Reactions between Tunichrome Mm-1, a Tunicate Blood Pigment, and Vanadium Ions in Acidic and Neutral Media†

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ABSTRACT: Tunichromes are yellow, polyphenolic tripeptides prevalent in blood cells of tunicates (suborders phlebobranchia and stolidobranchia). Spectrophotometric studies of reactions between tunichrome Mm-1 and V^{V} or V^{IV} ions were conducted *in vitro* in various media to crudely approximate cellular conditions: deionized water, aqueous methanol, and aqueous buffers at pH 2 and 7. Catechol was used in parallel studies for comparison to tunichrome and was found to be a good model for tunichrome reactivity. For V^{IV} in pH 7 buffer, both catechol and Mm-1 formed complexes with V^{IV} ions, and no redox products were found. For V^{V} in pH 2 buffer, both catechol and Mm-1 were oxidized by V^{V} ions. Room temperature EPR qualitatively showed that Mm-1 in pH 2 buffer reduced V^{V} ions to free V^{IV} ions. For V^{V} in pH 7 buffer, Mm-1 was oxidized by V^{V} ions and formed V^{IV} complexes. At higher concentrations, the V^{IV} complexes were observed by low temperature EPR [Grant, K. B. (1994) Dissertation, Columbia University; Grant, K. B., et al. (1996) *J. Inorg. Biochem.* (manuscript in preparation)]. Using a colorimetric assay for V^{III}, we found that reactions between Mm-1 and V^{V} or V^{IV} ions in pH 7 buffer clearly did not generate appreciable quantities of V^{III} products. Thus, the colorimetric V^{III} assay resolved the issue of V^{III} product formation raised in EPR studies of Mm-1 [cf. Ryan, D. E., et al. (1992) *Biochemistry 35*, 8651−8661]. Overall, the results provide insights into tunichrome-vanadium chemistry and identify conditions which promote complexation and/or redox reactions *in vitro*.

Tunicates (ascidians, sea squirts; phylum Chordata, class Ascidiacea) are sessile, marine filter-feeders that are intriguing to biologists as representatives of very early chordates and to chemists since Henze discovered that tunicate blood cells harbor large quantities of oxygen-sensitive V^{III} (Henze, 1911). Surveys of over 50 species found correlations between ascidian suborder and transition metal accumulation: phlebobranchs accumulate V^{III} predominantly, aplousobranchs accumulate V^{IV}, and stolidobranchs are Fe^{II} accumulators (Swinehart et al., 1974; Hawkins et al., 1983a). Recently, V^{III} accumulation was discovered in one other eukaryote, the marine fan worm *Pseudopotamilla occelata* (phylum Annelida, class Polychaeta), in which V^{III} is located in the epidermis of the branchial crown (Ishii et al., 1993). In the fan worm and in phlebobranchs, the biological role of V^{III} remains an enigma.

Vanadium-accumulating tunicates collect the metal ions from sea water, where low concentrations (10−40 nM) are present as monomeric vanadate ions ($H_2VO_4^-/HVO_4^{2-}$) in the V^{V} oxidation state (Kustin et al., 1975; Jaendel et al., 1987). Vanadate anions are transported into ascidian blood cells via facilitated diffusion utilizing the phosphate transport system (Dingley et al., 1981). After transport, vanadate anions are reduced to $V^{IV}O$ and/or V^{III} cations. Blood cells of *Ascidia nigra* and *Ascidia ceratodes* possess predominantly V^{III} ions, as well as low levels of V^{IV} ions (<10% of total vanadium) (Tullius et al., 1980; Dingley et al., 1981; Frank et al., 1986, 1995; Lee et al., 1988a; Smith et al., 1991). The phlebobranchs store endocytic vanadium at remarkably high concentrations—10^3-10^7-fold greater than the levels in sea water (Swinehart et al., 1974; Carlson, 1975; Agudelo et al., 1983; Michibata et al., 1986; Lane & Wilkes, 1988).

In many species of tunicates, the blood cells also harbor a yellow chromagen named tunichrome. Tunichrome was purified from blood cell lysates of phlebobranchs *A. ceratodes* (Swinehart et al., 1974) and *A. nigra* (Macara et al., 1979a,b) and the stolidobranch *Molgula manhattensis* (Macara et al., 1979c). Through a series of careful separations, tunichromes were isolated and their structures were determined (Chart 1): An-1, An-2, and An-3 **1a**−**c** from *A. nigra*; An-1 **1a** from *A. ceratodes*; and Mm-1 **2a** and Mm-2 **2b** from *M. manhattensis* (Bruening et al., 1985, 1986; Oltz et al., 1988). A survey of 11 species of tunicates found tunichromes in blood cells of phlebobranchs and a stolidobranch, but not aplousobranchs (Parry et al., 1992). In the phlebobranch *Phallusia mamillata*, dihydrotunichromes Pm-1, Pm-2, and Pm-3 corresponding to tunichromes **1** saturated at the C-11,12 olefin were isolated (Bayer et al., 1992). The tunichromes and dihydrotunichromes are structurally related to other marine natural products (Davidson, 1993). Although the function of tunichromes is not known, their importance is strikingly apparent by their abundance in *A. nigra* morula blood cells—constituting up to 50% of dry weight (Oltz et al., 1989).

The chemical structures of tunichromes, specifically the electron-rich catechol and pyrogallol rings, suggest that tunichromes may complex and/or reduce V^{V} upon transport into blood cells. Support for a connection between tunichrome and vanadium *in vivo* was found in FACS[1]-sorted blood cells of *A. nigra* and *A. ceratodes* (Oltz et al., 1989). Morula-type blood cells contained nearly all of the unoxidized tunichrome and up to 30% of the total vanadium,

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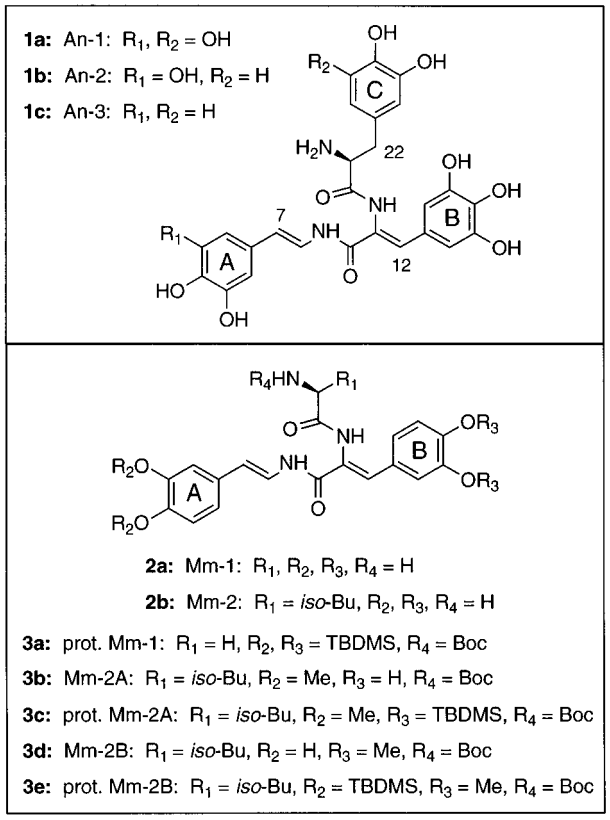
Chart 1



whereas the signet ring and compartment cells contained the majority of the vanadium[2] (cf. Botte et al., 1979; Michibata et al., 1987; Scippa et al., 1988; Brand et al., 1989). These findings suggest that tunichromes in morula cells may complex and reduce V^V ions and that morula cells may give rise to compartment cells and signet ring cells. If this hypothesis is correct, the V^{III} laden signet ring and compartment cells should also contain oxidized, cross-linked tunichrome (cf. Waite, 1990) which cannot be detected by the tunichrome assay of Oltz et al. (1989).

The well-studied redox and complexation chemistry of catechols and vanadium ions (Cooper et al., 1982) is especially relevant to considerations of tunichrome−vanadium chemistry. Catechol and pyrogallol form quite stable mono- and bis-ligated $V^{IV}O$ complexes in aqueous solution: the stability constants for the $V^{IV}O$ complexes of catechol (log K_1 17.7, log K_2 33.5) and pyrogallol (log K_1 15.0, log K_2 28.7) are the largest found among first row, divalent transition metals chelated by common bidentate ligands (Martell & Smith, 1979; Chasteen, 1981; Rehder, 1991). In methanol, the tris(catecholate) complex of V^V is stable and can be isolated (Cooper et al., 1982). In aqueous media, however, catechols complex V^V and reduce the metal to the V^{IV} state (Shnaiderman et al., 1972; Zelinka et al., 1974; Kustin et al., 1974a,b; Ferguson & Kustin, 1979). For vanadium and catechol redox reactions in aqueous media, standard reduction potentials E^o (volts vs NHE) under acidic conditions have been measured: 1.00 V for V^V/V^{IV}, 0.795 V for catechol/quinone, 0.680 V for pyrogallol/hydroxyquinone, and 0.337 V for V^{IV}/V^{III} (Lide, 1993; Horner & Geyer, 1965). For a series of catechols treated with vanadate (V^V) in acidic and mildly basic aqueous media (Kustin et al., 1974a,b; Ferguson & Kustin, 1979), the reaction kinetics indicated an initial complexation of V^V; the V^V−catecholate complex was then oxidized by free V^V ions to generate two $V^{IV}O$ ions per orthoquinone produced (orthoquinones are not stable in aqueous media). The rate of the redox reaction increased with decreasing pH, whereas the rate of initial complexation increased with increasing pH. Two electrons were transferred from catechol to a coordinated V^V pair, rather than to the initially chelated V^V ion. In order for one V^V ion to accept both electrons from a two-electron reductant, the reductant must be sufficiently potent to reduce V^{IV} to V^{III}. If so, the V^{III} products can persist if protected from V^V ions and other oxidants.

