

Potent Inhibition of Ribonuclease A by Oligo(vinylsulfonic Acid)*

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Ribonuclease A (RNase A) can make multiple contacts with an RNA substrate. In particular, the enzymatic active site and adjacent subsites bind sequential phosphoryl groups in the RNA backbone through Coulombic interactions. Here, oligomers of vinylsulfonic acid (OVS) are shown to be potent inhibitors of RNase A that exploit these interactions. Inhibition is competitive with substrate and has $K_i = 11$ μ M in assays at low salt concentration. The effect of salt concentration on inhibition indicates that nearly eight favorable Coulombic interactions occur in the RNase A-OVS complex. The phosphonic acid and sulfuric acid analogs of OVS are also potent inhibitors although slightly less effective. OVS is also shown to be a contaminant of MES and other buffers that contain sulfonylethyl groups. Oligomers greater than nine units in length can be isolated from commercial MES buffer. Inhibition by contaminating OVS is responsible for the apparent decrease in catalytic activity that has been observed in assays of RNase A at low salt concentration. Thus, OVS is both a useful inhibitor of RNase A and a potential bane to chemists and biochemists who use ethanesulfonic acid buffers.

RNA is the least stable of the biopolymers that effect information transfer in biology (1). Yet, the lifetime of RNA *in vivo* is most often determined by the rate of its enzymatic degradation (2). *In vitro*, ribonuclease inhibitors are often employed to mitigate damage to RNA from incidental contamination with secretory ribonucleases such as the human homolog of ribonuclease A (RNase A,¹ EC 3.1.27.5) (3). The abundance and diversity of natural ribonucleases has led to an ever-increasing interest in inhibitor design and discovery (4).

Similar to most known ribonucleases, RNase A can make multiple contacts with an RNA substrate (Fig. 1). The enzymatic active site and adjacent subsites bind sequential phosphoryl groups in the RNA backbone through Coulombic inter-

actions (5). The most potent RNase A inhibitors take advantage of this extended interface. For example, a pyrophosphate-linked oligonucleotide (pdUppA-3'-p), which occupies three subsites, is the tightest known small-molecule inhibitor of RNase A (4). Nature also uses this strategy to inhibit RNase A and its homologs. The 50-kDa ribonuclease inhibitor protein forms a tight 1:1 complex with RNase A ($K_d \sim 10^{-14}$ M) (6), chelating all of its phosphoryl group binding subsites (7). The utility of pyrophosphate-linked oligonucleotides and ribonuclease inhibitor is limited, both by the difficulty and expense of their production and by their intrinsic instability. For example, pyrophosphate-linked oligonucleotides are susceptible to hydrolysis (8), and ribonuclease inhibitor is readily inactivated by oxidation (9).

While studying RNase A catalysis as a function of salt concentration, we found that a contaminant in common biological buffers was a potent inhibitor in solutions of low salt concentrations (10). We estimated that K_i for this inhibitor was ~ 0.1 nM, which is 10^2 -fold lower than that for any other small-molecule RNase A inhibitor. Herein, we identified this inhibitor as a byproduct of the synthesis of commercial buffers containing sulfonylethyl groups. Next, we found that this inhibitor acts in a competitive manner and is the most potent known small-molecule inhibitor of a ribonuclease. In addition, we identified the number of Coulombic interactions that the inhibitor makes upon binding to RNase A. Finally, we examined RNase A inhibition by analogs of the inhibitor.

EXPERIMENTAL PROCEDURES

Materials—Poly(cytidylic acid) (poly(C)) was from Midland Certified Reagents (Midland, TX). Poly(C) was purified prior to use by precipitation in aqueous ethanol (70% v/v). The fluorogenic ribonuclease substrate 6-FAM~dArUdAdA~6-TAMRA (where 6-FAM is a 5'-6-carboxyfluorescein group and 6-TAMRA is a 3'-6-carboxytetramethylrhodamine group) was from Integrated DNA Technologies (Coralville, IA). Oligo(vinylsulfonic acid) ($M_r \sim 2,000$) and poly(vinylphosphonic acid) ($M_r \sim 20,000$) were from Polysciences (Warrington, PA). Vinylsulfonic acid and poly(vinylsulfuric acid) ($M_r \sim 170,000$) were from Aldrich. All other commercial chemicals and biochemicals were of reagent grade or better and were used without further purification.

