2′-*C*-Methyladenosine and 2′-*C*-Methyluridine 5′-Diphosphates Are Mechanism-Based Inhibitors of Ribonucleoside Diphosphate Reductase from *Corynebacterium nephridii*†

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ABSTRACT: The interaction of the adenosylcobalamin-dependent ribonucleoside diphosphate reductase of *Corynebacterium nephridii* with 2′-*C*-methyladenosine 5′-diphosphate (2′-MeADP) and 2′-*C*-methyluridine 5′-diphosphate (2′-MeUDP) has been investigated. The nucleotide analogs are converted to adenine and uracil, respectively, suggesting that they may be mechanism-based inhibitors. In addition, both analogs generate nucleotides with properties expected for the 2′-deoxy-2′-*C*-methylnucleotides. The nucleoside obtained after enzymatic dephosphorylation of the product formed from 2′-MeADP has been identified as 2′-deoxy-2′-*C*-methyladenosine by ^{1}H NMR and mass spectroscopies. Adenine is the major product derived from 2′-MeADP, indicating that the degradation pathway predominates. During the reaction, the carbon–cobalt bond of the coenzyme is cleaved irreversibly to yield 5′-deoxyadenosine and cob(II)alamin. 2′-MeADP is a potent competitive inhibitor of the reduction of the purine nucleotides ADP and GDP, while 2′-MeUDP competitively inhibits the reduction of the pyrimidine nucleotides UDP and CDP. 2′-MeADP is a very effective promoter of the tritium exchange reaction between [5′-$^3H_2$]adenosylcobalamin and the solvent, indicating that the exchange reaction is an integral part of the overall reduction. All these observations are consistent with the reaction mechanism proposed by Stubbe and co-workers [Harris, G., Ashley, G. W., Robins, M. J., Tolman, R. L., & Stubbe, J. (1987) *Biochemistry 26,* 1895–1902 (1987); Stubbe, J. (1990) *J. Biol. Chem. 265,* 5329–5332] in which they suggest that the partitioning between reduction and inactivation occurs at the level of the 2′-deoxy-3′-ketoribonucleotide intermediate.

Ribonucleotide reductases catalyze the irreversible reduction of ribonucleotides to their corresponding 2′-deoxyribonucleotides, the essential precursors for DNA synthesis. Ribonucleotide reductases have been purified to homogeneity from a number of sources. In spite of the diversity of the physical structures of the enzymes and their substrate, effector, and cofactor requirements, the mechanism of the reduction appears to be very similar for all reductases (Ashley & Stubbe, 1989). Each of the enzyme systems utilizes a unique method for the generation of a protein radical. Thus the R_2 subunit of the ribonucleotide reductase from *Escherichia coli* contains a binuclear iron center and a tyrosine residue at position 122 that is the site of the radical (Nordlund *et al.*, 1990). In the adenosylcobalamin-dependent ribonucleotide reductases, the homolysis of the carbon–cobalt bond generates the 5′-deoxyadenosyl radical and cob(II)alamin. However, this 5′-deoxyadenosyl radical is not directly involved in the enzymatic process (Ashley *et al.*, 1986). It generates a protein radical which initiates the reduction.

Stubbe and Ackles (1980) proposed a radical mechanism as a working hypothesis for the reduction catalyzed by ribonucleotide reductases. It is based on observations obtained with the 2′-deoxy-2′-halonucleoside 5′-diphosphates as mechanism-based inhibitors (Stubbe & Kozarich, 1980a,b), on analogies with chemical model systems (Walling & Johnson, 1975), and on the pivotal experiments using the [3′-^{3}H]uridine nucleotides with the *E. coli* reductase (Stubbe & Ackles, 1980) and the *Lactobacillus leichmannii* reductase (Stubbe *et al.*, 1981). Two thiol groups are directly invovled in substrate reduction and become oxidized during the reduction process (Thelander, 1974; Lin *et al.*, 1987). Recent site-directed mutagenesis studies with the reductase from *E. coli* have suggested that cysteines 225 and 462 are the redox-active residues in the active site (Mao *et al.*, 1992a). When a mutant reductase, cysteine-225-serine, was incubated with CDP, cytosine was formed as the major product, indicating cleavage of the glycosidic bond of the nucleotide and demonstrating that this single mutation causes the normal substrate to act as a mechanism-based inhibitor (Mao *et al.*, 1992b). Similar results were obtained earlier when 2′-chloro-2′-deoxyuridine 5′-diphosphate was found to be a mechanism-based inhibitor of the reductase. Stubbe proposed two pathways to account for the release of cytosine and the inactivation of the enzyme. Both pathways require a general acid catalyst, a role that was ascribed to an ammonium side chain of the enzyme. Stubbe modified her earlier mechanism to incorporate these findings, as shown in Scheme I. This mechanism involves the cleavage of the 3′-carbon–hydrogen bond of the ribonucleotide mediated by a protein-based radical and protonation of the 2′-hydroxyl function by an ammonium side chain of the enzyme. After water leaves, the resulting formylmethyl radical undergoes a one-electron-transfer reaction from the thiolate involved in protonating the amino group. The sequence of these reactions generates an anionic intermediate, which accepts a proton to yield a 2′-deoxy-3′-ketonucleotide intermediate. This anionic intermediate is a unique feature of the more recently proposed mechanism.

Earier, we described the syntheses of 3′-MeADP and 3′-MeUDP and their interaction with the adenosylcobalamin-dependent ribonucleotide reductase from *Corynebacterium nephridii* (Ong *et al.*, 1992). Neither nucleotide analog was a substrate for the reductase, however, they did function as

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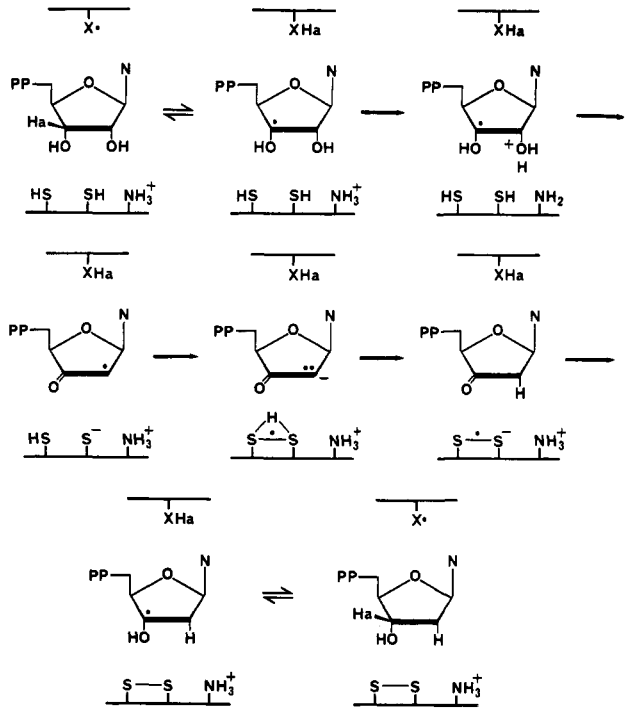
Scheme I[a]



[a] Adapted from Stubbe (1990). Ha is the 3′-H that is abstracted by the protein-based radical.

allosteric effectors and as inhibitors of the reduction of ADP and UDP, respectively. Furthermore, these 3′-*C*-methylnucleotides did not promote the hydrogen exchange reaction between [5′-$^3H_2$]adenosylcobalamin (AdoCbl) and the solvent.

