Interaction of Ribonucleotide Reductase with Ribonucleotide Analogs*

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ABSTRACT: The substrate specificity of ribonucleoside triphosphate reductase of *Lactobacillus leichmannii* has been studied using a series of ribonucleotide analogs modified in their sugar and base moieties. The 5′-triphosphates of 1-β-D-ribofuranosylbenzimidazole, 2′- and 3′-*O*-methyladenosine, 9-β-D-arabinofuranosyladenine, 1-β-D-arabinofuranosylcytosine, and 9-β-D-xylofuranosyladenine were synthesized by chemical procedures and tested for their ability to interact with the enzyme as substrates, inhibitors, or activators. Only benzimidazoleriboside triphosphate is reduced at a slow rate which is specifically stimulated by dGTP, while none of the other nucleotides containing methylated or epimeric sugars is a substrate of this reductase. However, both 2′-*O*-methyl- and 3′-*O*-methyl-ATP are able to stimulate specifically the reduction of CTP. While the 5′-triphosphates of 9-β-D-arbinofuranosyladenine and 1-β-D-arbinofuranosylcytosine both inhibit ribonucleotide reduction, they are nevertheless able to promote the tritium exchange reaction between deoxyadenosylcobalamin-5′-*t* and water. The other nucleotide analogs are unable to promote this exchange reaction.

These findings and our earlier results with ^{18}O-labeled substrates suggest that the substrate site of lactobacillus ribonucleotide reductase is highly specific for the ribose moiety of a nucleotide. The results also suggest that this reductase has *two* effector sites with different (although less stringent) requirements for the sugar moiety of nucleoside triphosphates.

Ribonucleotide reductase from *Lactobacillus leichmannii*, a deoxyadenosylcobalamin-dependent enzyme, catalyzes the reduction of ribonucleoside 5′-triphosphates to the corresponding 2′-deoxyribonucleotides while reductases from other bacterial or mammalian sources act upon ribonucleoside 5′-diphosphates and differ in their cofactor requirements. The specificity of these enzymes for the base portion of a substrate does not appear to be very stringent; because in addition to the common nucleotides CTP, UTP, ATP, GTP, and ITP the ribonucleotide reductase of *L. leichmannii* is also able to reduce the 5′-triphosphates of the pyrrolopyrimidine nucleosides tubercidin, toyocamycin, and sangivamycin (Suhadolnik *et al.*, 1968). On the other hand, the nature of the base greatly influences the reaction rate. The reduction of individual ribonucleotides in this system is further subject to specific activation by the 2′-deoxyribonucleotide products: the reduction of ATP is stimulated by dGTP, of CTP by dATP, and of UTP by dCTP. The enzyme is therefore assumed to possess both substrate and regulatory sites. (Vitols *et al.*, 1967).

The specificity of enzymatic nucleotide reduction with respect to the sugar moiety has not been studied in detail, although this question is of great importance for analyzing the biological action of analogs such as arabinosyl or xylosylnucleoside and nucleotides. For example, many arabinosyl derivatives are inhibitors of nucleic acid metabolism in various organisms (Balis, 1968; Cohen, 1966), but evidence for the nature of the enzymatic reactions affected or for a possible incorporation into RNA and DNA is quite incomplete and inconclusive. The 5′-triphosphate or -diphosphate of cordycepin, an analog of adenosine containing a 3′-deoxyribosyl moiety, is not reduced by the reductase systems of *L. leichmannii* or *Escherichia coli*, respectively (Suhadolnik *et al.*, 1968; Chassy and Suhadolnik, 1968). However, in previous studies using adenosine triphosphates labeled with ^{18}O in the 2′ and 3′ positions we have shown that the 3′-hydroxyl group does not participate in the reduction process itself (Follmann and Hogenkamp, 1969), suggesting that in the ribonucleotide substrates this hydroxyl group functions as another binding site to the enzyme.

In the present work, we have prepared the 5′-triphosphates of 1-β-D-ribofuranosylbenzimidazole,[1] 2′- and 3′-*O*-methyladenosine, 9-β-D-arabinofuranosyladenine, 1-β-D-arabinofuranosylcytosine, and 9-β-D-xylofuranosyladenine (Chart I, I–VI) and tested their effectiveness as substrates, inhibitors, or activators of ribonucleotide reduction. The interaction of these analogs with the lactobacillus enzyme was studied by three independent methods, namely by coupling the ribonucleotide reductase system with the thioredoxin system and TPNH as the ultimate reductant (Vitols *et al.*, 1967); by direct colorimetric or chromatographic determination of deoxyribosides with dihydrolipoate as reductant (Blakley, 1966); and by the tritium-exchange reaction between 5′-deoxyadenosylcobalamin-5′-*t* and water in the presence of dihydrolipoate (Hogenkamp *et al.*, 1968). The results demonstrate that the enzyme is only capable of reducing nucleoside triphosphates with unsubstituted ribofuranose as the sugar component; the analogs with modified sugars are, however, able to act as inhibitors or as activators of this reductase.

