Unraveling the Chemistry of Tunichrome

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Received October 4, 1990 (Revised Manuscript Received February 11, 1991)

The common sea squirt is an unusual animal that has intrigued scientists ever since Aristotle's time. Sea squirts or tunicates (Figure 1), so named because they squirt water when grabbed and for their resilient covering (tunic), are simple filter feeders that inhabit all oceans, at all depths. They are classified as lower chordates since their larvae possess a rudimentary spinal chord.

Tunicates are best known to chemists for their ability to store vanadium within blood cells, where extraordinary amounts were found over 80 years ago. It was postulated that such cells represent primitive erythrocytes (red blood cells present in higher vertebrates) and serve an analogous function of O_2 transport. The analogy was only tentative since the cells are vivid yellow-green rather than red, the difference ascribed to the presence of vanadium instead of iron. Actually, the pigmentation of blood in many dissimilar species is largely due to the air-sensitive hydroquinoid peptide "tunichrome",[1] whereas that of erythrocytes is from the protein hemoglobin.

This Account documents a substantial and growing body of evidence that a principal activity of tunichrome is as a reducing ligand. A variety of lines of evidence indicate that a green tunichrome–vanadium complex occurs naturally,[2,3] but would also be formed via blood cytolysis. To date, a physiological role for vanadium in animals remains obscure, though it is considered to be an essential trace element for mammals and avians.[4] A unified interpretation[5] for the prevalence of air-sensitive vanadium(III) and tunichrome in a respiring tunicate has now been derived from the organic, inorganic, biochemical, and ecological results reviewed here.

Tunichrome Isolation and Structure Determination

Direct Isolation. Early in the research a green chromatographic band containing tunichrome and vanadium was isolated from the species *Ascidia nigra*. It was eventually realized that the vivid yellow color of the tunichromes persisted longer when the pigments were separated from the green (vanadium-containing) constituents.[6] Attempts to purify unprotected pigments were also impeded by their tendency to decompose via oxidation, hydrolysis, polymerization, or exposure to mild alkaline conditions. Further complications arose since they adhere tenaciously to most solid-phase packing materials, including silica, alumina, cellulose, and reversed-phase and lipophilic matrices,[6] although this adhesive property reflects one aspect of tunichromes' proposed biological activity. A convenient chemical assay[3] for the isolation and structure determination work was acetylation of the pigments, even in fresh or freeze-dried blood pellets, to form stable tunichrome peracetates. Unfortunately, this assay is ineffectual for oxidized tunichrome or its metal complex since they resist acetylation, thus seriously hindering the detection of such compounds.

Several unusual steps were essential to purify this new class of polyphenolic blood pigments in their free form:[6] anhydrous, anaerobic Sephadex LH-20 column extrusion/extractions (thereby minimizing contact with air, vanadium, and water), centrifugal partition chromatography, and four-solvent reversed-phase high-performance liquid chromatography (HPLC) with diode array detection.[6b] This five-year effort came to fruition when spectroscopic and chemical studies on the free

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Koji Nakanishi was born in 1925 in Hong Kong and spent his childhood in Lyon, London, Alexandria, and Japan. He received his B.Sc. and Ph.D. from Nagoya University (Y. Hirata) in 1947 and 1954 and did postgraduate studies with L. F. Fieser, Harvard University, 1950–1952. He taught at Nagoya University, Tokyo Kyoiku University, and Tohoku University and moved to Columbia University in 1969, where he is Centennial Professor of Chemistry. He is engaged in structural and bioorganic studies of natural products, including biopolymers.

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(2) Oltz, E. M.; Pollack, S.; Delohery, T.; Smith, M. J.; Ojika, M.; Lee, S.; Kustin, K.; Nakanishi, K. *Experientia* **1989**, *45*, 186.
(3) Oltz, E. M.; Bruening, R. C.; Smith, M. J.; Kustin, K.; Nakanishi, K. *J. Am. Chem. Soc.* **1988**, *110*, 6162.
(4) Nielson, E. H. *Fed. Proc.* **1986**, *45*, 128.
(5) Smith, M. J. *Experientia* **1989**, *45*, 452.
(6) (a) Bruening, R. C.; Oltz, E. M.; Furukawa, J.; Nakanishi, K.; Kustin, K. *J. Am. Chem. Soc.* **1985**, *107*, 5298. (b) Bruening, R. C.; Oltz, E. M.; Furukawa, J.; Nakanishi, K.; Kustin, K. *J. Nat. Prod.* **1986**, *49*, 193.