Models of tunichrome structures have been composed of three pyrogallol or catechol moieties tethered by flexible linkages (Bulls et al., 1990; Kime-Hunt et al., 1991). These tethered models reduced V^V (ca. 2 mol equiv) to V^{IV} in aqueous methanol. In contrast, a tethered analog with catechol moieties produced a stable tris(catecholate)-type V^V complex which is unusual for maintaining the V^V state in aqueous media (Butler et al., 1992).

Catechols have reduced V^V and V^{IV} species to the V^{III} state in nonaqueous media only. In anhydrous THF, pyrogallol reduced $V^{IV}O(acac)_2$ and produced a pyrogallol-bridged V^{III} dimer with acetylacetonate ligands (Lee et al., 1988b). In acidic nonaqueous media (2 equiv of $HClO_4$ in CH_3CN), $V^{IV}O$(salen) complex disproportionated to V^V and V^{III} complexes under anaerobic conditions (Bonadies et al., 1987). Although the salen ligand is not a catechol, it has similar *ortho*-substituted phenol moieties. The other examples of catechols generating the V^{III} state are based on uncertain charge distributions within the complexes produced. In anaerobic toluene or anhydrous methanol, 3,5-di-*tert*-butyl-1,2-benzoquinone with $V(CO)_6$ gave the same product as 3,5-di-*tert*-butylcatechol with $V^{IV}O(acac)_2$; the product was formulated as the tris(semiquinone) complex of V^{III}, $V^{III}(DBSQ)_3$ (Cass et al., 1983, 1986). Although a semiquinone ligand was clearly evident, shifting electronic charge from V^{III} to one or two semiquinone ligands would give catecholate ligands and the metal in a higher oxidation state. Similarly, the reaction of 3,5-di-*tert*-butylcatechol with the heteropolyvanadate $[PV^V_{14}O_{42}]^{9-}$ in anaerobic acetonitrile gave a product formulated as the V^{III} dimer $[V^{III}(DBSQ)(DBcatH)_2]_2$; however, proof of structure was not given for this complex either (Tatsuno et al., 1987).

The earliest reaction *in vitro* between a tunichrome extract (from *A. ceratodes*) and vanadium ions found that tunichrome was oxidized by V^V; however, no chemical evidence was reported (Swinehart et al., 1974). More detailed spectrophotometric investigations found that tunichrome extracts from *A. nigra* and *M. manhattensis* readily reduced V^V and

[1] Abbreviations: acac, acetylacetonate; Boc, *tert*-butoxycarbonyl; *iso*-Bu, *iso*-butyl; CD, circular dichroism; DBcatH, 3,5-di-*tert*-butylcatechol; DBSQ, 3,5-di-*tert*-butylsemiquinone; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance spectroscopy; FACS, fluorescence-activated cell sorting; HPLC, high-performance liquid chromatography; Me, methyl; NHE, natural hydrogen electrode; NMR, nuclear magnetic resonance spectroscopy; ppt., precipitate; TBDMS, *tert*-butyldimethylsilyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; UV/vis, Ultraviolet/visible spectroscopy; XAS, X-ray absorption spectroscopy.

[2] Recent X-ray microanalysis of blood cells from the phlebobranch *Phallusia fumigata* found that morula cells contained higher levels of vanadium than levels found in signet ring or compartment cells (Martoja et al., 1994). The authors suggest that morula cells, especially, release their stored vanadium during cell sorting procedures, e.g., FACS or density gradient centrifugation.

Fe^{III} (Macara et al., 1979a,c). For additions of V^{IV} or V^{III}, however, no reaction was observed. These studies were conducted in aqueous acids (0.1 M HCl and 1.0 M H_2SO_4) which may not approximate cellular conditions. In blood cells, strongly acidic pH's (pH 1.8−2.0) (Frank et al., 1986, 1988; Michibata et al., 1991) were found as well as weakly acidic to neutral pH's (pH 4.6−7.1) (Hawkins et al., 1983b; Lee et al., 1990). As the redox and complexation equilibria for V^V, V^{IV}, and tunichromes are pH dependent, the reaction medium strongly influences which reactions are favored. Another source of ambiguity in the previous studies is the purity of the tunichrome extracts; for example, the high molar absorptivity of tunichromes could mask detection of co-eluted contaminants.

More accurate studies of tunichrome-vanadium chemistry became possible when pure tunichromes were obtained via synthesis (Horenstein & Nakanishi, 1989; Kim et al., 1990) or directly from tunicate blood via a protection−isolation−deprotection method (Kim et al., 1991). With synthetically pure tunichrome Mm-1 **2a** in hand, we investigated the complexation and redox chemistry between this simplest tunichrome and vanadium ions *in vitro*. Although Mm-1 is natively found in Fe-accumulating tunicates, it was selected for these studies as its complexation and redox properties pertain to the tunichromes generally. On the basis of our initial studies of Mm-1, we reported that reactions between Mm-1 and V^V or V^{IV} ions at neutral pH generated V^{III} products, according to indirect evidence obtained from EPR measurements (Ryan et al., 1992). These results encouraged the studies of Mm-1 reported here.

A large array of products was expected for tunichrome−vanadium reactions because of the various equilibrium distributions of free and complexed vanadium species in aqueous media, compounded by the variety of cross-linked Mm-1 products that result from oxidation of catechols. Catechols are oxidized to orthoquinones via loss of 2 equiv of electrons; however, orthoquinones in aqueous media are not stable and undergo dimerization (Alder & Berggren, 1960), oligomerization via cross-linking to unoxidized catechols (Waite, 1990; Sugumaran et al., 1987), and cross-linking to nucleophilic species (Schaefer et al., 1987; Christensen et al., 1991). Cross-linking to an orthoquinone generates a substituted catechol via tautomerization (e.g., Sugumaran et al., 1987), and further oxidation and cross-linking of such rings is conceivable. Thus, the cross-linked oxidation products of catechols and tunichromes possess catechol rings. In the presence of V^{IV} ions in aqueous media, catechols form mono, bis, and/or tris(catecholate)-type V^{IV} complexes (Branca et al., 1990; Cooper et al., 1982). Vanadium ions can also form metal−metal bonds. For example, in neutral aqueous media, V^{IV} ions are dimers, $[HOV^{IV}O]_2^{2+}$, and aggregates of $V^{IV}O(OH)_2$ (Francavilla & Chasteen, 1975); V^V ions are mixtures of mono-, di-, tetra-, and decavanadates with the degree of protonation dependent on pH (Rehder, 1995, 1991; Crans et al., 1990; Chasteen, 1983). Despite the complexity of products expected for tunichrome−vanadium reactions, the crude product mixtures were spectrophotometrically characterized by assigning peaks due to complexation products, redox products, or both.

In this report, anaerobic reactions between Mm-1 and V^V or V^{IV} ions were conducted in various media to crudely approximate cellular conditions: deionized water, aqueous methanol, and aqueous buffers at pH 2 and 7. As tunichromes display a large molar extinction coefficient at ca. 340 nm ($\pi \rightarrow \pi^*$ transition), the reactions were monitored spectrophotometrically in cuvettes under Ar. Crude product mixtures generated by Mm-1 and V^V or V^{IV} were characterized spectrophotometrically by adding EDTA to identify complexation products and by using sodium periodate, $NaIO_4$ ($E^o = 1.60$ V in acid; Lide, 1993), as a model for vanadate ($NaH_2V^VO_4$) in parallel reactions to identify tunichrome oxidation products. For comparison to tunichrome, catechol was used in analogous spectrophotometric studies. Similar reactions between Mm-1 and V^V or V^{IV} ions were analyzed using EPR spectroscopy to observe any V^{IV} products and using colorimetric V^{III} analysis to quantitate any V^{III} products.

