Vanadocytes, Cells hold the Key to Resolving the Highly Selective Accumulation and Reduction of Vanadium in Ascidians

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Keywords: Ascidian, Vanadium, Accumulation, Reduction, pH, Vacuolar H^+-ATPase, Monoclonal antibody.

ABSTRACT- Since Henze discovered vanadium in the blood (or coelomic) cells of an ascidian in 1911, this unusual phenomenon has attracted the interest of many investigators. The highest concentration of vanadium (350 mM) in the blood cells of *Ascidia gemmata*, which belongs to the suborder Phlebobranchia, is 10^7 times higher than that in sea water. Of the approximately ten types of blood cells, a combination of cell fractionation and neutron-activation analysis revealed that the signet ring cells were the true vanadocytes. In the vanadocytes, 97.6% of the vanadium is in the +3 oxidation state (III). The extremely low pH of 1.9 found in vanadocytes suggests that protons, concentrated by an H^+-ATPase, might be linked to the accumulation of vanadium energetically. The antigen recognized by a monoclonal antibody, S4D5, prepared to identify vanadocytes, was determined to be 6-PGDH in the pentose phosphate pathway. NADPH produced in the pentose phosphate pathway in vanadocytes is thought to participate in the reduction of vanadium(V) to vanadium(IV). During embryogenesis, a vanadocyte-specific antigen first appears in the body wall at the same time as significant accumulations of vanadium become apparent. Three different vanadium-associated proteins (VAPs) were extracted from the blood cells of vanadium-rich ascidians. These are 12.5, 15, and 16 kDa in size and are associated with vanadium in an approximate ratio of 1:16. The cDNA encoding the 12.5 and 15 kDa VAPs was isolated and the proteins encoded were found to be novel. Further biochemical and biophysical characterization of the VAPs is in progress.

Discovery of Vanadium-Containing Ascidians

In 1911, a German physiological chemist, Martin Henze, discovered high levels of vanadium in the blood (or coelomic) cells of an ascidian collected from the Bay of Naples (Henze, 1911). Ascidians, known as tunicates or sea squirts, possess a notochord, a dorsal nerve and pharyngeal gill slits and belong to the phylum Chordata, which is an evolutionary link between the Invertebrata and the Vertebrata. His discovery attracted the attention of chemists, physiologists, and biochemists for two reasons. There was initial interest in the extraordinarily high levels of vanadium, which had never been reported in other organisms. There was also considerable interest in the possible role of vanadium in oxygen transport as a third possible prosthetic group in respiratory pigments in addition to iron and copper. Much of the interest developed

because vanadium was found in ascidians, which are phylogenically intermediate between the Invertebrata and the Vertebrata.

Since many review articles on the accumulation of vanadium by ascidians have been published (Biggs *et al*, 1976; Boyd and Kustin, 1985; Goodbody, 1974; Kustin *et al*., 1983; Kustin and Robinson, 1995; Michibata, 1989, 1993, 1996; Michibata and Sakurai, 1990; Michibata and Kanamori, 1998; Michibata *et al*, 1998), we now focus attention on vanadocytes, the vanadium-containing blood cells, which must hold the key to resolving this extremely unusual phenomena of accumulating excessive amounts of vanadium.

Concentration of Vanadium in Ascidian Tissues

After Henze (1911) initially discovered vanadium in ascidian blood cells, several analytical chemists looked for vanadium in many species of ascidians. It is difficult, however, to quantitatively assay transition metals, including vanadium. A variety of analytical methods has been applied, including colorimetry, emission spectrometry, and atomic absorption spectrometry. Besides vanadium, niobium, chromium, tantalum, tungsten, and titanium have also been found in ascidians, although not all the results are reproducible (CantacuzÎne and Tchekirian, 1932; Vinogradov, 1934; Kobayashi, 1935; Webb, 1939; Noddack and Noddack, 1939; Bertrand 1950; Boeri, 1952; Lybing, 1953; Boeri and Ehrenberg, 1954; Webb, 1956; Levine, 1961; Bielig *et al*., 1954, 1961a, b, c, 1966; Kalk, 1963a, b; Ciereszko *et al*., 1963; Rummel *et al*., 1966; Carlisle, 1968; Swinehart *et al*., 1974; Danskin, 1978; Botte *et al*., 1979a, b; Hawkins *et al*., 1980a). Early data could not be compared directly, since the sensitivity and precision of the techniques varied considerably and the results were reported in terms of dry weight, wet weight, ash weight, inorganic dry weight, or amount of protein. This presented problems to researchers studying the physiology of vanadium accumulation in ascidians. About 20 years ago, we planned to quantify the vanadium levels in several tissues definitively using neutron-activation analysis, which is an extremely sensitive method for quantifying vanadium. We collected many species of ascidians, belonging to the Phlebobranchia and Stolidobranchia, two of the three suborders, from the Mediterranean and from the waters around Japan. Eight samples were taken from each specimen for analysis: blood cells, plasma, tunic, mantle (muscle),

branchial basket, stomach, hepatopancreas, and gonad. These were subjected to neutron-activation analysis in a nuclear reactor (Michibata, 1984; Michibata *et al*., 1986).

