Tunicate Research in Prof. Robinson's Lab

Tunicates are not particularly handsome, as is evident from these specimens of *Phallusia (Ascidia) nigra* and *Ciona intestinalis* to the right! Nevertheless, tunicates are amazing creatures. They are prominent members of the subtidal marine fouling community in both temperate and tropical regions. They appear to have few predators, due in part to the thickness, consistency, and chemical makeup of their outer protective covering, the "tunic" from which the group gets its common name. Although tunicates have a notochord and dorsal nerve chord in their larval stages (placing them squarely in the Phylum Chordata with us humans), these so-called "advanced" features are lost when the animals metamorphose into the adult. Even more curious than this, a number of tunicate species (primarily in the suborder Phlebobranchia; e.g. *P. nigra*) have the uncanny ability to concentrate the metal vanadium in several of their blood cells (Table 1, right; Kustin et al., 1990; Kustin & Robinson, 1995), at levels more than a million-fold higher than is found in the surrounding seawater, and in a highly reduced form (V^{III}; Lee et al., 1988; Kustin et al., 1996). Still other species (in the suborders Stolidobranchia and Phlebobranchia) concentrate reduced iron (Agudelo et al., 1983; Curtin et al., 1985). While there must be a reason for their having developed the ability to concentrate these metals, the answer to this riddle has eluded researchers since the high concentration of vanadium was first described by Henze in 1911.

Fig. 1. Specimens of *Phallusia nigra* (black pigment; each approximately 5 cm along the longest axis) and *Ciona intestinalis* (translucent yellow)

Table 1. Vanadium concentrations ($\mu g\ g^{-1}$ dry wt) and Bioconcentration Factors (BCF; $\mu g\ V\ g^{-1}$ wet tissue wt $\div\ \mu g\ V\ g^{-1}$ seawater) for *Phallusia nigra* tussues.

| Tissue	V conc.	BCF
Body (without tunic)	2,120	210,000
Tunic	320	32,000
Hemocytes	26,760	2,700,000

In collaboration with researchers from Brandeis University (Prof. Kenneth Kustin), Stanford University (Drs. Pat Frank and Keith Hodgson), Columbia University (Prof. Koji Nakanishi), and UMass Boston's Biology Dept. (Prof. Manickam Sugumaran), we have been trying to determine the function of vanadium in these tunicates. This is complicated by the fact that there are so many different types of blood cells. Depending on the species (and the investigator), eight to ten different types of blood cells have been described for various tunicate species (see Figure 2, below). The function of each cell type has yet to be determined. Recent vanadium K-edge X-ray Absorption Spectroscopy (XAS) studies on *P. nigra*, conducted by

Fig. 2. Blood cells from *Phallusia nigra*. (Glutaraldehyde fixed cells; light microscopy, Phase contrast, 400 x)
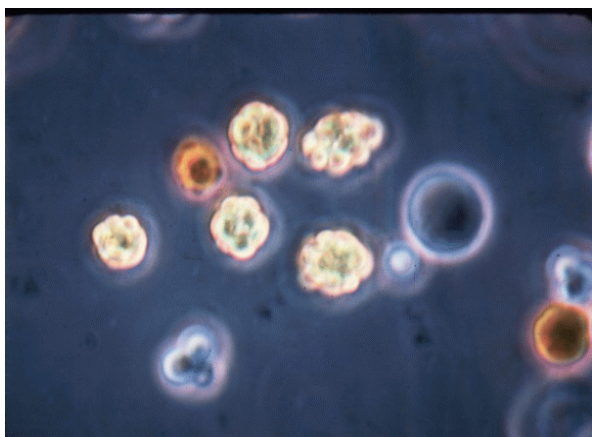
Frank and Hodgson at Stanford University in collaboration with our lab, have identified the presence of at least 3 separate V^{III} intracellular environments, plus at least 2 separate environments housing V^{IV}. At present, however, we cannot match up these separate intracellular environments with any particular blood cell type. We do know that, for the two species that we have conducted XAS studies on (*Ascidia cerratodes* and *Phallusia nigra*), over 90-95% of all the vanadium present in the blood cells is present as the highly reduced V^{III} (Frank et al., 1995; 1998; 2001), and we can identify some blood cell types that contain this highly reduced vanadium.

V^{III} is easily oxidized and can only be maintained intracellularly by either low pH or in the presence of a strong chelator. In 1979, Kustin's students at Brandeis isolated a yellow peptide from *P. nigra* blood cells, which they named tunichrome, that was present in approximately 1:1 ratio with vanadium. They hypothesized that tunichrome was the chelator of vanadium (e.g. Robinson et al., 1984). Nakanishi's group at Columbia then developed an improved isolation procedure for tunichrome, determined its structure, and eventually identified three forms

