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Mini-Review

Vanadium biochemistry: The unknown role of vanadium-containing cells in ascidians (sea squirts)

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Summary. This article reviews several new developments in vanadium biochemistry, as elucidated from studies of ascidians. A hypothesis correlating ascidian blood cell function to anaerobiosis, via two prominent redox constituents, namely vanadium(III) and the tunichromes, a family of metal ion complexing/reducing hydroquinonoid peptides, is presented.
Key words: Vanadium; ion pump; tunichrome; tunic; ascidian; respiration; oxygen; anaerobe.

Introduction

Organisms possess a variety of mechanisms for assimilating the particular transition metals needed for normal metabolic activity. Ascidians are one such case, for they display a remarkable ability to sequester and reduce vanadium in specialized blood cells termed vanadocytes; analogously, iron-accumulating species possess ferrocytes[1]. However, the function of this bioinorganic process has remained an enigma since 1911[2]. To date, a physiologic role for vanadium in animals remains conspicuously absent, even though it is considered to be an essential trace element[3]. In comparison, vanadium is integral to several algal bromoperoxidases[4] and to certain bacterial dinitrogenases[5]. Unlike previous attempts to explain the existence of oxygen sensitive V (III) in a living organism, the bioinorganic chemistry of vanadocytes is reviewed here in light of *anaerobic adaptation* as the crucial issue.

Background

Ascidians (class Ascidiaceae) are sessile, filter-feeding chordates which inhabit all of the oceans[6]. Two traits of these prolific creatures are their resilient mantle (i.e., tunic; hence tunicates), and their powerful siphons (hence sea squirts). It has been estimated that species such as *Ascidia ceratodes* store vanadium in cell vacuoles (vanadophores) at concentrations up to 1 M[7], while vanadium levels in vanadocytes approach 10 million times that of sea water[8]; the element is often found associated with intracellular membranes and granules[9]. In general, at least five varieties of circulating blood cells have been identified: lymphocytes, stem cells, leucocytes, pigment cells, and vacuolated cells[10]. Three types of the latter are commonly seen: green/grey signet ring cells (SRC), characterized by one large vacuole; green compartment cells (CC), containing several angular vacuoles; and bright yellow to yellow/green, mulberry-shaped

morula cells (MR), consisting of several spherical vacuoles. Traditionally, morula cells have been considered to be the major repositories for vanadium and iron, hence the vanadocytes and ferrocytes[11]. In many species they comprise a large but variable portion of the total blood cell population, approaching 55% in *A. ceratodes*[12]. A prominent chromogen in morula cells is tunichrome[13,14], which has been detected in roughly equimolar levels to vanadium within whole blood[13,15,16]. Strictly speaking, *the term vanadocyte encompasses all cells which assimilate vanadium*, thereby obviating any reference to a particular stage in cell development. In other words, the cells which begin to assimilate vanadium may be morphologically distinct from their mature counterparts.

Tunichrome complexation chemistry

The chemical characterization of 'tunichrome' by Nakanishi's group disclosed a family of hydroquinonoid peptides, members of which are present in both vanadium- and iron-accumulating species (fig. 1)[16]. Purification of the *metal-free form* of tunichrome necessitated several unusual chromatographic techniques, all performed in the presence of antioxidants[16]. Tunichrome had previously been found to reduce V (V) to V (IV) and Fe (III) to Fe (II) in vitro, making it a formidable contender for the long-sought metal ligand and reductant in vivo[14,15]. At pH values above 4, the vicinal hydroxy substituents of the catechol and pyrogallol rings confer potent chelating properties towards vanadium and iron[17–20]; the overall formation constants (K_f) of these ligands toward VO (IV), V (III), Fe (III), and Fe (II) are greater than 10^{10}, and several surpass 10^{28} (Martell and Smith[21]). Tunichrome could thus play a pivotal role in vanadium and iron sequestration, although little definitive evidence has been forthcoming. The characterization of labile metal complexes is no small feat, of course.