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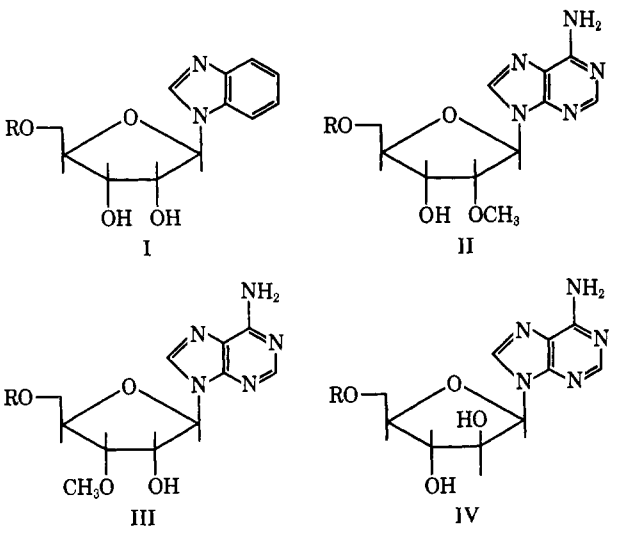
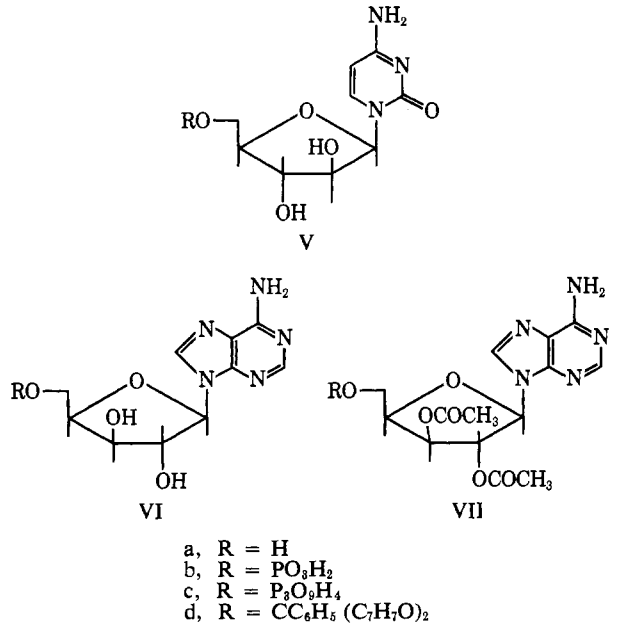
[1] Abbreviations used are: BzMP, 1-β-D-ribofuranosylbenzimidazole 5′-monophosphate; BzTP, 1-β-D-ribofuranosylbenzimidazole 5′-triphosphate. The prefixes *ara* and *xylo* are used to designate nucleotides containing a β-D-arabinofuranosyl or β-D-xylofuranosyl sugar moiety, respectively.

CHART I



a, R = H
b, R = PO_3H_2
c, R = $P_3O_9H_4$



a, R = H
b, R = PO_3H_2
c, R = $P_3O_9H_4$
d, R = $CC_6H_5(C_7H_7O)_2$

TABLE I: Paper Chromatography of Nucleosides and Nucleotides.

Compound	R_F Solvent A	R_F Solvent B	Compound	R_F Solvent A	R_F Solvent B
5′-AMP	0.16		IVc	0.06	
5′-ATP	0.05		Va	0.63	0.11
Ia	0.87	0.66	b	0.18	
b	0.53	0.10	c	0.08	
c	0.25		VIa	0.64	0.16
IIa	0.71	0.34	b	0.19	
b	0.29		c	0.08	
c	0.11		d		0.64
IIIa	0.71	0.33	VIIa	0.80	0.58
b	0.29		b	0.36	0.17
c	0.11		c	0.20	
IVa	0.64	0.16	d		0.90
b	0.18				

Experimental Section

Materials and General Methods. The following compounds were synthesized by published procedures: 1-β-D-ribofuranosylbenzimidazole (Davoll and Brown, 1951); 2′-*O*-methyl adenosine (Martin *et al.*, 1968); 3′-*O*-methyladenosine (Tong *et al.*, 1967); 9-β-D-arabinofuranosyladenine (Glaudemans and Fletcher, 1963); 9-β-D-xylofuranosyladenine (Baker and Hewson, 1957); deoxyadenosylcobalamin and deoxyadenosylcobalamin-5′-t_2 (Hogenkamp *et al.*, 1968a,b); dihydrolipoate (Gunsalus and Razzell, 1957). 1-β-D-arabinofuranosylcytosine hydrochloride was obtained from Pierce, *Crotalus adamanteus* 5′-nucleotidase from Sigma, and bacterial alkaline phosphatase from Worthington. Other nucleosides and nucleotides were purchased from P-L Biochemicals. Thioredoxin and thioredoxin reductase were isolated from *E. coli* B by the procedure of Laurent *et al.* (1964) and Moore *et al.* (1964) omitting the final gel filtration steps. Ribonucleotide reductase from *Lactobacillus leichmannii*, specific activity 68 μmoles/hr per mg of protein, was kindly provided by Dr. R. L. Blakley.

