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# Stereochemistry of the Hydrolysis of the Endo Isomer of Uridine 2',3'-Cyclic Phosphorothioate Catalyzed by the Nonspecific Phosphohydrolase from *Enterobacter aerogenes*<sup>†</sup>

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ABSTRACT: The nonspecific phosphohydrolase from *Enter*obacter aerogenes (ATCC 13048) requires divalent metal ions for activity, since zinc present in the isolated enzyme can be removed by extensive dialysis against 8-hydroxyquinoline-5-sulfonate at pH 7.5 to yield an inactive enzyme which can be reactivated by addition of  $Zn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , or  $Ni^{2+}$ ; six ions of either zinc or cadmium can be incorporated into the inactive enzyme, and this incorporation of metal ion can be correlated with the regaining of activity (J. A. Gerlt, R. Dhesi, and H. C. Hemmings, unpublished experiments). The cadmium-reactivated phosphohydrolase catalyzes the hydrolysis of the endo isomer of uridine 2',3'-cyclic phospho-

Enzymes which catalyze the hydrolysis of phosphodiester bonds are ubiquitous in nature and are crucial to many biochemical processes. For example, cAMP<sup>1</sup> phosphodiesterase catalyzes the hydrolysis of the 3'-ester bond in cAMP, providing the only known pathway by which this important regulatory nucleotide can be inactivated and returned to the acyclic adenine nucleotide pool. Nucleases are involved in a rothioate (U>pS) to yield uridine 3'-monophosphorothioate as the major product. After enzymatic hydrolysis of the cyclic phosphorothioate in 19.8%  $H_2^{18}O$  and chemical recyclization of the <sup>18</sup>O-labeled acyclic phosphorothioates to yield a mixture of the endo and exo isomers of U>pS, <sup>18</sup>O is found primarily in the exo isomer, as judged by examination of the 145.7-MHz phosphorus-31 nuclear magnetic resonance spectrum of the mixture. This observation indicates that the cadmium phosphohydrolase catalyzes hydrolysis of *endo*-U>pS with inversion of configuration, implying that the hydrolysis reaction proceeds by an in-line attack of water on the phosphorus.

wide variety of in vivo and in vitro processes, including repair of genetic damage induced by mutagens and the construction of recombinant DNA molecules which are useful in gene

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<sup>&</sup>lt;sup>1</sup> Abbreviations used: cAMP, adenosine 3',5'-cyclic monophosphate; NMR, nuclear magnetic resonance; EDTA, ethylenediamine-N,N'tetraacetic acid; Mops, morpholine-N-(3-propanesulfonic acid); Ches, cyclohexylamine-N-(2-ethanesulfonic acid); HQSA, 8-hydroxyquinoline-5-sulfonic acid; U>p, uridine 2',3'-cyclic monophosphate; U>pS, uridine 2',3'-cyclic phosphorothioate; UMP, uridine monophosphate; UMPS, uridine monophosphorothioate; TLC, thin-layer chromatography; ATP $\alpha$ S, adenosine 5'-O-(1-thiotriphosphate); ATP $\beta$ S, adenosine 5'-O-(2-thiotriphosphate); NaDodSO<sub>4</sub>, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

cloning and large-scale production of a variety of peptides and proteins.

With the exception of the ribonucleases, very little is known about the mechanisms by which phosphodiesterases catalyze the hydrolysis of phosphate ester bonds. In the hydrolysis of RNA by ribonucleases, hydrolysis of the internucleotide bonds occurs via formation of 2',3'-cyclic nucleotide intermediates, which are then enzymatically hydrolyzed to yield the acyclic nucleotide products (Richards & Wyckoff, 1971). Enzymes which catalyze the hydrolysis of DNA and the structurally rigid cAMP molecule, inter alia, cannot utilize such intramolecular catalysis, and these enzymes must provide all of the functional groups which are necessary for catalysis. Although cAMP phosphodiesterases and deoxyribonucleases have been purified to homogeneity from a number of sources and some can be obtained commercially, few details are known about their catalytic mechanisms. At least in the case of the nucleases, this lack of crucial information has resulted, in part, from the complex structures of their substrates, which discourages the use of substrate analogues, an approach which has proved useful with many other types of enzymes.

One approach to formulating mechanisms by which these enzymes catalyze the hydrolysis of phosphate ester bonds is to examine a phosphodiesterase whose substrate specificity will permit the use of a wide variety of easily synthesized substrates and substrate analogues. The success of the studies on alkaline phosphatase (Coleman & Chlebowski, 1979) is in part due to the broad substrate specificity of this enzyme which includes both phosphate and phosphorothioate esters. The only phosphodiesterase known which will hydrolyze a wide variety of phosphate esters is the phosphohydrolase produced by Enterobacter aerogenes when it is grown on a phosphodiester as the sole source of phosphorus (Gerlt & Whitman, 1975). This enzyme can catalyze the hydrolysis of a wide variety of acyclic and cyclic phosphodiesters, including the simplest diester, dimethyl phosphate. Unlike many phosphodiesterases, this enzyme is also able, under certain conditions, to catalyze the hydrolysis of phosphate monoesters, and as discussed in this paper this enzyme can catalyze the hydrolysis of a chiral phosphorothioate, which permits stereochemical studies. Thus, a variety of approaches are available for investigating the mechanism of this enzyme.

Although the original characterization of the phosphohydrolase from E. aerogenes suggested that the enzyme was not dependent on metal ions for activity, research currently in progress in this laboratory has established that divalent metal ions are required for catalytic activity (J. A. Gerlt, R. Dhesi, and H. C. Hemmings, unpublished experiments). Lengthy dialysis of the enzyme against 8-hydroxyquinoline-5-sulfonate (HQSA) at pH 7.5 results in complete inactivation of the enzyme and removal of zinc ions originally bound to the enzyme; following removal of the chelator by gel filtration, an inactive enzyme can be obtained. Addition of any of a number of divalent metal ions ( $Zn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , and  $Ni^{2+}$ ) to the inactive enzyme results in restoration of catalytic activity. At least in the cases of  $Zn^{2+}$  and  $Cd^{2+}$ . the restoration of activity can be correlated with the incorporation of one tightly bound metal ion per monomeric unit. Thus, this enzyme, like the nucleases, alkaline phosphatase, and most other phosphohydrolases, requires a divalent metal ion for activity, making detailed study of this enzyme exceedingly important to a more thorough understanding of enzymic phosphate ester hydrolysis.