We have now extended these studies and have synthesized the 2′-*C*-methylnucleotides 2′-MeADP and 2′-MeUDP and determined their interaction with the ribonucleotide reductase from *C. nephridii*. Their effects on both the reduction and the exchange reaction have been studied. One would expect the 2′-*C*-methyl group of these substrate analogs to stabilize the radical cation and to destabilize the anion intermediate (Scheme I). Both nucleotides should also promote the hydrogen exchange reaction between the coenzyme AdoCbl and the solvent because of the presence of the 3′-hydrogen in the analogs.

As expected, both nucleotide analogs are substrates for the reductase, and they are capable of promoting the exchange reaction. The glycosidic bonds of these nucleotide analogs are also cleaved, releasing adenine and uracil, respectively, suggesting that the 2′-*C*-methylnucleotides act as mechanism-based inhibitors.

MATERIALS AND METHODS

Uracil, N^6-benzoyladenine, triethylamine, sodium periodate, diatomaceous earth, 3,3-dimethylglutaric acid, AdoCbl, bovine intestinal mucosa alkaline phosphatase (2800 units/mg of protein), [2-^{14}C]UDP, tris(hydroxymethyl)aminomethane (Tris), ADP, and dGTP were purchased from Sigma Chemical Company. Diacetone-D-glucose, dry pyridine, dry dimethyl sulfoxide, dry toluene, dry 1,2-dichloroethane, dry dimethylformamide, dry ethanol, benzyl chloride, *p*-methylbenzoyl chloride, dibutyl stannoxane, methylmagnesium iodide, sodium methoxide, trifluoroacetic acid, 20% palladium hydroxide on carbon, cyclohexene, 4-dimethylaminopyridine, hexamethyldisilazane, trimethylsilyl trifluoromethanesulfonate, trimethyl phosphate, phosphorus oxychloride, phosphorus pentachloride, *m*-cresol, tributylamine, 1,1′-carbonyldiimidazole, and 99.99% D_2O were from Aldrich Chemical Company. [5-^{3}H]CDP and [8-^{3}H]GDP were from Amersham Corporation. [2,8-^{3}H]ADP was from Du Pont Company. Dithiothreitol (DTT) was obtained from Boehringer Mannheim. Methanol (HPLC grade), water (HPLC grade), and activated charcoal were from Mallinckrodt, Inc. Silica gel 60 and trichloroacetic acid were obtained from EM Science. Dowex 1-X2 and Dowex 50-X8 were products of Bio-Rad. SPIN-X centrifuge filter units were purchased from Costar. Pyrophosphoryl chloride was synthesized as described by Imai *et al.* (1969). [5′-$^3H_2$]AdoCbl was prepared as described before; sp act. = 10 000 cpm/nmol (Gleason & Hogenkamp, 1972). HPLC analyses were performed on a Beckman Model 332 instrument equipped with a Brownlee Aquapore RP-300 C-8 reversed-phase column (4.6 mm × 22 cm) or an Altex Ultrasphere ODS C-18 reversed-phase column (10 mm × 25 cm) and a 3-cm guard cartridge. UV spectra were recorded on a Hewlett-Packard diode array spectrophotometer. NMR spectra were obtained using a GE Omega 500-MHz spectrometer. Mass spectra were determined using a VG Analytical Ltd. high-resolution double-focusing mass spectrometer. Ribonucleotide reductase from *C. nephridii* was purified as described before; sp act. = 217 nmol of dCDP/min/mg of protein (Ong *et al.*, 1992).

Synthesis of 2′-MeADP and 2′-MeUDP. 2′-*C*-Methyladenosine and 2′-*C*-methyluridine were prepared by following the procedure of Beigelman *et al.* (1987, 1988). The nucleosides were purified using Dowex 1-X2 (hydroxide form) as described by Dekker (1965). 2′-*C*-Methyladenosine was eluted with 60% aqueous methanol, while the elution of 2′-*C*-methyluridine required 0.1 M ammonium bicarbonate. 2′-*C*-Methyluridine was converted to the monophosphate by the procedure of Yoshikawa *et al.* (1967). Since 2′-*C*-methyladenosine was not sufficiently soluble in trimethyl phosphate, it was converted to the monophosphate using pyrophosphoryl chloride in *m*-cresol as described by Imai *et al.* (1969). The desired 2′-*C*-methylnucleoside 5′-diphosphates were prepared from the monophosphates using the 1,1′-carbonyldiimidazole method of Hoard and Ott (1965). 2′-MeADP and 2′-MeUDP were homogeneous using the liquid chromatography system of Axelson *et al.* (1981). The UV spectra of the nucleotide analogs are identical to those of ADP and UDP, respectively.

2′-MeADP: ^{1}H NMR (500 MHz) (D_2O) 8.46 (s, 1 H, C_8H), 8.21 (s, 1 H, C_2H), 6.11 (s, 1 H, $C_{1'}H$), 4.36 (m, 1 H, $C_{4'}H$), 4.28 (d, 1 H, $J_{3'4'}$ = 8.8 Hz, $C_{3'}H$), 4.24 (m, 2 H, $C_{5'}H_{a,b}$), 0.89 ppm (s, 3 H, $C_{2'}$-Me); ^{31}P NMR (202 MHz) (D_2O, pH 3.5) −9.59 (d, $J_{\beta\alpha}$ = 19.22 Hz, P_β), −10.12 ppm (d, $J_{\alpha\beta}$ = 19.46 Hz, P_α).