Spectral measurements were made with a Cary Model 15 and a Zeiss PMQ II spectrophotometer; nuclear magnetic resonance spectra were recorded on a Varian A-60 spectrometer. Radioactivity was measured in a dioxane solution (Bray, 1960) using a Packard Tri-Carb Model 3003 liquid scintillation counter. The purity of nucleosides and nucleotides was determined by ultraviolet and nuclear magnetic resonance spectroscopy and by descending paper chromatography. Whatman No. 1 paper was used in the solvent systems: A, ethanol–1 M ammonium acetate, 5:2; B, 1-butanol–water, 86:14; and C, 1-butanol–7 M ammonium hydroxide, 86:14. R_F values are listed in Table I.

Synthesis of ATP Analogs. The nucleosides Ia–Va and VIIIa were phosphorylated to their corresponding 5′-monophosphates by the method of Imai *et al.* (1969). In a typical preparation 1 mmole of nucleoside was dissolved or suspended in 10 ml of *m*-cresol, and after addition of 0.7 ml (5 mmoles) of pyrophosphoryl chloride the mixture was stirred for 3 hr at 5°. Ice water was added, and cresol was removed by repeated extraction with ether. For desalting, the aqueous solution was adjusted to pH 3, treated with 10 g of acid-washed charcoal, and filtered, and the charcoal pad washed with water. Nucleotides were then eluted with ethanol–0.2 M ammonium hydroxide (1:1), and after concentration *in vacuo* the solution was applied to a column of Dowex 1-X2 (formate), 1.5 × 20 cm. The column was washed with water to remove unreacted nucleosides and then eluted with formic acid to obtain the desired nucleoside 5′-phosphates as follows: BzMP Ib, 0.02 N HCOOH (pH 2.7), yield, 50%; 2′-*O*-methyl-AMP and 3′-*O*-methyl-AMP IIb, IIIb, 0.1 N HCOOH (pH 2.4), 41% and 45%; *ara*-AMP IVb, 0.1 N HCOOH (pH 2.4), 61%; *ara*-CMP Vb, 0.02 N HCOOH (pH 2.7), 44%; and 2′,3′-di-*O*-acetyl-*xylo*-AMP VIIb, 0.2 N HCOOH (pH 2.2), 25%. In the latter preparation, desalting on charcoal had been omitted in order to avoid ammonia treatment of the alkali-labile protecting groups. All the nucleotides were obtained as amorphous powders by lyophilization.

In an alternative synthesis of Ib, 0.30 g (1.2 mmoles) of ribofuranosylbenzimidazole and cyanoethylphosphoric acid (10 mmoles) were stirred for 3 hr in a mixture of 7 ml of trimethylorthoformate and 5 ml of dimethylformamide. The resulting solution was neutralized with pyridine and after addition of 2 g of dicyclohexylcarbodiimide (10 mmoles) was stirred at room temperature overnight. The precipitate was removed by filteration and the solution evaporated *in vacuo*. The residue was first treated with 7 M ammonium hydroxide for 90 min at 60°, evaporated, redissolved in water, and then adjusted to pH 2 by addition of formic acid. After 1 hr at room temperature, the solution was filtered, lyophilized, and then chromatographed as described above to yield 0.21 g (52%) of the monophosphate Ib.

For the synthesis of nucleoside 5′-triphosphates the method of Hoard and Ott (1965) was used. The 5′-monophosphoric acids (0.5 mmoles) were neutralized with an equivalent amount of tri-*n*-butylamine and the salts dried exhaustively over P_2O_5 before 1,1′-carbonyldiimidazole (0.38 g, 2.5 mmoles) in 5 ml of anhydrous dimethylformamide was added. The mixture was stirred at room temperature overnight, treated with methanol (0.15 ml) for 30 min, and then treated with tri-*n*-butylammonium pyrophosphate (2.0 mmoles) in 10 ml of dimethyl sulfoxide. After 24 hr at room temperature the solution was filtered, concentrated *in vacuo* to a small volume, diluted with 50 ml of water, and applied to a column of DEAE-cellulose (bicarbonate), 2.5 × 40 cm. In the case of 2′,3′-di-*O*-acetyl-*xylo*-ATP (VIIc) the protecting groups were removed prior to chromatography by treatment of the dry residue with ammonia in methanol for 6 hr at room temperature. Elution of the column with a gradient of triethylammonium bicarbonate separated the triphosphates of benzimidazoleriboside and of the arabino- and xylonucleosides (Ic, IVc, Vc, VIc) from unreacted monophosphates but did not fully resolve the corresponding *O*-methyladenosine phosphate mixtures IIb–IIc and IIIb–IIIc. However, the latter fractions of the main elution peaks contained the pure triphosphates IIc and IIIc, and those fractions contaminated by the monophosphates were further purified by paper chromatography in solvent A. The triphosphates Ic–IVc, obtained in an average yield of 50%, were isolated by lyophilization as the amorphous triethylammonium salts of undefined water content.

