# Effect of Ribonucleoside–Vanadyl Complexes on Enzyme-Catalyzed Reactions Central to Recombinant Deoxyribonucleic Acid Technology†

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ABSTRACT: Ribonucleoside–vanadyl complexes are potent inhibitors of many enzymes with ribonucleolytic activity. Although the complexes have proved useful preparatively, their inhibitory spectrum has never been determined. A general survey showed that ribonucleases A, $N_1$, $T_1$, and $U_2$, human plasma ribonuclease, micrococcal nuclease, and polynucleotide phosphorylase were inhibited; $S_1$ nuclease and *Bacillus cereus* ribonuclease were not affected. These complexes also inhibited restriction endonucleases such as *Eco*RI, *Bam*HI, and *Hin*dIII. In contrast, they did not inhibit pancreatic deoxyribonuclease. Therefore, it was possible to degrade DNA without risk to RNA by using 10 mM vanadyl complexes together with deoxyribonuclease heavily contaminated with ribonuclease. Enzymes used for labeling termini, bacterial alkaline phosphatase, RNA ligase, poly(A) polymerase, and polynucleotide kinase, were affected by vanadyl complexes; the phosphatase was weakly inhibited by 10 mM complexes while the others were completely devoid of activity in reactions carried out with 5 mM complexes and limiting ATP. The inhibitory effects of the complexes could be overcome by increasing the ATP concentration in reactions catalyzed by the kinase, but ligation of diadenosine pyrophosphate to $(Ap)_4A$ with RNA ligase, an ATP-independent step in the overall reaction, was abolished by 2 mM vanadyl complexes. Inhibition of reverse transcriptase by 2 mM vanadyl complexes was apparently competitive with deoxyribonucleoside triphosphates; at high concentrations of triphosphates (1.5 mM), there was no inhibition, and molar yields of full-length globin cDNA were approximately 40% of the input mRNA. Vanadyl complexes were weak inhibitors of nucleases found in crude reverse transcriptase preparations. Studies of vanadyl complexes in protein-synthesizing systems showed that both the complexes at 1 mM and the uncomplexed oxovanadium ion at 0.2 mM were potent inhibitors of cell-free protein synthesis. Conversely, translation of interferon mRNA in *Xenopus laevis* oocytes was unchanged by 5 mM vanadyl complexes. Many common manipulations with RNA can be either improved or simplified by making use of the varied responses of enzymes and enzyme systems to vanadyl complexes.

Ribonucleoside–vanadyl complexes are stable analogues of the cyclic 2′,3′-monophosphate transition states that are believed to occur during catalysis by many ribonucleases (Lienhard et al., 1971). They are thought to bind tightly and noncovalently to a family of nucleases that shares a common mechanism of action with the well-studied ribonuclease A from bovine pancreas (Roberts et al., 1969; Usher et al., 1970). Since these substances are both gentle and specific, they can be utilized for investigations of RNA under conditions in which deproteinization and dissociation of protein–nucleic acid complexes are incompatible with experimental goals (Berger & Birkenmeier, 1979). They have been used to isolate leukocyte interferon mRNA from both unfractionated cytoplasm and polysomes of lymphoblastoid cells (Berger et al., 1980) and from the cytoplasm of normal leukocytes treated with Newcastle disease virus (Wallace et al., 1981). They have also been used to isolate immune interferon mRNA from the cytoplasm of mitogen-stimulated leukocytes (Wallace et al., 1981; Taniguchi et al., 1981; Gray et al., 1982).

The present study ascertains the spectrum of nucleolytic and nucleic acid utilizing enzymes that are affected by ribonucleoside–vanadyl complexes. The applicability of these substances to many reactions of importance in molecular biology such as RNA sequencing, DNA degradation in the presence of RNA, reverse transcription of mRNA, cell-free protein synthesis, and translation of mRNA in oocytes of *Xenopus laevis* has been examined.

## Materials and Methods

*Enzyme and Substrates.* Ribonucleases $U_2$ and $T_1$ and *Bacillus cereus* ribonuclease were obtained as part of an RNA sequencing kit from P-L Biochemicals (Milwaukee, WI). Polynucleotide phosphorylase (type 15) and two enzymes from $T_4$-infected *Escherichia coli*, RNA ligase and polynucleotide kinase, were purchased from the same source. Poly(A) polymerase was a product of BRL (Gaithersburg, MD). Micrococcal nuclease, bacterial alkaline phosphatase, ribonuclease A, and $S_1$ nuclease came from Sigma (St. Louis, MO), whereas ribonuclease $N_1$ came from Seikagaku Kogyo (Tokyo, Japan). Human plasma ribonuclease was supplied by Miles (Elkhart, IN), and reverse transcriptase was obtained from J. Beard and G. E. Houts of Life Sciences (Gulfport, FL). The restriction enzymes *Eco*RI, *Bam*HI, and *Hin*dIII were purchased from BRL; deoxyribonuclease I from bovine pancreas was a product of Worthington Biochemical Corp. (catalog no. S80P76E0, Freehold, NJ). Substrates and their suppliers were as follows: rabbit globin mRNA and λ DNA, BRL; oligo$(dT)_{12-18}$, oligo$(dT)_{25-60}$, $(Ap)_4A$, $(Ap)_5A$, and diadenosine pyrophosphate, P-L Biochemicals; deoxycytidine [$\alpha$-$^{32}$P]triphosphate (specific activity 605 Ci/mmol), [$\gamma$-$^{32}$P]ATP (specific activity 3000 Ci/mmol), and [$\alpha$-$^{32}$P]ATP (specific activity 50 Ci/mmol), Amersham (Arlington Heights, IL); cytidine 3′,5′-[5′-$^{32}$P]bisphosphate (specific activity 603 Ci/mmol), New England Nuclear (Boston, MA).