An exceedingly pertinent question to ask about the mechanism of this and all other phosphohydrolases is whether

catalysis requires formation of a covalent intermediate between the enzyme and phosphoryl group of the substrate, as has been established for alkaline phosphatase. Rather than initially attempting isolation of a potentially elusive intermediate, we have chosen to determine the stereochemical course of a reaction catalyzed by the phosphohydrolase from E. aerogenes. In the absence of pseudorotatory processes, the simplest explanation for an inversion of configuration at phosphorus is that a single displacement reaction has occurred; i.e., no intermediate is formed during catalysis. Retention of configuration would imply two displacement reactions and indicate that a covalent intermediate was involved in catalysis. Usher and Eckstein and their collaborators introduced the use of chiral phosphorothioates in stereochemical studies of phosphoryl-transfer enzymes (Usher et al., 1970), and this approach has been used to examine a variety of enzyme reactions. More recently, Knowles' group has reported a method for the synthesis and determination of the absolute configuration of [<sup>16</sup>O,<sup>17</sup>O,<sup>18</sup>O]phosphoryl groups (Abbott et al., 1978) and demonstrated the utility of this approach by examining the reactions catalyzed by alkaline phosphatase (Jones et al., 1978), glycerol kinase, and acetate kinase (Blättler & Knowles, 1979). Both the phosphorothioate and oxygen isotope approaches have demonstrated that stereochemical studies offer a very powerful and efficient method for determining whether covalent catalysis is important in phosphoryl-transfer enzymes.

In the research described in this paper, we utilized the phosphorothioate approach to determine the stereochemical course of a reaction catalyzed by the cadmium phosphohydrolase from E. aerogenes. In our experimental procedure, we incorporated Cohn's recent observation (Cohn & Hu, 1978) that <sup>18</sup>O induces a perturbation in the chemical shift of the phosphorus nuclei to which it is bonded to simplify the isotopic determinations required in this method. We found that the enzyme-catalyzed hydrolysis of the endo isomer of uridine 2'.3'-cyclic phosphorothioate (endo-U>pS) proceeds with inversion of configuration, suggesting that catalysis by this enzyme does not involve formation of a covalent intermediate. Thus, divalent metal ion requiring phosphohydrolases either may catalyze the direct attack of water on the phosphoryl group of the substrate (this enzyme) or may initially form a phosphoryl enzyme which is then attacked by water to yield the products (alkaline phosphatase).

#### **Experimental Section**

Methods. Melting points were measured in open capillaries with a Hoover melting point apparatus and were corrected. Phosphorus-31 NMR spectra at 32 MHz were obtained at ambient temperature with a Varian CFT-20 spectrometer equipped with a phosphorus probe. Spectra of <sup>18</sup>O-containing phosphorothioates at 145.7 MHz were obtained with the Bruker WH-360 spectrometer at the Middle Atlantic High Resolution NMR Facility at the University of Pennsylvania. Phosphorus chemical shifts are expressed relative to an external capillary containing 85% H<sub>3</sub>PO<sub>4</sub>; upfield chemical shifts are expressed as negative numbers. High-resolution proton spectra were obtained at 270 MHz with the Bruker HX-270 spectrometer at the Southern New England High Resolution NMR Facility at Yale University.

Buffers were prepared metal-free by extraction with 0.01% dithizone in CCl<sub>4</sub>, Sephadex by soaking in 0.01 M EDTA, pH 7.5, and glassware was prepared by soaking in a 1:1 mixture of nitric and sulfuric acids.

Materials. Uridine, imidazole, Mops, and Ches were purchased from Sigma. 2,6-Lutidine was obtained from Eastman. Diethyl phosphorochloridate and HQSA were products of Aldrich. Thiophosphoryl chloride was the product of Alfa Ventron. Specpure metal salts were purchased from Johnson Matthey. The 20.6% enriched  $H_2^{18}O$  was obtained from Prochem. All other reagents were the best grade commercially available.

Phosphohydrolase. The phosphohydrolase from E. aerogenes (ATCC 13048) was prepared with minor modification of the published procedure (Gerlt & Whitman, 1975). A 0.15-volume amount of a 2% solution of protamine sulfate, pH 7.5, was used to precipitate nucleic acids from the crude extract. The enzyme preparations employed in these studies were at least 95% pure as judged by NaDodSO<sub>4</sub> gel electrophoresis. The enzyme was routinely assayed by monitoring the rate of production of 4-nitrophenolate from 1 mM bis-(4-nitrophenyl) phosphate in a buffer containing 0.025 M Mops and 0.025 M Ches, pH 8.5, at 30 °C.

Zinc and Cadmium Phosphohydrolase. Inactive enzyme was prepared by dialysis of the phosphohydrolase against 10 mM HQSA-0.3 M NaCl, pH 7.5  $\pm$  0.5, for at least 2 weeks followed by gel filtration on metal-free Sephadex G-25 equilibrated in a metal-free buffer containing 0.025 M Mops and 0.025 M Ches, pH 8.0. The samples of inactivated enzyme utilized in this study routinely had specific activities less than 1% of the activity of the original enzyme.

The inactive enzyme ( $\sim 0.1 \text{ mM}$  in monomeric units) was reactivated by addition of either zinc sulfate or cadmium sulfate to a final concentration of 1 mM. The solution was incubated at 4 °C for 2 h and gel filtered on a column of metal-free Sephadex G-25 equilibrated in the pH 8.0 metal-free buffer to remove excess metal ions. Under these conditions, we routinely find that one divalent metal ion is incorporated per monomeric unit and that its presence can be correlated with restoration of catalytic activity to the inactive enzyme. This stoichiometry has been established by atomic absorption spectrometry, incorporation of <sup>109</sup>Cd and <sup>65</sup>Zn, and measurement of enzyme activity as a function of the added metal to protein ratio. A detailed description of these experiments as well as the physical and chemical properties of the zinc and cadmium enzymes will be reported in a forthcoming manuscript (J. A. Gerlt, R. Dhesi, and H. C. Hemmings, unpublished experiments).