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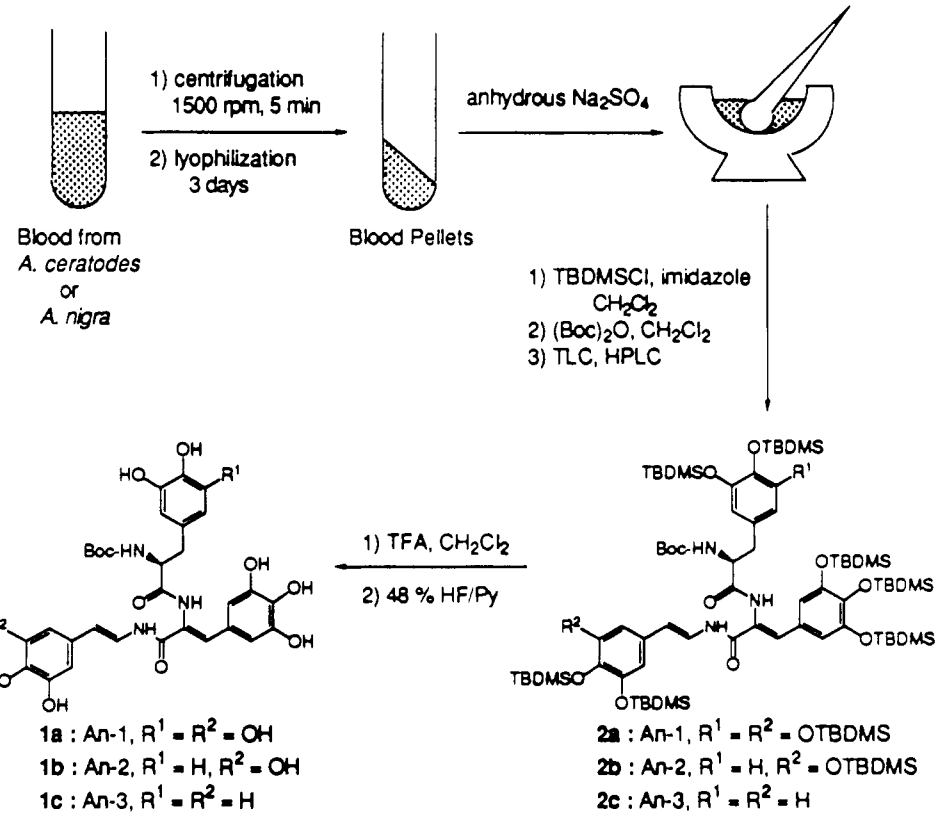
Scheme I
Isolation of Tunichromes by the Protection/Deprotection Scheme[a]



[a] The blood is collected into centrifuge tubes under a current of dry, oxygen-free Ar and centrifuged, and the centrifuged pellets are immersed in liquid N_2 to lyse and freeze the cells.[6b] The lyophilized blood pellet was ground with sodium sulfate, reacted with TBDMSCl and $(Boc)_2O$, and chromatographed. An *A. nigra* blood pellet (1.36 g) gave 42 mg, 17 mg, and 10 mg, respectively, of protected **2a**, **2b**, and **2c**;[7] after deprotection, 20 mg of **2a** yielded 4.1 mg of An-1, **1a**, 62% yield.

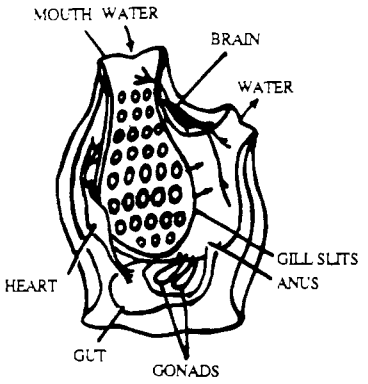


Figure 1. Drawing of a generic adult tunicate.

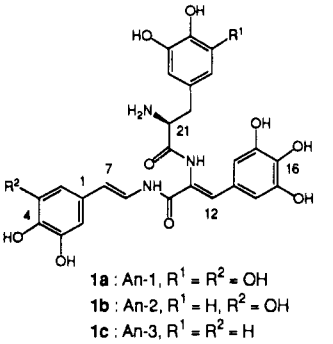


Figure 2. Tunichrome An-1 and related congeners isolated from *A. nigra*, a vanadium accumulator.

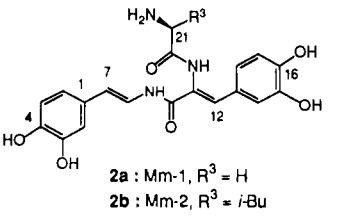


Figure 3. Tunichrome Mm-1 and -2 isolated from *M. manhattensis*, an iron accumulator.

pigment and its peracetate identified a new family of hydroxy-Dopa peptides (Figure 2).[3,6] Nuclear Overhauser effect experiments clarified the connectivities and elucidated the 11-ene geometry, while circular dichroism (CD) data established the absolute configuration at C-21 to be *S*.[3] The first compounds **1a–c** were characterized from vanadium-accumulating *A. nigra* (An-1, -2, and -3, Figure 2) and differed in their degree of ring hydroxylation, while subsequent identification of Mm-1 (**2a**) and Mm-2 (**2b**) from iron-accumulating *Molgula manhattensis* revealed varieties with only two catechol rings (Figure 3).[3] Evidently, two or three hydroquinoid rings, which could serve as chelation and polymerization centers, plus two conjugated olefinic bonds within a semirigid backbone constitute the basic functional framework of tunichrome.