*Ribonucleoside–Vanadyl Complexes.* Ribonucleoside–vanadyl complexes were prepared by the method of Berger & Birkenmeier (1979). Except where noted otherwise, an equimolar mixture of complexes made with cytidine, adenosine, guanosine, and uridine was employed. The stated concentration reflects the total vanadyl complex titer regardless of which ribonucleosides were used to form the adduct. Occasionally, ribonucleoside–vanadyl complexes from BRL (renamed by them vanadyl–ribonucleoside complexes or VRC) were employed.

*General Enzyme Assay Conditions.* The enzyme levels used in most experiments were adjusted to produce cleavage of not

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more than 20–30% of the substrate in 15 min unless noted otherwise. Under these conditions, it was highly likely that enzyme concentrations were catalytic and not stoichiometric. Since the inhibition of pancreatic ribonuclease A by vanadyl complexes is reversible with a $K_I$ value of approximately 10 $\mu$M (Lienhard et al., 1971), even enzymes sensitive to these substances would not be fully inhibited when analyzed at levels approaching the aforementioned dissociation constant. In the studies reported here, enzyme concentrations are expressed in units as defined by the supplier. The components of each assay, with the exception of the substrate, were mixed at 4 °C and incubated at the stated temperature for 15 min. Vanadyl complexes, where present, were used at concentrations between 2 and 20 mM as indicated. Each reaction was initiated by the addition of the appropriate substrate.

*Specific Assays.* Ribonucleases $U_2$ and $T_1$ and *B. cereus* ribonuclease at concentrations of 1500, 10, and 500 units/mL, respectively, were assayed essentially as described by Donis-Keller et al. (1977), Lockard et al. (1978), and the literature included in the RNA sequencing kit; urea was omitted. Each reaction contained 10 mM sodium citrate buffer at pH 5.0, tRNA, at 1–1.5 mg/mL, 20 mM vanadyl complexes where noted, and enzyme in a volume of 10 $\mu$L. Catalysis was allowed to proceed at 50 °C; reactions were terminated by boiling for approximately 1 min. Samples were stored at −20 °C and subsequently analyzed electrophoretically for degradation of tRNA without prior deproteinization.

Ribonucleases A and $N_1$, human plasma ribonuclease, and micrococcal nuclease at 0.04, 100, 5, and 10 units/mL, respectively, were assayed under similar conditions except for the use of 50 mM Tris-HCl at pH 7.5 and 37 °C. Nuclease $S_1$ activity was determined in 10 $\mu$L containing 30 mM sodium acetate at pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol, 1 mg/mL tRNA, and 0.1 unit/mL enzyme (Vogt, 1980). The reaction was carried out at 37 °C; vanadyl complexes were tested at 20 mM. In all cases, the products were analyzed electrophoretically without deproteinization.

RNA ligase was studied at 150 $\mu$g/mL as detailed by England & Uhlenbeck (1978) with 10 nM cytidine 3′,5′-[5′-$^{32}$P]bisphosphate as the donor and tRNA at 250 $\mu$g/mL as the acceptor in a complete ligation reaction at 4 °C. The effect of vanadyl complexes was tested at 5 mM. The final step of the RNA ligation reaction was examined in Tris-HCl buffer at pH 8.3 with diadenosine 5′,5′-pyrophosphate as the donor and pentaadenosine tetraphosphate [$(Ap)_4A$] as the acceptor. The enzyme concentration was 96 $\mu$g/mL in an incubation system previously described by England et al. (1977). Vanadyl complexes were tested at 2 mM. The predominant product after 1 h, $(Ap)_5A$, was separated from the substrate, $(Ap)_4A$, on a 12% polyacrylamide gel after phenol extracting the reaction mixture and labeling the oligonucleotides with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase essentially as detailed below.

Polynucleotide phosphorylase at 6 units/mL was examined in 50 $\mu$L of 50 mM Tris-HCl at pH 8.3 to which 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM sodium phosphate at pH 8 were added. Globin mRNA, 40 $\mu$g/mL, was the substrate for phosphorolysis which proceeded at 37 °C for 30 min. The effect of 2 mM vanadyl complexes on the reaction was tested. Subsequently, protein was removed by means of a phenol extraction, and the RNA products were characterized by gel electrophoresis as detailed below.

Reverse transcriptase at 672 units/mL was assayed under similar conditions in a total volume of 50 $\mu$L. In addition to the salts and the buffers included in the polynucleotide phosphorylase reaction, the reverse transcriptase system also contained 20 $\mu$g/mL globin mRNA, 5 $\mu$g/mL oligo$(dT)_{12-18}$ as primer, each of the four deoxyribonucleoside triphosphates at the stated concentration, and 20 $\mu$Ci/mL deoxycytidine triphosphate. The inorganic phosphate was omitted. The sizes of the cDNA transcripts were determined electrophoretically after deproteinizing the samples. The yield of cDNA in each reaction was obtained by excising the band of full-length globin cDNA from the gel and quantifying the Cerenkov radiation. Measurements were converted to dpm by means of an empirically determined constant. Nuclease activity in crude reverse transcriptase at 1180 units/mL was examined in the presence and absence of 8 mM vanadyl complexes under virtually the same conditions. However, oligo(dT) was omitted and 0.4 mg/mL tRNA was substituted for mRNA.

The effect of 10 mM vanadyl complexes on the ability of alkaline phosphatase at 0.055 unit/mL to remove the 5′-terminal phosphate of [$^{32}$P]tRNA was determined at 37 °C in 20 mM Tris-HCl at pH 8.0 made 0.2 mM in EDTA (Heppel et al., 1962). The radioactive substrate concentration was 0.6 $\mu$Ci/mL; 8.5 $\mu$g/mL 5′-dephospho-tRNA was also present. Acid-insoluble radioactivity was measured by withdrawing aliquots of approximately 4 $\mu$L and drying them on GF/C glass fiber filters (Millipore). The volume of each aliquot could be determined with precision from the Cerenkov radiation. Subsequently, filters were washed with 5% trichloroacetic acid, rinsed with ethanol, dried, and assayed. The data were normalized relative to the average Cerenkov radiation emitted by the entire set of unwashed filters.