2′-MeUDP: ^{1}H NMR (500 MHz) (D_2O) 7.98 (d, 1 H, $J_{6,5}$ = 8.3 Hz, C_6H), 6.01 (s, 1 H, $C_{1'}H$), 5.96 (d, 1 H, $J_{5,6}$ = 8.3 Hz, C_5H), 4.32–4.29 (m, 2 H, $C_{3'}H$, $C_{4'}H$), 4.12 (m, 2 H, $C_{5'}H_{a,b}$), 1.49 ppm (s, 3 H, $C_{2'}$-Me); ^{31}P NMR (202 MHz) (D_2O, pH 7.0) −5.23 (d, $J_{\beta\alpha}$ = 23.8 Hz, P_β), −10.21 ppm (d, $J_{\alpha\beta}$ = 21.7 Hz, P_α).

Reaction of 2′-MeADP with Ribonucleotide Reductase. The reaction mixtures contained 100 mM dimethyl glutarate (pH 7.2), 20 mM DTT, 50 μM AdoCbl, 200 μM 2′-MeADP, and 5.4 μg of ribonucleotide reductase in a final volume of 100 μL. Control reaction mixtures were identical except that 2′-MeADP was replaced by 100 μM ADP. The reactions were initiated by the addition of AdoCbl, and the mixtures were incubated for varying time periods up to 60 min. The experiments were also repeated with varying amounts of enzyme up to 5.4 μg with a 60-min incubation time. The reactions were stopped by boiling for 1 min. Each cooled

assay mixture was treated with 15 units of alkaline phosphatase in 50 μL of 0.5 M Tris-HCl (pH 8.5) containing 20 mM $MgCl_2$ and incubated for 1 h at 37 °C. The mixtures were boiled again for 1 min and centrifuged to remove denatured protein, and the supernatant was filtered through a Costar SPIN-X centrifuge filter unit (0.45-μm cellulose acetate). The filtrate was analyzed by HPLC on an Altex Ultrasphere ODS C-18 reversed-phased column (10 mm × 25 cm) using 20% methanol in 20 mM potassium phosphate (pH 5.6) as the eluent at a flow rate of 2 mL/min. The ribonucleosides were detected at 260 nm.

To ensure that the degradation products did not result from boiling the reaction mixtures, the assays were also stopped in a dry ice/2-propanol bath. In another set of experiments, the alkaline phosphatase treatment was omitted to determine the enzymatic formation of non-nucleotide products. Product formation was analyzed as described above.

In order to isolate larger quantities of the nucleotide product, ten reaction mixtures, each containing 50 mM Tris-HCl (pH 9.5),[1] 12 mM DTT, 50 μM AdoCbl, 1.2 mM 2′-MeADP, and 0.05 mg of reductase in a total volume of 0.85 mL, were incubated at 37 °C for 150 min. An additional 0.025 mg of enzyme and 1 μmol of 2′-MeADP were added to each reaction mixture, and the incubation was continued for another 2 h. The reaction mixtures were then processed as described above. The combined filtrates were concentrated to 2 mL by lyophilization, and the reaction products were separated as described above. The product eluting at 41.4 mL was collected from each of eight runs. The pooled fractions were concentrated to 1 mL by lyophilization, and the nucleoside was further purified and desalted by four runs on the same HPLC column, using 20% aqueous methanol as the eluent. The nucleoside (elution volume 40.4 mL; flow rate 2.0 mL/min) was collected from each run, pooled, and lyophilized to dryness (yield 546 nmol). For mass spectroscopy, a sample (approximately 50 nmol) was dissolved in water and lyophilized to dryness. For ^1H NMR spectroscopy, approximately 500 nmol of the nucleoside was dissolved in 1 mL of D_2O, lyophilized to dryness, redissolved in 1 mL of D_2O, lyophilized again, and finally dissolved in 1 mL of D_2O.

Reaction of 2′-MeUDP with Ribonucleotide Reductase. The reaction mixtures contained 100 mM dimethyl glutarate (pH 7.2), 15.4 mM DTT, 78 mM AdoCbl, 100 μM 2′-MeUDP, 1.4 mM dGTP, and either 15.7 or 31.3 μg of ribonucleotide reductase in a final volume of 130 μL. Control reactions contained 100 μM UDP instead of 2′-MeUDP. Experiments were also carried out without dGTP, the positive allosteric effector for UDP reduction. The reaction mixtures were incubated at 37 °C in the dark and processed as described above. The filtrate was analyzed by HPLC on a Brownlee Aquapore RP-300 C-8 reversed-phase column (4.6 mm × 22 cm) with 20 mM potassium phosphate (pH 5.6) as eluent.

Tritium Exchange from [5′-$^3H_2$]AdoCbl. These reaction mixtures contained 100 mM dimethyl glutarate (pH 7.2), 16 mM DTT, 50 μM [5′-$^3H_2$]AdoCbl, varying concentrations of 2′-MeADP or 2′-MeUDP up to 1 mM, and 2.5 μg of ribonucleotide reductase in a final volume of 200 μL. The reactions were initiated by the addition of the labeled coenzyme and incubated at 37 °C in the dark for 40 min. The reactions were terminated with 1 mL of 2% trichloroacetic acid containing 100 mg of activated charcoal. After being mixed thoroughly, the assays were centrifuged for 5 min. The amount of ^3H label transferred to the solvent was determined by liquid scintillation counting of a 400-μL aliquot of the supernatant in 10 mL of scintillation cocktail.

Effect of 2′-MeADP on the Reduction of ADP and GDP. The assay mixtures contained, in a final volume of 100 μL, 100 mM dimethyl glutarate (pH 7.2), 20 mM DTT, 100 μM AdoCbl, fixed concentrations of [^3H]ADP at 4, 8, 14, and 25 μM with varying concentrations of 2′-MeADP up to 25 μM, and 100 ng of ribonucleotide reductase. The reactions were started by the addition of AdoCbl and incubated in the dark at 37 °C for 10 min. They were then terminated, and the nucleotides were dephosphorylated as described above. [^3H]-Adenosine and [^3H]deoxyadenosine were separated by HPLC using a Brownlee Aquapore RP-300 column (4.6 mm × 22 cm) equipped with a 3-cm guard cartridge. The effluent was 14% methanol in 20 mM potassium phosphate (pH 5.6), at a flow rate of 0.8 mL/min. One-minute fractions were collected, and the radioactivity was determined by scintillation counting. The effect of 2′-MeADP on GDP reduction was determined in reaction mixtures containing the same concentrations of buffer, DTT, and AdoCbl, fixed concentrations of [^3H]GDP from 5 to 20 μM with varying concentrations of 2′-MeADP up to 25 μM, and 59 ng of ribonucleotide reductase in a final volume of 100 μL. [^3H]Guanosine and [^3H]-deoxyguanosine were separated by the same HPLC system using 20 mM potassium phosphate (pH 5.6) as the eluting buffer, at a flow rate of 0.7 mL/min. One-minute fractions were collected, and the radioactivity was determined by scintillation counting.