Isolation via Protection/Deprotection.[7] Adequate amounts of natural or synthetic tunichrome have been inaccessible because of low overall yields, nor has the *free* pigment been recovered from its peracetate.

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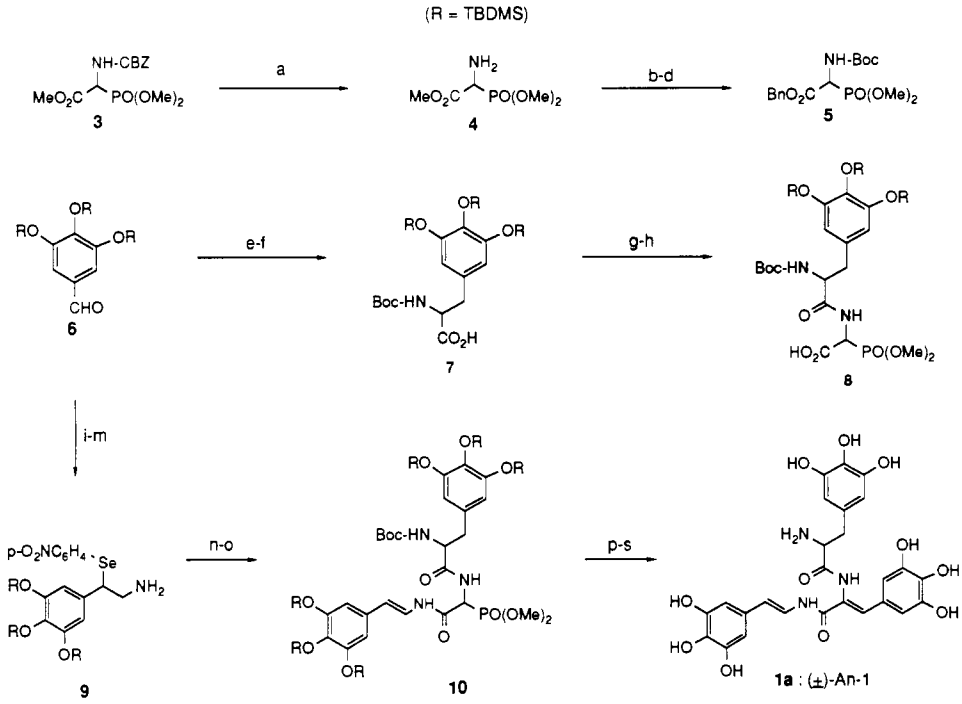
Scheme II
Synthesis of An-1[a]



[a] (a) H_2, 5% Pd/C, MeOH; (b) $(Boc)_2O$, CH_2Cl_2, (80% from **3**); (c) 1 N KOH, MeOH, 83%; (d) BnBr, DBU, CH_3CN, 75%, see ref 8c; (e) NaH, **5**, THF, 60% (E/Z = 1/3); (f) H_2, 5% Pd/C, MeOH, 92%; (g) **4**, DCC, CH_2Cl_2, DMF, 62%; (h) 1 N KOH, MeOH, 94%; (i) TMSCN, cat. ZnI_2; (j) LAH, THF; (k) $(Boc)_2O$, CH_2Cl_2 (67% from **4**); (l) p-$NO_2C_6H_4SeCN$, n-Bu_3P, THF, 75%; (m) TFA, CH_2Cl_2, quantitative; (n) **8**, DCC, EtOAc, 67%; (o) $NaIO_4$, dioxane/H_2O, 61%; (p) LDA, **6**, 82% (E/Z = 25/1); (q) $h\nu$ (300 nm), hexane, E/Z = 1/2, separation of Z isomer; (r) 20% TFA/CH_2Cl_2; (s) 48% aqueous HF, Py, 90%.

However, a recent breakthrough involving the protection/deprotection scheme from the synthetic[8] protocol, in conjunction with silica chromatography (Scheme I), affords pure, preparative quantities from blood pellets collected during our original isolation studies.[7]

The clean separation of An-1, -2, and -3, as well as Mm-1 and -2, via their *tert*-butyldimethylsilyl (TBDMS), *tert*-butyloxycarbonyl (Boc) derivatives is now routine, even by preparative thin-layer chromatography (TLC), whereas the corresponding unprotected compounds are poorly resolved by HPLC. This method should be applicable to other unstable compounds containing hydroxyl and/or amino groups and has improved analytical capabilities to the point where several new tunichromes have since been identified at trace levels in *A. nigra* (unpublished results).