Endo Isomer of Uridine 2',3'-Cyclic Phosphorothioate (endo-U > pS). 5'-Acetyluridine (Brown et al., 1956) and triimidazolylphosphine sulfide (Eckstein, 1970) were prepared according to literature methods and used for the synthesis of the endo and exo isomers of U>pS according to the procedure of Eckstein & Grindl (1968) with modifications suggested to us by Professor David Usher of Cornell University. In a typical preparation, 8 g of 5'-acetyluridine and 15 g of triimidazolylphosphine sulfide were dissolved under a dry nitrogen atmosphere in 500 mL of freshly distilled 2,6-lutidine and allowed to react overnight at room temperature. The reaction mixture was evaporated to dryness in vacuo, and the residue was dissolved in 200 mL of ammonium bicarbonate buffer (100 mL of 5 M NH<sub>4</sub>OH and 100 mL of 1 M ammonium bicarbonate) and stirred for 2 h at room temperature. The solution was evaporated to dryness, and the residue was dissolved in 150 mL of distilled water. This solution, which contained the product (as judged by <sup>31</sup>P NMR), was applied to a  $3 \times 100$  cm column of DEAE-Sephadex A-25 (HCO<sub>3</sub><sup>-</sup>), and the product was eluted with a 6-L gradient from 0.1 to 0.4 M triethylammonium bicarbonate, pH 8.5. The fractions containing the mixture of U>pS isomers were identified by TLC (silica gel; 2-propanol-concentrated ammonia-water, 7:1:2) and <sup>31</sup>P NMR, combined, and evaporated to dryness in vacuo. The dried residue was crystallized 4 times from 95% ethanol to yield 0.70 g (6%) of the crystalline triethylammonium salt of U>pS, mp 203.9-205.9 °C (lit. mp 197-201 °C), which was 96% endo and 4% exo, as judged by <sup>31</sup>P NMR.

Enzymatic Hydrolysis of Uridine 2',3'-Cyclic Phosphate (U>p). Kinetic constants for the hydrolysis of U>p by the cadmium enzyme were determined at pH 8.5 with a Radiometer pH stat.

The identities of the hydrolysis products obtained from U>p in the presence of the native, zinc, and cadmium enzymes were determined by <sup>31</sup>P NMR. In a typical procedure, 20 mg of U>p and enzyme were incubated for 2 h in a buffer of 0.025 M Mops and 0.025 M Ches, pH 8.5, containing 50% D<sub>2</sub>O. A 0.05-mL amount of a 0.1 M EDTA solution, pH 7.5, was added, and the <sup>31</sup>P NMR spectrum was obtained. Authentic 2'-UMP was then added to the enzymatic product, and the <sup>31</sup>P spectrum was again recorded.

Enzymatic Hydrolysis of endo-U>pS. Kinetic constants for the hydrolysis of endo-U>pS by the cadmium enzyme were determined at pH 8.5 with a pH stat.

The identity of the hydrolysis product obtained from *endo*-U>pS in the presence of the cadmium enzyme was determined by analysis of the proton spectra obtained at 270 MHz with and without selective proton ( $C_{1'}$ ) and phosphorus decoupling. The enzymatic product was the <sup>18</sup>O-labeled sample used in the stereochemical studies. The <sup>31</sup>P NMR spectrum of this sample at 32 MHz consisted of two major resonances, with the upfield resonance being larger (93:7).

Enzymatic Hydrolysis of exo-U>pS. A sample of exo-U>pS, contaminated by 11% of the endo isomer (as judged by <sup>31</sup>P NMR), was hydrolyzed by the cadmium enzyme in the pH 8.5 buffer containing 30% D<sub>2</sub>O. After the reaction was completed, EDTA was added and the <sup>31</sup>P NMR spectrum was obtained. Two resonances attributable to the acyclic phosphorothioates were obtained, with the upfield resonance being larger (85:15).

Enzymatic Hydrolysis of endo-U>pS in  $H_2^{18}O$ . Two identical reactions were separately carried out, and the hydrolysis products obtained were combined for subsequent chromatographic purification, chemical cyclization, and isotopic analysis.

In each of two 25-mL metal-free flasks, 40 mg of crystalline endo-U>pS and solid Mops, Ches, and cadmium sulfate were dissolved in 2.40 mL of 20.6% <sup>18</sup>O-enriched water (the labeled water was used as received). Each solution was brought to pH 8.5 by addition of a few microliters of a concentrated solution of sodium hydroxide. To each solution was added 4.5 mg of cadmium phosphohydrolase [specific activity 4.7  $\mu$ mol/(min mg) at pH 8.5] dissolved in 100  $\mu$ L of H<sub>2</sub><sup>16</sup>O buffer containing 25 mM Mops and 25 mM Ches, pH 8.5. The amounts of solid Mops, Ches, and cadmium sulfate originally placed in the flasks were such that their final concentrations were 25, 25, and 1 mM, respectively. The sealed flasks were incubated at 37 °C, and the pH values of the solutions were checked occasionally to ensure that they remained greater than 8.0, with microliter aliquots of concentrated sodium hydroxide solution being added to adjust the pH. After 22 h, the reactions were  $\sim$ 90% completed as judged by TLC; the reaction solutions were frozen, and the solvent was removed by lyophilization.

Each of the lyophilized residues was dissolved in 1 mL of deionized water and gel filtered on a  $1 \times 39$  cm column of Sephadex G-25 equilibrated in deionized water. The columns were eluted with water, and the fractions containing the

hydrolysis product were combined and evaporated to dryness. The residues were dissolved in water, combined, and applied to a  $1.2 \times 50$  cm column of DEAE-Sephadex A-25 (HCO<sub>3</sub><sup>-</sup>), and the uridine phosphorothioates were eluted with a 1.0-L gradient of 0.10–0.40 M triethylammonium bicarbonate, pH 8.5. Fractions containing the acyclic phosphorothioates were combined and concentrated: yield, 1196 OD<sub>260</sub> units or 60.0 mg.

Nonenzymatic Hydrolysis of endo-U>pS in  $H_2^{18}O$ . Forty milligrams of endo-U>pS was dissolved in 1.5 mL of the <sup>18</sup>O-labeled water lyophilized from the enzymatic reaction, and the solution was brought to pH 13.0 by addition of solid sodium hydroxide. After 14 h, the hydrolysis reaction was completed, as judged by <sup>31</sup>P NMR. The solution was lyophilized and the residue purified by ion-exchange chromatography as described for the enzymatic reaction: yield, 616 OD<sub>260</sub> units or 31.0 mg.