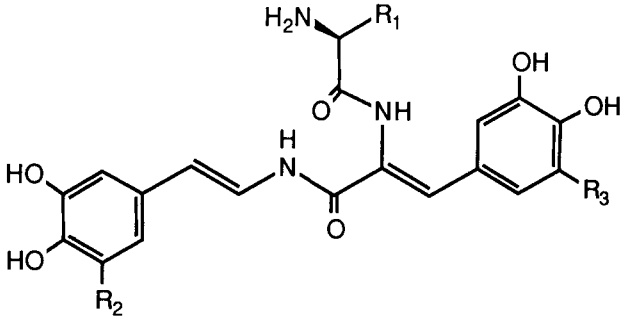

Figure 1. The tunichromes. 'An-' designates *Ascidia nigra*, a vanadium accumulator, while 'Mm-' designates Molgula manahattensis, an iron accumulator[16]. An-1: R_1 = hydroxy-Dopa; 'Dopa' designates 3-(3,4-dihydroxyphenyl)-L-alanine; $R_2 = R_3 = OH$; An-2: R_1 = Dopa, $R_2 = R_3 = OH$; An-3: R_1 = Dopa, $R_2 = H$, $R_3 = OH$; Mm-1: R_1 = glycine, $R_2 = R_3 = H$; Mm-2: R_1 = leucine, $R_2 = R_3 = H$.

Alternative vanadium complexes

One alternative and seemingly disparate view of vanadium sequestration in vanadocytes has been offered by Carlson, Hodgson and colleagues. Their ^{1}H-NMR[7], EPR[22], and X-ray absorption[23,24] analyses of *A. ceratodes* packed blood cells, which have prevalent tunichrome An-1 incidentally[13,16], suggests that the metal is coordinated as $[V(III)SO_4(H_2O)_{4-5}]^+$ within vanadophores[22,23]. These data strongly support this postulate, provided the structural integrity of the cells has remained intact. Significantly, the coordination of V (III) by water necessitates an *intravanadophoric* pH below 3. Evidence for an abnormally low pH was first obtained by Henze[2] and is still the subject of much debate[16], given the sensitivities of most pH indicators to environmental factors; those determinations which indicate a neutral cytosol[25] housing acidic vacuoles[7,22,25] may resolve several disparities, however. The vanadium sequestration controversy thus hinges on the conditional formation constants (K_{sp}^{cond}) of the putative ligands towards V (III, IV, and V); in turn, the values depend on pH and ligand lability.

Oxidative considerations

The distribution of *free* tunichrome and vanadium in *A. nigra* and *A. ceratodes* blood cells has been determined recently using fluorescence-activated cell sorting (FACS; i.e., flow cytometry), in conjunction with microanalyses[12]; somewhat unexpectedly, the morula cells contained nearly all of the *free* tunichrome detected and up to 30% of the vanadium, whereas the signet ring cells yielded only a trace of *free* tunichrome but a majority of the vanadium. In essence, when appropriate cell selection criteria were implemented (easily oxidized) tunichrome and (readily oxidizing) vanadium were detected in the same cells, thus improving the odds that this ligand sequesters vanadium in vivo. Again, it is no small wonder that *free* tunichrome could be detected at all, given its propensity to be oxidized by atmospheric oxygen, as well as by transition metals. Therefore, the cellular environment around *free* tunichrome must be *nonoxidative* to a significant degree. Considerations of blood cell maturation arise here also. Specifically, are the vacuolated cells (MRs, CCs, SRCs) part of one developmental sequence? Could certain cell types reflect abnormal decomposition events, such as oxidative damage? If cell differentiation is correlated to vanadium accumulation, the FACS results indicate that MRs give rise to SRCs; an earlier finding that SRCs contain a chromophore reminiscent of one present in reconstituted tunichrome-vanadium complexes[16], and of cross-linked catecholamine oxidation products[26], lends credence to this interpretation and reconciles the seemingly anomalous distribution of *free* tunichrome. Vanadocyte maturation may thus depend on vanadium uptake which, in turn, might occur at the ex-

pense of tunichrome oxidation. Admittedly, since this inference has yet to be proven an alternative interpretation that tunichrome is not involved in vanadium assimilation stands, although it rests on negative evidence. For instance, SRCs often yield negative results for assays based on autonomous fluorescence (i.e., *free* tunichrome)[27], and for assays of the tunichrome decaacetate derivative[13]. Again however, vanadium's presence will confound tunichrome's detection; specifically, paramagnetic species such as V (III and IV) or tunichrome semiquinones are likely to quench tunichrome's autonomous fluorescence and alter its acetylation pattern[13].