The data results are summarized in Table 1. Although vanadium was detected in samples from almost every species examined, the ascidians belonging to the suborder Phlebobranchia appeared to contain higher levels of vanadium than those belonging to the Stolidobranchia. Of the tissues examined, we confirmed that blood cells contain the highest amounts of vanadium. The highest concentration of vanadium (350 mM) was found in the blood cells of *Ascidia gemmata* belonging to the suborder Phlebobranchia (Michibata *et al*., 1991a). This concentration is 10^7 times higher than that in seawater (Cole *et al*., 1983; Collier, 1984). Levels of iron and manganese, determined simultaneously, did not vary much between the members of the two suborders (Michibata *et al*., 1991a).

Isolation of Blood Cells Containing Vanadium

Of the ascidian tissues examined, blood cells were confirmed to contain the highest amounts of vanadium. However, there are between nine and eleven different types of ascidian blood cells, which are grouped into six categories on the basis of their morphology: hemoblasts, lymphocytes, leukocytes, vacuolated cells, pigment cells, and nephrocytes (cf. Wright, 1981). The vacuolated cells can be further divided into at least four different types: morula cells, signet ring cells, compartment cells, and small compartment cells. Which of these cell types were the vanadium-containing blood cells (vanadocytes) was a subject of controversy. For many years, the morula cells were thought to be the so-called vanadocytes (Webb, 1939; Endean, 1960; Kalk, 1963a, b; Kustin *et al*., 1976). With the increasing availability of scanning transmission electron microscopes equipped with an energy dispersing X-ray detector at the end of the 1970's, it became possible to address the question of whether the morula cells were the vanadocytes with greater confidence. An Italian group was the first to demonstrate that the characteristic X-rays of vanadium were not detected in morula cells, but were seen in granular amoebocytes, signet ring cells, and compartment cells. Moreover, vanadium was selectively concentrated in the vacuolar membranes of these cells, and vanadium granules were present inside the vacuoles (Botte *et al*., 1979b; Scippa *et al*.,

1982, 1985; Rowley, 1982). Identifying the true vanadocytes became a matter of the highest priority to those interested in the mechanism for the accumulation of vanadium by ascidians.

We attempted to end the controversy over the identity of the true vanadocytes, using a combination of density gradient centrifugation, to isolate specific types of blood cells, and neutron-activation analysis, to quantify the vanadium content of the isolated subpopulations of blood cells (Michibata *et al*., 1987). When blood cells were loaded onto a discontinuous gradient that consisted of four different concentrations of Ficoll in artificial seawater and the gradient was centrifuged at 100 *g*, the blood cells were partitioned into four discrete layers. Neutron-activation analysis revealed that the subpopulation of cells in layer 4, dominated by signet ring cells, contained the highest level of vanadium. The same experiment was repeated with three different ascidian species and signet ring cells were found to be the true vanadocytes in all three species (Michibata *et al*., 1990, 1991a; Hirata and Michibata, 1991) (Fig. 1).

Preparing Monoclonal Antibodies against Ascidian Blood Cells

It is necessary to establish reliable cell markers that recognize different types of blood cells for two reasons. It is difficult to discriminate between several types of blood cells morphologically and our knowledge of the cell lineages from the so-called stem cells to the peripheral cells is inadequate. We prepared a monoclonal antibody, which we hoped might serve as a powerful tool for solving these problems, using a homogenate of the subpopulation of signet ring cells from *Ascidia sydneiensis samea* as the antigen (Uyama *et al*., 1991). The monoclonal antibody S4D5 reacted specifically with vanadocytes from *A. sydneiensis samea* and two additional species, *A. gemmata* and *A. ahodori*. Immunoblotting analysis showed that this antibody recognized a single polypeptide of approximately 45 kDa in all three species. The 45 kDa antigen was subsequently revealed to be 6-phosphogluconate dehydrogenase, localized in the cytoplasm of vanadocytes (Uyama *et al*, 1998a). S8E4 monoclonal antibody, also specific to vanadocytes, recognized a 100 kDa antigen in the cytoplasm (Fig. 2), which was identified as glycogen phosphorylase (Uyama *et al*., 1998b). We also obtained monoclonal antibodies against blood cells other than vanadocytes. C2A4 monoclonal antibody reacts specifically with vacuolar amoebocytes and recognizes a single 200 kDa

protein (Kaneko *et al*., 1995). V2C3 monoclonal antibody reacts with a 130 kDa polypeptide in the vacuolar membranes of vanadocytes (unpublished data).