Biosynthesis and Total Synthesis of Tunichrome

The structures of the tunichromes suggest that they are biosynthesized by the coupling together of three amino acids (or dopamine), enzymatic dehydrogenation, and sequential hydroxylation, although An-1 has been the sole tunichrome detected in *Ascidia ceratodes* and *Perophora viridis*.[3] Preliminary radiolabeling studies have shown, however, that [^{14}C]phenylalanine and [^{14}C]tyrosine are incorporated into An-1 of *A. ceratodes*.[9]

The total syntheses of *free* An-1,[8a] Mm-1[8b] and Mm-2[8b] have been completed; the strategy employed in the first synthesis of tunichrome (±)-An-1[8a] is summarized in Scheme II and can be readily modified to prepare a variety of tunichrome analogues (including isotopically enriched varieties). The latter should help clarify the complex chemistry and biological mode of action of tunichrome.

The phosphonoglycine unit **3**[8c] was selected as a key starting material and used twice in the entire scheme (to prepare hydroxy-Dopa **7** and peptide **8**). Sequential coupling of **4** with protected hydroxy-Dopa **7** and seleno amine **9** provided the desired framework **10**. The introduction of two olefinic bonds employed selenoxide elimination and Horner–Emmons Wittig reactions. The Boc and TBDMS protecting groups were removed by using dry 20% trifluoroacetic acid (TFA)/CH_2Cl_2 and 48% aqueous HF/pyridine, respectively (under Ar in degassed solvents). Fractional precipitation with a CH_2Cl_2-based solvent system yielded pure tunichrome (±)-An-1. The total syntheses[8b] of Mm-1 and -2 are outlined in Scheme III, a modified route that allows incorporation of a natural amino acid in the final condensation. A comparison of CD data on the peracetates of synthetic Mm-2 [containing (*S*)-(+)-leucine] and natural Mm-2 established the absolute configuration at C-21 of the latter to be *S*.[8b]

Distribution of Vanadium, Iron, and Tunichrome

All tunicates accumulate iron(II) to some degree and within a relatively narrow concentration range (<30-fold), whereas accumulated vanadium levels vary greatly (approximately 10^4-fold).[10] In vanadium-accumulating

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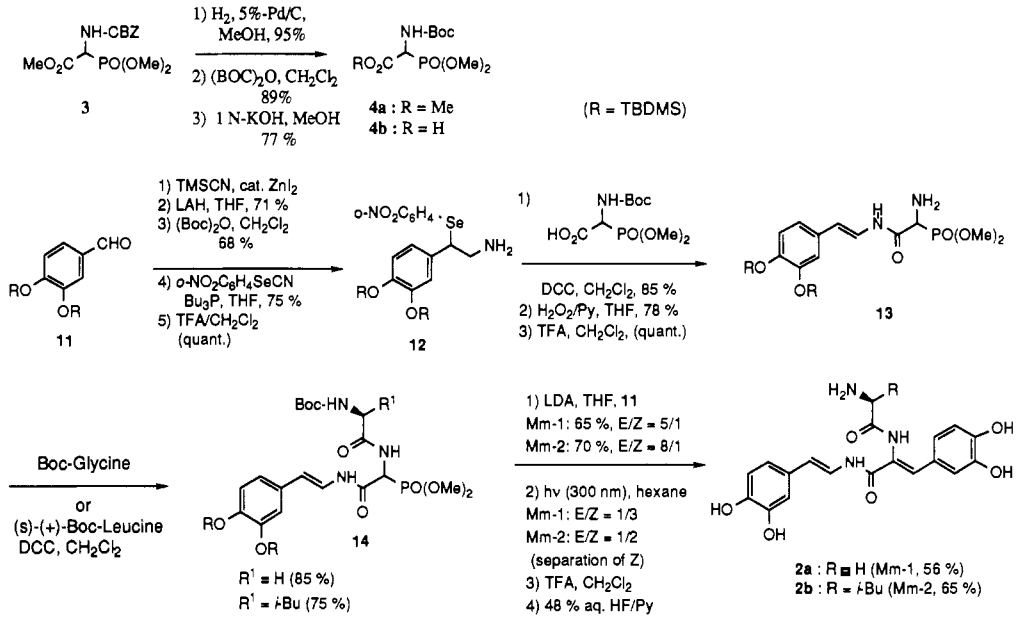
Scheme III
Synthesis of Mm-1 and Mm-2



species the element is stored in blood cells at levels as high as 0.5–1.1 M,[11] i.e., 5- to over 10-fold more vanadium than iron, and at roughly 10 million times its concentration in the ocean.[12]