Chemical Cyclization of <sup>18</sup>O-Acyclic Uridine Phosphorothioates. The enzymatic hydrolysis product, 996 OD<sub>260</sub> units (0.123 mmol), was dried in a 10-mL flask by storage over P<sub>2</sub>O<sub>5</sub> in vacuo. About 0.7 mL of pyridine freshly distilled from CaH<sub>2</sub> under a dry nitrogen atmosphere was used to dissolve the phosphorothioate. The solution was stirred under dry nitrogen, and 1 equiv of redistilled diethyl phosphorochloridate was added; after 15 min, the reaction mixture was evaporated to dryness. The residue was dissolved in 50 mL of water and purified by the usual ion-exchange chromatography on Sephadex A-25. The fractions containing the mixture of cyclic phosphorothioates were combined and concentrated. The conversion to cyclic esters was ~60% completed under these conditions, as judged by <sup>31</sup>P NMR.

An analogous procedure was used for the acyclic phosphorothioates obtained by nonenzymatic hydrolysis; 523  $OD_{260}$ units (0.0646 mmol) was dissolved in 0.5 mL of dry pyridine, cyclized for 15 min using 1 equiv of diethyl phosphorochloridate, and purified by ion-exchange chromatography.

Phosphorus-31 NMR of [<sup>18</sup>O]Uridine Phosphorothioates. The acyclic phosphorothioates obtained from the hydrolyses and the cyclic phosphorothioates obtained from the cyclization reactions were dissolved in 1.5-mL aliquots of D<sub>2</sub>O, and the solutions were made 2 mM in Na<sub>2</sub>EDTA. Proton-decoupled <sup>31</sup>P NMR spectra were obtained at either 32 or 145.7 MHz for phosphorus with either a Varian CFT-20 or a Bruker WH-360 NMR spectrometer, respectively. Approximately 2000 transients were required for acceptable spectra at 32 MHz, and ~100 transients were required at 145.7 MHz. The 145.7-MHz spectra were obtained with a 16-s acquisition time, so integrals of resonance intensities are accurate measurements of the relative amounts of the various species present.

#### Results

The enzyme obtained directly from the purification procedure is incapable of catalyzing the complete hydrolysis of endo-U>pS, even after long incubation times. While these stereochemical studies were in progress, it was discovered that the phosphohydrolase was dependent on metal ions for activity, since it could be inactivated by dialysis against HQSA and reactivated by a number of divalent metal ions. These studies revealed that when the inactivated enzyme was reactivated with either zinc or cadmium according to the procedure described in this manuscript, one metal ion was incorporated per monomeric unit (J. A. Gerlt, R. Dhesi, and H. C. Hemmings, unpublished experiments). In addition, samples of the purified enzyme have been found to contain approximately three zinc and three manganese ions per hexameric unit (see Discussion). We, therefore, examined the ability of the cadmium and zinc enzymes to hydrolyze endo-U>pS. Each

Table I:	Kinetic Constants for the Cadmium Phosphohydrolase <sup>a</sup>

substrate	V <sub>max</sub> [μmol/(min mg)]	$K_{\mathbf{m}}$ (mM)	
U>n	7.6	5.6	
U>pS	0.5	1.8	

<sup>a</sup> Initial velocity measurements were performed with a pH stat at 37  $^{\circ}$ C and pH 8.5.

Table II: <sup>18</sup> O Conte	<sup>18</sup> O Content of Nucleoside Phosphorothioates <sup>a</sup>					
	2'-UMPS (%)	3'-UMPS (%)	endo-U>pS (%)	exo-U>pS (%)		
enzyme hydrolysis	23.6	21.8	3.9 (18) <sup>b</sup>	17.5 (82) <sup>b</sup>		
chemical hydrolysis	16.8	16.1	3.9 (22) <sup>b</sup>	14.1 (78) <sup>b</sup>		

<sup>a</sup> Percentage of total area in <sup>18</sup>O-perturbed resonances shown in Figures 1-4. <sup>b</sup> Percentage of the total <sup>18</sup>O found in both isomers.

of these enzymes was capable of catalyzing at least partial hydrolysis of the cyclic ester  $(R_f 0.49)$  to yield acyclic uridine phosphorothioates  $(R_{f} 0.20)$ . However, the zinc enzyme also catalyzed subsequent hydrolysis of the P-S bond in the hydrolysis product to yield initially UMP ( $R_f 0.12$ ) and then more slowly uridine  $(R_f 0.49)$  and inorganic phosphate; the acyclic nucleoside phosphorothioate was inert in the presence of the cadmium enzyme. In addition, the hydrolysis catalyzed by the zinc enzyme did not proceed to more than  $\sim 25\%$  completion, even after an incubation period of 1 week. The incomplete hydrolysis is due to a potent inhibition of the zinc enzyme by the sulfide produced by enzymatic hydrolysis of the P-S bond in the acyclic phosphorothioate and the inorganic phosphate arising from hydrolysis of UMP (J. A. Gerlt and W. H. Y. Wan, unpublished experiments). For this reason, our stereochemical studies were performed with the cadmium enzyme.

Kinetic Parameters for the Hydrolysis of U>p and endo-U>pS. Values for the  $K_m$  and  $V_{max}$  for the hydrolysis of U>p and endo-U>pS by the cadmium enzyme are given in Table I.

Identity of Hydrolysis Products. U>p is hydrolyzed by the native enzyme and both the cadmium and zinc enzymes to yield predominately 3'-UMP (3'-UMP/2'-UMP = 94:6). No hydrolysis of UMP to uridine and inorganic phosphate by the zinc enzyme was observed in these experiments, in contrast to that observed in the hydrolysis of endo-U>pS. The explanation for this difference is that a smaller amount of enzyme and a shorter reaction time were used for the hydrolysis of U>p.

The endo isomer of U>pS was hydrolyzed by the cadmium and zinc enzymes to yield predominately 3'-UMPS (3'-UMPS/2'-UMPS = 93:7).

The exo isomer of U>pS, contaminated by 11% of the endo isomer, was hydrolyzed by the cadmium enzyme to yield a mixture of products in which the major product was 3'-UMPS (3'-UMPS/2'-UMPS = 85:15).