Synthesis of Diethanesulfonic Acid Ether (3)—2-Mercaptoethylether (5.0 g, 36.2 mmol, Caution: Stench!, Aldrich) was dissolved in glacial acetic acid (5 ml). The resulting solution was then cooled to 0 °C. While stirring at 0 °C, a mixture (50:45 ml) of glacial acetic acid and aqueous hydrogen peroxide (30% v/v) was added to the solution dropwise over 1 h. The reaction mixture was then heated at 60 °C for 90 min. The solvent was removed under reduced pressure. The addition of toluene enabled residual acetic acid to form azeotropes of low boiling point. The resulting yellow oil was used without further purification (spectral data: ¹H NMR (300 MHz, D₂O) δ 2.88 (t, $J = 7.1$ Hz, 4H), 2.17 (t, $J = 7.0$ Hz, 4H) ppm; mass spectrometry (electrospray ionization) m/z 232.9791 (M^+H^+ [$C_4H_9O_7S_2$] = 232.9795)).

Instruments—UV absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Palo Alto, CA). Fluorescence measurements were made with a QuantaMaster 1 photon counting fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ).

Production of RNase A—Plasmid pBXR (11) directs the production of RNase A in *Escherichia coli*. RNase A was produced and purified as

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¹ The abbreviations used are: RNase A, ribonuclease A; 6-FAM, 6-carboxyfluorescein; 6-TAMRA, 6-carboxytetramethylrhodamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; OVS, oligo(vinylsulfonic acid); poly(C), poly(cytidylic acid); PVP, poly(vinylphosphonic acid); PVOS, poly(vinylsulfuric acid); *F*, fluorescence; ddH₂O, double distilled H₂O; bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

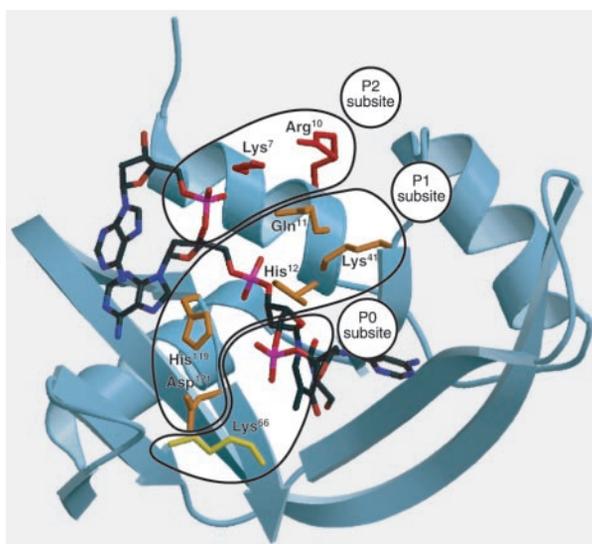


FIG. 1. Structure of the crystalline ribonuclease A-d(ATAAG) complex (PDB entry 1RCN) (5). Three phosphoryl group binding subsites are indicated. The guanidine residue and a fourth phosphoryl group binding subsite (Arg⁸⁵) (46) are not shown. Residues that comprise each subsite are colored as follows: P0 subsite (yellow), P1 subsite (orange), and P2 subsite (red).