Localizing Hematopoietic Tissues with Monoclonal Antibodies

Employing these monoclonal antibodies and the autonomous fluorescence emitted by each type of cell as cell markers (Wuchiyama and Michibata, 1995), we found that vanadocytes were distinct from morula cells, compartment cells and amoebocytes, and were localized in the connective tissues around the alimentary canal (Fig. 3) (Kaneko *et al*., 1995). According to earlier reports (Kalk, 1963a; Smith, 1970a; Ermak, 1975, 1976), hematogenic activity is observed in three main areas of ascidians: (1) the connective tissues around the alimentary canal, (2) the pharyngeal wall and transverse vessels of the branchial basket, and (3) in discrete nodules located in the body wall. Amoebocytes are also localized at site (2). As shown in Fig. 4, most of the amoebocytes were localized in the transverse vessels of the branchial basket, where C2A4 monoclonal antibody recognized the cells. Morula cells emitting blue-green fluorescence were localized just beneath the epidermis of the mantle around the visceral region (Kaneko *et al*., 1995). These results suggest that the precursors of vanadocytes develop in the connective tissues, while other types of blood cells develop at other sites.

Proliferation of Vanadocytes and Accumulation of Vanadium during Embryogenesis

Monoclonal antibodies are also useful tools for determining when vanadium starts to accumulate during embryogenesis. Since the amount of vanadium stored in embryos is below the limits of detection of conventional analytical methods, such as atomic absorption spectrometry, there are no reports on the direct determination of vanadium accumulated during ascidian embryogenesis. Using neutron-activation analysis and an immunofluorescence method, we found that the amount of vanadium per individual increased dramatically two weeks after fertilization. In *A. sydneiensis samea*, the amount accumulated in larvae after two months was about 600,000 times greater than that in unfertilized eggs (Michibata *et al*., 1992), as shown in Figure 5. A vanadocyte-specific antigen, recognized by a monoclonal antibody specific to the vanadocytes, first appears in the body wall at the same time as the first significant

accumulation of vanadium (Fig. 6) (Uyama *et al*., 1993).

Oxidation State of Vanadium in Ascidians

Although vanadium is in the +5 oxidation state in seawater, in ascidians almost all the vanadium is reduced to the +3 oxidation state via the +4 oxidation state and stored in vanadocyte vacuoles. Henze (1911) was the first to suggest the existence of vanadium in the +5 oxidation state. Later, Lybing (1953), Bielig *et al*. (1954), Boeri and Ehrenberg (1954), and Webb (1956) reported the +3 oxidation state of vanadium. More recently, noninvasive physical methods, including electron spin resonance spectrometry (ESR), extended X-ray absorption spectrometry (EXAFS), X-ray absorption spectrometry (XAS), nuclear magnetic resonance spectrometry (NMR), and superconducting quantum interference device (SQUID), have been used to determine the intracellular oxidation state of vanadium. These studies indicate that the vanadium ions in ascidian blood cells are predominantly in the +3 oxidation state, and a small amount is in the +4 oxidation state (Carlson, 1975; Tullius *et al*., 1980; Dingley *et al*., 1981; Frank *et al*., 1986; Lee *et al*., 1988; Brand *et al*., 1989).

However, these results were not derived from vanadocytes, but from the entire population of blood cells. Therefore, we made noninvasive ESR measurements of the oxidation state of vanadium in fractionated blood cells of *A. gemmata* under a reducing atmosphere after separating the various types of blood cells (Fig. 7)(Hirata and Michibata, 1991). Most of the vanadium (97.6%) in vanadocytes is in the +3 oxidation state, which is the most reduced state of vanadium in biological systems, while 2.4% of the vanadium is in the +4 oxidation state.