MATERIALS AND METHODS

Synthetic Mm-1 was previously prepared in protected form **3a** (Kim et al., 1990). Reagents and organic solvents were of the highest purity available from Aldrich and Sigma, including $VOSO_4 \cdot 3H_2O$ (99.99+%) and V_2O_5 (99.99%). Catechol was twice recrystallized from benzene using Norite charcoal. Water was distilled and deionized. Argon was prepurified grade (99.993%). Aqueous media were deoxygenated in Schlenk flasks by cooling to 4 °C and alternately evacuating (ca. 0.1 torr) and filling with Ar (12×). Organic solvents were similarly deoxygenated, but cooled to −78 °C. *All reactions were strictly carried out under Ar using airless techniques.* Additions were made via syringe equipped with custom 26- or 24-gauge, 10-inch needles (Hamilton); transfers were made via cannula.

UV/vis spectra were recorded on a Perkin-Elmer Model 320 spectrophotometer with background correction. X-band EPR spectra were recorded at room temperature on a Bruker ESP 300 spectrometer with OS 9 software. EPR instrument settings were 9.49 GHz frequency, 4.027 G modulation amplitude, 30.4 mW power, and 1200 G sweep width. Thin glass EPR tubes were prepared from 9-inch, disposable glass pipettes by flame-sealing the tips and sealing the tops with septa. EPR spectra of pentaaquovanadyl ions $V^{IV}O(H_2O)_5^{2+}$ were background corrected by subtracting a scaled cavity spectrum (recorded daily for 1:1 methanol/water in an EPR tube). To integrate a background corrected EPR spectrum, the intensities of the V^{IV} signal lines were measured with a ruler (in mm) and added together to give the integrated intensity (of the eight-line isotropic signal, the six lowest field lines were used: $^{-7}/_2$ to $^{+3}/_2$). To normalize an integrated spectrum for comparison to other spectra, the integrated intensity was divided by the scaling factor used for background correction.

Deprotection of Mm-1. The literature procedure (Horenstein & Nakanishi, 1989; Kim et al., 1990) was followed using 16.2 mg of protected Mm-1 **3a** (0.0172 mmol) and the following modifications. Teflon needles and cannula were used for all acids to avoid contamination with metal ions. After deprotection of the Boc-protected amine of **3a** using TFA (0.4 mL) in CH_2Cl_2 (1.6 mL), hexane (1.6 mL) was added. The solution was cooled to −78 °C and connected to a vacuum line with a 23-gauge needle to evaporate solvents. The residue was dissolved in CH_2Cl_2 (0.5 mL) and transferred to a 15-mL plastic centrifuge tube containing a flea-type stir bar. The solvent was evaporated as above using a 25-gauge needle. After deprotection of the TBDMS-protected phenols of **3a** using 48% HF(aq)/

pyridine with *tert*-butylsulfide (5 h), the reaction mixture was cooled to −78 °C. In an acid-rinsed flask, acetic acid (99.99+%) was measured to neutralize the pyridine. This was deoxygenated and transferred to the mixture at −78 °C (which then sat at 25 °C until thawed). The precipitation scheme was as follows: (1) added 2 mL of CH_2Cl_2, then 2 mL of hexane to precipitate a yellow oil; removed colorless supernatant via cannula; (2) added 0.5 mL of methanol, then 2 mL of CH_2Cl_2 (yellow ppt. formed, then became an oil), then 2 mL of hexane; removed colorless supernatant; (3) replaced old septum with new; cooled to −78 °C; and evaporated solvents under vacuum with gradual warming; (4) added 0.4 mL of methanol, then 2.5 mL of CH_2Cl_2 (yellow ppt. formed); centrifuged for 20 min at 1750 × *g*; and removed yellow supernatant; (5) twice repeated step 4 using sonication to partially dissolve the ppt. in methanol. The yellow ppt. was dried under vacuum. ^{1}H NMR (methanol-d_4/D_2O) showed the desired product **2a** as the acetate salt. [*Note:* anhydrous CH_2Cl_2 must be free of trace bases, such as CaH_2. Using 0.3 mL of methanol throughout the precipitation scheme is recommended for better yields.]

Spectrophotometric Studies. All reactions and air-sensitive ϵ determinations were carried out in custom-made quartz cuvettes (Wilmad) sealed with 8-mm septum screw caps (Wheaton) connected via 23-gauge needles to a vacuum/Ar line. To confirm that the sealed custom cuvettes were sufficiently airtight, V^{IV} in pH 7 buffer under Ar was stable over at least 2 h after disconnection from the Ar line. The reaction media were deionized water, methanol (HPLC grade), 50 mM phosphate buffer, pH 7.0, 50 mM bicarbonate buffer, pH 4.0, and 50 mM bisulfate buffer, pH 2.0. The following stock solutions were prepared in volumetric flasks and stored in Schlenk tubes: (i, ii) 0.5 mM and 5.0 mM V^{IV} in 10 mM bisulfate buffer, pH 2 (under Ar), (iii, iv) 0.5 mM and 5.0 mM V^V in 10 mM carbonate buffer, pH 10 (the V_2O_5 suspension was boiled to dissolve), (v, vi) 0.5 mM and 5.0 mM $NaIO_4$(aq), (vii, viii) 0.5 mM and 5.0 mM $NaIO_3$(aq) for reference cuvettes, (ix) 50 mM EDTA in 50 mM pH 7 buffer, and (x) 5 mM catechol in HCl(aq) pH 2.0. A stock solution of ca. 0.5 mM Mm-1 (below) in 20% aqueous methanol was prepared and stored in a 25-mL Schlenk flask at −70 °C.

Molar Extinction Coefficients (ϵ) for Mm-1 and Catechol in the Various Reaction Media. For determinations of ϵ for catechol (under Ar; solid handled in air), two 5.0 mM aqueous solutions were prepared in 25-mL volumetric flasks. For both solutions, ϵ was determined in water (950 μL + 50 μL addition). One solution was used to determine ϵ in pH 7 buffer (50 μL additions). For protected Mm-1 **3a** (handled in air), two hexane solutions were prepared (4.42 and 25.40 mg in 10- and 50-mL volumetric flasks, respectively), and ϵ was determined in hexane (960 μL + 40 μL addition). For the 50-mL solution, ϵ was determined in methanol and in acetonitrile (40 μL additions). The ϵ value for protected Mm-1 **3a** in acetonitrile was used for deprotected Mm-1 **2a** in acetonitrile (980 μL + 20 μL addition) to approximately calibrate the concentration of the Mm-1 stock solution. The calibrated Mm-1 stock solution was used to determine approximate values of ϵ in water, methanol, pH 7 buffer, and pH 2 buffer (20 μL additions under Ar). For another batch of protected Mm-1 **3a**, a hexane solution of 31.5 mg was prepared in a 100-mL volumetric flask. As above, ϵ was determined in methanol (940 μL + 60 μL addition). Values of ϵ were determined for partially protected tunichromes Mm-2A **3b** and Mm-2B **3d** (Grant, 1994).

Mm-1 Reactions. In a typical experiment, a 40 μL aliquot of the ca. 0.5 mM Mm-1 stock solution was added to 960 μL of the reaction medium in a sealed cuvette. The solution was mixed by Ar bubbling using a 26-gauge, 10-inch needle. The amount of Mm-1 added was chosen to give an absorbance of 0.5−1.0. The concentration of Mm-1 in the cuvette was calculated using ϵ determined for the reaction medium. Stepwise additions of V^{IV} or V^V were made such that the total V in the cuvette was 0.5, 1.0, 2.0, or 4.0 mol equiv (0.5 mM stock solution), or 8.0 mol equiv (5 mM stock solution). An absorption spectrum was acquired after each addition. The reference cuvette contained the reaction medium (1.0 mL). For the timed experiments, a single aliquot of V^{IV} or V^V stock solution was added at once, and absorption spectra were acquired over time. Because of the known oxygen sensitivity of catecholates and reduced vanadium species, all reactions were run twice to screen for artifacts.

Catechol Reactions. For the catechol reactions, the procedure was the same as for the Mm-1 reactions except for the following modifications. In a typical experiment, a 50-μL aliquot of the 5 mM stock solution was added to 950 μL of the reaction medium in a sealed cuvette. Stepwise additions of V^{IV} or V^V were made such that the total V in the cuvette was 0.5, 1.0, 2.0, or 4.0 mol equiv. The reference cuvette contained the reaction medium (1.0 mL) under Ar, and V^{IV} or V^V was added to obtain a corrected spectrum of the product mixture.

EPR Spectroscopic Studies. The reaction media were 50 mM bisulfate buffer, pH 2.0, and 50 mM phosphate buffer, pH 7.0, 6.0, or 5.0. Stock solutions of reactants were prepared in volumetric flasks and stored in Schlenk tubes: (i) 5.0 mM V^{IV} in 17.6 mM bisulfate buffer, pH 2.0 (under Ar), and (ii) 5.0 mM V^V in 10 mM carbonate buffer, pH 8.2 (V_2O_5 was boiled to dissolve). A stock solution of ca. 0.5 mM Mm-1 (Kim et al., 1990) in methanol was prepared and stored in a Schlenk flask at −70 °C. The Mm-1 stock solution was calibrated periodically by UV/vis analysis in methanol (ca. 340 nm, 32 500 M^{-1} cm^{-1}). Reactions were conducted in 1- or 2-mL test tubes sealed with rubber septa.