described previously (12) with the following modifications. *E. coli* strain BL21(DE3) transformed with pBXR was grown to an optical density of 1.8 at 600 nm in terrific broth medium containing ampicillin (0.40 mg/ml). The expression of the RNase A cDNA was induced by the addition of isopropyl-1-thio- β -D-galactopyranoside to 0.5 mM. Cells were collected 4 h after induction and lysed with a French pressure cell. Inclusion bodies were recovered by centrifugation and resuspended for 2 h in 20 mM Tris-HCl buffer, pH 8.0, containing guanidine-HCl (7 M), dithiothreitol (0.10 M), and EDTA (10 mM). The protein solution was diluted 10-fold with aqueous acetic acid (20 mM), subjected to centrifugation to remove any precipitate, and dialyzed overnight against aqueous acetic acid (20 mM). Any precipitate was removed again by centrifugation. The supernatant was diluted to a protein concentration near 0.5 mg/ml in a refolding solution of 0.10 M Tris-HCl buffer, pH 8.0, containing NaCl (0.10 M), reduced glutathione (1.0 mM), and oxidized glutathione (0.2 mM). RNase A was refolded for 16 h and concentrated by ultrafiltration with a YM10 membrane (M_r 10,000 cut-off, Millipore, Bedford, MA). Concentrated RNase A was applied to a Superdex G-75 gel filtration fast protein liquid chromatography column (Amersham Biosciences) in 50 mM sodium acetate buffer, pH 5.0, containing NaCl (0.10 M) and NaN_3 (0.02% w/v). Protein from the major A_{280} peak was collected and applied to a Mono S cation-exchange fast protein liquid chromatography column. RNase A was eluted from the column with a linear gradient of NaCl (0.2–0.4 M) in 50 mM sodium acetate buffer, pH 5.0. Protein concentration was determined by UV spectroscopy using $\epsilon = 0.72 \text{ ml mg}^{-1} \text{ cm}^{-1}$ at 278 nm (13).

Inhibition of RNase A Catalysis—Inhibition of ribonucleolytic activity was measured by using either poly(C) or a fluorogenic substrate. The total cytidyl concentration of poly(C) was quantitated using $\epsilon = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$ at 268 nm (14). The cleavage of poly(C) was monitored by the decrease in ultraviolet hypochromicity. The $\Delta\epsilon$ value for this reaction calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product was $2,380 \text{ M}^{-1} \text{ cm}^{-1}$ at 250 nm (15). Assays were performed at 25 °C in 50 mM imidazole-HCl buffer, pH 6.0, containing NaCl (0.10 M), poly(C) (10 μM –1.5 mM), OVS (0–1.43 μM), and enzyme (1.0 nM). Molar values of OVS were calculated by using its average molecular mass of 2,000 g/mol. It is possible that a polymer of this size could bind two enzymes. Thus, the actual K_i values could be 2-fold higher. Kinetic parameters were determined from initial velocity data with the program DELTA-GRAPH 4.0 (DeltaPoint, Monterey, CA).

For the fluorescence assay, the inhibition of ribonucleolytic activity was assessed at 25 °C in 2.0 ml of 50 mM imidazole-HCl buffer, pH 6.0, containing NaCl (0–0.25 M), 6-FAM~dArUdAdA~6-TAMRA (60 nM), and RNase A (1–5 μM) as described previously (16, 17). Fluorescence (F) was measured using 493 and 515 nm as the excitation and emission wavelengths, respectively. The value of $\Delta F/\Delta t$ was measured for 3 min after the addition of RNase A. An aliquot of inhibitor (I) dissolved in the

assay buffer was added next, and $\Delta F/\Delta t$ was measured in the presence of the inhibitor for 3 min. The concentration of inhibitor in the assay was doubled repeatedly in 3-min intervals. Excess RNase A was then added to the mixture to ensure that <10% substrate had been cleaved prior to completion of the inhibition assay. Apparent changes in ribonucleolytic activity due to dilution were corrected by comparing values to an assay in which aliquots of buffer were added to the assay. Values of K_i were determined by non-linear least squares regression analysis of data fitted to Equation 1 (16, 17).

$$\Delta F/\Delta t = (\Delta F/\Delta t)_0 (K_i / (K_i + [I])) \quad (\text{Eq. 1})$$

At 0 M NaCl, the enzyme concentration ($[E]_{\text{total}}$) caused a significant depletion in inhibitor concentration, thus the data were fitted to Equation 2, which describes tight-binding inhibition (18).

$$\Delta F/\Delta t = ((\Delta F/\Delta t)_0 / 2[E]_{\text{total}}) \{ [(K_i + [I] - [E]_{\text{total}})^2 + 4K_i[E]_{\text{total}}]^{1/2} - (K_i + [I] - [E]_{\text{total}}) \} \quad (\text{Eq. 2})$$

In Equations 1 and 2, $(\Delta F/\Delta t)_0$ was the ribonucleolytic activity prior to inhibitor addition.