Correspondingly, acetylation assays have shown that tunichrome constitutes 20–50% of the blood (dry weight) in *A. nigra* and other species; these values equate to ca. 0.3–0.4 mmol of An and ca. 0.16 mmol of vanadium per milligram of lyophilized blood cells.[3] Roughly equimolar levels of *free* tunichrome and vanadium are also found in *A. ceratodes*.[2,3] Estimates on the endogenous composition of *free* tunichrome have been made with *A. nigra* specimens collected in September 1984: An-1 Ac (acetate) (30%), An-2 Ac (50%), An-3 Ac (17%), minor components (ca. 1–2% each).[3] Preliminary surveys[3] have disclosed that four out of 12 species collected from coastal waters around the United States gave positive assays for tunichrome: three vanadium-accumulating species (*A. nigra*, *A. ceratodes*, and *P. viridis*) and one iron accumulator (*M. manhattensis*). *P. viridis* is a colonial tunicate, while the others are solitary species. More recent surveys have widened tunichrome's geographic and taxonomic distribution. Peracetylated pigments with the same TLC R_f value and the pumpkin-colored fluorescence of tunichrome standards are present in four vanadium accumulators from the Great Barrier Reef in Australia (*Phallusia julinea*, *Ascidia liberata*, two varieties of *Ascidia glabra*, and *Ecteinscidia nexa*; unpublished results). Tunichrome is thus affiliated with vanadium- and iron-accumulating species, as well as solitary and colonial species, many of which live in dissimilar marine habitats. Even so, the diversity of endogenous organic ligands in nature raises the possibility that tunichrome is not ubiquitous to tunicates; assessment of tunichrome's ubiquity is again hindered by the fact that exposure of tunichrome to paramagnetic ions or oxidants quenches its detection via fluorescence[13] or acetylation.

While morphological cell classification is often fraught with subtle ambiguities, tunicates appear to possess at least five varieties of circulating blood cells.[14] The distribution of *free* tunichrome and total vanadium in *A. nigra* and *A. ceratodes* blood cell subpopulations has been estimated via fluorescence-activated cell sorting (FACS), together with acetylation assays and atomic absorption elemental analyses.[2] This approach minimizes errors due to cell lysis or extracellular contamination because FACS selects only intact cells, the integrity of which are reflected by the optical parameters monitored. Somewhat surprisingly, the yellow to yellow-green morula cells (containing several spherical vacuoles) held nearly all the *free* tunichrome and up to 30% of the vanadium found, whereas the green-grey signet ring cells (characterized by one large vacuole) yielded only a trace of *free* tunichrome but the bulk of vanadium.[2] This is the first evidence that tunichrome and vanadium were present in the same intracellular compartment. The above distribution pattern for vanadium agrees with earlier X-ray microanalyses[15] and more recent staining techniques using 2,2′-bipyridine and 1,10-phenanthroline.[16]

An intracellular excess of *free* tunichrome (over vanadium) in morula cells may thus prove to be the mechanistic[1,5] key whereby *vanadium influx would occur at the expense of tunichrome oxidation* and give rise to signet ring cells possessing abundant vanadium but little *free* tunichrome. Accordingly, the inference that "*signet ring cells give rise to morula cells*", though commonplace, is difficult to reconcile with our data. If

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true for either *A. nigra* or *A. ceratodes*, it also necessitates that signet ring cells excrete virtually all vanadium (sequestered at no small expense) and accumulate *free* tunichrome to become morula cells.

Intracellular Vanadium Environment

Investigations by numerous groups[2,10a,16,17] indicate that endogenous organic ligands may be instrumental in vanadium sequestration for tunicates. As for tunichrome, its structure, prevalence, and distribution make it a prime candidate, though insoluble green to green-black complexes form[3] when V^{IV} or V^{V} is added to An-1, -2, or -3. Tunichrome's tendency to generate insoluble vanadium complexes in vitro is significant because this metal is often associated with intracellular membranes and granules within blood cells.[15a,16] Tunichrome, singly and as an intracellular collection of homologues, provides an extensive array of overlapping chelation centers connected through a semirigid backbone, thereby predisposing it to form heterogeneous, polynuclear metal complexes, as demonstrated by Job's plot An:V determinations of a 2–3:1 binding stoichiometry;[3] its hydroquinoid, styrene-like moieties also confer susceptibilities to many oxidatively catalyzed cross-linking reactions,[18] enhancing the formation of insoluble polymers.[3]

Now that an array of natural[7] and synthetic[8a,b] tunichromes and analogues[19] are available (affiliated with both vanadium and iron accumulators), the bioinorganic chemistry can be further simulated via reconstitution studies. Because of the complications outlined, no stability constants for tunichrome–metal complexes have been determined, though preliminary work indicates that soluble chelates are formed when vanadium(IV or III) or iron(II) is added to Mm-1; structural studies are ongoing. The catechol or pyrogallol constituents of tunichrome confer strong chelating properties toward vanadium and iron (at basic to mildly acidic pH values), with overall formation constants[20,21] (K_f) for $V^{IV}O$, V^{III}, Fe^{III}, and Fe^{II} ranging between 10^{10} and 10^{28} M^{-1}. Yet even though tunichrome is a prime candidate to mediate metal ion assimilation,[3,5] *no direct, definitive evidence* linking this or any other compound[11,17,22] has been secured, and understandably so; the unambiguous identification of a specific, air-sensitive metal complex among a dynamic array of homologues in fragile, developing cells is no small feat.