Preparation of 2′,3′-Di-O-acetylxylofuranosyladenine. Anhydrous xylofuranosyladenine (VIa, 1.34 g, 5 mmoles) was dissolved in 10 ml of puridine and 2 ml of dimethylformamide and was treated with dimethoxytrityl chloride (2.7 g, 8 mmoles) for 15 hr at room temperature. The reaction mixture was then treated with ice water and the product extracted with benzene. The benzene solution was concentrated to a small volume and chromatographed on a column (2 × 40 cm) of alumina (15% deactivated) in benzene. The column was washed with 500 ml of benzene to remove dimethoxytritanol and the desired product was eluted with 800 ml of benzene containing 5% methanol. 5′-*O*-Dimethoxytritylxylofuranosyladenine (VId) was obtained in 1.74 g yield (63%) as a yellow solid, λ_{max} (ethanol) 265, 235 mμ.

A solution of VId (2.75 g, 5 mmoles) in 25 ml of pyridine was treated with acetic anhydride (4 ml, 40 mmoles) at room temperature for 3 hr. The mixture was diluted with water, the product extracted with chloroform, and the chloroform layer washed with sodium bicarbonate solution. Evaporation of the solvent afforded 2.7 g (85%) of 5′-*O*-dimethoxytrityl-2′,3′-di-*O*-acetylxylofuranosyladenine (VIId) which was used for the next step without further purification.

A solution of VIId (2.7 g, 4.2 mmoles) in 100 ml of 80% acetic acid was kept at room temperature for 30 min, and the acid was then removed by repeated coevaporation with ethanol. The syrupy residue was suspended in ether and the product extracted with five portions of water. The combined aqueous solutions were lyophilized to yield 0.87 g (58%) of 2′,3′-di-*O*-acetylxylofuranosyladenine (VIIa) as a white powder which failed to crystallize from water, alcohols, acetone, or their mixtures: λ_{max} (water) 260 mμ; the nuclear magnetic resonance spectrum (D_2O, *vs.* Me_4Si) showed the presence of two acetoxy groups at τ 7.79 (3, s) and 7.85 (3, s) besides the sugar protons (H 1′ at τ 3.85, 1, d, J = 4 Hz; others at 4.6–5.5, 5, m) and base protons (1.70 and 1.81, 1, s).

Enzyme Assays. Ribonucleotide reduction was measured at 25° by the coupled spectrophotometric assay described by Vitols *et al.* (1967). In order to avoid unspecific TPNH oxidation, two separate solutions were prepared, solution I containing ribonucleotide reductase (0.4 mg/ml) and thioredoxin reductase (0.3 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.5, and 2 mM EDTA, solution II containing thioredoxin (0.012 mM), TPNH (0.5 mM), and deoxyadenosylcobalamin (0.03 mM) in the same buffer. Before each assay, equal volumes of I and II were mixed and 1.0 ml of the mixture was placed into both the reference and experimental cuvet of a Cary 15 spectrophotometer; the reaction was started by addition of substrate and/or effector to the experimental cuvet. The absorption decrease at 340 mμ was linear for at least 4 min under these conditions. To test for reduction of the analogs alone, solutions with tenfold increased enzyme and coenzyme concentrations were used.

The colorimetric assay of deoxyribonucleotide production with diphenylamine and the tritium-exchange reaction between deoxyadenosylcobalamin-5′-*t* and water were carried out essentially as described by Blakley (1966) and by Hogenkamp *et al.* (1968).

For the identification of 1-β-D-2′-deoxyribofuranosylbenzimidazole a reaction mixture containing 7 mM BzTP (Ic), 30 mM dihydrolipoate, 0.3 mM deoxyadenosylcobalamin, and 1.5 mg of ribonucleotide reductase in 0.30 ml of 0.1 M dimethylglutarate buffer, pH 7.3, was incubated in the dark at 37°; a second identical reaction mixture also contained 3 mM dGTP. After 6 and 12 hr 0.1-ml aliquots were removed, heated in a boiling water bath for 5 min, adjusted to pH 8 and treated with 0.5 mg of alkaline phosphatase for 2 hr. They were then spotted directly on Whatman No. 1 paper pretreated with sodium tetraborate. Chromatography in solvent C revealed the presence of a new compound of R_F 0.87 besides ribofuranosylbenzimidazole (0.37), deoxyguanosine (0.13), and dihydrolipoate (0.75); this material was identified as 2′-deoxyribofuranosylbenzimidazole by its spectrum (λ_{max} 279, 272, 245 mμ, pH 7) and by its inability to react with sodium periodate. The deoxyriboside could be detected after 6-hr incubation in the dGTP-containing reaction mixture, however in the absence of this effector a 12-hr incubation was necessary to reduce approximately 25% of the substrate.

Results

Preparation of Nucleoside Triphosphates. Several nucleoside 5′-phosphates containing 2′-*O*-methylribose, arabinose, and xylose have previously been obtained in rather low yields by a variety of chemical and enzymatic methods (Rottmann and Heinlein, 1968; Cohen, 1966; Ellis and LePage, 1965).