Agents that Reduce Vanadium

Reducing agents must participate in the accumulation of vanadium in vanadocytes. Several candidates for the reduction of vanadium in ascidian blood cells have been proposed: tunichromes, a class of hydroxy-Dopa containing tripeptides (Bruening *et al*, 1985), glutathione, H_2S, NADPH, dithiothreitol (Ryan *et al*., 1996), and thiols such as cysteine (Frank *et al*., 1987). Of these, the presence of a pyrogallol (1,2,3-trihydroxybenzene) moiety in tunichromes suggested that tunichromes act as both a reducing agent and a complexing agent. Early studies showed, however, that *in vitro*

tunichromes are only able to reduce vanadium(V) to vanadium(IV) and not vanadium(III) (Macara 1979a, b; Kime-Hunt *et al*., 1988). The discovery that vanadium and tunichromes are located in separate blood cells raised further doubt about the participation of tunichromes in the vanadium reduction process (Michibata *et al*., 1988, 1990; Oltz *et al*., 1988). Furthermore, the very acidic environment of vanadium in tunicate blood cells (*videinfra*) is not good for coordination with phenolic ligands. It is also predicted that catechol (cat: 1,2-dihydroxybenzene) cannot stabilize the vanadium(III) oxidation state. Conversely, hard catechol-type ligands stabilize the highest oxidation state of the metal, and in fact $[V(cat)_3]^{3-}$ is very sensitive to oxidation (Cooper *et al*., 1982). Therefore, it is very unlikely that tunichromes play simultaneous roles as reducing and complexing agents, although they may still act as a reducing agent.

Despite the identification of tunichromes as a potential reducing agent it is still now known how vanadium(V) is reduced to vanadium(III) in ascidians. Recently, we discovered that cysteine methyl ester can reduce vanadium(IV) to vanadium(III) in the presence of aminopolycarboxylate in water (Kanamori *et al*., 1997).

Localization of the Pentose Phosphate Pathway in Vanadocytes

Recently, it was revealed that the antigen of the S4D5 monoclonal antibody specific to vanadocytes, is 6-phosphogluconate dehydrogenase (6-PGDH: EC1.1.1.44) localized in the cytoplasm of vanadocytes (Uyama *et al*., 1998a). 6-PGDH is the third enzyme in the pentose phosphate pathway. Western blot analysis confirmed the abundance of 6-PGDH in vanadocytes and the soluble protein extracted from the blood cells also had correspondingly high levels of 6-PGDH enzymatic activity (Uyama *et al*, 1998a). Glucose-6-phosphate dehydrogenase (G6PDH: EC1.1.1.49), the first enzyme in the pentose phosphate pathway, was also localized immunocytologically (Fig. 8) and enzymatic activity in the cytoplasm of vanadocytes was confirmed (Uyama *et al*., 1998b). These two enzymes are known to produce 2 mols of NADPH in the pentose phosphate pathway.

On the other hand, it has been reported that vanadium(V) stimulates the oxidation of NAD(P)H; specifically, vanadium(V) is reduced to vanadium(IV) in the presence of NAD(P)H *in vitro.* Erdmann *et al* (1979) first noted that vanadium(V)

stimulated the oxidation of NADH by plasma membranes and attributed this effect to a membrane-containing NAD(P)H-dependent vanadium(V) reductase. Liochev and Fridovich (1990) proposed that NAD(P)H dehydrogenases or oxidases produce $O_2^{\cdot-}$, which causes vanadium(V) to stimulate NAD(P)H oxidation, and endogenous superoxide plays a central role in this reaction. Shi and Dalal (1991, 1993) demonstrated that $O_2^{\cdot-}$ radicals do not play a significant role in generating vanadium(IV), but they pointed out that vanadium(IV) is generated by the microsomal reduction of vanadium(V) in the presence of NAD(P)H and that vanadium(IV) formation exhibits typical enzymatic kinetics. In fact, our preliminary data showed that NADPH could reduce vanadium(V) to vanadium(IV) *in vitro* (to be published elsewhere). These observations suggest that NADPH conjugates the reduction of vanadium(V) to vanadium(IV) in the vanadocytes of ascidians, although there is controversy over the mechanism involved. The pentose phosphate pathway consists of oxidative and nonoxidative parts. The oxidative part converts glucose-6-phosphate into ribulose-5-phosphate and CO_2 and generates NADPH for use in reductive biosynthesis at the same time. The nonoxidative part isomerizes ribulose 5-phosphate into xylulose 5-phoshate and ribose 5-phosphate, which are converted into fructose 6-phosphate and glyceraldehyde 3-phosphate by a sugar rearrangement system. In addition to the two enzymes, 6-PGDH and G6PDH, in the oxidative part of the pentose phosphate pathway, we found that transketolase (TKL: EC2.2.1.1) (manuscript in preparation) and glycogen phosphorylase (EC 2.4.1.1) (Uyama *et al*., 1998b) are also localized in vanadocytes exclusively. The former is an enzyme in the nonoxidative part of the pentose phosphate pathway and the latter is an enzyme that catalyzes the phosphorolysis of glycogen to produce glucose 1-phosphate, which is interconverted to glucose 6-phosphate, the initial substrate in both the pentose phosphate and Embden-Meyerhof pathways.

