This activity can be regenerated by incubation of Fe³⁺, O₂, and ascorbate with the inactive protein. Return of the activity is accompanied by an increase in the absorbance at 410 nm as well as return of the EPR signal. These elegant studies establish that the radical is necessary for activity, although there is no evidence for its direct involvement in the reduction reaction.

Additional evidence for potential radical mechanisms of reduction involve studies with ribonucleotide reductase from *Lactobacillus leichmannii*. This enzyme, studied extensively by Blakley and co-workers and Hogenkamp and co-workers, catalyzes the conversion of NTPs to dNTPs and uses adenosylcobalamin as a cofactor (18, 19). Stopped-flow kinetic studies by Tamao and Blakley (20) and rapid quench EPR studies by Orme-Johnson et al. (21) have shown that homolytic cleavage of the carbon cobalt bond of AdoCbl is sufficiently rapid to be kinetically competent in the reduction reaction. Evidence is also emerging that the function of AdoCbl may be as a radical chain initiator; the 5'-deoxyadenosyl radical abstracts a H from a residue on the protein generating a protein radical which may then participate in the reduction reaction (22, 19).

These observations have led us to postulate the following radical mechanism as a working hypothesis for the cleavage of the 3'-C-H bond catalyzed by both *E. coli* ribonucleotide reductase and *L. leichmannii* ribonucleotide reductase (Scheme I). This hypothesis predicts that a residue on the protein, X, can abstract a "H" from position 3' of the nucleotide to generate a 3'-nucleotide radical IV. Generation of this intermediate would facilitate acid-catalyzed cleavage of the 3'-C-OH bond, producing a radical cation intermediate V. This intermediate could then be reduced concomitantly with oxidation of two thiol groups on the protein. The resulting 3'-deoxynucleotide radical VI then reabstracts a "H" from the protein residue HX, regenerating X.

This mechanism is based on the chemical model studies of Walling and Johnson (23) and Gilbert et al. (24), who examined the interaction of Fenton's reagent with CH₂OHCHOH₂OH (Scheme II).

In this reaction, Fenton's reagent is a source of HO· which abstracts a "H" from ethylene glycol to form VII. When X = OH, acidic conditions are required to make OH a better leaving group. Under these conditions the C-X bond is cleaved rapidly and radical cation VIII is generated. The driving force for this reaction is the stabilization of the carbonium ion by the unshared pair of e's on the HO group. In this model system, the intermediate radical cation can be reduced by Fe²⁺ to form acetaldehyde.

The enzymatic model (Scheme I), based on this chemical model (Scheme II), makes two predictions that are experimentally testable: 1) cleavage of the 2'-C-OH bond of substrate NDP requires cleavage of the 3'-C-H bond of the substrate; 2) the H removed from position 3' in the substrate is returned to position 3' in the product.

To examine the first prediction, a pyrimidine, [3'-3H]UDP, and a purine, [3'-3H]ADP, were synthesized. If cleavage of the 3'-C-H bond occurs and is rate-determining or partially rate-determining an isotope effect ought to be observed on the ribonucleotide reductase catalyzed reaction. In both cases the isotope effect was examined as a function of pH and the trends on the isotope effects were the same: the lower the pH, the larger the isotope effect (Tables I and II). With [3'-3H]UDP at pH 6.6, the largest isotope effect was observed. The magnitude of the isotope effect on [3'-3H]UDP reduction at low pH can only be interpreted in terms of a primary effect, resulting from enzyme-catalyzed cleavage of the 3'-C-H bond.

The purine nucleotide [3'-3H]ADP, which is allosterically regulated by dGTP, was examined with ribonucleotide reductase, with the hope that a more fully expressed primary isotope effect might be observed. As indicated in Table II, the isotope effects were, in fact, very small. At pH 6.6, ADP gave an isotope effect of only 1.90. While this number is larger than any β-H, 2° isotope effect reported, to our knowledge, in the chemical literature, it is very small for a primary effect (25). However, 3H₂O release data, discussed subsequently, clearly establishes that this is a 1° effect.

The model proposed in Scheme I also suggests that protein residue X might be able to exchange the 3'-H abstracted from [3'-3H]NDP with the solvent. H₂O release was, therefore, examined as a function of extent of reaction and at various pH values (Figs. 4 and 5). The total amount of H₂O released is small, 0.5 to 1.25% (50% reaction) and increases with increasing pH. Separate control experiments in which the B₁ subunit was inactivated with CldUDP and the B₂ subunit was inactivated with NacUDP indicate that H₂O release is dependent on the presence of both active subunits. These results indicate that ribonucleotide reductase catalyzes cleavage of the 3'-C-H bond of NDP substrate, confirming the interpretation
of the isotope effect studies. Furthermore, the group in the protein responsible for abstraction of the H from substrate is able to exchange with bulk solvent.

The second prediction made by this model (Scheme I) is that the 3'-H abstracted from the substrate is returned to position 3' in the product. Experiments using [3-3H]UDP confirm this prediction (Figs. 5 and 6).

Finally an alternative mechanism which is consistent with all of the experimental data ought to be considered (Scheme III). This mechanism involves heterolytic cleavage of the 3'-C—H bond of NDP to form enol IX which can tautomerize to ketone X. The ketone must then be reduced, concomitant with thiol group oxidation and the abstracted H from position 3' in substrate returned to position 3' in product. At present we favor a radical rather than heterolytic mechanism based on what is known about the structure of the cofactor. However, until evidence for radical substrate involvement is obtained, this alternative mechanism must be seriously considered.

To summarize, evidence is presented that ribonucleotide reductase catalyzes cleavage of the 3'-C—H bond of purine and pyrimidine NDP substrates. This cleavage is accompanied by a small but significant amount of 3H2O release to the solvent. Finally, the H removed from position 3' in substrate NDP is returned to position 3' in product dNDP.

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