In nucleosides lacking a cis-diol system specific protection of the 2′- or 3′-hydroxyl functions is difficult and direct phosphorylation with standard reagents such as cyanoethylphosphate is inefficient. In the present systematic study we have successfully employed pyrophosphorylchloride in *m*-cresol (Imai *et al.*, 1969) to convert unprotected ribofuranosylbenzimidazole (Ia) 2′- and 3′-*O*-methyladenosine (IIa, IIIa), arabinofuransyladenine, and arabinofuranosylcytosine (IVa, Va), and 2′,3′-di-*O*-acetylxylofuranosyladenine (VIIa) into their 5′-monophosphates which were purified by anion-exchange chromatography and obtained in 40–60% yield. Ribofuranosylbenzimidazole phosphate was also conveniently synthesized *via* its 2′,3′-cyclic orthoformate and condensation with cyanoethylphosphate (Darlix *et al.*, 1967). Selective phosphorylation at the 5′-hydroxyl groups of the benzimidazole and arabinofuransyl nucleotides Ib, IVb, and Vb was confirmed by their complete hydrolysis with snake venom 5′-nucleotidase at pH 8.4; 3′-*O*-methyl-AMP was resistant to this enzyme as has been observed for the 2′ isomer (Rottman and Heinlein, 1968).

xylo-AMP cannot be obtained from unprotected xylofuranosyladenine because in the presence of condensing agents the 3′,5′-cyclic phosphate is formed. Therefore, in this case it was necessary to prepare 2′,3′-di-*O*-acetylxylofuranosyladenine (VIIa) *via* the 5′-dimethoxytrityl derivatives VId and VIId, none of which could be crystallized but which were characterized by their chromatographic and spectral properties. Although xylose derivatives are prone to acetyl migration (Johnston, 1968) this did not occur at room temperature during the various reaction steps.

Condensation of the monophosphates Ib–VIIb with carbonyldiimidazole and tri-*n*-butylammonium pyrophosphate in organic solvents (Hoard and Ott, 1965), followed by paper or column chromatography, finally afforded the nucleoside triphosphates Ic–VIc pure and in good yields.

Activity of Nucleoside Triphosphates with Ribonucleotide Reductase as Substrates. The ATP and CTP analogs containing 2-*O*-methylribose, 3-*O*-methylribose, arabinose, or xylose did *not* function as substrates in a complete ribonucleotide reductase system with enzyme concentrations ranging from 2.6×10^{-6} M to 6×10^{-5} M (based upon a molecular weight of 76,000). Furthermore, the addition of dGTP or dATP, the activators of ATP and CTP reduction, did not result in TPNH oxidation. In contrast BzTP was reduced by the lactobacillus reductase albeit at a rate less than 1% of the rate of ATP reduction. This low reduction rate was barely detected under the standard assay conditions, but was stimulated more than tenfold by the addition of dGTP while dATP, dCTP, and TTP had no effect upon the reaction (Table II). The reduction of BzTP was unambiguously confirmed by isolating 2′-deoxyribofuranosylbenzimidazole from a reaction mixture containing the substrate, enzyme, deoxyadenosylcobalamin, and dihydrolipoate as reductant after incubation with or without dGTP for a suitable time and treatment with alkaline phosphatase.

As Activators or Inhibitors. In Table II are presented the effects of the ATP and CTP analogs upon the reduction of various ribonucleotide substrates. Under the assay conditions dGTP caused a 3-fold increase in the rate of ATP reduction, dATP stimulated CTP reduction approximately 2.4-fold and dCTP stimulated UTP reduction approximately 1.5-fold, in close agreement with earlier findings (Vitols *et al.*, 1967). Among the analogs both O-methylated ATPs stimulated the rate of CTP reduction 2-fold, suggesting that they can replace either dATP or ATP, since the latter is also capable of enhancing the rate of CTP reduction (Beck *et al.*, 1966). In contrast, neither *ara*- nor *xylo*-ATP activated the reduction of CTP nor did these analogs compete with dATP for its stimulatory action; likewise *ara*-CTP could not activate UTP reduction.

TABLE II: Ribonucleotide Reduction in the Presence of Nucleotide Analogs.[a]

Addition	Substrate				
	ATP	CTP	GTP	UTP	Bz-TP[b]
None	9	13	50; 40[c]	8	<0.1
Bz-TP	9	13	50	7	
dGTP	28				1.0
dATP	8	31			<0.1
2′-*O*-CH_3-ATP	8	25			
3′-*O*-CH_3-ATP	8	26			
ara-ATP	5	13	50	8	
xylo-ATP	8	13			
dCTP		10		12	<0.1
ara-CTP	6	10	50	8	
CTP			27[c]		

[a] Results are expressed as nmoles of TPNH oxidized per minute in the 1.0-ml standard assay mixture at 25° (enzyme, 200 μg; coenzyme, 20 μM; substrates and effectors, 1 mM). [b] Corrected values; substrate tested at tenfold higher enzyme and coenzyme concentrations. [c] Values obtained from colorimetric assays (15 min, 46 μg of enzyme/ml) and corrected; GTP concentration 1 mM, CTP concentration 0.6 mM.