Stereochemical Analysis. A sample of the endo isomer of U>pS was hydrolyzed by the cadmium enzyme in 19.8% <sup>18</sup>O-enriched water to yield a mixture of the two hydrolysis products, of which the 3' isomer was the major product (93:7). For both products we observed an unperturbed [<sup>16</sup>O]phosphorus resonance and a single <sup>18</sup>O-perturbed resonance (Cohn & Hu, 1978), the intensities of which were those expected on the basis of the isotopic composition of the water (Figure 1; isotopic enrichment data are tabulated in Table II). Only a single <sup>18</sup>O-perturbed resonance was present for each product,



FIGURE 1: 145.7-MHz <sup>31</sup>P NMR spectrum of acyclic nucleoside phosphorothioates obtained by enzymatic hydrolysis of *endo*-U>pS in 19.8%  $H_2^{18}O$ . The chemical shift of 2'-UMPS is 44.20 ppm, and that of 3'-UMPS is 43.45 ppm.



FIGURE 2: 145.7-MHz <sup>31</sup>P NMR spectrum of acyclic nucleoside phosphorothioates obtained by base-catalyzed hydrolysis of *endo*-U>pS in  $H_2^{18}O$ .

indicating that the enzyme did not catalyze a nonstereospecific exchange of the phosphorothioate oxygen atoms of the product with the solvent.

The alkaline hydrolysis of *endo*-U>pS yielded an approximately equimolar mixture of the 2'- and 3'-phosphorothioates (Figure 2). The isotopic enrichment data are given in Table II.

The hydrolysis products obtained from the enzymatic and alkaline hydrolyses were cyclized as described in the previous



FIGURE 3: 145.7-MHz  $^{31}$ P NMR spectrum of cyclic nucleoside phosphorothioates obtained by chemical cyclization of the acyclic products resulting from enzymatic hydrolysis of *endo*-U>pS. The chemical shift of the endo isomer is 75.00 ppm, and that of the exo isomer is 76.37 ppm.



FIGURE 4: 145.7-MHz <sup>31</sup>P NMR spectrum of cyclic nucleoside phosphorothioates obtained by chemical cyclization of the acyclic products resulting from base-catalyzed hydrolysis of *endo*-U>pS.

section, i.e., by using 1 equiv of diethyl phosphorochloridate and a reaction time of 15 min. When larger amounts of the phosphorochloridate and longer reaction times were used, the cyclization was more complete but both loss of <sup>18</sup>O label and racemization were observed.

Chemical cyclization of the <sup>18</sup>O-labeled hydrolysis products obtained from the enzymatic and the alkaline hydrolyses of *endo*-U>pS resulted in formation of nearly equal amounts of the endo and exo isomers of U>pS (Figures 3 and 4). In each isomeric mixture of <sup>18</sup>O-labeled U>pS, ~80% of the <sup>18</sup>O was associated with the exo isomer (Table II). This distribution of label between the two diastereomeric species is similar to those reported for the uridine phosphorothioate ring closure reaction by Usher et al. (1970) and for the glycerate phosphorothioate ring closure reaction reported by Orr et al. (1978). Negligible loss of <sup>18</sup>O label occurred during the cyclization reaction.

The chemical shift difference between the <sup>16</sup>O and <sup>18</sup>O resonances was 0.032 ppm for the acyclic phosphorothioates and 0.041 ppm for the cyclic phosphorothioates. At 32 MHz these differences are readily observable, but the two resonances are not cleanly separated; at 145.7 MHz the two resonances are nearly base-line separated, as illustrated in the figures.

#### Discussion

A major research objective of this laboratory is to elucidate the mechanisms of phosphodiesterases which catalyze the hydrolyses of substrates with structures that cannot facilitate catalysis by intramolecular assistance. Deoxyribonucleases constitute the major class of enzymes in which we are interested, since, in contrast to the ribonucleases, their mechanisms remain obscure despite considerable effort in other laboratories. We are also studying cAMP phosphodiesterase, which has received considerably less mechanistic attention. Most of these enzymes require divalent metal ions for activity, and it is for this reason that we believe that a detailed understanding of the mechanism of the phosphohydrolase from E. aerogenes will be important to our studies of the other enzymes. The broad substrate specificity of this enzyme, which includes acyclic and cyclic phosphodiesters, but not DNA (W. H. Y. Wan and M. Leahy, unpublished experiments), and phosphorothioates, facilitates this undertaking. Stereochemical investigations of the mechanisms of a number of phosphoryland nucleotidyl-transfer enzymes have been reported (Usher et al., 1970, 1972; Eckstein et al., 1972; Sheu & Frey, 1978; Richard & Frey, 1978; Li et al., 1978; Burgers & Eckstein, 1978, 1979; Jones et al., 1978; Orr et al., 1978; Midelfort & Sarton-Miller, 1978; Blättler & Knowles, 1979; Bryant & Benkovic, 1979), and the results of these studies indicate that this approach is extraordinarily useful in determining whether the catalytic mechanisms involve formation of a covalent intermediate between the enzyme and phosphoryl group of the substrate. Thus, we have chosen to examine the stereochemistry of a reaction catalyzed by the phosphohydrolase from E. aerogenes at an early stage of the detailed chemical study of this enzyme to provide evidence as to whether a transitory covalent intermediate is mechanistically significant.

The stereochemical studies we have described in this paper utilized the cadmium phosphohydrolase, which was prepared from enzyme inactivated by dialysis against HQSA. The zinc enzyme did not bring about complete hydrolysis of endo-U>pS, even after prolonged incubation with the enzyme; this problem did not occur with the cadmium enzyme. In addition, the zinc enzyme, but not the cadmium enzyme, catalyzed hydrolysis of the P-S bond in the acyclic phosphorothioate product. The problem of incomplete hydrolysis also occurred with what might be termed "purified" rather than "native" enzyme. We have chosen to refer to the enzyme obtained from the purification as purified rather than native enzyme since, despite considerable effort, we do not know the in vivo metal composition of the enzyme. Samples of homogeneous purified enzyme contain about three zinc ions and three manganese ions per hexameric unit, whereas the zinc and cadmium enzymes prepared from the inactive enzyme contain six catalytically essential zinc or cadmium ions per hexameric unit. We believe that the native enzyme should contain only one kind of metal ion in an amount stoichiometric with the number of subunits. A possible explanation for the metal content of the purified enzyme is that the purification procedure includes a necessary heat transfer at 60 °C for 10 min; under these conditions, zinc enzyme prepared from inactive enzyme can be activated by manganese, with the activation being accompanied by loss of zinc from and incorporation of manganese into the enzyme (J. A. Gerlt and R. Dhesi, unpublished experiments). Our work with the phosphohydrolyase has been plagued by isolation of homogeneous samples of purified enzyme with capriciously variable specific activities, and a reasonable explanation for this behavior is heat-promoted exchange during purification of the in vivo metal ion with other divalent metal ions which are present in the crude extracts. (Of the metal ions which restore activity to the apoenzyme, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, and Co<sup>2+</sup>, we would assume that zinc is the most likely candidate for the in vivo metal.) Since the purified enzyme does not catalyze the hydrolysis of endo-U>pS to completion and since it apparently does not contain one kind of essential metal ion, we chose to work with enzyme samples containing stoichiometric amounts of a specific metal ion in the present studies.