RESULTS

Purification of Inhibitor from a Commercial Buffer—Previously, we reported that a contaminant in MES buffer inhibits catalysis by RNase A, especially in solutions of low salt concentration (10). We subsequently found that other ethanesulfonic acid buffers (*i.e.* “Good” buffers) (19), such as BES, CHES, and PIPES, similarly inhibited RNase A (data not shown). Thus, we speculated that the inhibitor was a byproduct of ethanesulfonic acid buffer synthesis. We have shown that the byproduct responsible for RNase A inhibition would probably be anionic (10) because the RNase A active site and RNA binding sites are cationic ($pI = 9.3$) (20). Thus, we chose to purify the inhibitor by anion-exchange chromatography. The low concentration of this inhibitor in MES buffer (~2 ppm) (10) necessitated purifying the contaminant from a large amount of MES buffer. We first tested the inhibitory activity of a number of different commercial lots of MES buffer. All of the MES buffers tested exhibited substantial RNase A inhibition at low salt concentrations, but the inhibition per mol of MES did vary by 20-fold in different lots. Thus, we passed 0.50 kg of the most inhibitory MES buffer (Sigma, 5.0 liters of a 0.50 M solution, pH 3.0) through a column containing 50 g of AG[®] 1-X8 anion-exchange resin (chloride form, Bio-Rad). No inhibitory activity was detected in the flow-through, indicating that the inhibitor was anionic and could be purified with anion-exchange chromatography.² Likewise, no inhibitory activity was observed in a 0.1 M HCl wash of the column. The inhibitor was eluted with a 1–4 M linear gradient of HCl. Inhibitory activity was found in fractions corresponding to 1.7–4 M HCl. These fractions were pooled and evaporated to dryness, yielding 40 mg of material.

Identification of Inhibitor—In ethanesulfonic acid buffer synthesis, a nucleophile attacks 2-bromoethanesulfonic acid in H_2O to yield the buffer product (Fig. 2). Hydrolysis or β -elimination of 2-bromoethanesulfonic acid could yield 2-hydroxyethanesulfonic acid (1) or vinylsulfonic acid (2). Nucleophilic attack of 2-hydroxyethanesulfonic acid on 2-bromoethanesulfonic acid or Michael addition to vinylsulfonic acid could generate diethanesulfonic acid ether (3). Indeed, all three of these byproducts were identified by NMR spectroscopy and mass spectrometry in the material purified from MES buffer (data not shown).

Commercial 2-hydroxyethanesulfonic acid (1) and vinylsulfonic acid (2) were tested as inhibitors of RNase A. Neither was a potent inhibitor in solutions of low salt concentration (Fig.

² Of note, the flow-through of this column can be recrystallized from water to yield MES buffer that is devoid of inhibitor.

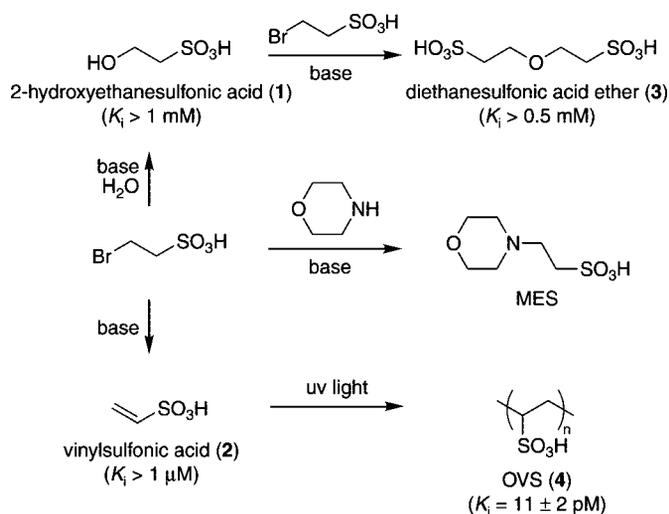


FIG. 2. **Byproducts of MES buffer synthesis.** Values of K_i are listed for inhibition of catalysis of 6-FAM~dArUdAdA~6-TAMRA cleavage by ribonuclease A in 50 mM imidazole-HCl buffer, pH 6.0. The K_i for OVS inhibition is calculated based on a molecular mass of 2,000 g/mol.

2).³ Diethanesulfonic acid ether (3) was synthesized (*vide supra*) but likewise failed to inhibit RNase A. Thus, the sought-after inhibitor was not byproduct 1, 2, or 3.