Polynucleotide kinase at 100 units/mL was assayed in 50 mM Tris-HCl at pH 8.0, 10 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM potassium phosphate at pH 8.0, 6 $\mu$M [$\gamma$-$^{32}$P]ATP, 50 $\mu$g/mL bovine serum albumin, and 15 $\mu$g/mL dephospho-oligo$(dT)_{25-60}$ at 37 °C at the stated times (Sgaramella & Khorana, 1972). Trichloroacetic acid insoluble material in 2-$\mu$L aliquots was quantified on GF/C glass fiber filters as described for alkaline phosphatase. The effect of vanadyl complexes at 5 mM was determined. The enzyme was also analyzed at 500 units/mL by using 1 mg/mL hexaadenosine pentaphosphate [$(Ap)_5A$] and 2 mM [$\gamma$-$^{32}$P]ATP (specific activity 1 Ci/mmol) as substrates in the presence and absence of 2 mM vanadyl complexes. Radioactive products after 20 min of reaction were analyzed without deproteinzation on 12% polyacrylamide gels and visualized on X-ray film.

Poly(A) polymerase activity was measured at 37 °C in reaction mixtures containing 50 mM Tris-HCl at pH 8.0, 10 mM $MgCl_2$, 0.25 M NaCl, 0.5 mg/mL bovine serum albumin, and 10 units/mL enzyme by using two protocols (Sippel, 1973). In the first, 100 $\mu$M [$\alpha$-$^{32}$P]ATP was the donor with 10 $\mu$g/mL tRNA acting as the acceptor. Where present, vanadyl complexes were 5 mM. At the stated intervals, the amount of acid-insoluble $^{32}$P in a 5-$\mu$L aliquot was determined on filters as indicated above for alkaline phosphatase. In the second procedure, the vanadyl complexes were tested at 2 mM with 2 mM [$\alpha$-$^{32}$P]ATP (100 Ci/mol), using 20 $\mu$g/mL globin mRNA as the acceptor. The radioactive products were analyzed electrophoretically on 12% polyacrylamide gels and visualized as detailed for polynucleotide kinase.

Deoxyribonuclease I was assayed in a 20-$\mu$L reaction mix composed of 200 mM potassium phosphate buffer at pH 4.5, 10 mM $MgCl_2$, 150 $\mu$g/mL $\lambda$ DNA, and 7 units/mL enzyme. The stock solution of the latter (2800 units/mL) was diluted with 0.15% NaCl to 56 units/mL immediately before use. After a 10-min incubation at 37 °C in the presence and ab-

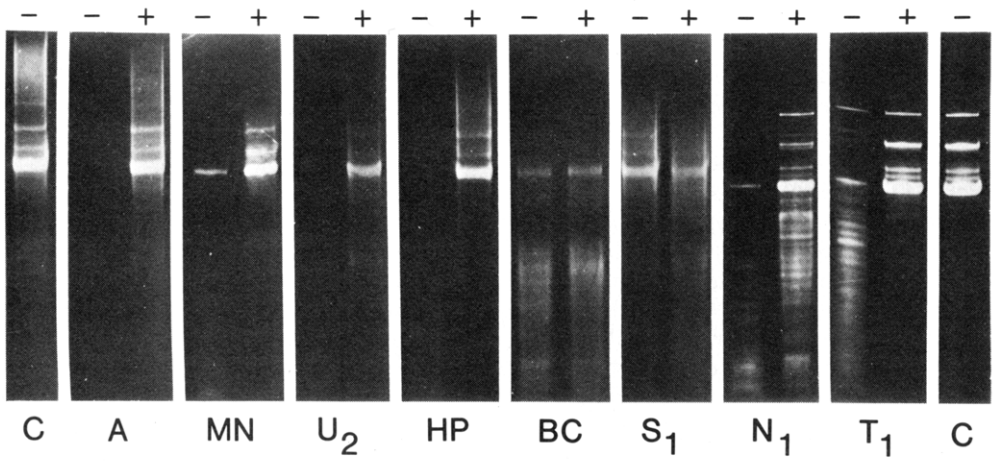


FIGURE 1: Effect of vanadyl complexes on ribonuclease activities. *E. coli* tRNA at 1 mg/mL was treated with the stated enzyme in the presence and absence of vanadyl complexes. Reactions were terminated by boiling and analyzed electrophoretically on 12% polyacrylamide gels. Enzymes tested were ribonuclease A (A), micrococcal nuclease (MN), ribonuclease $U_2$ ($U_2$), human plasma ribonucleases (HP), *B. cereus* ribonuclease (BC), $S_1$ nuclease ($S_1$), ribonuclease $N_1$ ($N_1$), and ribonuclease $T_1$ ($T_1$). Lanes labeled C contain tRNA in the absence of either enzymes or vanadyl complexes. The first 13 lanes from left to right were photographed together as were the remaining lanes. The presence or absence of vanadyl complexes during catalysis and electrophoresis is indicated by + or −, respectively, above the lane. Details of individual reactions are included under Materials and Methods.

sence of 10 mM vanadyl complexes, the reaction was stopped by the addition of 90 μL of buffer containing 10 mM sodium acetate at pH 5.1, 3 mM EDTA, and 50 mM NaCl (ACE buffer). Deproteinization was carried out by phenol extraction, and the resultant samples were analyzed electrophoretically.