Fig. 3. Tunichrome molecules from *Phallusia nigra* (An) and *Molgula manhattensis* (Mn)

of a tripeptide tunichrome (An) from *P. nigra* and a dipeptide tunichrome (Mn) from *Molgula manhattensis* (Figure 3, above right). Subsequently, they were able to synthesize tunichrome An *de novo*.
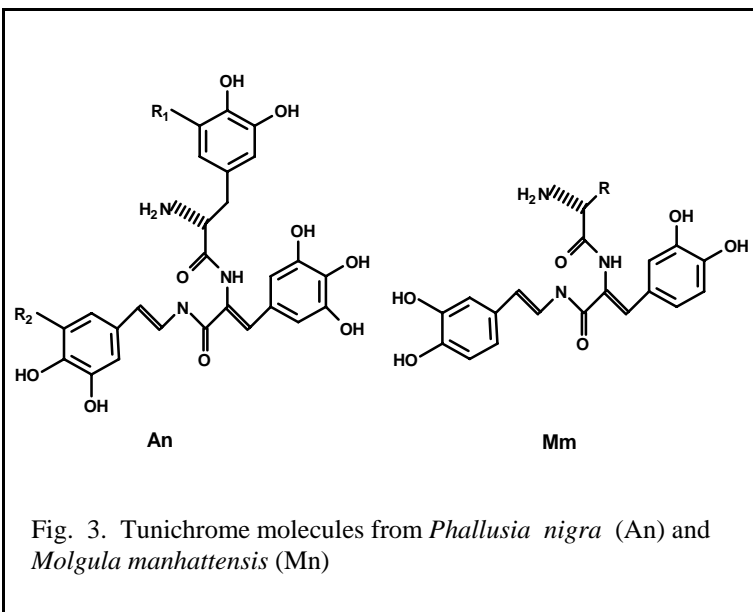
Work in our lab has shown that radiolabeled tyrosine and phenylalanine can be incorporated directly into tunichrome *in vivo* (He et al., 1992; Robinson et al., 1996). Although several additional tunichromes have been isolated from various tunicate species, their biosynthetic pathway remains unknown. Taylor and coworkers (1993) have hypothesized that tunichrome is an artifact of sample preparation, being formed when cells are lysed and vanadium is released. They further hypothesize that tunichome is a hydrolysis product of a tyrosine-rich protein. Using similar spectroscopic techniques as used by Taylor and coworkers, followed by TLC identification of tunichrome, we were able to detect free tunichrome in intact cells of *P.*

nigra (unpublished data). While our results do not rule out the possibility that tunichrome is formed as a hydrolysis product of a pre-tunichrome protein, it does demonstrate that *P. nigra* retains the tunichrome molecule in some of its blood cells in a concentrated form, indicating that this peptide must have a role to play in tunicate biochemistry.

Several lines of evidence indicate that tunichrome may not be the long-sought-after primary chelator of vanadium. First, Nakanishi's group found that little vanadium is co-located with tunichrome (at least, the uncomplexed form of tunichrome that could be assayed with the techniques developed in their lab) in *P. nigra* and *Ascidia ceratodes* blood cells. Using Fluorescence-Activated Cell Sorting (FACS), they demonstrated that the morula cell contained almost all of the tunichrome, but only a small percentage of vanadium; signet ring and compartment cells contained the vast majority of vanadium but no measurable tunichrome. Staining hemocytes with BIPY corroborates this finding – vanadium (dark deposits, Figure 4, right) is only found in signet ring and compartment cells, not in the morula cells that contain the tunichrome. Secondly, tunichrome is not capable of reducing either V^V or V^{IV} to V^{III} in vitro (Ryan et al., 1996). Thirdly, the recent K-edge XAS work mentioned above has demonstrated that the majority of vanadium is present as hexaaquovanadium^{III} in both *P. nigra* and *A. ceratodes* (Frank et al., 1995, 1998, 2001). In *A. ceratodes*, however, there is also a significant percentage of vanadium present as a sulfate complex, implying a low pH environment, and a very minor (insignificant) percentage of V^{III} complexed to a catecholate-like compound. The situation is more complex in *P. nigra*, however. While no sulfate is present, chloride is found, possibly as hydrochloric acid. In addition, a significant (though still small) proportion of the vanadium is associated with a catecholate-like compound, suggesting that a vanadium-tunichrome complex may be present in one of the blood cells in this species (possibly a scavenging phagocytic cell?).

Fig. 4. Blood cells of *Ascidia cerrotodes* stained with BIPY to demonstrate the localization of vanadium.
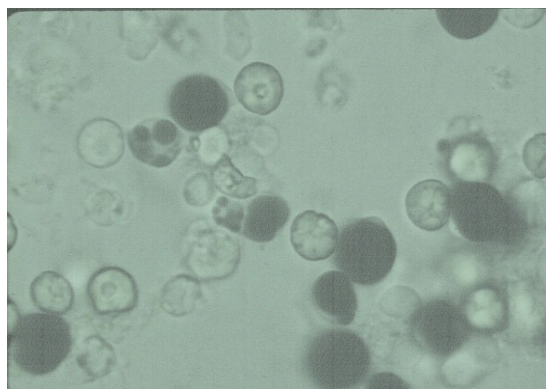
Our elusive search for the role of vanadium and tunichrome in tunicates is therefore complicated by the findings that these two entities are not typically co-located in intact blood cells (excepting the XAS findings for *P. nigra*!). It is possible, however, that these two substances combine *extracellularly*, either to form a useful product or else to provide a needed function by means of the chemical reaction itself.   This raises the question of whether vanadium and tunichrome could be involved in immunological defense or in the formation of a byproduct such as a component of the tunic. Both of these hypotheses have been variously proposed over the last 90 years since Henze's discovery of vanadium in tunicate blood cells, but neither has been convincingly demonstrated. Our work with embryonic *Ascidia callosa* suggest that a tunichrome-like compound plays an important role in the formation of the tunic and fins of the

tadpole larvae (Robinson et al., 1986, 1991), lending further credence to the idea that tunichrome is involved in generating a component of the tunic.