Within the ocean, vanadium exists in the 5+ oxidation state as monomeric vanadate (i.e., $H_2VO_4^-/HVO_4^{2-}$; pK 8.3, sea water pH ca. 8.3), whereas tunicates store this element in reduced states. Vanadium(III) and vanadium(IV) in *A. nigra* blood have been measured directly via magnetic susceptibility determinations at variable temperatures and magnetic fields up to 50 kOe with a superconducting quantum interference device (SQUID susceptometer). This method is diagnostic of *both* 3+ and 4+ oxidation states and does not depend on comparisons with model compounds. Analysis of the data indicate that ca. 90% and 10% are in the 3+ and 4+ oxidation states, respectively.[23a] This agrees with earlier magnetic susceptibility[23b] and X-ray absorption spectroscopy (XAS)[23c] results from additional species in the order Phlebobranchia, such as *A. ceratodes*.

Another seemingly distinct but mutually compatible view of vanadium complexation considers the metal to be coordinated as the aqua ion $[V^{III}SO_4(H_2O)_{4-5}]^+$ on the basis of extensive ^1H NMR,[11] electron paramagnetic resonance (EPR),[22a] and XAS[22b,23b] analyses of *A. ceratodes* blood cells. These data strongly support this postulate, provided the structural integrity of the associated cell has remained intact. Even though an aqua vanadium(III) ion may exist *naturally*, it is even more likely to be generated whenever the associated cell's vitality is compromised, just as tunichrome–vanadium complexes are, in whole blood cytolysates. For this reason, although the lability of tunicate blood is self-evident, reemphasis is warranted. Upon exposure to air, gently packed *A. nigra* blood cells turn from vivid yellow-green to red and olive green within a few minutes, while purified green, vanadium-laden signet ring cells are bleached instantaneously.

An aqua vanadium(III) ion necessitates a strongly acidic milieu because above pH 3, in the absence of other stabilizing ligands, V^{III} exists as insoluble black V_2O_3.[24] Since the cytosolic pH of virtually all *living* cells is maintained near neutrality, the postulated existence of an intracellular acidity corresponding to 0.01 M H_2SO_4 is extraordinary. A variety of pitfalls and inferences are associated with determining the pH of the "vanadium milieu"; given the sensitivities of most indicators to environmental factors.[11,25] For instance, the studies cited above were carried out on whole blood, which consists of cells with and without vanadium, while two varieties of the former are commonplace. Again, persuasive evidence for the presence of a highly acidic intracellular milieu is found in a fraction of the EPR-active vanadium complexes that may be safely assigned to an aqua vanadyl(IV) complex. A milieu stabilizing simple hydrated, lower valence vanadium can be rationalized by postulating the existence of an intracellular "pH < 3 compartment" in (1) a subcellular vacuole,[11] (2) a hydrophobic region like the interior of a membrane, and/or (3) a damaged or senescent cell itself.

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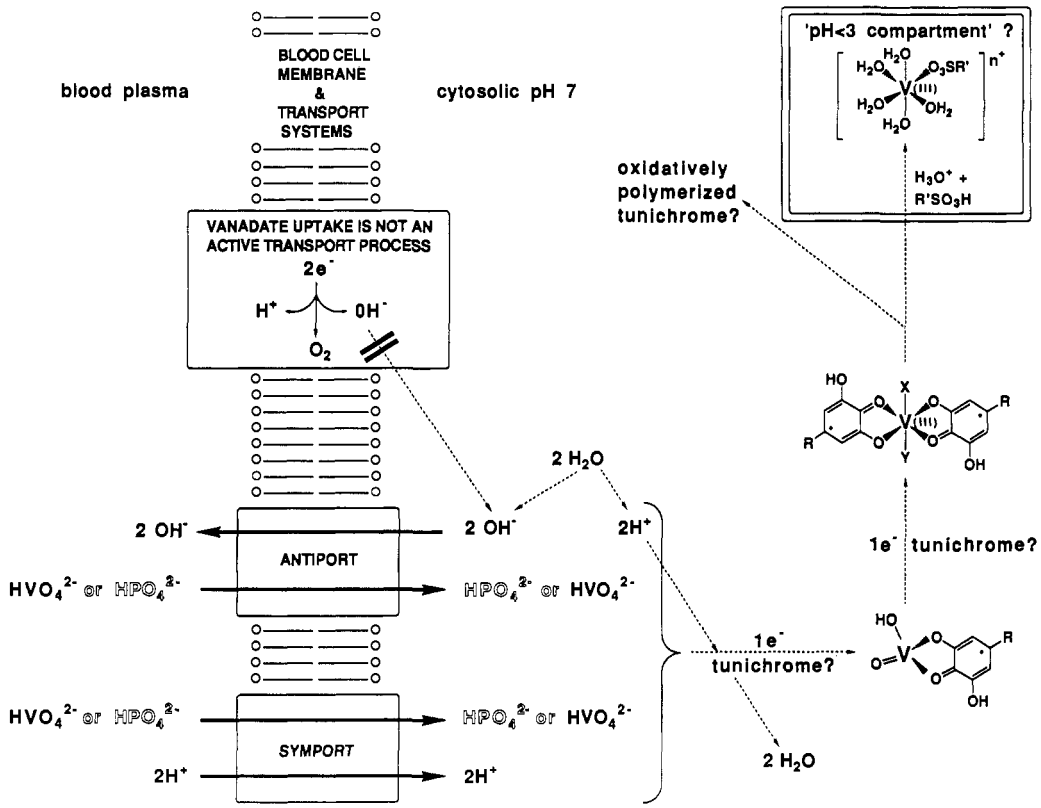