Restriction endonuclease assays using λ DNA at 40 μg/mL as substrate were carried out in a total volume of 50 μL according to the literature supplied by the manufacturer with each enzyme. Enzymes tested were, respectively, *Hin*dIII, 20 units/mL; *Eco*RI, 400 units/mL; and *Bam*HI, 360 units/mL. The effect of 5 mM vanadyl complexes was investigated in reaction mixes incubated for 1 h at 37 °C. Catalysis was terminated by the addition of 5 μL of restriction endonuclease stop mix (400 mM EDTA, 50% glycerol, 0.05% bromphenol blue, and 0.05% xylene cyanol FF). The DNA was characterized electrophoretically without additional purification.

*Phenol Extraction Procedure.* Samples that required deproteinization were brought to 200 μL in 1.5-mL Eppendorf tubes with ACE buffer and extracted with an equal volume of phenol by agitating the tube vigorously for 2 min with a vortex mixer. The phenol used throughout this study was freshly distilled, made 0.1% in 8-hydroxyquinoline, saturated with ACE buffer, and stored at −20 °C in small aliquots which were thawed shortly before use. The sample was centrifuged 2 min in an Eppendorf microfuge (Model 5412), and the aqueous phase was recovered. A second extraction of the aqueous layer was performed similarly with an equal volume composed of 10 parts buffer-saturated phenol, 9.4 parts chloroform, and 0.6 part isoamyl alcohol. Nucleic acids in the aqueous phase were precipitated at −70 °C with 0.15 volume of 2 M sodium acetate and 3 volumes of 100% ethanol in a bath of dry ice–ethanol. Carrier tRNA (5–10 μg) was added to DNA samples and to globin mRNA. After the mixture was cooled a minimum of 15 min, nucleic acids were pelleted in the microfuge by 5 min of centrifugation; the supernatant fluid was discarded.

RNA and cDNA precipitates were dissolved in 20 μL containing 5 M urea, 0.05% bromphenol blue, and 0.05% xylene cyanol FF. After being heated to 65 °C for 10 min, the samples were cooled rapidly on ice, warmed slowly to room temperature, and analyzed in 150 × 105 × 1.5 mm polyacrylamide–urea gels. The gels, either 6% or 12% acrylamide, contained 7 M urea and an acrylamide:bis(acrylamide) ratio of 30:1. Electrophoresis was performed at 200–400 V. Gels were either stained with 1 μg/mL ethidium bromide at room temperature for 30 min and photographed with Polaroid positive/negative land film 665 or visualized with X-Omat XR-5 or XAR-2 X-ray film for 2 min to several days, depending on the yield of radioactive products.

DNA samples were dissolved in 5% sucrose, 0.05% bromphenol blue, and 0.05% xylene cyanol FF and analyzed in 1% agarose gels (150 × 130 × 3 mm) by submerged gel electrophoresis at 100 V for 3 h. Buffer was not recirculated.

*Protein Synthesis.* Cell-free protein synthesis was performed with nuclease-treated reticulocytes lysate (BRL). Globin mRNA was translated by using the protocol suggested by the manufacturer.

Protein synthesis was also investigated in oocytes from *Xenopus laevis*. Crude lymphoblastoid α-interferon mRNA was isolated from cultured Namalva cells by the method of Berger & Birkenmeier (1979) and Berger et al. (1980) and injected into oocytes (Hitchcock & Friedman, 1980). Interferon in the oocyte bathing medium was assayed by determining the cytopathic effect on GM 2504 human fibroblasts challenged with encephalomyocarditis virus (Berger et al., 1980).

## Results

*Effects of Vanadyl Complexes on Ribonucleases.* Vanadyl complexes inhibit a broad range of ribonucleases acting on *Escherichia coli* tRNA and on the RNA impurities in tRNA preparations. As shown in Figure 1, ribonuclease A from bovine pancreas, the "classical" enzyme used for testing each new preparation of vanadyl complexes, was completely inhibited as were micrococcal nuclease and human plasma ribonuclease. Ribonucleases $U_2$, $N_1$, and $T_1$ were also inhibited by 20 mM complexes (Figure 1). Since ribonucleases A [reviewed in Richards & Wyckoff (1971)], $N_1$, $T_1$, and $U_2$ [reviewed in Uchida & Egami (1971)] and human plasma ribonuclease (Schmukler et al., 1975) are all known to form 2′,3′-cyclic ribonucleotide intermediates and since vanadyl complexes are transition-state analogues of enzyme-bound cyclic phosphates, inhibition of the activity of these enzymes by vanadyl complexes was expected. In comparison, micrococcal nuclease forms 3′-monophosphates but not cyclic 2′,3′-phosphates [reviewed in Anfinsen et al. (1971)].