Both *ara*-nucleoside triphosphates were found to be inhibitors of ATP reduction in the presence or absence of dGTP; no significant inhibition or reduction of the other substrates was observed. All the other analogs including dATP and dCTP, the natural reduction products, caused very slight inhibition of substrate reduction, if any. In the coupled assay system, it was not possible to accurately determine kinetic constants for the inhibition of ATP reduction by the *ara* nucleotides. However, our present preliminary data indicate that the inhibition by *ara*-ATP is entirely different from the mutual inhibition between two substrates like ATP and GTP which was previously reported (Beck *et al.*, 1966) and which we could confirm by using CTP and GTP under different assay conditions (Table II). The inhibition of GTP reduction by CTP was readily measured with the colorimetric method of Blakley (1966) because the more acid-labile purine deoxyribonucleotide can be detected without interference from the pyrimidine deoxyribonucleotide formed at the same time. The kinetic data obtained with this assay suggest that the inhibition of GTP reduction by CTP is indeed competitive.

As Promoters of Tritium Exchange. The effect of the analogs on the ribonucleotide reductase catalyzed exchange reaction between 5′-deoxyadenosylcobalamin-5′-t_2 and water is shown in Table III. Only *ara*-ATP and *ara*-CTP promote this exchange at a rate comparable with that of ATP and 2′-dATP. The exchange in the presence of BzTP and *xylo*-ATP is very low although consistently higher than the exchange in the absence of a nucleotide. The O-methylated ATPs do not promote tritium transfer. In order to determine the

TABLE III: Effect of Nucleoside Triphosphates on the Hydrogen Exchange between Deoxyadenosylcobalamin-5′-t_2 and Water.

Addition to Standard System[a]	Radioactivity of Water (cpm)	Addition to Standard System[b]	Radioactivity of Water (cpm)
ATP	36,150	ATP	9,432
Bz-TP	305	ATP + Bz-TP	4,701
2′-*O*-CH_3-ATP	260	ATP + 2′-*O*-CH_3-ATP	9,519
3′-*O*-CH_3-ATP	205	ATP + 3′-*O*-CH_3-ATP	7,826
ara-ATP	4,915	ATP + *ara*-ATP	10,366
xylo-ATP	470	ATP + *xylo*-ATP	6,687
2′-dATP	12,555	ATP + 2′-dATP	18,075
ara-CTP	4,577		
None	230		

[a] The standard reaction system contained 28 μg of ribonucleotide reductase, 45 μM deoxyadenosylcobalamin-t_2 (4.7 mCi/mmoles), 30 mM dihydrolipoate, and 1 mM EDTA in a total volume of 0.5 ml of dimethylglutarate buffer, pH 7.3, and was incubated at 37° for 10 min. Nucleotides were present in a concentration of 2 mM. [b] ATP was present in a concentration of 0.2 mM and the analogs in a concentration of 2 mM.

ability of the analogs to compete with the exchange reaction promoted by ATP they were also tested in combination with this substrate; in these experiments the analog was present in a tenfold excess over ATP. The data included in Table III show that the O-methylated ATPs do not significantly effect the ATP-promoted tritium exchange while the benzimidazolenucleotide and *xylo*-ATP inhibit this reaction. It is also evident that dATP and *ara*-ATP compete with ATP in the exchange reaction since the tritium released in the presence of both nucleotides is less than the sum of the radioactivities in reaction mixtures containing ATP and dATP or *ara*-ATP separately. This effect had been observed before with combinations of the natural nucleotides (Hogenkamp *et al.*, 1968).

Discussion

Substrate Specificity of the Enzyme. The reduction of BzTP by ribonucleotide reductase and the lack of reduction of all the other analogs illustrate that the β-D-ribofuranose moiety of the nucleotide is a basic requirement for a substrate of this enzyme. Although benzimidazole differs from all the natural purines or pyrimidines in its strongly hydrophobic character and does not have any site for hydrogen bond formation, the presence of this aglycone only retards reduction but does not entirely prevent it. The slow reaction rate and the stimulation by dGTP of this substrate closely resemble the data reported for the two analogs, toyocamycin and sangivamycin triphosphate (Suhadolnik *et al.*, 1968) in which the N-7 atom of adenosine is replaced by either a CCN or a $CCONH_2$ group. There is no striking relationship between reduction rates and the base structure in all the known substrates. However, the fact that GTP and CTP are reduced most rapidly suggests that both the amino and the carbonyl functions may mediate additional binding with protein groups at this end of the substrate molecule. Preparation of further model compounds such as modified GTP derivatives is necessary to test this hypothesis.