In a typical reaction between Mm-1 and V^V, an aliquot (100 μL) of the Mm-1 solution was transferred to a sealed test tube. Then pH 7 buffer (20 μL) was added followed by V^V reactant (10 μL for 1 mol equiv). After ca. 1.5 h at pH 7, pH 2 buffer (70 μL) was added. This addition dissolved the greenish black precipitate that sometimes formed at pH 7. After an additional hour, the solution was transferred via cannula to an EPR tube, and a pre-O_2 spectrum was acquired. Subsequently, O_2 was slowly bubbled through the sample (10 min) using a 10-inch, 26-gauge needle. The septum was replaced, and Ar was bubbled through the sample (15 min) using a 10-inch needle inlet and a mineral oil bubbler outlet. A post-O_2 EPR spectrum was then acquired. Parallel V^{IV} controls were prepared and analyzed similarly using V^{IV} solution instead of V^V solution and using methanol instead of the Mm-1 solution.

Colorimetric Assay for V^{III}. The method is described elsewhere (Ryan et al., 1996).

Table 1: Molar Extinction Coefficients (ϵ) of Mm-1 and Related Compounds

compound	solvent	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})
catechol	water	275	2300 ± 7
catechol	pH 7 buffer	275	2312
prot. Mm-1 **3a**	hexane	330	29400 ± 500
prot. Mm-1 **3a**	methanol	340	35100 ± 600
prot. Mm-1 **3a**	acetonitrile	338	35800
Mm-2A **3b**	acetonitrile	341	34200
prot. Mm-2A **3c**	acetonitrile	339	31700
Mm-2B **3d**	acetonitrile	331	33900
prot. Mm-2B **3e**	acetonitrile	339	33800
Mm-1 **2a**	acetonitrile	335	~35800[a]
Mm-1 **2a**	water	336	~30000
Mm-1 **2a**	methanol	339	~32500
Mm-1 **2a**	pH 7 buffer	339	~30000
Mm-1 **2a**	pH 2 buffer	336	~31500

[a] Approximated from ϵ for **3a** in acetonitile (see text); approximate ϵ values for **2a** in other solvents were determined relative to this ϵ.

RESULTS AND DISCUSSION

Synthetic tunichrome Mm-1 **2a** was obtained in its fully protected form **3a** (Kim et al., 1990). In our hands, deprotection of **3a** usually resulted in a yellow oil rather than a precipitate as reported, and the product often contained unacceptable amounts of impurities, most likely due to oxidation during the isolation procedure. To deter oxidation, the pyridine used in the final deprotection step was neutralized with acetic acid. The product work-up produced a yellow oil that settled to the bottom of a centrifuge tube when sufficient hexane was added. The supernatant was removed, and the procedure was repeated to extract HF/pyridine. Subsequently, volatile components were evaporated from the yellow oil under vacuum, and a yellow precipitate was obtained as reported and purified by repeated precipitation to give Mm-1 of acceptable purity (by ^{1}H NMR analysis).

Spectrophotometric Studies. To make stoichiometric additions (of V^V, V^{IV}, etc.) to Mm-1 and catechol in spectrophotometric studies, it was necessary to determine their molar extinction coefficients, ϵ (M^{-1} cm^{-1}), in the various reaction media (Table 1). For catechol and fully protected tunichromes **3a,c,e**, ϵ determinations were straightforward because the material could be weighed in the air. For partially protected tunichromes **3b,d** (and the fully protected compounds **3c,e** as controls), constant weights were obtained in Schlenk flasks (Teflon stopcocks) under N_2 at constant pressure (mercury bubbler outlet). In acetonitrile, the ϵ values for the partially protected tunichromes **3b,d** were nearly the same as those for the fully protected compounds **3c,e** (cf. Table 1). Based on this result, as well as the similarity of λ_{max} values for free Mm-1 **2a** and fully protected Mm-1 **3a** in mutual solvents (Table 1), the ϵ for protected Mm-1 **3a** in acetonitrile (338−339 nm, 35 800) was used as an approximation of ϵ for free Mm-1 **2a** in acetonitrile (λ_{max} 335 nm). The concentration of an Mm-1 stock solution was calibrated spectrophotometrically in acetonitrile under Ar, and the calibrated solution was used to determine the approximate ϵ values for free Mm-1 in the various reaction media (cf. Table 1).

Anaerobic reactions between Mm-1 (or catechol) and V^V or V^{IV} ions were conducted in deionized water, aqueous methanol, and aqueous buffers at pH 2 and 7. For reactions in nonbuffered water or methanol, the pH of the reaction mixtures was affected by the buffered V^{IV} or V^V solutions introduced. For all reactions, the V^{IV} solution (10 mM bisulfate buffer pH 2) or the V^V solution (10 mM carbonate buffer, pH 10) constituted from 2% to 17% of the total sample volume (first to last addition, respectively). For reactions in water or methanol, the final pH of the product mixtures was ca. pH 2 for V^{IV} additions and ca. pH 10 for V^V additions (except for catechol + V^V, in which pH <10 was likely because the catechol stock solution consisted of catechol in HCl(aq) pH 2 and constituted 4% of the final sample volume).

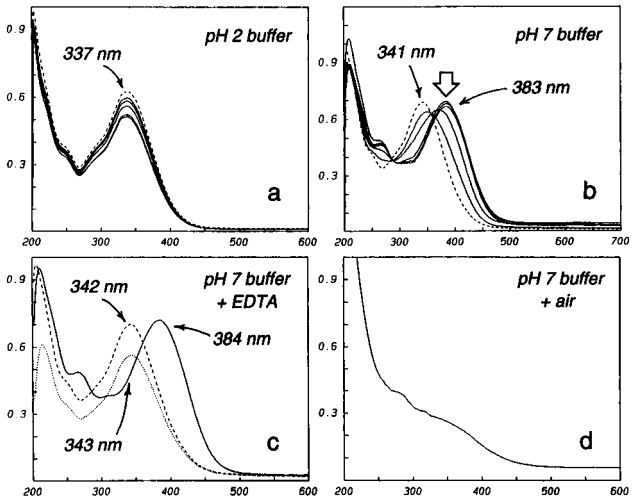


FIGURE 1: Absorbance vs wavelength (nm) for Mm-1 treated with stepwise additions of V^{IV} ions (0.5, 1, 2, 4, and 8 total mol equiv) in (a) pH 2 buffer (20 μM Mm-1 in 50 mM bisulfate buffer, pH 2), or (b) pH 7 buffer (23 μM Mm-1 in 50 mM phosphate buffer, pH 7). Overlays: initial Mm-1 (- - -), V^{IV} additions (—). The down outlined arrow indicates attenuation of the peak for latter V^{IV} additions. (c) pH 7 buffer + EDTA: Absorbance vs wavelength (nm) for Mm-1 (23 μM) in 50 mM phosphate buffer, pH 7, treated with V^{IV} ions (4 mol equiv) for 10 min under Ar followed by excess EDTA (40 mol equiv added to both cuvettes). Overlays: initial Mm-1 (- - -), V^{IV} addition (—), 1.5 h after EDTA addition (···). (d) pH 7 buffer + air: Absorbance vs wavelength (nm) for Mm-1 (18 μM) in 50 mM phosphate buffer, pH 7, treated with V^{IV} ions (4 mol equiv) and exposed to air for 2 h.

Reactions of V^{IV} with Catechol or Mm-1. For spectrophotometric studies of catechol−vanadium chemistry in various media, catechol was treated with V^{IV} (up to 4 mol equiv) in deionized water, pH 2 buffer, and pH 7 buffer. No reaction was apparent in water or pH 2 buffer. However, in pH 7 buffer, addition of 1 mol equiv (or more) of V^{IV} produced a new peak at 292−293 nm that was mostly stable over time (cf. 275 nm for catechol). This product mixture (from catechol + 2 mol equiv V^{IV}) was demonstrated to be V^{IV} complexes when excess EDTA (10 mol equiv) was added and the original peak for free catechol was immediately restored. The observed 1:1 V^{IV}/catechol stoichiometry suggests that the peak at 292−293 nm represents primarily 1:1 complexes, denoted simplistically as $V^{IV}O$-(catecholate); however, the product mixture is undoubtedly an equilibrium mixture of various species.