Low pH and Energetics

Henze (1911), the discoverer of extremely high levels of vanadium in the blood cells of ascidians, also reported that the homogenate of the blood cells was extremely acidic (Henze, 1911, 1912, 1913, 1932). This unusual phenomenon has also attracted the interest of investigators because of the possible role of the highly acidic environment in changing or maintaining the redox potential. We found a correlation between the

concentration of vanadium(III) ions and the pH within the vacuole (Michibata *et al*, 1991a), as shown in Table 2. In *Ascidia gemmata*, which contains the highest concentration of vanadium (350 mM), the vacuoles have the lowest pH (1.86). Vacuoles of *A. ahodori* containing 60 mM vanadium have a pH of 2.67, and those of *A. sydneiensis samea* containing 13 mM vanadium have a pH of 4.20 (Michibata *et al*., 1991a).

Immunocytological studies, using antibodies against subunits *A* and *B* of the vacuolar-type H^+-ATPases (V-ATPases) developed from bovine chromaffin granules, show that V-ATPases are localized in the vacuolar membranes of vanadocytes (Uyama *et al*., 1994). A specific V-ATPase inhibitor inhibits the proton pump in the vanadocyte vacuoles, neutralizing the vacuoles' contents, as shown in Fig. 9 (Uyama *et al*., 1994). Therefore, one definite function of V-ATPases is to accumulate protons in the vanadocytes. However, it is difficult to explain the extremely low pH observed in ascidian vacuoles only by the action of V-ATPases, since the maximum ΔpH that a V-ATPase can generate under typical physiological conditions is around 4 pH units, based on measured H^+/ATP stoichiometry (Rea and Sanders, 1987).

We proposed that two mechanisms are responsible for the proton accumulation in vanadocytes. One is the hydrolysis of the water molecules coordinating to the vanadium(III) ions. In a recent study, we showed that an extremely low pH could be achieved by hydrolyzing the water molecules coordinating to vanadium(III) ions (Kanamori *et al*., unpublished data). The other mechanism involves the extremely tight coupling of ATP hydrolysis and proton pumping by V-ATPase in the vanadocytes. In general, V-ATPase is composed of at least five different subunits, denoted as subunits *A* to *E*. Of these, direct chemical labeling and sequence homology studies show that subunits *A* and *B* play an important role in binding and catalyzing ATP. Therefore, as a first step to assess the second mechanism, we isolated and analyzed the cDNA of subunits *A* and *B* of V-ATPase from the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*. The nucleotide sequences of the cDNA of subunits *A* and *B* encoded proteins of 619 and 509 amino acids, respectively. Both of these are highly conserved in ascidian species (Ueki *et al*., 1999). So far, we have not found any evidence of other isoforms of subunits *A* or *B* in the V-ATPase from vanadocytes. However, it is still possible that the V-ATPase in vanadocytes is unusually tightly

coupled to ATPase activity and proton pumping and can generate very low pH values in the vacuole. Recently, the subunit composition of V-ATPase in the lemon juice sac, whose vacuoles have a pH of 2.5, was reported to differ from that in other organs, and the authors suggested that this may be responsible for the low pH (M¸ller *et al*., 1997).

Sulfate in Vanadocytes

A considerable amount of sulfate is always associated with the vanadium in ascidian blood cells (Henze, 1932; Califano and Boeri, 1950; Bielig *et al*., 1954; Levine, 1961; Botte *et al*., 1979a, b; Scippa *et al*., 1982, 1985, 1988; Bell *et al*., 1982; Pirie and Bell, 1984; Lane and Wilkes, 1988; Frank *et al*., 1986, 1987, 1994, 1995; Anderson and Swinehart, 1991). This suggests that sulfate might participate in the accumulation and reduction of vanadium or play an unknown biological role. Frank *et al* (1987) suggested the existence of a non-sulfate sulfur compound, an aliphatic sulfonic acid, in ascidian blood cells. As the first step in studying the correlation between the accumulation and reduction of vanadium and sulfate, we determined the ratio of the levels of sulfate and vanadium in blood cells from the ascidian *Ascidia gemmata* by Raman spectroscopy, as shown in Figure 10. The ratio was approximately 1.5, which is expected if the sulfate ions are present as the counter ions of vanadium ions in the +3 oxidation state. We also found evidence of an aliphatic sulfonic acid in the blood cells (Kanamori and Michibata, 1994).