In view of the mutual inhibition of both pyrimidine and purine nucleotides mentioned above, it appears likely that ribonucleotide reductase has only one catalytic site capable of reducing any type of 5′-triphospho-β-D-riboside. While the triphosphate group is a prerequisite for reduction and cannot be replaced by phosphonate analogs with a β,γ-methylene bridge (R. L. Blakley, personal communication), this part of the molecule cannot be the only nor the main interaction site with the protein because the products, deoxyribonucleoside triphosphates, and most of the epimeric and methylated triphosphates described above are poor inhibitors of the enzyme system. Most attention must instead by given to the role of the two adjacent 2′- and 3′-hydroxyl groups in substrate binding and catalysis. From the inactivity of 3′-*O*-methyl-ATP, 3′-dATP, and *xylo*-ATP it seems clear that the free 3′-hydroxyl group is required for interaction with the protein, presumably *via* a hydrogen bridge. Likewise, the 2′-hydroxyl group has to interact with the enzyme in order to be replaced by a hydride ion, presumably after protonation of this hydroxyl function. The fact that only ribosides fulfill both these requirements and the lack of significant inhibition by any of the other compounds suggest that substrate binding and reduction proceed in a cooperative or concerted fashion: binding of a triphosphate cannot occur without concommitant reduction at the 2′-OH group nor can reduction occur without prior fixation at the 3′-OH group. Such an interdependence is in complete agreement with our previous finding that ATP-2′-^{18}O does not exchange its label in a system containing enzyme and coenzyme but no reductant (Follmann and Hogenkamp, 1969).

Interaction of the Effectors with the Enzyme. The interactions between ribonucleotide reductase and ribo- or deoxyribonucleotides that lead to the specific activation of individual reduction rates are more difficult to assess. The effectors almost certainly do not only affect enzyme–substrate binding, but also cause tighter binding of the coenzyme to the protein (H. P. C. Hogenkamp and C. Brownson, unpublished results).

The structure specificity of the effector site(s) differs greatly from that of the substrate site. Since the activation shows base specificity, binding of an effector to its site must result in rather specific protein–base interactions. Accordingly, BzTP with its abnormal base cannot serve as an activator for the reduction of any other substrate either because it does not bind to an effector site or because when bound it does not cause the desired conformational changes at the sub-

strate site. The latter possibility seems more reasonable because BzTP does inhibit the tritium exchange promoted by ATP and thus is able to bind to an effector site. On the other hand, the base specificity in the activation process is not absolute since dGTP stimulates not only the reduction of ATP and the pyrrolopyrimidine nucleotides but also of the benzimidazole nucleotide.

It is evident from the data in Tables II and III that in a series of analogous nucleotides with the same base, namely adenine, the capabilities of these analogs to function as activators of CTP reduction and to promote hydrogen exchange do not necessarily coincide. ATP and dATP are active, while *xylo*-ATP is inactive in *both* reactions. In contrast, the O-methylated ATP's are good activators without promoting tritium exchange, while *ara*-ATP is active in the exchange reaction but inhibits rather than stimulates CTP reduction. CTP reduction is also stimulated by 3′-dATP, but the activity of this nucleotide in the exchange reaction is not known (Suhadolnik *et al.*, 1968). These results suggest that the enzyme has at least two different effector sites for interaction with these nucleotides. One site is able to accommodate ribonucleotides, 2′- and 3′-deoxyribonucleotides, and 2′- and 3′-*O*-methylribonucleotides; binding of these nucleotides at this site causes specific stimulation of substrate reduction. The second site is able to accept ribonucleotides, 2′-deoxyribonucleotides, and arabino nucleotides; binding of these nucleotides affects enzyme–coenzyme interaction and thus promotes tritium exchange between 5′-deoxyadenosylcobalamin-5′-t_2 and water, (*via* a dithiol). It is, however, difficult to discern which specific properties of the sugar moieties control the different affinities for these two sites. There may be binding between the hydroxyl functions and the protein as is the case at the substrate site. Furthermore the orientation of the nucleotide base may be significantly altered by the sugar conformation in a methylribose-, arabinose-, or xylose-containing nucleotide. Unfortunately, the conformation of such uncommon nucleotides is unknown.

Arabinonucleotides simulate both ribo- and 2′-deoxyribonucleotides in their sugar structure. This property may account for the activity of *ara*-ATP and *ara*-CTP in the tritium-exchange reaction at the second effector site. The inhibitory action of these two nucleotides is probably not due to binding at the substrate site because both *ara*-ATP and *ara*-CTP only effectively inhibit ATP reduction. It is conceivable that in addition to occupying the second effector site, these *ara* nucleotides are able to bind at the first effector site where their abnormal structure induces such adverse changes in the enzyme–coenzyme–substrate complex that the reduction cannot take place.

While no effective natural inhibitors of the lactobacillus reductase are known, ribo- and deoxyribonucleotides can cause both activation or inhibition of the reductase of *E. coli;* for example, ATP stimulates the reduction of CDP and UDP in this system but dATP inhibits substrate reduction. This enzyme also has two different kinds of regulatory sites (Brown and Reichard, 1969).

The inhibitory effects of *ara*-ATP and *ara*-CTP upon the ribonucleotide reductase of *Lactobacillus leichmannii in vitro* are of general interest. They emphasize the antagonistic role of arabinose-containing analogs which have been described for DNA-connected enzyme systems in *E. coli* and other organisms (Cohen, 1966; Balis, 1968).