For analogous studies of tunichrome−vanadium chemistry, Mm-1 was treated with V^{IV} (up to 8 mol equiv) in deionized water, methanol, pH 2 buffer, and pH 7 buffer. Tunichrome Mm-1 displayed the same pattern of reactivity as catechol in these media. For V^{IV} additions to Mm-1, no reaction was found in water, methanol, or pH 2 buffer (Figure 1a; same for water or methanol). Identical spectra were obtained for parallel controls that lacked V^{IV} (10 mM pH 2 buffer was

used instead of V^{IV} solution); therefore, the observed attenuation of the Mm-1 absorption was due to dilution of the sample. However, in pH 7 buffer, addition of 2 mol equiv (or more) of V^{IV} generated a new peak at 382−383 nm (Figure 1b). The final product mixture was the same for the addition of excess V^{IV} (40 mol equiv). This product mixture (from Mm-1 + 4 mol equiv V^{IV}) was demonstrated to be V^{IV} complexes when excess EDTA (40 mol equiv) was added and the original peak for free Mm-1 was restored after 1−1.5 h (Figure 1c, dotted line). (Control experiments showed that the observed attenuation of the Mm-1 absorption was due to dilution of the sample.) The observed 2:1 V^{IV}/Mm-1 stoichiometry suggests that the peak at 382−383 nm represents primarily 2:1 complexes, denoted simplistically as OV^{IV}(Mm-1)$V^{IV}O$; however, the product mixture is undoubtedly an equilibrium mixture of various species. Unfortunately, it was found to be too dilute for low temperature EPR analysis at 130−145 K. The much slower rate of EDTA exchange for Mm-1 relative to catechol implies that the Mm-1/V^{IV} complex is kinetically more stable than the catechol/V^{IV} complex in reactions with EDTA.

The absorption peak for the Mm-1/V^{IV} complex was strongly attenuated by air exposure over 2 h (Figure 1d). The resultant UV absorption is notably similar to previously reported UV/vis spectra of the following: a chromatographed An-2/V complex, signet ring blood cells lysed under Ar, and lysed morula blood cells exposed to air (Oltz et al., 1988). It is reasonable that all of these contained oxidized, polymerized tunichrome.

To summarize, no reactions were apparent for V^{IV} additions to catechol or Mm-1 in water, methanol, or pH 2 buffer. However, both catechol and Mm-1 complexed V^{IV} ions in pH 7 buffer selectively.

Reactions of V^{V} with Catechol or Mm-1. In further spectrophotometric studies, catechol was treated with V^{V} (up to 4 mol equiv) in deionized water, pH 2 buffer, pH 4 buffer, and pH 7 buffer. In water, pH 4 buffer, and pH 7 buffer, a peak at ca. 275 nm for unreacted catechol was apparent after mixing. It was necessary to add V^{V} to the reference cuvettes because the UV absorption of V^{V} is quite strong relative to that of catechol. However, well corrected spectra could not be obtained for the V^{V} series with catechol, perhaps due to differently equilibrated V^{V} species in sample versus reference cuvettes. For comparison, $NaIO_4$ was used to oxidize catechol in pH 7 buffer (cf. Alder & Magnusson, 1959). The addition of $NaIO_4$ (1 mol equiv) produced a peak for *ortho*-benzoquinone (λ_{max} 389 mn) (Mason, 1949) which disappeared over ca. 1 h. The final product showed a peak with the same λ_{max} as catechol but significantly broadened. Subsequent addition of V^{IV} (1 mol equiv) generated a mono-(catecholate)-type V^{IV} complex (λ_{max} 293 mn) of the oxidized catechol. In contrast, the mixtures of V^{V} and catechol in water, pH 4 buffer, and pH 7 buffer were quite stable over at least 1 h. Although no reaction was apparent between catechol and V^{V} in these media, minor reactions were possible. In similar studies of V^{V} additions to substituted catechols (tiron, 6,7-dihydroxy-2-naphthalenesulfonic acid, and norepinephrine) at pH 8−9, redox reactions were determined to be thermodynamically unfavorable (Ferguson & Kustin, 1979). As pH decreased, the rates of the redox reactions were found to increase. In our studies of V^{V} with catechol, the observations for pH 4 buffer, pH 7 buffer and water were in contrast to an obvious redox reaction in pH 2 buffer (see below).

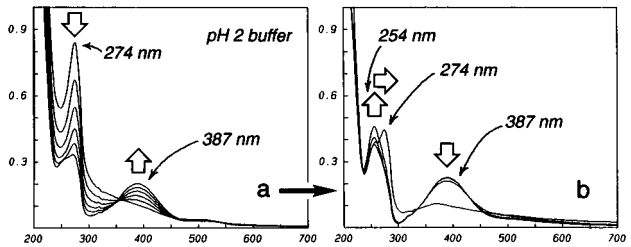


FIGURE 2: Absorbance vs wavelength (nm) for a timed reaction between catechol (250 μM; λ_{max} 274−275 nm) and V^{V} ions (2 mol equiv) in 50 mM bisulfate buffer, pH 2: (a) at 0, 5, 10, 15, 22, 30 min after mixing; V^{V} (1 mol equiv) in reference cuvette; (b) at 62, 76, 98 min, and 11.25 h after mixing; no V^{V} in reference cuvette. The outlined arrows indicate the direction of evolution of a particular spectral feature over time (e.g., down arrow indicates attenuation of the peak).

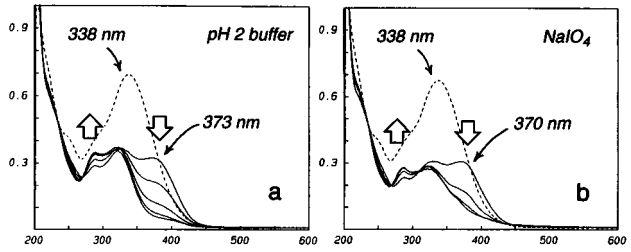


FIGURE 3: (a) pH 2 buffer: Absorbance vs wavelength (nm) for a timed reaction between Mm-1 (22 μM) and V^{V} ions (4 mol equiv) in 50 mM bisulfate buffer, pH 2. Overlays: initial Mm-1 (- - -), at 0, 4, 9, 15, and 32 min after mixing (—). (b) $NaIO_4$: Timed reaction between Mm-1 (21 μM) and $NaIO_4$ (2 mol equiv) in 50 mM bisulfate buffer, pH 2. Overlays: initial Mm-1 (- - -), at 0, 8, 21, and 47 min after mixing (—). The outlined arrows indicate the direction of evolution of a particular spectral feature over time.

For V^{V} additions to catechol in pH 2 buffer (Figure 2), oxidation of catechol to *ortho*-benzoquinone was evident from the peak at 385−392 nm (cf. spectrum of authentic sample; Mason, 1949) that developed over ca. 1 h and then diminished with broadening as a peak developed at 254−255 nm over ca. 2 h. Over several hours, the 254−255 nm peak diminished as an additional peak developed at 274 nm, similar to the original catechol absorption. [The same oxidation products were reported for oxidation of catechol by tyrosinase (Mason, 1949).] Addition of $NaIO_4$ (1 mol equiv) to catechol in pH 2 buffer generated the *ortho*-benzoquinone peak instantaneously. Similarly, the quinone peak diminished with broadening as the 254−255 nm peak developed over ca. 2 h.

Analogous to catechol, Mm-1 was treated with V^{V} (up to 8 mol equiv) in deionized water, methanol, pH 2 buffer, and pH 7 buffer. Like catechol, tunichrome was oxidized by V^{V} in pH 2 buffer as follows. For Mm-1 treated with V^{V} (4 mol equiv) in pH 2 buffer (Figure 3a), the tunichrome peak became strongly attenuated, and the final product showed two peaks of similar intensity at 285 and 318 nm with a weak shoulder at ca. 380 nm. Under parallel conditions, treating Mm-1 in pH 2 buffer with $NaIO_4$ (2 mol equiv) generated a strikingly similar curve with two major peaks at 286 and 321 nm (Figure 3b). For both reactions, a transient shoulder was initially apparent at 370−373 nm in the region of *ortho*-benzoquinone absorption. The lifetime of this transient was ca. 45 min for a tunichrome concentration of 0.021 mM. The final oxidation products have