We next used a Vivaspin concentrator (5,000 molecular weight cut-off, Vivascience AG, Hannover, Germany) to purify the inhibitor based on its affinity for RNase A. RNase A (10 mg) was mixed in ddH₂O (10 ml) with the inhibitor (10 mg) that had been purified by anion-exchange chromatography. The sample was subjected to centrifugation at 6,000 rpm for 15 min, washed with ddH₂O (3 × 15 ml), and subjected again to centrifugation. Molecules that bind tightly to RNase A remained in the retentate, whereas impurities were washed into the eluate. Matrix-assisted laser desorption ionization mass spectrometry of the retentate containing RNase A and the inhibitor revealed a heterogeneous mixture of small molecules of a molecular mass of 900–2,000 g/mol. The inhibitor was then separated from RNase A by adding a solution of ammonium acetate (0.10 M) to the mixture. After repeatedly concentrating and adding ammonium acetate solution to the mixture, unbound inhibitor moved to the eluate, whereas RNase A remained in the retentate. Matrix-assisted laser desorption ionization mass spectrometry of the free inhibitor revealed the same heterogeneous distribution of molecular mass with individual peaks separated by 108 g/mol (Fig. 3). Because the molecular mass of vinylsulfonic acid (2) is 108 g/mol, we reasoned that the inhibitor was probably an oligomer of vinylsulfonic acid (OVS, 4). Similar to byproducts 1–3, OVS is probably a byproduct of ethanesulfonic acid buffer synthesis with ultraviolet light possibly initiating the radical-mediated polymerization of vinylsulfonic acid (3) (Fig. 2) (23).

Characterization of Inhibition by OVS—Inhibition of RNase A activity was measured in 0.05 M imidazole-HCl buffer, pH 6.0, containing NaCl (0.10 M) and commercial OVS ($M_r \sim 2,000$, 0–1.43 μM). OVS inhibition of RNase A is not time-dependent (data not shown). The addition of NaCl diminishes the OVS inhibition of RNase A, indicating that OVS is a reversible inhibitor of the enzyme. OVS inhibits RNase A at concentrations well below that of substrate; thus, inhibition by OVS is not attributed to its sequestering of RNA. Double-reciprocal

³ In contrast, divinylsulfone ($\text{CH}_2\text{CHS}(\text{O})_2\text{CHCH}_2$) is an irreversible inhibitor of RNase A, forming covalent bonds to active-site residues by Michael addition (21). Mechanism-based inactivation of RNase A by Michael addition has also been described previously (22).

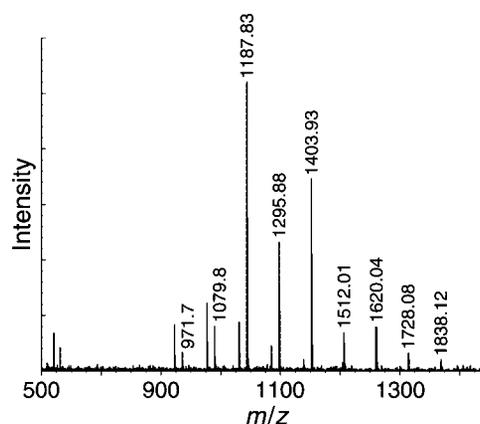


FIG. 3. **Mass spectrum of oligo(vinylsulfonic acid) purified from MES buffer.** The molecular mass of vinylsulfonic acid ($\text{C}_2\text{H}_4\text{O}_3\text{S}$) is 108 g/mol.

plots of RNase A catalytic activity *versus* the concentration of poly(C) at different OVS concentrations reveal that OVS inhibits RNase A in a competitive manner (Fig. 4). With poly(C) as a substrate, the apparent $K_i = (0.40 \pm 0.03) \text{ }\mu\text{M}$ at 0.10 M NaCl. A replot of $(K_m/V_{\text{max}})_{\text{app}}$ *versus* [OVS] reveals a straight line, which is indicative of simple competitive inhibition (24).