Vanadium-Associated Proteins

It seems likely that some proteins participate in the pathway for the accumulation of vanadium from seawater, even though the results reported to date seem to indicate that in ascidians vanadium is present as either a free, non-complexed form or in association with low-molecular-weight components. The route for accumulating vanadium ions from seawater into the blood system is still unknown. Previous studies were designed to clarify the direct uptake of vanadium ions from the surrounding seawater. These used radioactive vanadium ions (^{48}V) and were, therefore, limited to examining how much vanadium was incorporated into certain tissues (Goldberg *et al*., 1951; Bielig *et al*., 1963; Dingley *et al*., 1981; Michibata *et al*., 1991b), although there were a few exceptions (Hawkins *et al*., 1980b; Roman *et al*., 1988). The majority of

the vanadium incorporated by ascidians was thought to be dissolved as ionic species or associated with low-molecular-weight substances rather than proteins (cf. Kustin and Robinson, 1995).

Heavy metal ions incorporated into the tissues of living organisms generally bind to macromolecules such as proteins. Therefore, we searched for vanadium-binding proteins in ascidian blood cells. Using a combination of anion-exchange columns and flameless atomic absorption spectrometry, we succeeded in extracting a vanadium-associated protein (VAP), which was estimated to associate with vanadium in an approximate ratio of 1:16. SDS-PAGE revealed that the peak contained three peptides whose molecular weights were estimated to be 12.5, 15, and 16 kDa (Fig. 11) (Kanda *et al*, 1997). We raised a monoclonal antibody against VAP that recognized the related 15 and 16 kDa peptides. Using this antibody, VAP was found in the cytoplasm of vanadocytes and compartment cells (Wuchiyama *et al*., 1997). Recently, we isolated the cDNA encoding the 12.5 and 15 kDa VAP (manuscript in preparation). A search of the sequence database for similar peptides showed that VAP is a novel protein. Further biochemical and biophysical characterization of VAP is in progress.

Physiological Roles of Vanadium in Ascidians

The physiological roles of vanadium remain unknown. Recently, the polychaete *Pseudopotamilla occelata* was reported to accumulate high levels of vanadium (Ishii *et al*., 1993). *P. occelata* possess two antigens that are also found in the ascidian *Ascidia sydneiensis samea*. These antigens are recognized by a polyclonal antibody against VAP extracted from blood cells and a monoclonal antibody against vanadocytes in the vanadium-rich ascidian *A. sydneiensis samea*. Therefore, it is likely that a similar mechanism causes vanadium accumulation in the Polychaeta and the Ascidiidae (Uyama *et al*, 1997). The characterization of these phenomena should help to elucidate the reason for the unusual accumulation of vanadium by one class of marine organisms.

Conclusions

As mentioned above, ascidian vanadocytes are unusual cells that contain high levels of both vanadium and protons in their vacuoles. Almost all of this vanadium is

reduced to the +3 oxidation state and stored in the vacuoles with high levels of sulfate. Enzymes from the pentose phosphate pathway are localized in the cytoplasm (Fig. 12). How ascidians accumulate high levels of vanadium and what purpose this serves are two unanswered questions that should be resolved by elucidating the functions of the vanadocytes.

Acknowledgments

We thank our associates, whose names are included in our original articles, for their unstinting assistance. We thank Dr. T. Numakunai, Mr. S. Tamura, and Mr. M. Washio of the Marine Biological Station of Tohoku University, Mr. K. Morita of the Ootsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Prof. M. Yamamoto and Mr. W. Godo of the Marine Biological Station of Okayama University, and Mr. N. Abo of our laboratory for their kind cooperation in collecting specimens for so many years. Early work preparing the monoclonal antibodies was performed in collaboration with Prof. N. Satoh of Kyoto University.

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Table 1. Concentrations of Vanadium in the Tissues of Several Ascidians (mM)

	Tunic	Mantle	Branchial basket	Serum	Blood cells
Phlebobranchia					
Ascidia gemmata	N.D.	N.D.	N.D.	N.D.	347.2
A. ahodori	2.4	11.2	12.9	1.0	59.9
A. sydneiensis	0.06	0.7	1.4	0.05	12.8
Phallusia mammillata	0.03	0.9	2.9	N.D.	19.3
Ciona intestinalis	0.003	0.7	0.7	0.008	0.6
Stolidobranchia					
Styela plicata	0.005	0.001	0.001	0.003	0.007
Halocynthia roretzi	0.01	0.001	0.004	0.001	0.007
H. aurantium	0.002	0.002	0.002	N.D.	0.004

N.D.: not determined.