That *yellow-green* ascidian blood is unusual and unstable to the atmosphere[10,28] deserves emphasis. In particular, *no reversible oxygen-binding* by the blood of *A. nigra* could be demonstrated[28], in contrast to one undocumented reference[29] on a different species to the contrary. In fact, at the onset of studies on tunichrome one predominating belief was that the compound contained metal and pyrrole groups, hence comparisons to heme structure and function prevailed. A green pigment of unknown relationship to tunichrome, investigated previously, was designated hemovanadin for this reason[30]. Henze, the discoverer of vanadium in ascidians, pointed out that since the pigment is a strong reducing agent the oxidation potential at which it could act as a potentially reversible redox agent would probably be very low and, thus, beyond the normal physiological range[31]. Correspondingly, the hydroquinonoid groups of tunichrome are effective *wet-oxygen scavengers*, based on the fact that aqueous alkaline pyrogallate solutions are implemented as *oxygen scrubbers* during experimental manipulations requiring an inert-atmosphere[32,33]. Nevertheless, the recent structure elucidation of tunichlorin[34], an unusual nickel-containing chlorin isolated from the ascidian *T. solidum* living commensally with a cyanobacterium, renews the issue of hemovanadin, although the macrocycle might be biosynthesized by the prokaryote. Several cyclic peptides with potential metal binding activity have also been isolated from ascidians recently[35–37].

Overview of vanadium assimilation

Three key considerations concerning ascidian vanadium assimilation are the pH, the energy requirements, and the ligands associated with the process. The series of chemical reactions driving vanadium uptake against a steep gradient and reducing it to V (III) are the crux of the issue. Further, given the fact that the cytosolic pH of virtually all living cells is maintained near neutrality[38], should the *final* vanadium complex prove to be $[V(III)SO_4(H_2O)_{4-5}]^+$, it must somehow reach an acidic vacuole. Whether or not one grants merit to this interpretation, the role and mechanism behind vanadium assimilation still warrant disclosure. So how might this element be driven into a cell and reduced to V (III)?

The finding that vanadocytes[39], as well as human red blood cells[40], translocate vanadate ion (e.g., $H_2VO_4^-$) via specific anion transport systems utilized by phosphate ion (e.g., $H_2PO_4^-$) suggests that some variant of Peter Mitchell's chemiosmotic model[41,42] is engaged (fig. 2). Recognition of this biochemical feature led Kustin, Macara and coworkers to propose a vanadium "trapping" model, comprised of two components: a membrane transport system and tunichrome[14,39]; importantly, inhibitors of glycolysis and of oxidative phosphorylation did not block vanadate uptake. This result suggests that ascidian vanadium assimilation is not an active transport process, requiring a direct energy input (e.g., ATP hydrolysis). Rather, in the absence of a direct energy input, the 'passive' sequestration, or facilitated diffusion, of vanadate against an *apparent* 10 million-fold concentration gradient can be achieved in two ways[43]: 1) vanadate uptake is coupled to the flux of a secondary ion (e.g., phosphate/OH^- antiport); 2) vanadate is removed from solution by being bound or converted to some other chemical form. Both alternatives may pertain since vanadate utilizes the 'phosphate transporter' and undergoes a two-step reduction to V (III)[39]. Should the vanadium complexes so incorporated become 'trapped' as insoluble polymers or membrane-bound, this would further shift the solution equilibrium in favor of vanadate uptake. In contrast, respiring mitochondria accumulate phosphate ion by utilizing energy derived from electron transport to drive the formation of a proton (pH) gradient across a membrane (e.g., 'active' phosphate/OH^- antiport). In comparison, while traditional (mitochondrial) phosphate accumulation requires a direct energy input, resulting in acidification of the phosphate repository, ascidian vanadate uptake does not require a direct energy source. Hence, a low vanadophoric pH, if present, may bear no *direct* relationship to the mechanism in question. Nevertheless, the sheer magnitude of vanadium and tunichrome accumulation would still indicate a sizeable (indirect) metabolic burden.