Studies recently reported by Jaffe and Cohn demonstrate that metal ion coordination with thiophosphates depends on the identity of the metal ion (Jaffe & Cohn, 1978). For example, Mg<sup>2+</sup> preferentially chelates with the oxygen atom of the  $\beta$ -phosphorus of ATP $\beta$ S whereas Cd<sup>2+</sup> chelates to the sulfur atom. Since our stereochemical study utilized the cadmium phosphohydrolase and the metal ion could participate in catalysis by coordination with the anionic substrate, we determined the identity of the products obtained from the enzyme-catalyzed hydrolysis of U>p, the endo isomer of U>pS(contaminated by 4% of the exo isomer), and the exo isomer of U>pS (contaminated by 11% of the endo isomer). In each case, the predominant product was the 3' isomer of the acyclic nucleotide. Thus, if cadmium is chelating to the substrate, this coordination does not affect the stereospecificity of the hydrolysis reaction.

The observation that the zinc phosphohydrolase catalyzed P–S bond cleavage in UMPS whereas the cadmium enzyme did not might be explained by the coordination preferences of the metal ions, if the metal ion does in fact directly coordinate to the substrate at the active site. Cadmium is known to prefer sulfide ligands more than zinc does (Cotton & Wilkinson, 1972), and depending on the required relationship between the metal ion and leaving group this effect could prevent hydrolysis of the P–S bond in the cadmium-catalyzed hydrolysis. At this time, we have no unequivocal evidence that the metal ion participates in catalysis by coordination with the substrate.

The stereochemical result which we report in this paper demonstrates that the hydrolysis of *endo*-U>pS catalyzed by the cadmium phosphohydrolase is accompanied by *inversion* of configuration at phosphorus. We interpret this result to indicate that the enzyme catalyzes the direct attack of water on the cyclic phosphorothioate to directly yield the acyclic product, i.e., without initial formation of a covalent intermediate between the acyclic nucleotide moiety and enzyme followed by attack of water on the phosphorus to yield the hydrolysis product. In the hydrolysis of a phosphate or phosphorothioate ester, the principle of microscopic reversibility predicts that in a two-step mechanism the formation and breakdown of the intermediate would occur by steps which were the reverse of one another, leading to an overall retention of configuration. Such results were obtained for the two steps of the ribonuclease reaction (Usher et al., 1970, 1972), and Knowles' group has recently reported that a reaction catalyzed by alkaline phosphatase, in which a two-step mechanism has been unambiguously established by other techniques, occurs by overall retention of configuration at phosphorus (Jones et al., 1978). We, therefore, believe that it is unlikely that our results should be explained by a two-step mechanism in which one nucleophilic displacement occurs with inversion and the second with retention of configuration. A single-step displacement which was accompanied by retention of configuration would imply the participation of pseudorotation, and the evidence accumulated to this time indicates that such processes may not be important for phosphoryl and nucleotidyl group transfer reactions involving monoanionic phosphoryl groups. However, as will be discussed in this section, pseudorotation could be important in the transfer of dianionic phosphoryl groups.

The phosphorothioate approach to determining the stereochemical course of a reaction often requires that the <sup>18</sup>O composition of a diastereomeric pair of phosphorothioates be determined. In the past this requirement has necessitated physical separation of the mixture, either by crystallization (Usher et al., 1970) or by gas chromatography, if the phosphorothioates could be modified to be volatile (Orr et al., 1978). Moreover, if the components of the pair cannot be readily volatilized, e.g., the isomers of U>pS, the isotopic analysis can be accomplished only after a tedious combustion procedure. In this study we have taken advantage of the <sup>18</sup>O perturbation of phosphorus chemical shifts (Cohn & Hu, 1978; Lowe & Sproat, 1978) so that isotopic analysis of the diastereomeric pair could be directly and quickly determined. Since the phosphorus chemical shifts of the endo and exo isomers of U>pS can be confidently assigned and since their chemical shifts are relatively close to one another, the <sup>18</sup>O composition of each diastereomer can be readily determined by examination of the proton-decoupled high-field <sup>31</sup>P NMR spectrum of the pair. We assume that <sup>18</sup>O-perturbed chemical shifts [be they of phosphorus or carbon (Van Etten, 1979)] will be extraordinarily useful in future stereochemical studies.

That the phosphohydrolase is absolutely dependent on divalent metal ions for activity and that the stereochemical course of the hydrolysis reaction is inversion suggest a hypothesis about the mechanism for this enzyme which is similar to those advanced about the mechanisms of other hydrolytic enzymes, including staphylococcal nuclease (Mildvan, 1974), carbonic anhydrase (Bauer et al., 1976), and chymotrypsin (Makinen et al., 1979); i.e., the metal ion promotes the acidity of a water molecule to provide the hydroxide ion nucleophile required for the hydrolysis reaction. This proposal is consistent with the pH dependence of the kinetic parameters for the hydrolysis of bis(4-nitrophenyl) phosphate by the zinc and cadmium enzymes; the  $k_{cat}/K_m$ -pH profiles have maxima which differ by 3 pH units, with the zinc enzyme being a better catalyst at acidic pH than the cadmium enzyme (J. A. Gerlt, R. Dhesi, and H. C. Hemmings, unpublished experiments). This 3 pH unit difference is the same as that reported for the relative acidities of water coordinated to these metal ions in solution (Chabarek et al., 1952). The pH dependence of this kinetic parameter can have other explanations, e.g., displacement of chloride (or another) anion from the coordination sphere of the metal as the pH is increased, and further work on this enzyme will focus on distinguishing the possible alternatives.