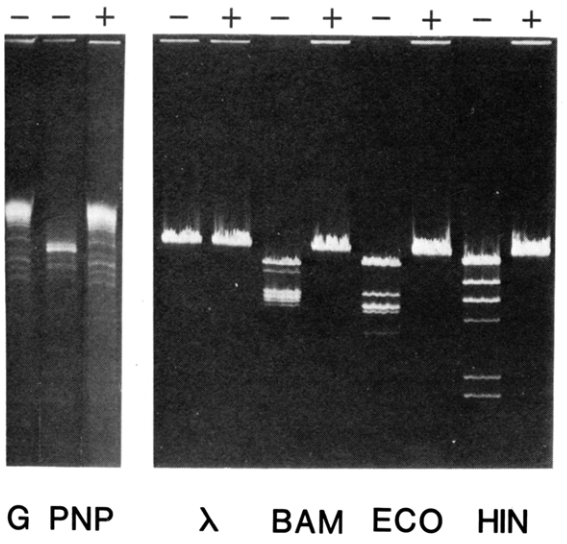


FIGURE 2: Effect of vanadyl complexes on polynucleotide phosphorylase (left) and on selected restriction endonucleases (right). Globin mRNA (first three lanes from left to right) was treated with polynucleotide phosphorylase in the presence and absence of vanadyl complexes, phenol extracted, and analyzed on a 6% polyacrylamide gel. Lane G, untreated (control) globin mRNA; lanes labeled PNP, catalysis with polynucleotide phosphorylase in the absence (−) and presence (+), respectively, of 2 mM vanadyl complexes. λ DNA (remaining eight lanes) was treated with the stated restriction endonuclease in the presence (+) and absence (−) of vanadyl complexes and examined electrophoretically on an agarose gel. Restriction enzymes tested were *Bam*HI (BAM), *Eco*RI (ECO), and *Hin*dIII (HIN). Lanes labeled λ are λ DNA in the presence (+) and absence (−) of vanadyl complexes. Details can be found under Materials and Methods.

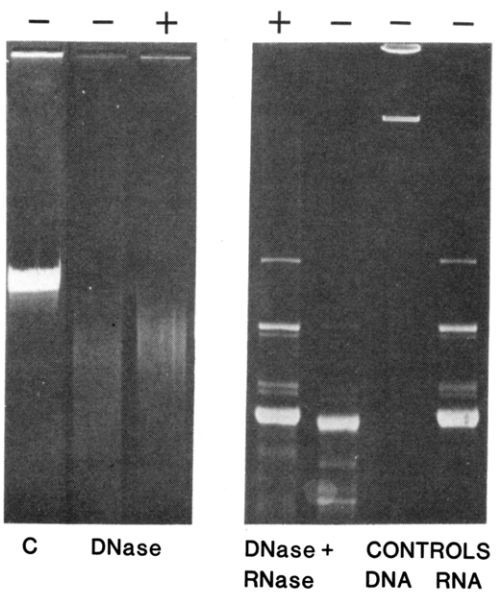


FIGURE 3: Effect of vanadyl complexes on deoxyribonuclease (left) and on deoxyribonuclease in the presence of ribonuclease (right). (Left) λ DNA was treated with deoxyribonuclease (lanes labeled DNase) in the presence (+) and absence (−) of vanadyl complexes. Samples were phenol extracted and analyzed on an agarose gel. The lane labeled C contains untreated (control) DNA. (Right) λ DNA (lane labeled Controls, DNA) and *E. coli* tRNA at 1 mg/mL (lane labeled Controls, RNA) were mixed and hydrolyzed with a combination of pancreatic deoxyribonuclease (7 units/mL) and pancreatic ribonuclease (0.4 units/mL) (lanes labeled DNase + RNase) in the presence (+) and absence (−) of 10 mM vanadyl complexes. Samples were phenol extracted and analyzed on a 12% polyacrylamide gel. Details can be found under Materials and Methods.

Therefore, the complete protection of RNA afforded by the complexes was of interest. $S_1$ nuclease and *B. cereus* ribonuclease were not affected by 20 mM vanadyl complexes. Although the mechanism of the latter enzyme is unknown (Lockhard et al., 1978), the implication is that catalysis by *B. cereus* ribonuclease does not involve a cyclic 2′,3′-monophosphate. However, as will be seen below, the assignment of mechanism based on the inhibition pattern with vanadyl complexes is premature.

Polynucleotide phosphorylase, unlike the other ribonucleases in this study, is an exonuclease. The enzyme has little activity on DNA substrates but degrades RNA molecules processively from the 3′-hydroxyl terminus in a phosphorolytic rather than a hydrolytic cleavage reaction [reviewed in Godefroy-Colburn & Grunberg-Manago (1972)]. As shown in Figure 2, polynucleotide phosphorylase is capable of shortening globin mRNA in the absence of vanadyl complexes; in the presence of 2 mM vanadyl complexes, complete inhibition is observed.

*Vanadyl Complexes as Inhibitors of Restriction Enzymes.* The effect of vanadyl complexes on catalysis by several representative restriction endonucleases was studied by using λ DNA as the substrate. These data, also illustrated in Figure 2, demonstrate that cleavage of DNA by *Bam*HI, *Eco*RI, and *Hin*dIII was completely prevented by the complexes. Since the complexes are reputed to deplete $Mg^{2+}$ (Egberts et al., 1977), *Hin*dIII was also analyzed in the presence of 17 mM $Mg^{2+}$. The inhibition by vanadyl complexes was not reversed by this manipulation (data not shown) nor did the complexes, themselves, affect the electrophoretic behavior of λ DNA (Figure 2). Since the formation of a cyclic 2′,3′-phosphate is impossible with a DNA substrate, inhibition by ribonucleoside–vanadyl complexes is clearly not a simple means for elucidating the mechanism of action. The reason for the inhibition of restriction endonucleases is unclear.

*Effect of Vanadyl Complexes on Deoxyribonuclease.* Pancreatic deoxyribonuclease I was not inhibited by vanadyl complexes (Figure 3); in qualitative experiments, the enzyme sometimes appeared to be more active when the vanadyl complexes were included in the reaction mixture. The lack of sensitivity of deoxyribonuclease to vanadyl complexes is of major importance to procedures that require the removal of DNA while preserving RNA in an intact state. A model set of experiments which demonstrates selective degradation of DNA is displayed in Figure 3. For these experiments, "ribonuclease free" pancreatic deoxyribonuclease was adulterated with 0.4 unit/mL pancreatic ribonuclease A, a level of contamination probably well beyond that usually encountered. The mixture of enzymes was then analyzed in the presence and absence of the vanadyl complexes by using a mixture of λ DNA and a crude preparation of *E. coli* tRNA as substrates. The DNA was completely degraded in the presence of the enzyme mixture regardless of whether or not the vanadyl complexes were present. In contrast, in the absence of vanadyl complexes, ribonuclease removed some bands entirely, shortened others, and created new smaller products from larger molecules. Such degradation of RNA was completely prevented by including ribonucleoside–vanadyl complexes in a system designed for optimal activity of deoxyribonuclease. (Inhibition of pancreatic ribonuclease A under conditions which favor that enzyme was demonstrated in Figure 1.) Thus, the vanadyl complexes convert crude deoxyribonuclease containing ribonuclease to a preparation acceptable for quantitative removal of DNA without risk to RNA.