Figure 4. A model for the facilitated diffusion of vanadate into vanadocytes. Symport and antiport are formally equivalent processes.[28] The putative ligands depicted are tunichrome semiquinones, sulfate, and water, while X and Y are indeterminate. Reference 1 presents the original "trapping" model. Adapted from ref 5.

Bearing the above in mind, the pH of unsorted and sorted *A. ceratodes* blood cells has been reexamined recently by using equilibration experiments with radiolabeled 5,5-dimethyloxazolidine-2,4-dione and methylamine.[25g] Exacting conditions were required to sustain physiologically normal buffering capacities within the cell preparations. The average pH of *resuspended* blood cells was 6.98 ± 0.15, whereas that for the cytosol and vacuoles of morula subpopulations was 7.1 ± 0.2 and 5.0 ± 0.1, respectively.[25g] The current absence of similar pH determinations on the vanadium-laden signet ring cells, owing to their ease of lysis and resulting low recovery, admits the possible existence of a "pH < 3 compartment" therein. Nevertheless, a considerable fraction of the intracellular vanadium(III and IV) still appears to be *stabilized by chelation* at a neutral pH, at least in morula cells.

Mechanistic Considerations of Vanadium Assimilation

The sheer magnitude of vanadium and tunichrome incorporation constitutes a sizable metabolic allocation, while the crux of the former consists of the sequence of events that drive vanadium up a 10 million fold concentration gradient and reduce it to V^{III}. Three key considerations are the energy requirements, the ligands involved, and the attendant pH. The presence of free anionic vanadate(V) in tunicate blood plasma of nearly neutral pH,[25h] together with the fact that vanadocytes[25b] (i.e., cells accumulating vanadium) and human erythrocytes[26] translocate vanadate via phosphate transport systems, indicates that some variant of a chemiosmotic mechanism[27] is engaged. Recognition of this gave rise to the so-called "vanadium trapping model",[1] comprising a membrane "translocator" and tunichrome, a refined version[5] of which is diagramed in Figure 4. Correspondingly, the altered specificity of iron-accumulating systems would be conferred by the ligands and/or translocator subcomponents.

Moreover, vanadium influx does not appear to be an "active transport" process in tunicates, i.e., requiring a direct energy input, since inhibitors of glycolysis and uncouplers of oxidative phosphorylation have no effect on the rate.[25b] Instead, the "passive sequestration" or "facilitated diffusion" of vanadate against an *apparent* 10 million fold concentration gradient can be achieved in two ways:[28] (1) vanadate influx is coupled to the virtual efflux of ions with equivalent charge, e.g., symport; (2) the internal *vanadate* concentration is effectually diminished following influx. Both alternatives may pertain since vanadate utilizes the phosphate translocator and undergoes a two-step reduction to vanadium(III).[25b] Should the vanadate incorporated become trapped as impermeable ions, precipitates, or insoluble polymers or become membrane bound, this would further shift its distribution in favor of influx. Such is actually the case for human erythrocytes.[26]

The above interpretation is supported by tunichrome's ability to form insoluble metal complexes and reduce vanadium(V) as well as iron(III);[1,10a,29] however, such experiments have yet to demonstrate that it can reduce vanadium(IV) to vanadium(III) without further

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assistance. Nevertheless pyrogallol, a constituent of An-1, -2, and -3, facilitates the reduction of vanadium(IV) to vanadium(III) in vitro,[30a] thereby indicating that tunichrome might facilitate a similar feat in vivo, either directly or via acid-catalyzed disproportionation[30b] of vanadium(IV) into vanadium(III) and vanadium(V), despite inferences[19b] derived from tunichrome models to the contrary. Thereafter the vanadium(III) aqua ion could be generated naturally and/or artifactually via hydrolysis of a tunichrome[(semi)quinone]–V(III) complex, as depicted in Figure 4; hydrolytic dissociation of the two may result via loss of cell integrity, concomitant acidification, and/or decreased affinity of the (oxidized) ligand for the reduced metal ion. Accordingly, the postulated "pH < 3 compartment" may have little or no bearing on vanadium influx, whereas an entirely different mechanism would have to be formulated if tunichrome and vanadium were to reside exclusively in separate subcellular compartments.