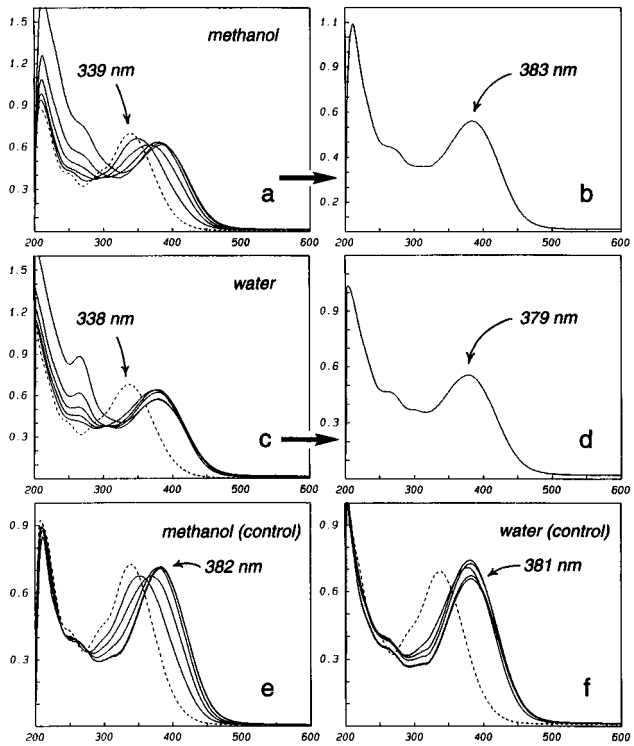


FIGURE 4: (a and b) Methanol: Absorbance vs wavelength (nm) for Mm-1 (21 μM) in methanol treated with stepwise additions of V^V ions (0.5, 1, 2, 4, and 8 total mol equiv). (a) Overlays: initial Mm-1 (- - -), V^V additions (—); no V^V in reference cuvette; (b) after final V^V addition (ca. 1 h after first addition); V^V (6 mol equiv) in reference cuvette. (c and d) Water: Absorbance vs wavelength (nm) for Mm-1 (23 μM) in deionized water treated with stepwise additions of V^V ions (0.5, 1, 2, 4, and 8 total mol equiv). (c) Overlays: initial Mm-1 (- - -), V^V additions (—); no V^V in reference cuvette; (d) after final V^V addition (ca. 1 hr after first addition); V^V (6 mol equiv) in reference cuvette. [Note: For reactions in water or methanol, the pH of the reaction mixtures was affected by the pH of the V^V solution (10 mM carbonate buffer, pH 10) constituting from 2% to 17% of the total sample volume (first to last addition, respectively).] (e and f) Controls: Absorbance vs wavelength (nm) for (e) Mm-1 (22 μM) in methanol or (f) Mm-1 (23 μM) in deionized water treated with stepwise additions of 10 mM carbonate buffer, pH 10, (the media for V^V solutions) in a parallel manner to the V^V additions (a and c). Overlays: initial Mm-1 (- - -), pH 10 buffer additions (—).

absorption features similar to those of Mm-1 itself. Studies of the tyrosinase oxidation of α,β-unsaturated *N*-acetyldopamine, a compound analogous to the A-ring amide of Mm-1 **2a**, showed a transient quinonoid product (λ_{max} 500 nm; Sugumaran et al., 1987). The absorption curve of the final product (λ_{max} 280 nm, shoulder at 310 nm) was quite similar to that of the dopamine reactant, but attenuated. A single product was isolated, characterized, and found to be a dimer produced by intermolecular Michael addition of dopamine reactant to the quinonoid oxidation product. By analogy, oxidation products of tunichrome may be intermolecular Michael adducts as well.[3]

For V^V additions to Mm-1 in methanol or water (Figure 4), the Mm-1 absorption curves were red-shifted to 379−383 nm due to deprotonation of Mm-1 by the V^V solution

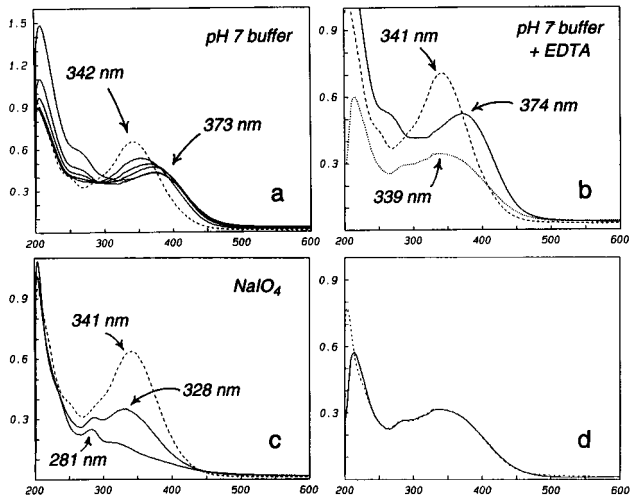


FIGURE 5: (a) pH 7 buffer: Absorbance vs wavelength (nm) for Mm-1 (22 μM) in 50 mM phosphate buffer, pH 7, treated with stepwise additions of V^V ions (0.5, 1, 2, 4, and 8 total mol equiv). Overlays: initial Mm-1 (- - -), V^V additions (—). (b) pH 7 buffer + EDTA: Absorbance vs wavelength (nm) for Mm-1 (23 μM) in 50 mM phosphate buffer, pH 7, treated with V^V ions (4 mol equiv) for 1 h under Ar followed by excess EDTA (40 mol equiv added to both cuvettes). Overlays: initial Mm-1 (- - -), V^V addition (—), 1.5 h after EDTA addition (•••). (c) $NaIO_4$: Absorbance vs wavelength (nm) for Mm-1 (21 μM) in 50 mM phosphate buffer, pH 7, treated with stepwise additions of $NaIO_4$ (1 and 2 total mol equiv). Overlays: initial Mm-1 (- - -), $NaIO_4$ additions (—). (d) Fit (- - -) to the post-EDTA absorption curve (—) from b, as described in text.

buffer (pH 10), as demonstrated by parallel controls that lacked V^V (10 mM pH 10 buffer was used instead of V^V solution) (Figure 4). However, the red-shifted Mm-1 absorptions were slightly attenuated in the presence of V^V relative to the controls, most likely due to low level oxidation of Mm-1.

For V^V additions to Mm-1 in pH 7 buffer (Figure 5a), addition of 2 mol equiv (or more) of V^V produced a peak at 372−375 nm. The final product mixture was the same for the addition of excess V^V (40 mol equiv). This product mixture (from Mm-1 + 4 mol equiv of V^V) was treated with excess EDTA (40 mol equiv). After 1.5 h, the post-EDTA spectrum (Figure 5b, dotted line) was similar to the spectrum of free Mm-1 (dashed line) but was significantly broadened and attenuated (relative to controls that accounted for Mm-1 dilution). To identify Mm-1 oxidation products, Mm-1 in pH 7 buffer was treated with $NaIO_4$ (1 and 2 mol equiv) to give partially oxidized[4] and fully oxidized product mixtures (Figure 5c).[3] Using these and other spectra, the post-EDTA spectrum (Figure 5b, dotted line; Figure 5d, solid line) was fit by a curve (Figure 5d, dashed line) whose summed components were partially oxidized Mm-1 products (0.53 mol equiv; Figure 5c), fully oxidized Mm-1 products (0.15 mol equiv; Figure 5c), complexed products with λ_{max} 372−375 nm (0.29 mol equiv; Figure 5b, solid line), and unreacted V^V (1.56 mol equiv; Figure 6a, solid line). The poor fit below 250 nm is attributed to a lack of V^{IV} in the fit and to imperfectly matched EDTA concentrations (cf. Figure 6b). Adjusting the fit by adding V^{IV} (3.0 mol equiv; Figure 6b, solid line) and using less unreacted V^V (1.0 mol equiv)

[3] Although the structures of tunichrome oxidation products are not known, they are expected to arise from unstable orthoquinonoid intermediates by undergoing intermolecular cross-linking to produce polymers with rearomatized catechol-type rings (Sugumaran et al., 1987; Waite, 1990).

[4] The partially oxidized Mm-1 mixture may consist of fully oxidized Mm-1 products, half-oxidized Mm-1 products, and unreacted Mm-1 (1:2:1 expected).

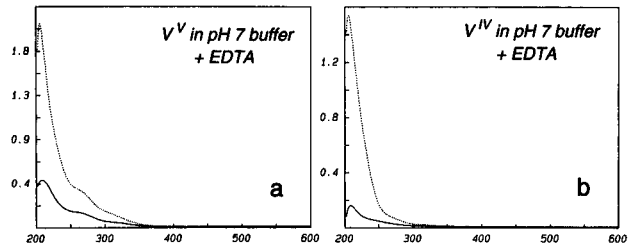


FIGURE 6: (a) V^V in pH 7 buffer + EDTA: Absorbance vs wavelength (nm) for V^V ions (78 μM, comparable to 4 mol equiv additions of V^V) in 50 mM phosphate buffer, pH 7, treated with EDTA (40 mol equiv). Overlays: V^V (—), after EDTA addition (···). (b) V^{IV} in pH 7 buffer + EDTA: Absorbance vs wavelength (nm) for V^{IV} ions (78 μM, comparable to 4 mol equiv additions of V^{IV}) in 50 mM phosphate buffer, pH 7, treated with EDTA (40 mol equiv). Overlays: V^{IV} (—), after EDTA addition (···).

improves the fit below 250 nm while maintaining the same fit above 250 nm (not shown); however, the strong absorption of EDTA below 250 nm interferes with the accuracy of fits to this region.