*Effect of Vanadyl Complexes on Enzymes Used for End Labeling.* Other enzymes which were tested for activity in the presence of vanadyl complexes were poly(A) polymerase, polynucleotide kinase, RNA ligase, and alkaline phosphatase.

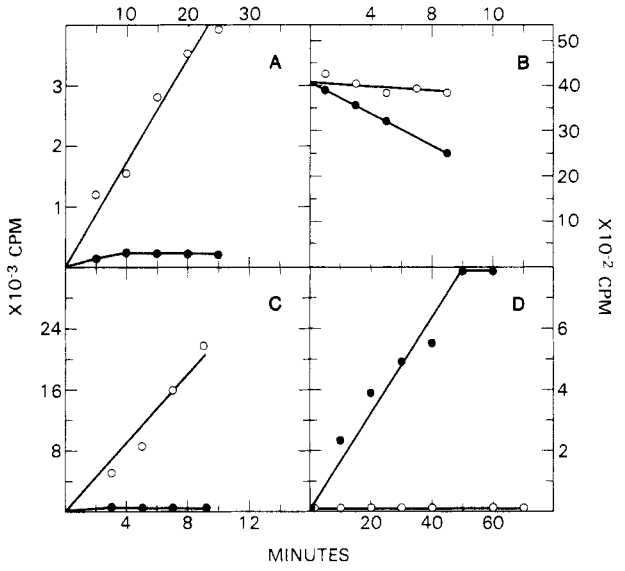


FIGURE 4: Effect of vanadyl complexes on (A) polynucleotide kinase, (B) alkaline phosphatase, (C) poly(A) polymerase, and (D) RNA ligase. Open circles in (A) and (C) represent enzymatic reactions in the absence of vanadyl complexes; closed circles in (A) and (C) are enzymatic reactions in the presence of vanadyl complexes. Open circles in (B) and (D) are reactions in the presence of vanadyl complexes; closed circles in (B) and (D) are reactions in the absence of vanadyl complexes. The particulars of each reaction are included under Materials and Methods.

The first three enzymes were strongly inhibited (Figure 4A,C,D). Alkaline phosphatase was only weakly inhibited by 10 mM vanadyl complexes (Figure 4B). In a practical sense, inhibition of the phosphatase could be overcome either by reducing the concentration of complexes or by increasing the level of enzyme, both of which serve to increase phosphatase activity (data not shown).

The strongly inhibited enzymes, unlike alkaline phosphatase, require ATP, and the ATP concentrations that are routinely used are low (Sgaramella & Khorana, 1972; England & Uhlenbeck, 1978; Sippel, 1973) relative to 2 mM, the lowest effective concentration of vanadyl complexes in crude systems (Berger & Birkenmeier, 1979). When the ATP concentration was increased to a level approximating that of the vanadyl complexes, a different picture emerged. With 2 mM vanadyl complexes, 2 mM [$\gamma$-$^{32}$P]ATP (specific activity 1 Ci/mmol), and $(Ap)_5A$ as substrate, polynucleotide kinase was not inhibited relative to an untreated control. Autoradiograms of the radioactive products separated electrophoretically from [$^{32}$P]ATP appeared by inspection to be identical in density (data not shown). Hence, the inhibition of polynucleotide kinase by vanadyl complexes is apparently competitive with ATP. The same approach taken with poly(A) polymerase was unsuccessful. Adenylation of globin mRNA in the presence of 2 mM [$\alpha$-$^{32}$P]ATP was completely inhibited by 2 mM vanadyl complexes; whereas a pronounced band of labeled globin mRNA was visible on a gel in the absence of the complexes, none was detectable when they were included. Apparently, the inhibition by complexes was noncompetitive with ATP. RNA ligase poses a different problem because the high concentration of ATP needed to attempt competition with the vanadyl complexes is, itself, inhibitory to the enzyme [reviewed in Higgins & Cozzarelli (1979)]. It was nevertheless possible to test the effect of the vanadyl complexes on the ATP-independent ligation step by using diadenosine pyrophosphate as the activated donor. The reaction with $(Ap)_4A$ did not progress under conditions in which approximately half the substrate was converted to $(Ap)_5A$ when the complexes were omitted (data not shown).

Table I: Effect of Vanadyl Complexes on Reverse Transcription[a]

| vanadyl complexes | [dNTP] (mM) | sp act. of dCTP ($\mu$Ci/$\mu$mol) | full-length globin cDNA cpm (% yield) 60 min | 120 min |
|---|---|---|---|---|
| − | 0.175 | 831 | 54 200 (27) | |
| + | 0.175 | 831 | 19 800 (10) | |
| − | 0.875 | 216 | 21 100 (41) | 24 600 (48) |
| + | 0.875 | 216 | 18 000 (35) | 21 700 (42) |
| − | 1.5 | 126 | 11 400 (38) | 11 800 (39) |
| + | 1.5 | 126 | 10 800 (36) | 12 800 (43) |

[a] Globin mRNA was transcribed with reverse transcriptase as described under Materials and Methods in the presence of the stated concentration of each deoxyribonucleoside triphosphate (dNTP) and 2 mM vanadyl complexes where indicated. Products after 60 or 120 min of reaction were separated electrophoretically on 6% polyacrylamide gels. The percent yield of full-length cDNA defined as (moles of cDNA/moles of mRNA) × 100 was determined by excising the band from the appropriate gel in each case. In making the calculations, it was assumed that the sequence of deadenylated globin mRNA was 25% guanylic acid residues.