Table 2. Correlation between the Vanadium Concentration and pH in Ascidian Blood Cells

Species	Vanadium Concentration	pH
Ascidia gemmata	350mM	1.86
A. ahodori	60mM	2.67
A. sydneiensis samea	13mM	4.20

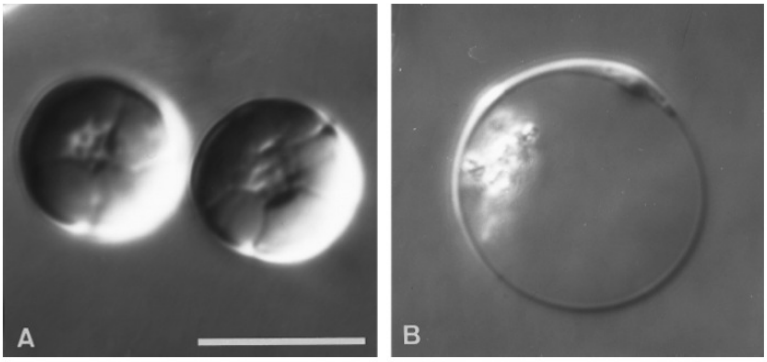

Fig. 1. Morula cells (A), initially misidentified as vanadocytes, and signet ring cells (B), recently identified as vanadocytes, in the ascidian, *Ascidia ahodori*. Scale bar indicates 10 micro meter.

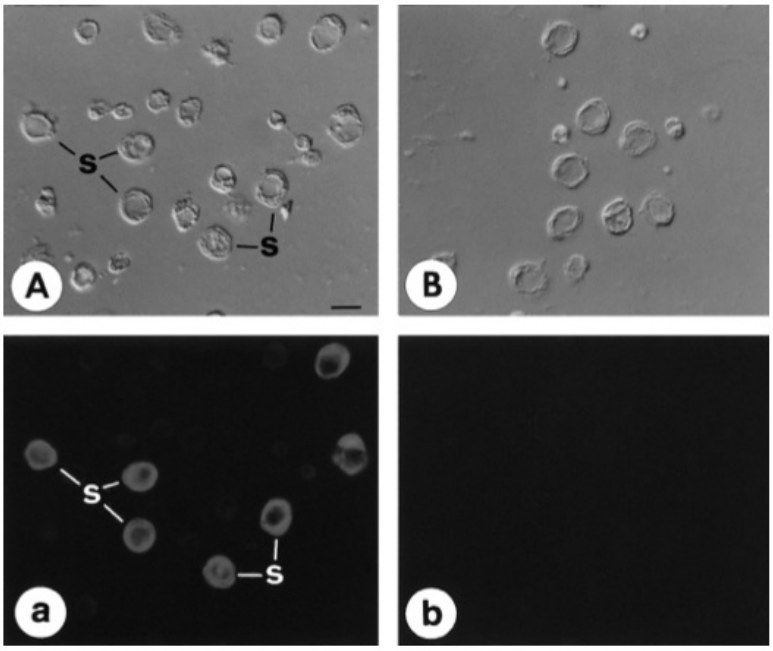

Fig. 2. Immunocytological detection of S8E4 monoclonal antibody in blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea*.

The blood cells shown in panels (A) and (a) were reacted with S8E4. The blood cells in panels (B) and (b) were reacted with nonimmune mouse serum as a negative control. The upper (A and B) and lower (a and b) panels were visualized by Nomarski differential-interference and fluorescence microscopy, respectively. Vanadocytes (signet ring cells) were recognized by S8E4 exclusively and fluoresced with FITC. No immunoreactivity was observed in the other types of blood cells. Morula cells emitted autofluorescence faintly. s, vanadocyte (signet ring cell); m,

morula cell. Scale bar indicates 10 micro meter.