Further analysis of the pre-EDTA spectrum for Mm-1 treated with V^V (Figure 5b, solid line) found that the peak at 372−375 nm was reproduced by treating the partially oxidized Mm-1 mixture (Figure 5c) with 2 mol equiv of V^{IV} to generate V^{IV} complexes (λ_{max} 374 nm; not shown) (decomplexation with 40 mol equiv of EDTA liberated the partially oxidized Mm-1 after 1.5 h). To allow comparisons of the peaks at 372−375 nm, the molar absorptivities of these peaks were normalized relative to the concentrations of Mm-1 starting material and relative to the sample volumes. Thus, the normalized molar absorptivity of the 372−375 nm peak for the pre-EDTA product mixture (Figure 5b, solid line) was only 2−4% greater than that for the partially oxidized Mm-1/V^{IV} complexes (374 nm peak). In contrast to these V^{IV} complexes, treating the partially oxidized Mm-1 mixture (Figure 5c) with 2 mol equiv of V^V produced marked attenuation and a smaller red-shift (to 360 nm) suggestive of further oxidation of Mm-1 accompanied by V^{IV} complexation (not shown). Similarly, treating the fully oxidized Mm-1 mixture (Figure 5c) with V^{IV} or V^V (2 mol equiv) produced only slight red-shifts (<5 nm; not shown) suggestive of very limited or weak complexation. (The V^{IV} results were the same with and without $NaHSO_3$ addition to destroy any residual $NaIO_4$ oxidant prior to V^{IV} addition.) Therefore, the product mixture generated by Mm-1 and V^V (4 mol equiv) consisted of a major component of partially oxidized Mm-1 products complexed to nascent V^{IV}, presumably at the unoxidized catecholate rings of Mm-1, and a minor component of fully oxidized Mm-1 products. This product mixture was too dilute for low temperature EPR analysis at 130−145 K (20−25 μM Mm-1). However, at 100-fold higher concentrations in aqueous methanol instead of pH 7 buffer, Mm-1 and V^V (1 mol equiv; $NaCO_3$(aq) pH 8.1) produced bis- and tris(catecholate)-type complexes of V^{IV} observed directly by EPR at 130−145 K (Grant, 1994; Grant et al., 1996). These findings corroborate the spectrophotometric observations of V^{IV} complexes produced by Mm-1 and V^V in pH 7 buffer.

The post-EDTA fit (Figure 5d, dashed line) accounts for 0.97 mol equiv of the Mm-1 used or 97%. An inventory of electrons (e^-) donated by Mm-1 components of the fit totals 2.24 mol equiv of e^- (2 mol equiv from partially oxidized Mm-1, including residual V^{IV} complex, and 4 mol equiv from fully oxidized Mm-1). For comparison, the amount of reduced V^V based on the fit is 2.44−3.0 mol equiv. The electron balance is fairly well accounted for; therefore, the fit represents reasonable redox products from Mm-1 and V^V.

The pre-EDTA absorption curve became strongly attenuated over 2 h of air exposure, presumably due to further oxidation and polymerization of the tunichrome catalyzed by V^V species under neutral, aerobic conditions. The broad UV absorption produced looked the same as for the Mm-1/V^{IV} complex in pH 7 buffer exposed to air (Figure 1d).

To summarize, V^V oxidized catechol and Mm-1 in pH 2 buffer and *ortho*-benzoquinone-type intermediates were observed. For mixtures of V^V and catechol in water, pH 4 buffer, or pH 7 buffer, the UV/vis spectra suggest that catechol remained unreacted, although minor reactions were possible. For mixtures of V^V and Mm-1 in water or methanol, Mm-1 was deprotonated by the V^V solution buffer (pH 10), and minor reactions were indicated. Interestingly, V^V oxidized Mm-1 in pH 7 buffer, and complexes formed between nascent V^{IV} and partially oxidized Mm-1 products, presumably at the unoxidized catecholate rings. The results are consistent with other studies of catechol and substituted catechols treated with V^V in acidic (pH ≤1) and mildly basic (pH 8−9) aqueous media: the rates of redox reactions increase as pH decreases, whereas the rates of complexation increase as pH increases (Kustin et al., 1974a,b; Ferguson & Kustin, 1979). Further, our results demonstrate that tunichrome−vanadium redox reactions are promoted at neutral pH in buffered aqueous media, presumably proceeding via tunichrome−V^V complexation under the neutral conditions.

EPR Spectroscopic Studies. EPR spectroscopy was employed to observe V^{IV} products from reactions between Mm-1 and V^V ions. Paramagnetic V^{IV} species can be observed by EPR at room temperature due to slow relaxation of the $3d^1$ electronic configuration. Paramagnetic V^{III} is EPR silent above 4 K due to rapid electronic relaxation (Wertz & Bolton, 1972); V^V is diamagnetic and not observable by EPR. A room temperature EPR spectrum of pentaaquovanadyl ions $V^{IV}O(H_2O)_5^{2+}$ was obtained for the product mixture generated by treating Mm-1 with V^V (1 mol equiv) in pH 2 buffer at a 10-fold higher concentration than for the UV/vis studies above. The quantity of V^{IV} produced in a 1.5 h reaction was ca. 80% of the total vanadium (calibrated using a parallel V^{IV} control that lacked Mm-1) (Table 2, pH 2 entry). From other similar samples, the error for such a measurement at 0.25 mM total vanadium is ca. ±35% (Table 2). This EPR result qualitatively shows that Mm-1 in pH 2 buffer reduced V^V ions to free V^{IV} ions, consistent with reactions between Mm-1 extracts and V^V ions in acidic media which produced V^{IV} ions (Macara et al., 1979c).

Previously, we reported that reactions between Mm-1 and V^V or V^{IV} ions in pH 7 buffer generated V^{III} products, according to an EPR method developed to indirectly assay V^{III} products (Ryan et al., 1992). In those studies, Mm-1 in methanolic aqueous buffer (pH 7) was treated with V^V or V^{IV} (1, 2, or 4 mol equiv) under Ar. After 1.5 h, the reaction mixtures were acidified to dissociate V^{IV} dimers and aggregates, bubbled with O_2, and purged with Ar. Room temperature EPR spectra of pentaaquovanadyl ions $V^{IV}O(H_2O)_5^{2+}$ were acquired before and after O_2/Ar treatment. The ratio of post-O_2 signal intensity vs pre-O_2 intensity indicated whether the post-O_2 signal was greater than the

Table 2: EPR Analysis of Mm-1 Treated with V^V in Methanolic Aqueous Buffer

no. of trials	pH[a]	reactants	mol equiv of Mm-1	[V][b] (mM)	ratio of V^{IV} EPR signal intensities[c] pre-O_2/control[d]	ratio of V^{IV} EPR signal intensities[c] post-O_2/pre-O_2[e]
1	2	V^{IV} control	0	0.25		124%
1	2	V^V + Mm-1	1	0.25	80%	133%
3	7	V^{IV} control	0	0.25		107 ± 6%[f]
10	7	V^{IV} control	0	0.50		111 ± 7%[f]
1	7	V^V + Mm-1	2	0.25	123%	101%
4	7	V^V + Mm-1	1	0.25	102 ± 35%[e]	118 ± 9%[f]
3	7	V^V + Mm-1	1	0.50	114 ± 20%[e]	113 ± 3%[f]
1	7	V^V + Mm-1	0.25	0.25	85%	102%
1	6	V^{IV} control	0	0.25		127%
1	6	V^V + Mm-1	1	0.25	122%	114%
1	5	V^{IV} control	0	0.25		137%
1	5	V^V + Mm-1	1	0.25	116%	154%

[a] The pH of the reaction buffer; samples were acidified with excess pH 2 buffer prior to EPR measurements. [b] Concentration of total vanadium in EPR sample. [c] Ratio of two integrated V^{IV} EPR signal intensities recorded on the same day. [d] Ratio of integrated V^{IV} signal intensity for V/Mm-1 product mixture versus signal intensity for V^{IV} control, both acquired prior to O_2 treatment. [e] Ratio of integrated V^{IV} signal intensity for a sample (V/Mm-1 product mixture or V^{IV} control) acquired prior to O_2 treatment versus signal intensity of the same sample acquired after O_2 treatment. [f] Mean ratio ± standard deviation.

pre-O_2 signal due to oxidation of V^{III} in the sample; i.e., the portion of the ratio above 100% represented oxidized V^{III}. The highest level of V^{III} products was indicated for Mm-1 treated with 1 mol equiv of V^V in pH 7 buffer. Notably, we have since been unable to reproduce our previously reported EPR results. In fact, the more recent EPR results indicate that reactions between Mm-1 and V^V in methanolic aqueous buffer (pH 7, 6, or 5) did not generate significant levels of V^{III} products (Table 2). Inspection of Table 2 (all entries) reveals that the post-O_2/pre-O_2 ratios for product mixtures are not significantly greater than such ratios for the parallel V^{IV} controls; therefore, V^{III} products were not found.

The cause of the irreproducibility was determined to be the O_2/Ar treatment step. In the previously reported experiments, the post-O_2 signal intensities for two V^{IV} controls were 83 and 85% of the pre-O_2 intensities. The post-O_2/pre-O_2 ratios for these controls were dramatically lower than ratios determined for samples which showed increased post-O_2 intensities; thus significant percentages of V^{III} products were indicated for some samples relative to the controls. In contrast, the V^{IV} controls in Table 2 invariably showed increased post-O_2 signal intensities, presumably due to evaporative concentration of the controls during O_2/Ar bubbling. Therefore, the O_2/Ar bubbling step irreproducibly gave both increased and decreased post-O_2 signal intensities for identical V^{IV} controls, even though the standard deviation for this step was relatively low (±6−7%) for all of the recent V^{IV} controls (Table 2).