Salt Dependence of Inhibition by OVS—The K_i of OVS was measured at four different salt concentrations in 50 mM imidazole-HCl buffer, pH 6.0. Because OVS inhibits RNase A in a competitive manner, we were able to use a sensitive fluorescent assay to assess inhibition by OVS. OVS inhibition of RNase A is highly salt-dependent (Fig. 5A). At 0 M NaCl, OVS inhibits catalysis by RNase A with an astonishingly low inhibition constant of $K_i = (11 \pm 2) \text{ pM}$. At 0 M NaCl, the inhibition curve was fitted to a tight-binding inhibitor equation, yet the curve still exhibits some cooperativity. At 0.10 M NaCl, OVS inhibits RNase A with an inhibition constant of $K_i = (120 \pm 10) \text{ nM}$.⁴

According to polyelectrolyte theory, the slope of a plot of $\log(K_i)$ *versus* $\log([\text{cation}])$ reveals the number of Coulombic interactions between a ligand and a polyanion (28). OVS makes on average 7.8 ionic interactions with RNase A (Fig. 5B). Poly(vinylsulfuric acid), an OVS analog, exhibits a similar salt dependence (data not shown).

RNase A Inhibition by OVS Analogs—To assess the importance of the sulfonic acid group for RNase A inhibition, we tested poly(vinylphosphonic acid) (PVP) and poly(vinylsulfuric acid) (PVOS) for inhibition of ribonucleolytic activity in our fluorescent assay. These analogs are also good inhibitors of RNase A but are slightly less effective than is OVS (Table I). The average molecular masses of PVP and PVOS were 20,000 and 170,000 g/mol, respectively. Nevertheless, by mass spectrometry, the minimum number of OVS units that bound tightly to RNase A was nine. Thus, each chain of commercial OVS ($\sim 2,000$ g/mol) could tightly bind to two RNase A molecules per chain, whereas each chain of PVP or PVOS could tightly bind to more. Hence, to enable a direct comparison of inhibition by OVS, PVP, and PVOS, the data listed in Table I are in units of mass rather than moles.

DISCUSSION

Purification and Characterization of Inhibitor—OVS is an extremely low level contaminant in MES buffer (~ 2 ppm) (10).

⁴ In theory, the value of K_i for a competitive inhibitor should be independent of the substrate used in the assay. Yet, the observed value of K_i for OVS is 3-fold higher when poly(C) rather than 6-FAM~dArUdAdA~6-TAMRA is the substrate for RNase A. This effect of polymeric substrates has much precedence and several proposed explanations (25–27).

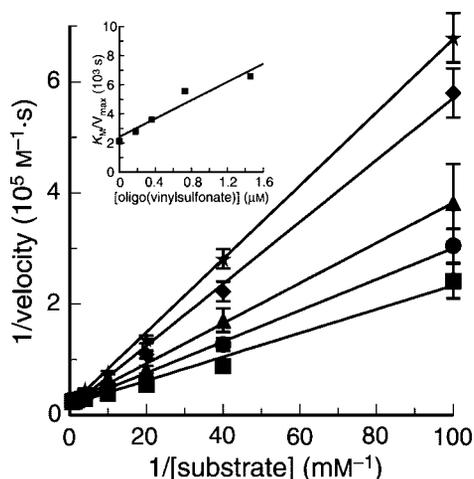


FIG. 4. Effect of commercial oligo(vinylsulfonic acid) on catalysis of poly(cytidylic acid) cleavage by ribonuclease A. Lineweaver-Burk plots are shown for five concentrations of oligo(vinylsulfonic acid): 0.0 (■), 0.35 (●), 0.7 (▲), 1.4 (◆), and 2.8 μM (*). Assays were performed at 25 °C in 50 mM imidazole-HCl buffer, pH 6.0, containing NaCl (0.10 M). Inset, slope replot of the kinetic data.

Thus, its purification is problematic. We were able to isolate <2 mg of OVS from 0.5 kg of MES buffer. This material was difficult to separate from other anionic byproducts of ethanesulfonic acid buffer synthesis (Fig. 2). OVS has no distinct properties that allow it to be detected during purification. NMR spectroscopy failed to detect OVS in the material purified from MES, because <5% of that material was OVS. However, mass spectrometry did enable the identification of OVS in MES buffer as the cause of RNase A inhibition in solutions of low salt concentration (Fig. 3). Specifically, oligomers of 9–17 units were responsible for the inhibition. It is probable that oligomers shorter than 9 units in length are also present in MES buffer, but these were not observed after anion-exchange chromatography and affinity purification. Because purification of the inhibitor was monitored by RNase A inhibition assays, oligomers of fewer than 9 units are probably less-effective inhibitors of RNase A.