Our stereochemical result demonstrates that a mode of rate acceleration potentially available to metal-dependent phosphohydrolases is not important in the hydrolysis of U>pScatalyzed by the E. aerogenes enzyme. Formation of a four-membered ring bidentate chelate between the substrate and metal could lead to significant rate acceleration, since it would be expected to induce significant strain in the substrate. Sargeson and his collagues recently documented the formation of such a complex between Co3+ and inorganic phosphate and reasoned that the Co<sup>3+</sup>-promoted hydrolysis of 4-nitrophenyl phosphate in base occurs by nucleophilic attack of hydroxide on the strained chelate (Anderson et al., 1977). The rate acceleration observed in very basic solution (pH 13.5) was 10<sup>9</sup> relative to the rate observed for the uncoordinated ester at the same pH. However, strain induction by bidentate chelate formation should have important stereochemical implications. Sargeson's observations would suggest that the rate acceleration could be explained in analogy to that reasoning used to explain the rapid rate of hydrolysis of methyl ethylene phosphate, i.e., relief of strain upon attack of the nucleophile by formation of a trigonal-bipyramidal intermediate (Westheimer, 1968). If the usual rule of apical attack-apical expulsion would be applicable to the Co<sup>3+</sup>-4-nitrophenyl phosphate chelate, the 4-nitrophenolate anion could leave only after a pseudorotation had occurred; i.e., the hydrolysis reaction would have to occur with retention of configuration at phosphorus. Whether this is true cannot be immediately ascertained since the product of the reaction that Sargeson's group studied was inorganic phosphate.

For endo-U>pS, such a chelate might not be expected if the metal ion does coordinate the substrate since it would induce additional destabilization in an already strained structure (a five-membered ring diester). The possibility does remain, however, that this type of coordination could be important in the hydrolysis of acyclic phosphodiesters catalyzed by the enzyme.<sup>2</sup> Although bidentate coordination should be more important for dianionic rather than for monoanionic phosphoryl groups by virtue of their greater charge, we do not feel that we can dismiss this interesting consideration without additional experimentation. The most direct approach is a stereochemical one, but that the enzyme apparently does not catalyze the hydrolysis of acyclic phosphorothioate O,O-diesters (N. Cataldo and F. H. Westheimer, personal communication) indicates that only the oxygen isotope method can be used, and such a study is underway in this laboratory.

That endo-U>pS is hydrolyzed with inversion of configuration by the cadmium enzyme strongly suggests that its hydrolysis, and, by analogy, that of any other substrate, does not involve covalent catalysis. As pointed out earlier in this section, this stereochemical study was only possible with the cadmium enzyme. However, we believe that the result obtained for this enzyme, i.e., hydrolysis occurring without covalent catalysis, should be true for enzyme containing any of the other divalent metal ions which can activate chelator-inactivated enzyme,  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , or Ni<sup>2+</sup>. At the present time we have no direct evidence that the metal ion is

<sup>&</sup>lt;sup>2</sup> A referee pointed out that it is unlikely that the metal ion would both promote the acidity of a water molecule to provide the nucleophile and also neutralize the phosphate ester charge if the metal ion is intimately involved in catalysis. We agree with this supposition, but in view of the limited amount of information available concerning the mechanism of this enzyme, it is impossible for us to clearly define the relationship between the metal, substrate, and water. For this reason, we have described the mechanistic implications our stereochemical result would have for each of the possible functions, recognizing that they are probably mutually exclusive.

intimately involved in catalysis, but even if it does play a direct role in catalysis we certainly do not anticipate that catalysis accompanied by formation of a covalent intermediate should occur with some metals but not with others. Therefore, we are confident that the hydrolysis of phosphate esters catalyzed by this enzyme always involves a single displacement reaction at phosphorus. If we should find that acyclic diesters are hydrolyzed with retention of configuration, the best explanation would therefore be a single displacement reactions.

Sargeson's results on the Co<sup>3+</sup>-promoted hydrolysis of 4-nitrophenyl phosphate suggest that the assumption that pseudorotation will not occur in enzymatic reactions need not be correct for the metal-dependent enzyme-catalyzed hydrolysis of phosphate monoesters, for which bidentate coordination would be more likely than for diesters or monoanionic phosphoryl groups in pyrophosphates. An interesting question raised by this consideration is whether the hydrolyses of monoesters catalyzed by phosphomonoesterases proceed with retention rather than with inversion of configuration. Such a hypothesis cannot be easily tested with the phosphohydrolase, which catalyzes monoester hydrolyses at rates  $\sim 10\%$  or less of those observed for diesters, since we are not aware of any transphosphorylation reactions that it can catalyze. However, alkaline phosphatase does catalyze both hydrolysis and transphosphorylation reactions of monoesters, including phosphoryl transfer to the enzyme to form the required covalent intermediate. Perhaps both enzyme phosphorylation and dephosphorylation steps are promoted by bidentate chelate formation. Evidence for a strained phosphoryl group as the covalent intermediate has been obtained from the <sup>31</sup>P NMR chemical shifts of the intermediate (Chlebowski et al., 1976) and supports the idea that the metal ion promotes hydrolysis by the strained chelate mechanism. Thus, the overall retention of configuration which Knowles' group found for this enzyme (Jones et al., 1978) may be the result of two phosphoryltransfer steps which are both accompanied by retention of configuration.

This mechanistic consideration indicates that stereochemical studies on enzymes which utilize dianionic phosphoryl groups as substrates may not necessarily allow distinction between mechanisms which involve a covalent intermediate and those involving a necessary pseudorotation which accompanies catalysis via bidentate chelate formation. However, since the bidentate mechanism is not expected to be important for reactions involving monoanionic phosphoryl groups (subject to the results of our studies in progress), the vast majority of phosphoryl- and nucleotidyl-transfer enzymes should yield mechanistically useful information when studied by stereochemical techniques: inversion of configuration indicating direct nucleophilic attack on phosphorus and retention of configuration indicating formation of a covalent intermediate between the enzyme and substrate.