We have recently pointed out that the reaction mechanisms for the ribonucleotide reductases from *L. leichmannii* and *E. coli* are very similar, if not identical, despite the different substrate phosphorylation levels and cofactor requirements (Follman and Hogenkamp, 1969). *A priori*, such a similarity need not exist for other aspects of the substrate specificities because the relative reaction rates of various substrates and the pattern of activation or inhibition by other natural nucleotides differ widely among the enzymes isolated from different organisms. Nevertheless in the two well-studied bacterial reductase systems all the ribonucleotides are reduced by one enzyme, and there is no evidence for the existence of more than one enzyme in the less purified preparations from rat tumors (Moore and Hurlbert, 1966), *Rhizobium* (Cowles and Evans, 1968), or *Euglena* (Gleason and Hogenkamp, 1970). In all these enzymes the rather complex reduction process at C-2′ will require a highly ordered arrangement of substrate, enzyme, and cofactor groups at or near the catalytic site. The substrate specificities of most ribonucleotide reductases with respect to the sugar and sugar–base linkage may well be the same as that found for the *L. leichmannii* enzyme.

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Synthesis of Muramic Acid 6-Phosphate [2-Amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-(Dihydrogen Phosphate)]*

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ABSTRACT: Muramic acid 6-phosphate [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-(dihydrogen phosphate)] was synthesized starting from benzyl 2-acetamido-3-*O*-[D-1-(methoxycarbonyl)ethyl]-2-deoxy-β-D-glucopyranoside. Tritylation at C-6, acetylation at C-4, detritylation, dibenzyl phosphorylation, followed by catalytic hydrogenolysis, acid hydrolysis, and column chromatography gave crystalline muramic acid 6-phosphate. The same series of reactions was also performed in the α series.

The synthetic muramic acid 6-phosphate was characterized by infrared spectroscopy, X-ray powder diffraction, Elson–Morgan reaction, and by paper and thin-layer chromatography, and it was found to be identical with the natural muramic acid phosphates isolated from *Micrococcus lysodeikticus* cell wall and also from *Streptococcus pyogenes* cell wall.

The first evidence of the presence of an acid-stable muramic acid phosphate in bacteria was presented by Ågren and de Verdier (1958) who isolated the crystalline phosphate from the acid hydrolysate of *Lactobacillus casei*. Subsequently, an identical muramic acid phosphate was isolated from the cell walls of various Gram-positive bacteria (Hall and Knox, 1965; Heymann *et al.*, 1967; Liu and Gotschlich, 1967; Montague and Moulds, 1967; Grant and Wicken, 1968; Knox and Holmwood, 1968; Hungerer *et al.*, 1969). On the basis of the acid stability of the phosphate and of the results of the periodate oxidation, Ågren and de Verdier proposed the structure of a 6-phosphate, but the results of the periodate oxidation have in the past led to erroneous interpretations of structures containing muramic acid (Salton and Ghuysen, 1959, 1960; Ghuysen and Strominger, 1963). Since muramic acid 6-phosphate may serve as a link between the peptidoglycan structure and the cell wall polysaccharides or teichoic acids, it was of interest to obtain synthetically 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose 6-*O*-(dihydrogen phosphate) and compare it to the muramic acid phosphate isolated from natural products.

Results and Discussion

Since complete removal of the benzyl aglycon group can be effected by mild catalytic hydrogenolysis, the benzyl glycoside derivatives of *N*-acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] were selected as starting materials for the synthesis of muramic acid 6-phosphate (**7**) (see Scheme I). Benzyl 2-acetamido-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**1**) (Jeanloz *et al.*, 1968) was tritylated at C-6 and then acetylated at C-4 to give benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-6-*O*-triphenylmethyl-β-D glucopyranoside (**2**). Detritylation of compound **2** with hot 60% acetic acid gave benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside (**3**) which was subsequently phosphorylated with dibenzyl phosphorochloridate (Smith, 1961) to give benzyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-β-D-glucopyranoside 6-(dibenzyl phosphate) (**4**). Phosphorylation of compound **5** (the α anomer of compound **3**; Flowers and Jeanloz, 1963) with dibenzyl phosphorochloridate in the same fashion gave compound **6**. Hydrogenolysis of the benzyl glycosides of muramic acid is a very sluggish process (Flowers and Jeanloz, 1963; Osawa and Jeanloz, 1965; Osawa *et al.*, 1969), and the complete removal of the benzyl aglycon group of compound **4** or **6** required catalytic hydrogenolysis for 1 week in the presence of 10% Pd/C. In order to find out the optimal conditions of acid hydrolysis of the acetyl groups at C-2 and C-4, and of the methyl ester group at the lactyl moiety, the hydrogenolysates of compound **4** and **6** were treated with various concentrations of hydrochloric acid at 100°. The resulting hydrolysates were analyzed by paper electrophoresis and paper chromatography, and the results are reported in Table I. The hydrolysate with 1 M hydrochloric acid for 6 hr showed two spots; by treatment with acid phosphatase, as shown in Table I, one of the spots was identified

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