Effect of 2′-MeUDP on the Reduction of UDP and CDP. The reaction mixtures contained, in a final volume of 100 μL, 100 mM dimethyl glutarate (pH 7.2), 20 mM DTT, 100 μM AdoCbl, fixed concentrations of [^{14}C]UDP at 5, 10, and 20 μM with varying concentrations of 2′-MeUDP up to 500 μM, and 200 ng of ribonucleotide reductase. The reactions were initiated and incubated in the dark at 37°C for 10 min and then processed as described above. [^{14}C]Uridine and [^{14}C]-deoxyuridine were separated by HPLC on an Altex Ultrasphere ODS C-18 reversed-phase column (10 mm × 25 cm) using 8% methanol in 20 mM potassium phosphate (pH 5.6) as the eluting buffer at a flow rate of 2 mL/min. One-minute fractions were collected, and the radioactivity was determined by scintillation counting. The effect of 2′-MeUDP on CDP reduction was determined in reaction mixtures containing the same concentrations of buffer, DTT, and AdoCbl, fixed concentrations of [^3H]CDP from 5 to 20 μM with varying concentrations of 2′-MeUDP up to 500 μM, and 78 ng of ribonucleotide reductase in a total volume of 100 μL. [^3H]-Cytidine and [^3H]deoxycytidine were separated by the same HPLC system using 4.5% methanol in 20 mM potassium phosphate (pH 5.6) as the eluting buffer, at a flow rate of 2 mL/min. The eluent was collected and counted as described above.

RESULTS

2′-C-Methylnucleotides Are Substrates for Ribonucleotide Reductase. Incubation of the ribonucleotide reductase from *C. nephridii* with 2′-MeADP generates two products. HPLC analyses of reaction mixtures after dephosphorylation show the disappearance of 2′-*C*-methyladenosine (elution volume = 28.2 mL) and the formation of a major product which elutes earlier than 2′-*C*-methyladenosine at 22.4 mL and a minor one with an elution volume of 41.4 mL (Figure 1). Neither product is formed in controls without enzyme or coenzyme. The formation of these products depends on the concentration of the reductase and on the incubation time.

[1] The optimum pH for the reaction of 2′-MeADP with the reductase.

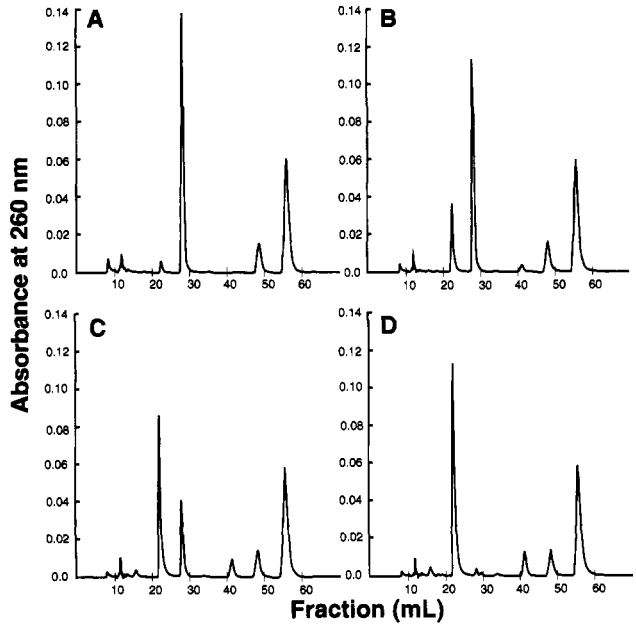


FIGURE 1: Time course of the reaction of ribonucleotide reductase with 2′-MeADP. HPLC elution profiles of the reaction products after incubation for 0 (A), 10 (B), 30 (C), and 60 min (D). Elution volumes: adenine, 22.4 mL; 2′-*C*-methyladenosine, 28.2 mL; 2′-deoxy-2′-*C*-methyladenosine, 41.4 mL; 5′-deoxyadenosine, 48.6 mL. Experimental details are described under Materials and Methods.

Addition of dGTP, the positive allosteric effector of ADP reduction, does not increase the rate of the reaction or change the nature of the products. The elution volume of the first product is identical to that of adenine, and indeed its spectral properties identify it as adenine (λ_{max} = 262 nm, λ_{min} = 228 nm, 250/260 = 0.75, 280/260 = 0.31, pH < 3; λ_{max} = 268 nm, λ_{min} = 237 nm, 250/260 = 0.66, 280/260 = 0.54, pH > 11). The second product has the UV spectral properties of an adenine nucleoside (λ_{max} = 258 nm, λ_{min} = 229 nm, 250/260 = 0.77, 280/260 = 0.19, pH < 3; λ_{max} = 260 nm, λ_{min} = 233 nm, 250/260 = 0.79, 280/260 = 0.13, pH > 11). Its mass spectrum (Figure 2) showed the following molecular ions: *m/e* (relative intensity) 266.3 (5.1), 248.3 (3.7), 176.3 (13.9), 164.2 (31.1), 150.2 (12.0), 136.2 (100), 135.2 (18.7), 131.2 (14.5). The ^{1}H NMR spectrum (500 MHz) is shown in Figure 3: 8.32 (s, 1 H, C_8H), 8.17 (s, 1 H, C_2H), 6.42 (d, 1 H, $J_{1'2'}$ = 7.3 Hz, $C_{1'}$H), 4.14 (t, 1 H, $J_{3'2'} = J_{3'4'}$ = 8.8 Hz, $C_{3'}$H), 3.86 (m, 1 H, $C_{4'}$H), 3.58 (m, 2 H, $C_{5'}H_{a,b}$), 2.75 (m, 1 H, $C_{2'}$H), 0.66 ppm (d, 3 H, $J_{Me\text{-}2'}$ = 7.0 Hz, $C_{2'}$-Me). Adenine is the product of the ribonucleotide reductase reaction and not the consequence of the heating steps or the alkaline phosphatase reaction. HPLC analyses of reaction mixtures that were stopped by cooling in a dry ice/2-propanol bath and were not treated with the phosphatase showed just one product with a retention time identical to that of adenine. The second minor peak was not seen in these chromatograms, suggesting that this product is an adenine nucleotide. During the processing of the reaction mixtures, 5′-deoxyadenosine (elution volume 48.6 mL) is formed from the decomposition of the coenzyme. The last peak at 56.9 mL has an absorbance maximum at 280 nm and is derived from the enzyme and oxidized DTT.