A comparison of the *primary* ligand contenders reveals that most are ill-suited to the perceived needs: prevalence and a strong affinity for vanadium at a neutral pH. For example, while membrane-bound alkyl sulfates are abundant in ascidian blood cells[24,44], they are poor ligands for V (IV or V), exhibiting K_fs ca 10^1 and $10^{2.5}$, respectively[21]. Likewise, although another vanadium-binding substance termed vanadobin is considered to be a more likely ligand than tunichrome, the thrust of this interpretation rests on the negative evidence discussed above[27]; vanadobin's chemical structure and affinity for vanadium warrants disclosure, however. Based on the data available tunichrome appears to be the most suitable mediator for the facilitated diffusion of vanadate; further, its high reactivity to transition metals may also prefigure biochemical coupling to oxidatively catalyzed self-polymerizations, and/or $[V(III)SO_4(H_2O)_{4-5}]^+$ coordination, and/or anaerobic redox reactions, amongst

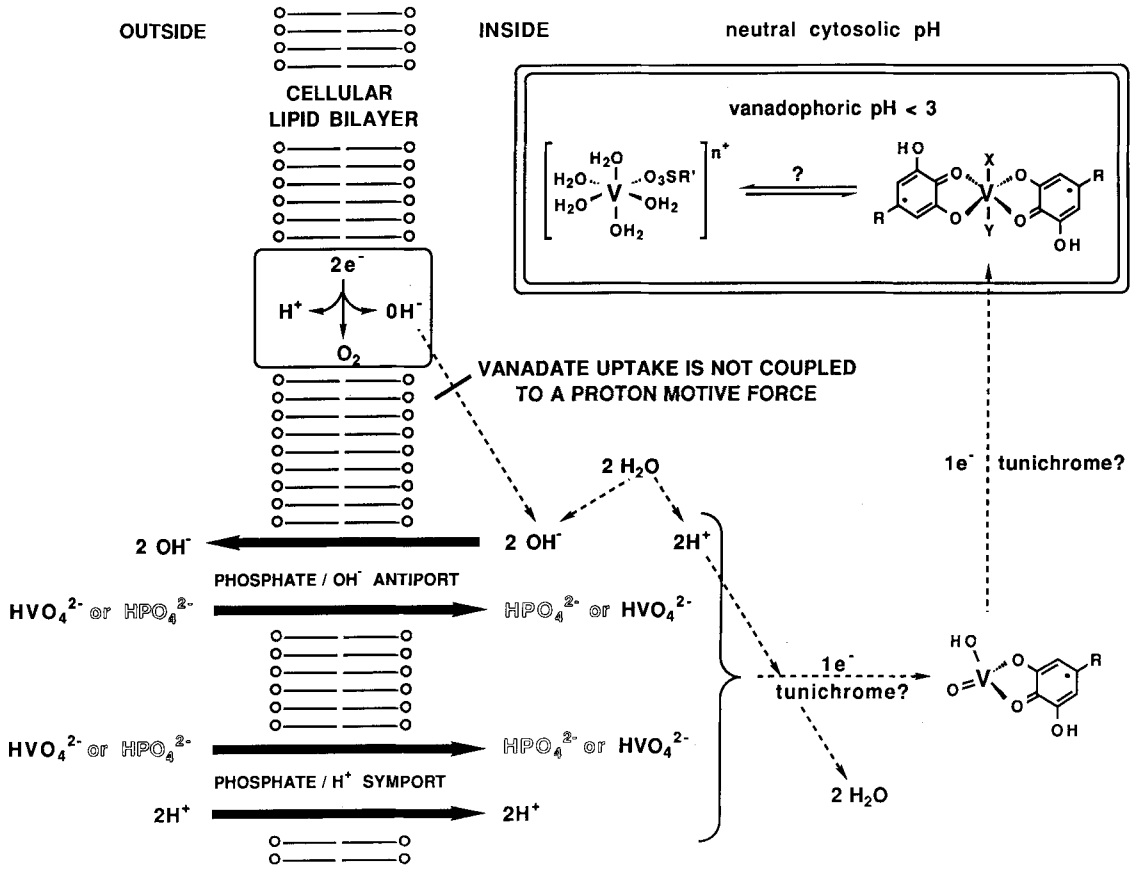

Figure 2. A model for the facilitated diffusion of vanadate into vanadocytes. Phosphate symport and antiport are formally equivalent processes, yet differ in mechanistic details[43]. The location, solubility, and ligands of the 'ultimate vanadium complex' are equivocal. The putative ligands depicted are tunichrome semi-quinones, alkyl sulfates and water. Reference 14 presents the original 'trapping' model.