*Effect of Vanadyl Complexes on Reverse Transcriptase.* The synthesis of complementary DNA from mRNA by using reverse transcriptase is a key step in cloning. Since it is the first procedure to be performed after mRNA has been isolated, low yields sustained at this stage place subsequent manipulations at a disadvantage. In order to optimize the synthesis of cDNA by inhibiting putative contaminating ribonucleases, we and others (Paterson & Roberts, 1981) examined the transcription reaction in the presence of vanadyl complexes. Although in some cases unqualified use of these inhibitors without supporting data was advocated, the results presented in Table I do not support such methodology. In the presence of 2 mM vanadyl complexes, the yield of full-length globin cDNA after 1 h was 37% of that in the absence of the complexes when the concentration of each triphosphate was 175 $\mu$M. Molar yields of globin cDNA relative to mRNA were low in both cases. With an increase in the levels of the deoxyribonucleoside triphosphates to 875 $\mu$M, the yield of cDNA with vanadyl complexes present was 85% of the uninhibited reaction. With the addition of fresh enzyme and incubation for an additional hour, the results with complexes were even closer to that of the untreated sample. At a concentration of 1.5 mM for each of the triphosphates, the production of cDNA in 2 h in the presence of 2 mM vanadyl complexes exceeded that in their absence. Given the inherent errors in cutting bands from gels for quantitation, the data indicate that the vanadyl complexes have virtually no effect on transcription at high triphosphate concentrations. The data also demonstrated that molar yields of full-length globin cDNA of approximately 40% of the input mRNA can be anticipated.

The ribonucleases found in crude preparations of reverse transcriptase from avian myeloblastosis virus were partially inhibited by 8 mM vanadyl complexes (Figure 5). With tRNA as the substrate in a model reverse transcription reaction mix made without oligo(dT), the RNA was completely degraded by crude reverse transcriptase unless vanadyl complexes were available to reduce the activity of the endogenous ribonucleases (Figure 5). From these data, it can be inferred that at least one ribonuclease in the crude reverse transcriptase preparation was not completely inhibited by vanadyl complexes. When purified reverse transcriptase was made from the crude enzyme studied here, nuclease activity was not detected, and vanadyl complexes had no effect on the electrophoretic pattern of RNA (Figure 5). However, in the event

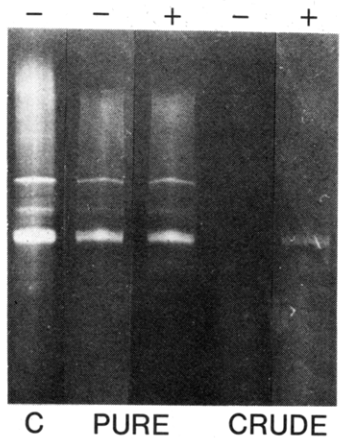


FIGURE 5: Effect of vanadyl complexes on the ribonucleases of avian myeloblastosis virus. *E. coli* tRNA, 0.4 mg/mL, was treated for 1 h at 37 °C with the stated concentration and type of reverse transcriptase in a volume of 5 μL containing 50 mM Tris-HCl at pH 8.3, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl, 1 mM EDTA, 1.5 mM of each deoxyribonucleoside triphosphate, and 8 mM vanadyl complexes where indicated. The reaction was terminated by boiling, and the samples were analyzed on a 12% polyacrylamide gel without prior deproteinization. Crude and purified reverse transcriptases were gifts of G. E. Houts. Purification of the crude enzyme was halted after the DE-52 column chromatography step (Houts et al., 1979). Purified enzyme was obtained from the crude material by completing the purification scheme. Lane labeled C, 4 μg of tRNA, untreated; remaining lanes, 2 μg of tRNA treated with 1640 units/mL purified reverse transcriptase (PURE) or 1180 units/mL crude reverse transcriptase (CRUDE) in the presence (+) or absence (−) of vanadyl complexes.

that contaminants remain in some purified enzyme preparations, or that they are introduced with other components of the system, the complexes may improve the yield and/or the quality of the products. Clearly, the benefit to be derived from vanadyl complexes depends on the nature of the contaminants.

*Protein Synthesis in the Presence of Vanadyl Complexes.* The ability to evaluate the effect on protein synthesis of protein-containing moieties such as messenger ribonucleoprotein particles depends on reliable methods for removing or inhibiting ribonucleases in the sample under scrutiny. Toward this end, we tested cell-free protein synthesis in the reticulocyte lysate system in the presence of vanadyl complexes prepared with KOH to avoid introducing sodium ion into the system. All four ribonucleoside–vanadyl complexes were tested separately with the hope of finding one or more that would not be inhibitory (Table II). At a concentration of 2 mM, the vanadyl complexes, individually, severely depressed the levels of protein synthesis (experiment 1). Increasing the level of magnesium ion relative to the standard magnesium concentration (0.65 mM) in order to compensate for the putative binding of this ion by vanadyl complexes served only to decrease translation still further (experiment 2). A partial explanation is also found in Table II. When $VOSO_4$, neutralized with KOH, was added to a cell-free protein synthesizing system in the absence of vanadyl complexes, inhibition of translation was observed at concentrations from 0.2 to 2 mM. According to our interpretation, replacement of the divalent oxovanadium cation in the complexes by $Mg^{2+}$, at high $Mg^{2+}$ concentration, results in the production of another inhibitor, the oxovanadium ion, itself. Clearly, vanadyl complexes are incompatible with cell-free protein synthesis since similar results were also observed in the wheat germ system (data not shown).