Incubation of the reductase with 2′-MeUDP also generates two products. However, this reaction is very slow, and consequently higher concentrations of enzyme and longer incubation times are required. HPLC analyses of the reactions after phosphatase treatment show two peaks that are not seen in the absence of either enzyme or coenzyme (Figure 4). The first product elutes earlier than 2′-*C*-methyluridine (elution volume = 9.4 mL) with an elution volume of 7.8 mL, identical to uracil, and its UV spectral properties identify it as uracil (λ_{max} = 258 nm, λ_{min} = 235 nm, 250/260 = 0.92, 280/260 = 0.31, 6 M HCl; λ_{max} = 274 nm, λ_{min} = 245 nm, 250/260 = 0.93, 280/260 = 1.02, pH 14). The second product elutes later at 10.4 mL; unfortunately its paucity precluded definite identification. It should be noted that the reaction mixtures with 2′-MeUDP also contain the positive allosteric effector, dGTP. In the absence of dGTP, both products are still formed but at an even lower rate.

2′-C-Methylnucleotides Are Promoters of the Exchange Reaction. 2′-MeADP is an effective promoter of tritium exchange between [5′-$^3H_2$]AdoCbl and the solvent (Figure 5). In contrast, 2′-MeUDP stimulates this reaction only marginally, and indeed, at higher concentrations (>50 μM) it inhibits the low level of exchange seen in the absence of a nucleotide. These results are similar to our earlier observations using ADP and UDP as promoters of the exchange reaction (Ong *et al.*, 1992).

2′-C-Methylnucleotides Inhibit the Reduction of Ribonucleoside Diphosphates. Both nucleotide analogs inhibit the reduction of ribonucleotides. 2′-MeADP is a potent competitive inhibitor of ADP and GDP reduction. 2′-MeUDP inhibits the reduction of UDP and CDP. The kinetic parameters were determined at less than 15% substrate turnover by fitting the data to the Michaelis–Menten equation with nonlinear regression analysis using the Trinity Software program *Enzyme Kinetics* by Jacek Stanislawski or by linear regression analysis of Dixon plots. The resulting $K_{i_{app}}$ values are presented in Table I.

DISCUSSION

The interaction of the adenosylcobalamin-dependent ribonucleoside diphosphate reductase of *C. nephridii* with 2′-MeADP and 2′-MeUDP generates two products from each nucleotide analog. The major and minor products from 2′-MeADP have been identified as adenine and 2′-deoxy-2′-*C*-methyladenosine 5′-diphosphate, respectively. The mass spectrum of the minor product after treatment with alkaline phosphatase shows a mass of 266.3 corresponding to the parent ion + H. As pointed out by Shaw *et al.* (1970), the low relative intensity of the parent mass is expected for nucleosides containing methyl branching in the sugar moiety. The masses at 136.2 and 135.2 correspond to the adenine + 2H and adenine + H ions. Loss of a hydroxyl radical yields the ion at 248.3; the 131.2 mass corresponds to the 2′-deoxy-2′-*C*-methylribose moiety. The ^{1}H NMR spectrum of the nucleoside establishes its structure as 2′-deoxy-2′-*C*-methyladenosine. The presence of a proton at the 2′-position generates a doublet for the anomeric proton at 6.42 ppm, a broad triplet (doublet of doublets) for the 3′-proton at 4.14 ppm, and a doublet for the methyl protons at 0.66 ppm. The 2′-proton appears as a broad octet at 2.75 ppm due to the coupling of this proton with the protons at C-1′ and C-3′ and the methyl protons. A one-dimensional differential NOE experiment with irradiation of the anomeric proton as well as a two-dimensional NOESY with a wide range of mixing times showed no connectivity between the anomeric proton and the 2′-*C*-methyl group, indicating that 2′-*C*-MeADP is reduced to 2′-deoxy-2′-*C*-MeADP with retention of configuration. Uracil was identified as the major product derived from 2′-*C*-MeUDP; the minor product elutes later, as would be expected for a deoxyribonucleoside. Because only minute quantities of this product could be isolated, its structure was not established. These observations are reminiscent of those of Harris *et al.* (1987)