After our identification of OVS in MES buffer, we found a previous report (29) that large polymers (~50,000 g/mol) of poly(vinylsulfonic acid) comprised ~1% of a single lot of MES buffer (29). This lot of MES buffer inhibited the catalytic activity of 6-phosphogluconate dehydrogenase. Other lots of MES buffer failed to inhibit the enzyme because only long polymers were inhibitory (30). We suspect that oligo(vinylsulfonic acid) and occasionally poly(vinylsulfonic acid) contaminate commercial MES buffer and other ethanesulfonic acid buffers and that the amount of these contaminants varies from lot to lot.

Kinetic Analyses—OVS inhibition of RNase A follows a simple competitive model (Fig. 4). Because OVS (~2000 g/mol) has on average only 18 monomer units per molecule, it is on the cusp of consideration as a polyelectrolyte (28). Nonetheless, a double-log plot of K_i versus [cation] indicates that OVS forms 7.8 Coulombic interactions with RNase A (Fig. 5B). The inhibition of RNase A by poly(vinylsulfuric acid) (~170,000 g/mol) shows a similar salt dependence (data not shown). The number of Coulombic interactions between OVS and RNase A is in gratifying agreement with a previous report (28) that single-stranded DNA forms 7 Coulombic interactions with RNase A. Thus, OVS probably saturates the same phosphoryl group binding subsites as does a single-stranded nucleic acid (Fig. 1).

Multivalent Inhibition—Polyanions are known to be effective inhibitors of RNase A (27). Heparin, tyrosine-glutamate copolymers, and many different polysulfates and polyphosphates

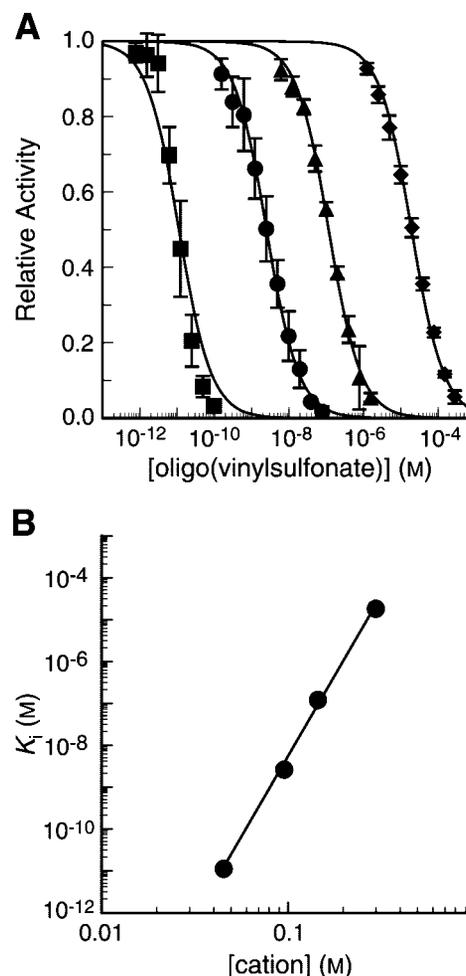


FIG. 5. Salt dependence of commercial oligo(vinylsulfonic acid) inhibition of catalysis of 6-FAM~dArUdAdA~6-TAMRA cleavage by ribonuclease A. A, assays were performed at 25 °C in 50 mM imidazole-HCl buffer, pH 6.0, containing NaCl (■, 0; ●, 0.05; ▲, 0.10; and ◆, 0.25 M). Rates determined at 0.05–0.25 M NaCl were fitted to Equation 1. Rates determined at 0 M NaCl were fitted to Equation 2. B, values of K_i were calculated from the data in A. [Cation] refers to the concentration of Na^+ plus imidazolium ion.