After this manuscript had been submitted for publication, the stereochemical course of the hydrolysis of the  $R_p$  isomer of ATP $\alpha$ S catalyzed by snake venom phosphodiesterase was reported to be retention of configuration (Bryant & Benkovic, 1979). Snake venom phosphodiesterases only recently have been purified to homogeneity (Frischanf & Eckstein, 1973), and some of these enzymes are reported to require divalent metal ions (magnesium, manganese, or cobalt) for activity (Philipps, 1975). Although the retention of configuration found for this enzyme was presumed to indicate formation of a covalent intermediate during the course of catalysis, the stereochemical implication of a mechanism involving metal ion chelation with the substrate discussed earlier in this section does suggest that the reaction catalyzed by the snake venom could proceed with a necessary pseudorotation. If bidentate chelation of the substrate by the venom enzyme is not important, the first two stereochemical investigations of phosphodiesterases which cannot utilize substrate functionality to assist catalysis demonstrate that the reactions catalyzed by these enzymes proceed by different mechanisms, even though they have similar metal ion requirements and catalyze chemically similar reactions. Further investigations of these and other metal-requiring phosphodiesterases are required and will provide important information about their mechanisms of catalysis.

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## Activation of Saccharide Binding in Demetalized Concanavalin A by Transition Metal Ions<sup>†</sup>

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ABSTRACT: Saccharide-binding activity is induced when apoconcanavalin A binds only one Mn<sup>2+</sup> ion near pH 6.5 at 5 or 23 °C. The affinity of Mn<sup>2+</sup>-concanavalin A for 4methylumbelliferyl  $\alpha$ -D-mannopyranoside is about one-half that of Ca<sup>2+</sup>-concanavalin A under similar conditions. Equilibrium dialysis studies with  ${}^{54}Mn^{2+}$  show that a single Mn<sup>2+</sup> binds per concanavalin A monomer in the presence and absence of saccharide. One Mn<sup>2+</sup> is sufficient to activate sugar binding by each monomer. This result is similar to our earlier finding which showed that a single Ca<sup>2+</sup> ion per protein monomer is most likely responsible for sugar-binding activation of Ca<sup>2+</sup>-concanavalin A near physiological pH [Christie, D. J., Alter, G. M., & Magnuson, J. A. (1978) Biochemistry 17, 4425]. Water proton relaxation rates for Mn<sup>2+</sup>-concanavalin A measured at 20.5 MHz, pH 6.4, and 23 °C decrease as the  $Mn^{2+}$  concentration is increased from 1 to 2 equiv of  $Mn^{2+}$ per monomer. This confirms similar observations made recently by others [Brown, R. D., III, Brewer, C. F., & Koenig,

Concanavalin A<sup>1</sup> (Con A), the lectin isolated from jack bean (*Conavalia ensiformis*), has been the subject of major biochemical investigations for nearly a decade. Con A has been used in affinity purification of glycoproteins, glycopeptides, and polysaccharides, and a similar protocol has been used to separate viruses and bacteria (Bittiger & Schnebli, 1976). The mitogenic response elicited by Con A in cells such as lymphocytes (Wecksler et al., 1968; Powell & Leon, 1970; Beckert & Sharkey, 1970) is studied extensively. The lectin agglutinates or binds to many types of cells (Nicolson, 1974) and is widely used as a probe for studying membrane structure and function (Bittiger & Schnebli, 1976). Transformed cells are agglutinated more readily than normal cells (Inbar & Sachs, 1969a,b). The property of Con A which makes it useful as a probe is its ability to bind to carbohydrates which possess

S. H. (1977) Biochemistry 16, 3883]. No decrease in relaxation rates occurs when concanavalin A is incubated with Mn<sup>2+</sup> at 5 °C. At least two different conformational states exist, one near 5 °C and one near 23 °C. The form at 5 °C converts, upon incubation at 23 °C, to the higher temperature form. Both have full saccharide-binding activity, which only requires one bound  $Mn^{2+}$  per monomer.  $Mn^{2+}$  interaction with apoconcanavalin A is different than Ni<sup>2+</sup>, Co<sup>2+</sup>, and Zn<sup>2+</sup> interaction. While binding with a stoichiometry of one metal ion per monomer, these other three transition metal ions do not induce saccharide-binding activity. Inactive Ni<sup>2+-</sup> concanavalin A, however, is activated in the presence of  $Mn^{2+}$ , and no  $Ni^{2+}$  is displaced. This suggests that  $Mn^{2+}$  is not binding at exactly the same site as  $Ni^{2+}$ ,  $Co^{2+}$ , and  $Zn^{2+}$ , but may be at a hybrid site which overlaps in some way with both the specific S1 transition metal ion site and the S2  $Ca^{2+}$  ion site.

the D-arabinopyranoside ring structure (Goldstein et al., 1965, 1973).

The nature of the binding of divalent metal ions to Con A is of interest because the ions are necessary for saccharidebinding activity. It is generally accepted that near pH 5 each Con A subunit first binds a transition metal ion such as Mn<sup>2+</sup> at a specific binding site S1 and then sequentially binds a Ca<sup>2+</sup> ion at site S2 (Kalb & Levitzki, 1968; Shoham et al., 1973; Sherry et al., 1975). The sites have been identified in X-ray studies (Hardman & Ainsworth, 1976; Becker et al., 1975), and both the X-ray and NMR studies (Alter & Magnuson, 1974; Brewer et al., 1973a,b; Villafranca & Viola, 1974) show that the distance between a bound Mn<sup>2+</sup> ion and the specific carbohydrate-binding site is  $\sim 10-14$  Å. We recently demonstrated that near pH 7 Ca<sup>2+</sup> ion binding to apo-Con A does not require the prior binding of a transition metal ion and that  $Ca^{2+}$  induces a cooperative effect on the binding of  $Mn^{2+}$  to Con A (Alter et al., 1977). Further studies have shown that

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<sup>&</sup>lt;sup>1</sup> Abbreviations used: Con A, concanavalin A; apo-Con A, demetalized concanavalin A;  $Mn^{2+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Zn^{2+}$ , and  $Ca^{2+}$ -Con A, demetalized concanavalin A remetalized with the respective metal ion; MUM, 4-methylumbelliferyl  $\alpha$ -D-mannopyranoside; Mops, 3-(*N*-morpholino)propanesulfonate; EDTA, ethylenediaminetetraacetate; NMR, nuclear magnetic resonance; ESR, electron spin resonance.