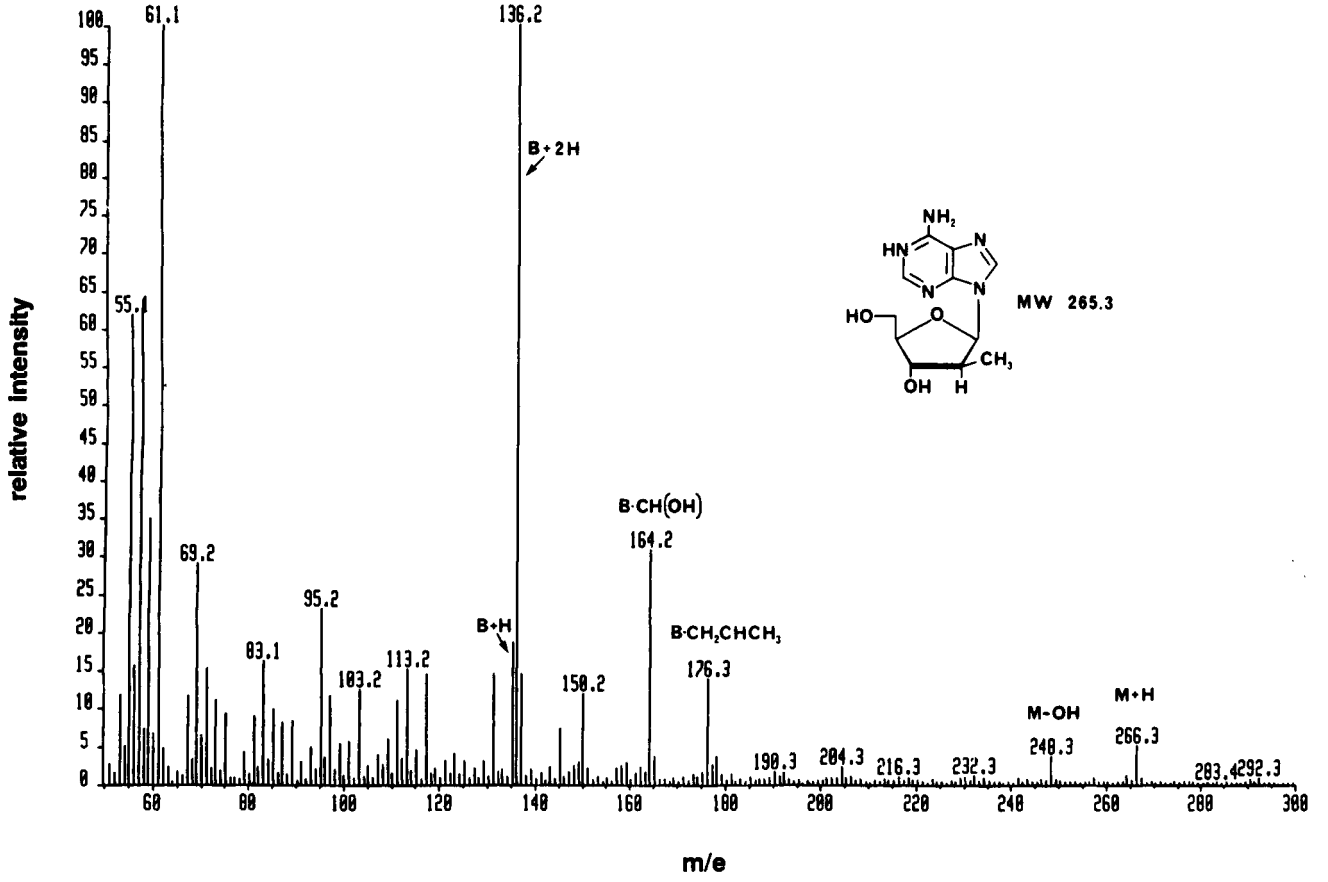


FIGURE 2: Mass spectrum of 2′-deoxy-2′-C-methyladenosine.

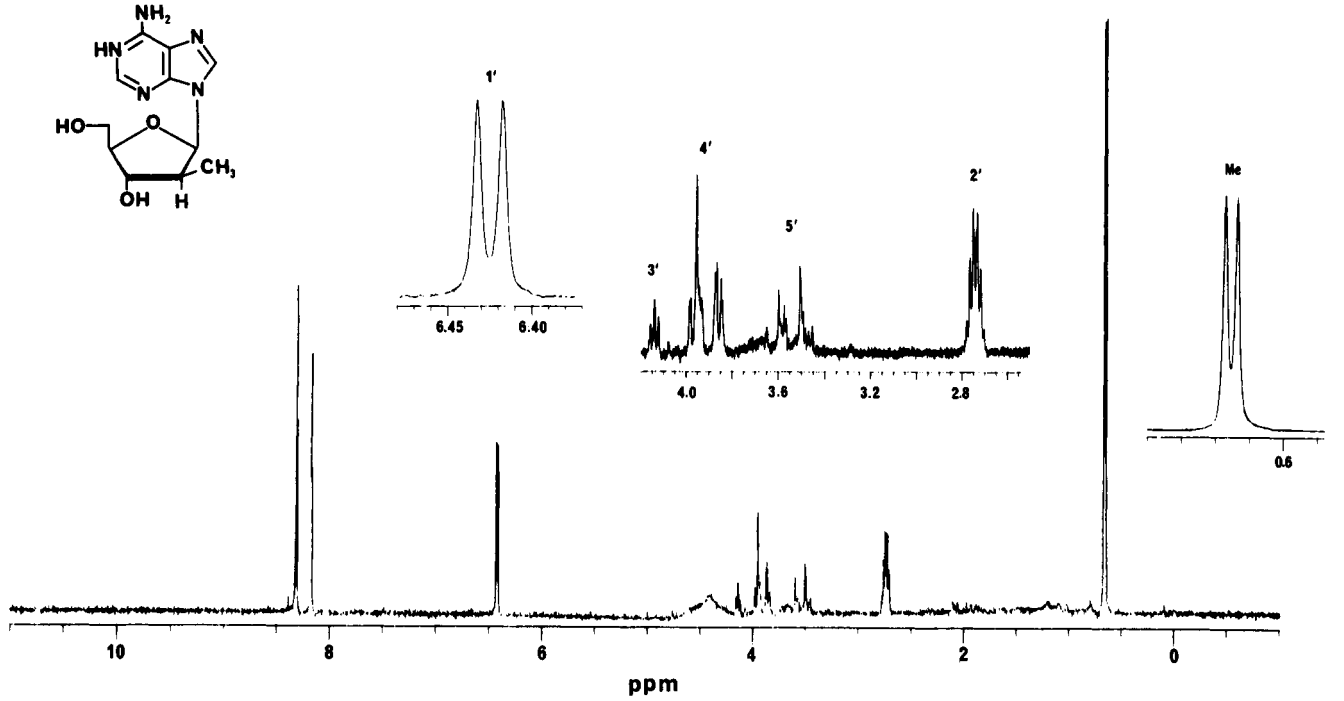


FIGURE 3: ^{1}H NMR spectrum (500 MHz) of 2′-deoxy-2′-C-methyladenosine.

who investigated the interaction of several 2′-halogenated nucleoside triphosphates with the ribonucleoside triphosphate reductase of *L. leichmannii*. They found that these nucleotides partitioned between normal reduction to the 2′-deoxyribonucleotides and decomposition to the nucleotide base, pyrophosphate, and 2-methylene-3(2*H*)-furanone. They suggested that the 2′-deoxy-3′-ketoribonucleotides collapsed to these products. The behavior of the 2′-C-methylnucleotides is similar to that of the halogenated nucleotides, and indeed, our results are in accord with the proposed mechanism of Stubbe (Scheme I). In the case of the 2′-C-methylnucleotides, the positive inductive effect of the methyl group should facilitate the protonation of the 2′-hydroxyl moiety, its departure as water, and the stabilization of the 2′-cationic intermediate (Scheme II). This cationic intermediate then isomerizes to the 2′-C-methyl-3′-ketonucleotide radical. At this juncture