TABLE I
Inhibition of ribonuclease A catalysis by commercial oligo(vinylsulfonic acid) and its phosphonic acid and sulfuric acid analogs

Inhibitor	K_i^a
	$\mu\text{g/ml}$
Oligo(vinylsulfonic acid)	0.24 ± 0.02
Poly(vinylphosphonic acid)	0.35 ± 0.02
Poly(vinylsulfuric acid)	0.38 ± 0.06

^a Values of K_i were obtained in 0.05 mM imidazole-HCl buffer, pH 6.0, containing NaCl (0.10 M). Values of K_i are in units of $\mu\text{g/ml}$ to account for the different average molecular mass of each polymer.

have been shown previously to inhibit catalysis by the enzyme (26, 31, 32). We were surprised to learn that 40 years ago, even poly(vinylsulfonic acid) had been tested as an inhibitor of RNase A. Those data suggested that poly(vinylsulfonic acid) was a worse inhibitor of RNase A than other polyanions (33, 34), or alternatively, that only long polymers (>9,000 g/mol) were good inhibitors of RNase A (35). We do not know the basis for the disparity with our data.

OVS, like PVP and PVOS, is similar to a nucleic acid backbone in having anionic non-bridging oxygen atoms. In addition, the phosphorous atoms in a nucleic acid and alternating sulfur atoms in OVS are separated by five other atoms. However,

there is a major difference between OVS and a nucleic acid. With its three non-bridging oxygens per monomer unit, OVS provides many more opportunities to form strong hydrogen bonds than does a nucleic acid. Pyrophosphate-linked ribonuclease inhibitors also display extra non-bridging oxygens, which probably enhance their affinity for RNase A (8).

OVS compares favorably with the most potent known small-molecule inhibitor of RNase A, a pyrophosphate-linked oligonucleotide, pdUppA-3'-p (4). Under similar buffer conditions with 0.10 M NaCl, each has a K_i near 120 nM. Yet, unlike pdUppA-3'-p, OVS is simple to prepare and is extremely stable. Accordingly, OVS could be useful in preventing incidental ribonuclease contamination and RNA degradation in experiments involving RNA. Indeed, poly(vinylsulfuric acid), an OVS analog, has been added to experiments involving the isolation of mRNA (36) or cell-free translation (37).

Other enzymes are known to be inhibited by poly(vinylsulfonic acid). For example, poly(vinylsulfonic acid) inhibits catalysis by RNA polymerase and reverse transcriptase (38, 39). We believe that OVS could be an inhibitor of any enzyme that binds strands of RNA or DNA.

Buffer Contamination—The presence of OVS in all lots of MES buffer tested herein and in many other ethanesulfonic acid buffers is troubling. The amount of OVS varies from lot to lot, and thus, some lots of buffers could contain high concentrations of OVS. We recommend that all ethanesulfonic acid buffers be purified by anion-exchange chromatography prior to their use in assays of enzymatic activity. Alternatively, an OVS-free buffer should be used instead. Imidazole, bis-tris, and Tris buffer are suitable alternatives, depending on the pH of the assay.

MES buffer has been the buffer of choice in assays of the catalytic activity of RNase A, as the pK_a of MES buffer ($pK_a = 6.15$) (19) is near the pH of maximal activity (pH = 6.0) (40). Many RNase A assays are performed in the presence of 0.10 M NaCl at which the K_i of OVS is ~ 120 nM (Fig. 5A). We find that the OVS concentration in many lots of MES buffer is near 2 ppm. In 0.10 M MES buffer, the concentration of OVS is near 0.2 μ M, which is greater than its K_i value. Historically, RNase A has been reported to have a bell-shaped salt-rate profile with an optimum salt concentration near 0.1 M NaCl (41–43). We believe that this observed bell shape is an artifact because of contaminating OVS in MES buffer. Indeed, the salt-rate profile of RNase A has been measured recently in bis-tris buffer, revealing that ribonucleolytic activity increases to the diffusion limit as salt concentration decreases (10, 44, 45).

CONCLUSIONS

We have found that OVS is a common contaminant of ethanesulfonic acid buffers. Although present in only ppm concentrations, OVS is a potent inhibitor of RNase A, making nearly 8 favorable Coulombic interactions with the enzyme. OVS is inexpensive and extremely stable, unlike other known ribonuclease inhibitors. Accordingly, OVS has the potential to be a useful prophylactic in many chemical, biochemical, and

biotechnical experiments involving RNA. Finally, we note that the purity of the buffer used to assay RNase A and other enzymes deserves special consideration.

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