Recent studies by a number of investigators have identified phenoloxidase in tunicate blood cells. We have also observed phenoloxidase activity in *Phallusia nigra* blood cells in our lab. Since this enzyme produces reactive oxygen species (e.g. oxygen free radicals) that are used by some species to destroy infectious bacteria, the presence of phenoloxidase in tunicate blood cells, if it could be linked with either tunichrome or vanadium, would support the immunological defense hypothesis. On the other hand, 20+ years of extensive research in Sugumaran's laboratory at UMass Boston has demonstrated the importance of phenoloxidase-like enzymes in transforming DOPA-containing peptides as part of the sclerotization process in insect cuticle (Sugumaran, 1998). Since tunichromes are essentially tri- or di-DOPA peptides, it is possible for the phenoloxidase, released from blood cells, to react with tunichrome to form polymers that are used as structural components of the tunic. It is also possible that vanadium could be incorporated into this polymer as an additional crosslinking agent (Figure 5. below)

Fig. 5. Diagram of possible pathways, involving tunichrome, vanadium and phenoloxidase, leading to tunic formation
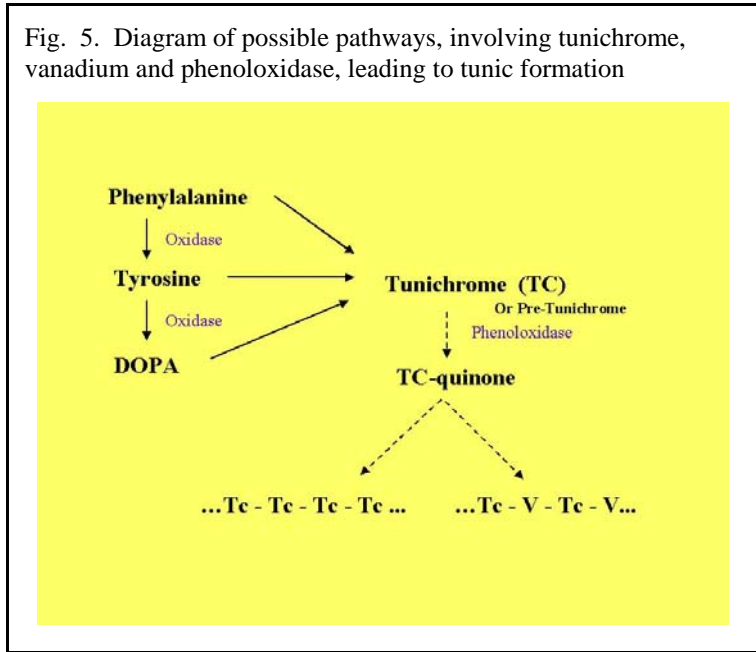
We are currently pursuing research to confirm the role of tunichrome and vanadium in tunic formation. Tunichrome will be isolated, and tunichrome derivatives and model tripeptides (e.g. gly-dopa-dopa, gly-dopa methylester) will be prepared using published procedures. All dehydro derivatives will be subjected to oxidative polymerization studies with and without test proteins (e.g. ribonuclease, albumin, etc.) cellulose, mucopolysaccharides, or DNA. The progress of polymerization will be monitored by SDS-PAGE, agarose electrophoresis, HPLC and other related techniques. The possible incorporation of vanadium ions into the tunichrome-polymerized biopolymers will be investigated using ICP-MS. All of these compounds will also be tested for antibiotic properties since similar DOPA-containing natural products that have been isolated from various tunicate species exhibit strong antimicrobial action. Using standard antimicrobial assays, two potential mechanisms of toxic action will be investigated: (1) as cross linkers, tunichromes could bind to cellular macromolecules and interfere with physiological function; or (2) as generators of reactive oxygen species (i.e. oxygen free radicals), tunichromes could cause oxidative stress.

Tunichromes and their derivatives could form potentially useful biomaterials that possess both antibacterial and adhesive/polymerization properties since these two apparently diverse

characteristics are actually closely-linked biochemically. These related characteristics would make tunichrome a highly useful compound for the biomedical industry. We envision the *in situ* synthesis of biomembranes with inherent antimicrobial properties that would find applications in accident triage, emergency medical treatment and surgery, as well as for minor wound repair. Non-medical uses could entail the production of antifouling panels for marine and freshwater construction. Moreover, the detailed mechanisms that we will learn from our studies will be directly applicable for the "Green" formulation of environmentally safe products with inherent antipredator, antibacterial and antifouling properties.

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