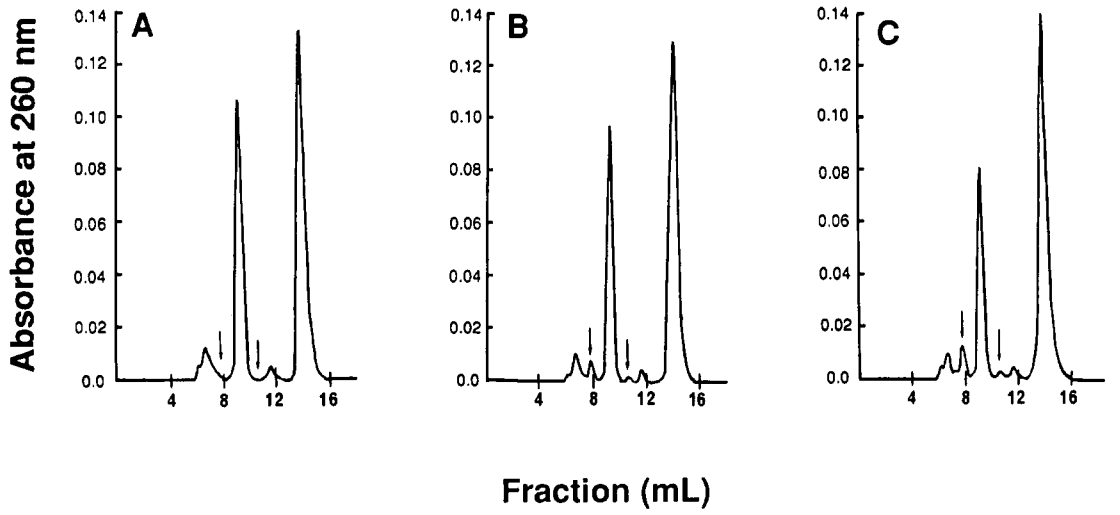


FIGURE 4: Time course of the reaction of ribonucleotide reductase with 2′-MeUDP: HPLC elution profiles of the reaction products after incubation for 0 (A), 30 (B), and 120 min (C). Elution volumes: uracil, 7.8 mL; 2′-*C*-methyluridine, 9.4 mL; uracil nucleoside, 10.4 mL; 2′-deoxyguanosine, 14.5 mL. Experimental details are described under Materials and Methods.

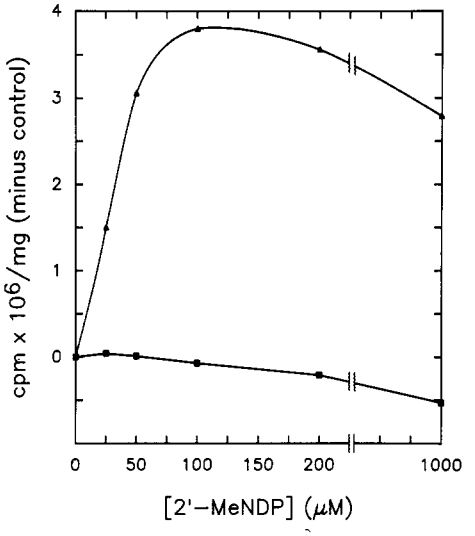


FIGURE 5: Effect of 2′-MeNDPs on the tritium exchange between [5′-$^3H_2$]AdoCbl and the solvent. See Materials and Methods for experimental conditions. The low level of tritium exchange observed in the absence of a nucleotide was set at 0: (▲) 2′-MeADP and (■) 2′-MeUDP.

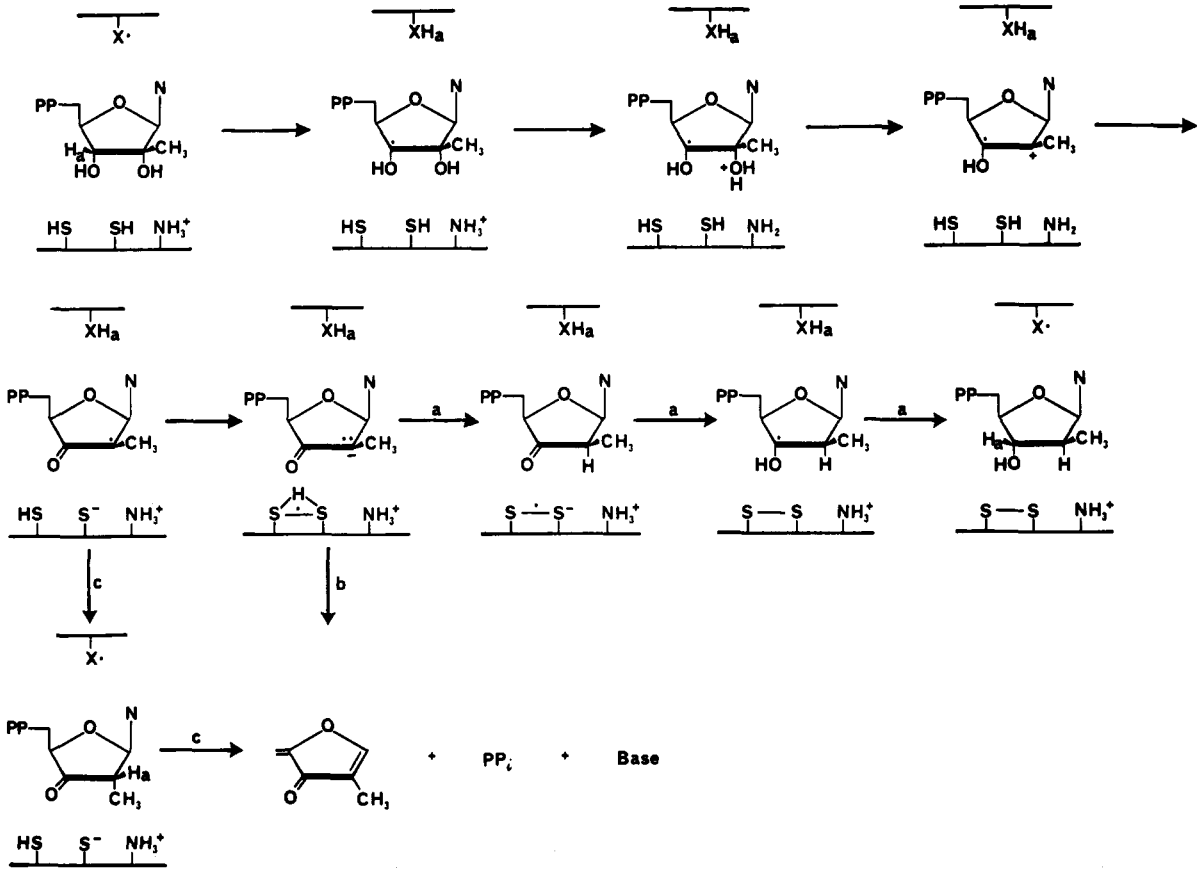
Table I: Inhibition Constants for the 2′-MeNDPs