numerous other possibilities. Accordingly, vanadium assimilation is best perceived as several discrete coordination events occurring in concert, *one plausible scenario being that tunichrome acts as the primary, transient ligand and initial reductant of V(V), leading to its ultimate storage as V(III) in vanadophoric granules of low pH* (fig. 2). Again sinced tunichrome's delicate structure is susceptible to a multitude of oxidatively-catalyzed polymerizations, the ultimate vanadium complex may not be monomeric. From a teleological standpoint, a much more potent ligand could have evolved should tunichrome's sole role be to secure metal ions; because of its rigid backbone tunichrome is not disposed to form stable, monomeric vanadium (or iron) complexes[16]. In contrast, a flexible bacterial tris-catechol analogue of tunichrome is enterobactin; it bears the distinction of being the most thermodynamically stable natural iron chelate known, exhibiting a $K_f = 10^{52}$ for the monomeric Fe(III) complex[45,46]!

Proposed roles for vanadocytes

Considerable effort has been devoted to unraveling the biological role(s) of the vanadocyte, hence vanadium and tunichrome. Moreover, a unique bioinorganic process may not pertain should ferrocytes fulfill an analogous function. It is remarkable that Henze also pioneered the idea that tunic biogenesis might be the ultimate answer[31]. Several refinements on this theme have since appeared[47–50], some of which specify that tunic formation may be analogous to the maturation (sclerotization) of insect cuticle[16,22,51], via oxidation of catecholamine cross-linking agents. Alternative proposals include antifeeding activity[52], histo-incompatibility responses[53], and peroxide production/sterilization reactions[53], any of which may be compatible with a more fundamental activity. A paramount condideration then, is the oxidative lability of tunichrome, vanadium(III), and the vanadocytes they are contained within. *Therefore, I submit that vanadocytes enable ascidians to sustain periods of oxygen deprivation (anoxia), in contradistinction to proposals suggesting that they serve as oxygen carriers.* I have noted a similar correlation between bacterial high affinity metal ion transport, anaerobiosis, and unusual imino acids acting as alternative electron and proton sinks during respiration[54,55]; in general, such imino acids, termed opines, are reductive amination products of α-keto acids and L-amino acids. Significantly, Hochachka and colleagues had previously outlined how marine invertebrate imino acid dehydrogenases, hence imino acid levels, appear to

modulate the redox status of certain cells during anoxia[38, 56]. Evidently, in spite of the high efficiency of oxygen-based respiration, many organisms cope with anoxia by engaging one of a variety of alternative redox pathways[38, 56, 57].

Anaerobic adaptation

The basic role of vanadocytes could thus prove to be anaerobically adaptative in nature. Several salient points supporting this hypothesis are outlined below:

1) Oxygen-binding studies of *A. nigra* blood failed to demonstrate that it could serve as an oxygen carrier[28].
2) Oxygen uptake by *A. nigra*[47] (i.e., V_{O_2} consumption) is comparable to other so-called 'good anaerobes' or 'oxygen conformers', such as the mussel *Mytilus edulis*, another filter-feeder, which has been estimated to be able to survive without oxygen for up to 150 days[38]!
3) Upon exposure to the atmosphere (i.e., after the tide recedes and the siphons close) pharyngeal cavity pO_2 levels for the intertidal ascidian *Phallusia mamillata* (a vanadium accumulator) decline from 150 mm Hg to 20 mm after 1 h, and drop to 4–5 mm after 2 h[29]. A marine organism living in a sealed mantle without an oxygen carrier may face a dire dilemma.
4) Both V (III), the predominant form present in *A. nigra* blood[58], and tunichrome[16] are very labile to oxygen. It has also been shown that pyrogallol facilitates the reduction of V (IV) to V (III) in vitro[59], thereby suggesting that tunichrome might perform a similar feat in vivo. Again, vanadocytes seem to serve as electron and proton sinks [e.g., V (III) storage, acidic vacuoles]; their ultimate fate appears to involve migration to, and lysis within, the outer test (disposal?), whereby tunic biogenesis may ensue[47, 50, 60, 61]. Ironically, vanadocytes may thus redound as an anaerobic 'cause and cure'.
5) The sessile lifestyle of adult ascidians may actually elevate the anoxic dilemma, for such creatures cannot avoid it via relocation. Innumerable prokaryotes and eukaryotes rely critically on unique metabolic strategies directed toward sustained anaerobic activity. Ascidian vanadocytes, containing large glycogen stores but few mitochondria[60], may utilize 'a novel solution to an old dilemma'. Analogously, iron and manganese are two of several substrates that the facultative anaerobic bacterium *Alteromonas putrefaciens* can utilize as a terminal electron acceptor for growth (i.e., Fe^{3+}, MnO_2, NO_3^-, NO_2^-, $S_2O_3^{2-}$, SO_3^{2-}, glycine, fumarate, or O_2)[62].
6) As primitive chordates, ascidians may have evolved at a time when conditions were less oxidizing. The appearance of oxygen in the atmosphere is considered to have led to the precipitation of oxidizable metal ions such as vanadium and iron as hydroxide polymers[63]. One organismal response to decreases in the availability of essential metal ions appears to have been to elaborate high affinity metal ion transport systems[63].

In essence, vanadocytes, and by analogy ferrocytes, may accomodate the 'end products problem' during anoxia by providing an alternative electron and proton sink, the final result being to generate a resilient tunic from polymerized tunichrome. It is hoped that this review will be of heuristic value to future studies on vanadocyte function, for many important details merit investigation.

Note added in proof: The recent report[64] that an endogenous orange pigment demarcates the muscle-lineage cell progenitors in *Boltenia villosa* ascidian embryos is germane to the above hypothesis. At 13 hours after fertilization the pigment is obscured by the appearance of a brown pigment, presumably melanin[65]. This phenomenon bears a striking resemblance to the anticipated fate of yellow tunichrome, Dopa being a constituent of both natural products.

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Research Articles

Subcutaneous transposition of the spleen enhances the survival rate following 90% hepatectomy in rats

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Summary. The effects of subcutaneous transposition of the spleen (STS) on the survival rate following 90% hepatectomy were investigated in rats. The survival rate was significantly higher in the STS group than in the non-STS group. Light microscopy enabled us to note that congestion in the terminal portal veins and sinusoids occurred either slightly earlier or to a higher degree in the non-STS group.
Key words. 90% hepatectomy; spleen transposition; portal congestion; portal-systemic shunt; hepatic failure.

Ninety percent hepatectomy in rats is fatal within 40 h without regeneration of the liver remnant[1]. When rats are given 20% glucose to prevent hypoglycemia[2], or given testosterone to enhance protein synthesis[3], the survival rate is over 80%.

Subcutaneous transposition of the spleen (STS) was attempted, to produce portal-systemic shunting for the decompression of the portal blood flow[4,5]. An overload of metabolites in the liver remnant has been considered to be one of the main factors causing the high mortality rate following 90% hepatectomy in rats[6]. The effect of STS in increasing the survival rate following 90% hepatectomy is reported.

Materials and methods. Male Wistar rats, weighing 250–300 g at the time of 90% hepatectomy, were used. They were fed a standard laboratory chow and water ad libitum. The animals were divided into 2 groups with or without the subcutaneous transposition of the spleen (STS).

STS was undertaken as follows: A left-sided longitudinal abdominal incision was made under light ether anesthesia. The fine peritoneal attachments between the stomach and the spleen were divided. Through a split in the abdominal muscle the spleen was pulled out and placed under the skin. The muscle was approximated carefully and the skin closed. The splenic artery and vein were not damaged[4,5].

Ninety percent hepatectomy was performed as follows: The rats of both groups were deprived of solid food for the 12 h prior to surgery and 5% glucose was given ad libitum. 90% hepatectomy consisted of Higgins-Anderson's partial hepatectomy[7] plus a resection of the lower