As an alternative to cell-free protein synthesis, the effect of vanadyl complexes on translation in *Xenopus laevis* oocytes was tested. Bulk deproteinized mRNA containing α-interferon mRNA was isolated from induced lymphoblastoid cells, mixed with the stated concentration of vanadyl complexes, and injected into oocytes at a mRNA concentration of 125 μg/mL. As shown in Table III, the introduction of 5 mM mixed vanadyl complexes into the oocyte together with the mRNA in a total volume of 50 nL per oocyte was not inhibitory. However, 10 mM vanadyl complexes were not well tolerated. The 2-fold reading uncertainty associated with interferon assays must be considered in viewing the data; differences must exceed 100% in order to be significant.

Table II: Effect of Ribonucleoside–Vanadyl Complexes on Cell-Free Protein Synthesis[a]

| expt | reaction mixture | cpm in protein at 30 min | cpm in protein at 60 min |
|---|---|---|---|
| 1 | complete | 27000 | |
| | complete − mRNA | 2300 | |
| | complete + V-A[b] | 3900 | |
| | complete + V-C[b] | 4500 | |
| | complete + V-G[b] | 7800 | |
| | complete + V-U[b] | 4300 | |
| | complete[b] | 21000 | |
| 2 | complete | 29000 | 45000 |
| | complete − mRNA | 2600 | 2200 |
| | complete + V-C | 9300 | 12000 |
| | complete + V-C[c] | 4800 | 7200 |
| | complete + 2 mM $VOSO_4$ | 7600 | 8700 |
| | complete + 0.2 mM $VOSO_4$ | 9000 | 7800 |

[a] Globin mRNA was translated in reticulocyte lysate as described under Materials and Methods in a volume of 25 μL with 0.62 mCi/mL [$^3$H]leucine (145 Ci/mmol) and 0.65 mM magnesium acetate unless noted otherwise. At the stated time, an aliquot of 5 μL was withdrawn into 0.2 mL of 0.3 N potassium hydroxide. Samples were heated at 37 °C for 20 min, diluted by the addition of 2 mL of 10% trichloroacetic acid, and precipitated on ice for 30 min. Precipitates were collected on GF/C glass fiber filters; radioactivity on the filters was determined in a scintillation counter. The concentration of each vanadyl complex was 1 mM in experiment 1 and 2 mM in experiment 2. The vanadyl complex with adenosine, cytidine, guanosine, or uridine is designated V-A, V-C, V-G, or V-U, respectively. [b] 1.65 mM magnesium. [c] 1.5 mM magnesium.

Table III: Effect of Ribonucleoside–Vanadyl Complexes on Protein Synthesis in *Xenopus laevis* Oocytes[a]

| sample injected | α-interferon mRNA activity | | |
|---|---|---|---|
| | group 1 | group 2 | group 3 |
| interferon mRNA | 384 | 512 | 512 |
| interferon mRNA + 5 mM complexes | 192 | 256 | 768 |
| interferon mRNA + 10 mM complexes | 64 | 48 | <4 |

[a] Messenger RNA from induced lymphoblastoid cells was injected into *Xenopus laevis* oocytes as indicated under Materials and Methods. Briefly, a 50-nL aliquot of each sample containing 125 μg/mL mRNA and the stated concentration of vanadyl complexes was introduced semiautomatically into each of 30 oocytes. Subsequently, the oocytes were incubated in Barth's solution in groups of 10 per 18 h at room temperature. Numbers represent the amount of interferon in laboratory units per milliliter produced and secreted by groups of 10 oocytes in response to the mRNA. Controls injected with water synthesized no interferon. One international unit of interferon defined by the NIH standard is equivalent to approximately 20 laboratory units under the conditions used.

## Discussion

Ribonucleoside–vanadyl complexes are potent inhibitors of all ribonucleases tested with the exception of the ribonuclease

from *Bacillus cereus*. The list of enzymes that are inhibited includes one phosphorolytic enzyme and many hydrolytic enzymes together with uncharacterized enzymes such as those in lymphocyte cytoplasm or avian myeloblastosis virus preparations. $S_1$ nuclease, an enzyme capable of degrading single-stranded DNA as well as RNA (Vogt, 1980) is not affected by vanadyl complexes. However, unlike most ribonucleases, $S_1$ gives rise to 5′-mononucleotides.

The inhibition patterns exhibited by enzymes in the presence of vanadyl complexes can be used productively. In some instances, ease of handling is the result. For example, in sequential reactions, the complexes can be used to stop an initial step while a subsequent step proceeds. An RNA can be ligated and then "kinased" in the absence of RNA ligase activity. A mRNA without a poly(A) tail can be adenylated with poly(A) polymerase and, after adenylation has been halted, translated in frog oocytes etc. The complexes can also be used to protect RNA molecules from endogenous or exogenous nucleases. Of particular importance are the ability to degrade DNA with deoxyribonuclease in the presence of RNA and the ability to reverse transcribe RNA. In both cases, the complexes do not inhibit the desired reaction while inhibiting undesirable side effects resulting from ribonucleases.

Storage of high molecular weight labile RNAs in solutions containing vanadyl complexes is also advocated. If necessary, the complexes can readily be removed by phenol extraction. In this laboratory, hydroxyquinoline added to phenol as an antioxidant has been used as a simple indicator of the presence of complexes. When the phenol layer remains bright yellow, not black, in a phenol extraction, the complexes have been removed. However, many functions do not require their removal. For example, marker RNAs can be analyzed electrophoretically without eliminating the complexes. Finally, complexes which have previously been used preparatively to obtain polysomes or bulk cytoplasmic mRNAs (Berger et al., 1980) are also suitable for protection of RNA during all phases of whole cell extractions. Because they are inexpensive and easy to make, the need for large amounts in large-scale operations should not be a deterrent.

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