	analog			
	2′-MeADP		2′-MeUDP	
substrate	ADP	GDP	UDP	CDP
K_i (μM)	3.7	7.9	84	115

three pathways are possible that lead to the decomposition of the nucleotide analogs and to the formation of the 2′-deoxy-2′-*C*-methylnucleotide products. In pathway a the 2′-*C*-methyl-3′-ketonucleotide radical accepts an electron from one of the thiols of the enzyme to yield the 2′-*C*-methyl-3′-ketonucleotide anion, analogous to the reaction sequence shown in Scheme I. This nucleotide anion can accept a proton and continue with the normal reaction sequence that leads to the 2′-deoxy-2′-*C*-methylribonucleotide product, or the anion can collapse to the base, pyrophosphate, and 2-methylene-3(2*H*)-4-methylfuranone (pathway b). In the normal reaction sequence the protein-based radical, essential for catalysis, is regenerated, while in the decomposition sequence this is not the case. However, the positive inductive effect of the 2′-*C*-methyl group should destabilize the nucleotide anion, hindering the one-electron-transfer reaction. As a consequence, the 2′-*C*-methyl-3′-ketonucleotide radical could react by abstracting a hydrogen from the enzyme to generate the protein-based radical and form the 2′-*C*-methyl-3′-ketonucleotide, which decomposes by elimination of the base and pyrophosphate (pathway c). This reaction sequence is very similar to that proposed for the collapse of the 2′-chloro-2′-deoxyribonucleotides (Harris *et al.*, 1987). Our results indicate that approximately 5% of 2′-MeADP is reduced to 2′-deoxy-2′-*C*-MeADP, while the bulk of the nucleotide analog decomposes to adenine and other yet unidentified products. During the processing of the reaction mixtures the coenzyme decomposes, and thus it is not yet possible to establish the stoichiometry between the reaction products from 2′-*C*-MeADP, 5′-deoxyadenosine, and cob(II)alamin. Hamilton *et al.* (1971) and Yamada *et al.* (1971) found that incubation of the ribonucleotide reductase from *L. leichmanii* with the coenzyme, dihydrolipoate, and a nucleotide triphosphate resulted in the formation of 5′-deoxyadenosine and a paramagnetic corrinoid.

Harris *et al.* (1987) suggested that the partitioning between product formation and mechanism-based inhibition is a function of the pK_a of the leaving group at the C-2′ position of the substrate. They postulated that "as the leaving group ability increases and the requirement for general acid assistance decreases, the abnormal reaction to produce the 3′-keto-2′-deoxynucleotide intermediate becomes more important." In the case of 2′-*C*-methylnucleotides, the positive inductive effect of the 2′-*C*-methyl group should facilitate the departure of the 2′-hydroxyl group as water and, thus, our current studies clearly support their postulate. Space-filling models of the 2′-*C*-methylnucleotides show a highly crowded region at the C-2′ position of the analogs. Consequently, the glycosidic bonds of these nucleotide analogs are strained and will lead to a more facile collapse of the postulated 2′-*C*-methyl-3′-ketoribonucleotide. Both 2′-*C*-methylnucleotides are potent competitive inhibitors of ribonucleotide reduction, indicating that they interact strongly with the substrate site of the enzyme. As shown in Table I, the apparent K_i value of 2′-*C*-MeADP for GDP reduction is higher than that for ADP reduction (7.9 versus 3.7 μM). Similarly the apparent K_i of 2′-*C*-MeUDP for CDP reduction is higher than that for UDP reduction (115 versus 84 μM). The apparent K_m values for the substrates ADP and UDP are 2.2 and 27 μM, respectively.

Scheme II



These observations and our earlier studies using the 3′-*C*-methylnucleotides suggest that the spatial requirements for the interaction between the enzyme and its substrates are more stringent at the 3′-position of the sugar moiety than at the 2′-position. Follmann and Hogenkamp (1971) and Ludwig and Follmann (1978) reported that the 2′- and 3′-*O*-methylnucleotides as well as the *xylo*- and 3′-deoxyribonucleotides were neither substrates nor inhibitors of ribonuclotide reduction. In contrast, the diastereomeric *arabino* nucleotides were weak competitive inhibitors or mixed-type inhibitors, indicating that modifications at the 2′-position of the substrate can be accommodated by the enzyme. In accord with these observations, we have shown in our earlier paper (Ong *et al.*, 1992) that the 3′-*C*-methylribonucleotides, 3′-MeADP and 3′-MeUDP, do not function as substrates and are moderate and weak inhibitors, respectively.

In the reaction sequence proposed by Stubbe (Scheme I), the interaction of the reductase with the 3′-carbon of the nucleotide involves just one amino acid residue, the residue that harbors the protein-based radical. During the first step this protein-based radical abstracts the hydrogen at C-3′ of the substrate, while in the last step this hydrogen is returned to C-3′ of the 2′-deoxyribonucleotide radical. In contrast, the actual reduction occurs at C-2′ of the substrate and involves the interaction with several amino acid residues and rather sophisticated chemistry. Consequently, more flexibility is required at the active site for reaction at C-2′ of the substrate, and thus nucleotides with larger substituents at this position can be accommodated.

2′-MeADP is an effective promoter of the tritium exchange reaction between [5′-$^3H_2$]AdoCbl and water; its action is very much like that seen for ADP. 2′-MeUDP stimulates the exchange, albeit only marginally, at low concentrations (<50 μM). We have reported earlier (Ong *et al.*, 1992) that the deoxyribonucleotide products (dADP, dGDP, and dCDP) are much more effective promoters of the exchange reaction, suggesting that product formation is required for this reaction. Indeed, the lower level of exchange seen in the presence of the normal substrates (ADP, GDP, and CDP) may be due to the reduction of the ribonucleotide substrates within the 10-min incubation period. The resulting deoxynucleotides would then be the actual promoters of the exchange reaction. However, the exchange reactions promoted by both UDP and dUDP are complex and distinct from those promoted by the other ribonucleotide substrates or the deoxyribonucleotide products. UDP is an effective promoter at low concentrations (<100 μM), while at higher concentrations it inhibits the exchange reaction. 2′-MeUDP shows a similar pattern in its exchange reaction. It is a marginal stimulator of the exchange reaction up to 50 μM and inhibits the low level of exchange observed in the absence of a promoter at higher concentrations. The poor activity as a stimulator of exchange parallels its low reactivity as a substrate.

All of these observations are in accord with our earlier conclusion that the presence of an abstractable hydrogen at the C-3′ position is essential not only for the overall reduction but also for the exchange reaction. Indeed, our current studies with the 2′-*C*-methylnucleotides have extended our findings to include product formation as an additional prerequisite for the exchange reaction.

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