Colorimetric Assay for V^{III}. To clarify the issue of V^{III} product formation in reactions between Mm-1 and vanadium ions in aqueous media, we employed a direct method for detecting V^{III} itself. A colorimetric assay for V^{III} using ammonium thiocyanate (1.8 M NH_4SCN/1 M HCl in 6:4 acetone/water) was adapted for air-sensitive, microscale reactions from literature procedures (Crouthamel et al., 1955; Zolotavin et al., 1962). The yellow $V^{III}(SCN)_6$ complex absorbs maximally at 396−399 nm (8630 M^{-1} cm^{-1}). In colorimetric studies comparable to the EPR studies, Mm-1 in methanolic aqueous buffer (pH 7 or 2) was treated with V^V or V^{IV} ions for 20 min or 1.5 h (Table 3). Since the previously reported EPR results suggested that 1 mol equiv of V^V and 4 mol equiv of V^{IV} in pH 7 buffer produced the most V^{III} (Ryan et al., 1992), these conditions were tested in the colorimetric studies. The concentration of vanadium ions in the colorimetric studies (50 or 200 μM) was well above the detection limit (2 μM) for the V^{III} assay. The assay results clearly show that Mm-1 did not generate detectable quantities of V^{III} products under the conditions tested (Table 3). For positive control assays, an aliquot of V^{III} stock solution (1 mol equiv) was injected into the completely assayed product mixtures, and the peak for $V^{III}(SCN)_6$ invariably appeared as expected.

Table 3: Colorimetric Assay for V^{III} in Mm-1 Reactions

reactant	[V] (μM)[a]	buffer	time	[V^{III}] (μM)
V^{IV} (4 equiv)	200	pH 7	20 min	0
V^{IV} (4 equiv)	200	pH 7	1.5 h	0
V^V (1 equiv)	50	pH 7	20 min	0
V^V (1 equiv)	50	pH 7	1.5 h	0
V^V (1 equiv)	50	pH 2	1.5 h	0

[a] Concentration of total vanadium in the colorimetric sample.

CONCLUDING REMARKS

The spectrophotometric and EPR studies of Mm-1, the simplest tunichrome, support the following conclusions. (i) In pH 2 buffer, both catechol and Mm-1 were oxidized by V^V ions. Room temperature EPR qualitatively showed that Mm-1 in pH 2 buffer reduced V^V ions to free V^{IV} ions. (ii) In pH 7 buffer, both catechol and Mm-1 complexed V^{IV} ions introduced. (iii) In pH 7 buffer, Mm-1 was oxidized by V^V ions, and complexes formed between nascent V^{IV} and partially oxidized Mm-1 products, presumably at the unoxidized catecholate rings. [At higher concentrations, the V^{IV} complexes were observed by low temperature EPR (Grant, 1994; Grant et al., 1996).] (iv) Catechol was found to be a good model for tunichrome, although tunichrome appears to be a more protective chelator of V^{IV} in pH 7 buffer based on the relative rates of EDTA exchange.

Using a colorimetric assay for V^{III}, we found that reactions between Mm-1 and V^V or V^{IV} ions in pH 7 buffer clearly did not generate appreciable quantities of V^{III} products. The same conditions had previously been studied using an EPR method to indirectly assay V^{III} products (Ryan et al., 1992). However, the reported EPR results could not be reproduced in the more recent EPR studies presented here. The colorimetric V^{III} assay resolved the issue of V^{III} product formation in our studies of Mm-1.

Notably, the Mm-1 studies identified conditions which promote tunichrome−vanadium complexation and redox reactions *in vitro*. Thus, tunichromes in *A. nigra* and *A. ceratodes* could complex V^V under similar conditions *in vivo* and reduce the complexed metal to (at least) the V^{IV} state. In subsequent work (Ryan et al., 1996), we utilized the conditions identified here for *in vitro* studies of An-1 **1a**, a tunichrome from V-accumulating tunicates. The colorimetric V^{III} assay was used to survey An-1 and various biogenic thiols for V^{III} product formation.

Recently, several papers have questioned whether tunichromes exist in blood cells prior to lysis (Taylor et al., 1991, 1993; Hawkins, 1991; Bayer et al., 1992). In one

report, the authors failed to recognize a species variation for their dihydrotunichromes isolated from *P. mamillata* (Bayer et al., 1992). The spectrophotometric characteristics of the dihydrotunichromes (λ_{max} 301 nm) match the intense absorption band for hemolysates from this species (Carlson, 1977). Likewise, the absorption curve for tunichrome An-1 (λ_{max} 335−340 nm) matches the intense absorption band for hemolysates from *A. ceratodes* (Carlson, 1977) and *A. nigra* (Macara et al., 1979b). Hence, the difference between dihydrotunichromes and tunichromes does not result from blood cell lysis but is instead an intriguing species variation (cf. Biggs & Swinehart, 1979).

In an interesting study of blood cells from *A. ceratodes* and *P. julinea* (which also produces tunichromes; Parry et al., 1992), spectroscopic analyses did not provide evidence of tunichromes in intact cells by UV/vis, circular dichroism, or fluorescence (Taylor et al., 1993). Considering the fluorescence properties of tunichromes and the high concentrations expected in blood cells, the different spectroscopic characteristics for intact versus lysed cells can be easily rationalized. The tunichromes constitute up to 50% of morula blood cells (dry weight); therefore, high concentrations are expected *in vivo*. Spectrophotometric characteristics (λ_{max}) of compounds are dependent on media and concentration. Although Beer's law applies to tunichromes in dilute solutions (Taylor et al., 1993), at high concentrations in cellular media the position of λ_{max} may be quite different. The CD spectra are especially influenced by concentration and media effects on molecular conformations. The fluorescence properties of tunichromes are influenced by subtle electronic effects; for example, peracetate derivatives of tunichromes display orange fluorescence (Oltz et al., 1988, 1989) whereas TBDMS/Boc protected tunichromes do not fluoresce on TLC plates under UV light. Free An-1 was reported to display orange fluorescence (Bruening et al., 1986); however, this fluorescence may be quenched in blood cells (Kustin et al., 1976). Hence, proving the existence of tunichromes and many native compounds is quite challenging in intact cells.

The method of tunichrome isolation is quite preservative (Kim et al., 1991; Ryan et al., 1996). The fresh cell pellet under Ar is rapidly frozen in liquid nitrogen then lyophilized immediately. (If the pellet warms before high vacuum is attained, thawing occurs at a low temperature causing the pellet to blacken. In this case, the sample is ruined and must be discarded.) Successfully lyophilized blood pellets maintain their pale yellow color. The dry pellets are kept under Ar and treated directly with protecting reagents under strictly anaerobic, anhydrous conditions. It is difficult to imagine that selective peptide bond hydrolysis of a precursor protein occurs (e.g., ferreascidin) and/or that introduction of one or both olefin bonds occurs during the isolation procedure as the recent papers have suggested.

The *in vitro* studies presented here characterize reactions between a representative tunichrome (Mm-1) and V^{IV} or V^{V} ions in aqueous media that crudely approximate cellular conditions. However, the results may or may not correlate with reactivity *in vivo*, where no relationship between tunichrome and vanadium has been proved. Vanadium K-edge X-ray absorption spectroscopy (XAS) of intact blood cells from *A. ceratodes* did not show evidence of a tunichrome−vanadium complex (Tullius et al., 1980; Frank et al., 1995). Rather, the spectra are most consistent with V^{III} surrounded by six oxygen ligands from water in an octahedral array, $(V-O)_{ave}$ = 1.99 Å. As the spectra represent the average of all vanadium species in unsorted blood cells, minor vanadium species (≤10%) may not be detected. An example of such a minor species may be $V^{III}-O-V^{III}$ dimers, present in some samples of intact *A. ceratodes* blood cells at ca. 15% of total vanadium (Anderson & Swinehart, 1991). Another example of a minor species could be a transient or stable tunichrome−vanadium complex. Interestingly, vanadium may be stored in different forms in different species. In *P. mamillata*, for example, X-ray microanalysis of blood cells has identified concentrated vanadium deposits within vacuolar membranes, vacuolar granules, and cytoplasmic granules in amoebocytes, signet ring cells, and a type of compartment cell (Scippa et al., 1988). Whether tunichromes are located in these granular deposits is not known.

Alternatively, the biological role of tunichrome may be as a quinone-tanning agent for tunic strengthening, wound healing, and/or for adhesion of tunicates to an underwater surface (Waite, 1990; Endean, 1955; Barrington & Thorpe, 1968). In continuing studies, we explored the tunichrome-vanadium complexation and redox chemistry of An-1 from V-accumulating tunicates (Ryan et al., 1996).

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