Biological Roles of Tunichrome and Vanadium

Teleologically, a much more suitable ligand could have evolved should tunichrome's sole role be to complex metal ions; for example, a structurally similar yet flexible tris-hydroquinoid peptide with the distinction of forming the most thermodynamically stable natural iron chelate known is enterobactin ($K_f = 10^{52}$ M^{-1} for the monomeric Fe(III) complex).[31] The biogenesis of a resilient tunic for tunicates has long been postulated to be the raison d'être for the vanadium-accumulating blood cells (vanadocytes).[32,33] Correspondingly, the mode of action of vanadocytes includes circulation through the hemocoel and lysis within tissue thought to form the tunic.[33a,d,34] Tunic biogenesis[3,33e] may thus be analogous to the hardening or maturation (sclerotization) of insect cuticle, which involves oxidation of hydroquinoid cross-linking agents[18] not unlike tunichrome. Indeed, the high reactivity that makes tunichrome relatively intractable in vitro may foreshadow its mode of action in vivo; specifically, this mobile ligand's hydroquinoid, styrene-like moieties predispose it to generate markedly adhesive,[3,6] insoluble[3] polymers.

Additional insight into the physiological role of cells storing vanadium(III) and tunichrome may be near at hand, especially now that an earlier postulate[35] involving O_2 transport is no longer tenable. It is astounding that vanadium(III) and tunichrome even persist in a respiring organism, given their susceptibility to air oxidation. Since the cellular milieu around these two reducing agents must be *nonoxidative* to a high degree, a new hypothesis[5] correlates their redox activity to *anaerobic metabolism*. Namely, despite the efficiency of O_2-based (aerobic) respiration, many organisms cope with chronic O_2 deprivation (hypoxia) by shifting metabolically generated reducing equivalents toward chemical respiration.[36] Correspondingly, no reversible O_2 binding ability has been experimentally found in blood extracted from healthy vanadium-accumulating[37a] or iron-accumulating[37b] tunicates. The O_2 tension of tunicate blood plasma has since been reported to be very low, and if it is raised artificially, vanadium(IV) levels rise concomitantly [i.e., via vanadium(III) oxidation].[16] Vanadocytes might thus enable tunicates to tolerate hypoxia, in contradistinction to the aforementioned postulate[35] suggesting that they serve as O_2 carriers. Biological precedence for such a metabolic option is provided by facultative anaerobes that substitute transition metals for O_2 as terminal electron acceptors.[38] Similarly, vanadium- and iron-accumulating cells may accommodate the metabolic "end products problem" by providing the animal with an alternative electron and proton sink,[36] e.g., via NAD(P)H oxidation, the final result being to harden tunic with oxidatively polymerized tunichrome, the latter being formally analogous to melanin. Accordingly, vanadate-catalyzed oxidation of NAD(P)H merits attention.[39] Likewise, the appearance of a brown pigment,[40] evidently melanin,[41] at 13 h after fertilization of *Boltenia villosa* (tunicate) embryos is also pertinent, for it obscures an endogenous "orange chromogen" which demarcates muscle-lineage cell progenitors. Therefore, hydroquinoid oxidation may be crucial to the development of tunicate embryos,[33d] as well as signet ring cells, and transition metals catalyze melanogenic reactions.[42]

Viable alternative roles for vanadocytes involve histoincompatibility responses[43] and peroxide production/sterilization reactions,[43] either of which may be compatible with a more fundamental activity. As the most primitive chordates in existence, tunicates may have evolved when conditions on earth were less oxidizing (more anaerobic). The primordial appearance of O_2 in the atmosphere created a double jeopardy for many forms of life: (a) a diminished availability of essential metal ions like vanadium and iron, due to their precipitation as hydroxide polymers, and (b) exposure to a hazardous oxidizing agent, O_2. Three organismal responses to this dilemma appear to have been to (1) elaborate high affinity metal ion transport systems;[44] (2) maintain alternative respiratory options;[36] and (3)

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protect cells from overexposure to O_2.[36] These unobtrusive creatures thus provide ample cause to pause and reflect upon the secret behind their perpetual success, for all three responses may be engaged.

Conclusions

Two hallmarks of tunicates are their tunic and anomalous blood. Extraordinary quantities of tunichrome and vanadium are often stored within the latter and give rise to its high reactivity and vivid pigmentation. Clarification of the unusual chemical ecology of tunicates may thus derive from continued efforts to disclose the mode of action of these constituents. To this end, the isolation, characterization, and synthesis of a prevalent reducing ligand, tunichrome, have been instrumental. Now that adequate quantities of analytically pure, synthetic and natural tunichromes are available, several key investigations may proceed.

Much of the data presented is from the Columbia University Ph.D. Theses of Eugene Oltz (natural tunichrome), Benjamin Horenstein (synthetic tunichrome), and Seunghee Lee (bioinorganic studies) and from studies performed by Drs. Giuseppe Ruberto and William E. Robinson at the New England Aquarium (biosynthetic studies). Funding was provided by National Institutes of Health Grants AI 10187 (K.N.) and F32 CA 08057-02 (M.J.S.) and from National Science Foundation Grant DCB 8500309 (K.K.). We are grateful to the referees for their comments.