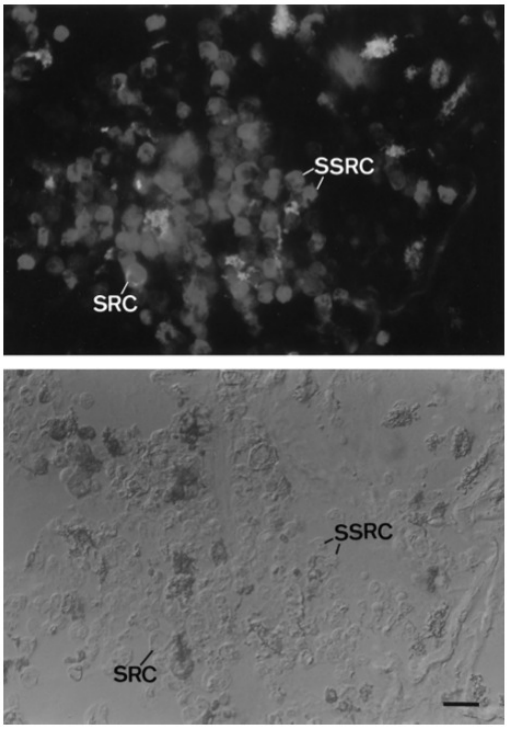

Fig. 3. The localization of signet ring cells as revealed by indirect immunofluorescence microscopy. Clusters of dozens of signet ring cells that reacted with the monoclonal antibody S4D5 are observed in the connective tissues around the alimentary canal. Smaller blood cells, resembling signet ring cells, are found in this area, but were less reactive with S4D5 monoclonal antibody. Autonomous orange-yellow fluorescence is emitted from a type of pigment cell. The upper photograph is a fluorescence micrograph and the lower one is a Nomarski differential-interference micrograph. SRC, signet ring cells; SSRC, smaller signet ring cells. Scale bar = 10 micro meter.

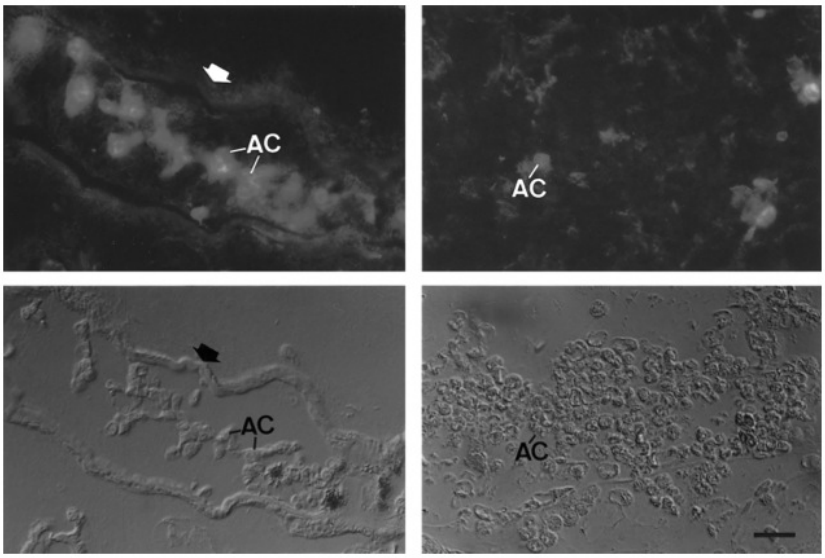

Fig. 4. Blood cells, flowing in the transverse vessels of the branchial basket (the photographs on the left), are stained for indirect fluorescence with a monoclonal antibody specific to amoebocytes (C2A4). A small number of vacuolar amoebocytes are also observed in the connective tissue around the alimentary canal (the photograph on the right). The upper photographs are fluorescence micrographs and the lower ones are Nomarski differential-interference micrographs. AC, vacuolar amoebocytes. The arrow indicates the transverse vessels. Scale bar = 10 micro meter.

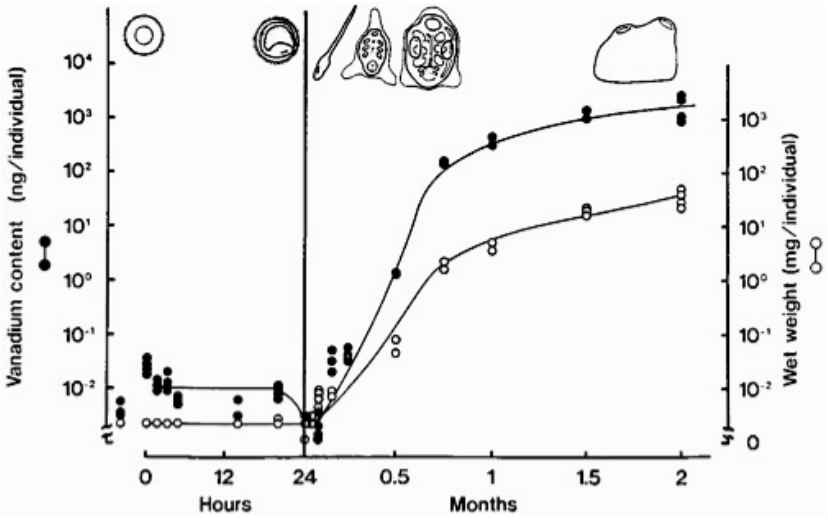

Fig. 5. Accumulation of vanadium during embryogenesis in the ascidian, *Ascidia sydneiensis samea.* To determine when the accumulation of vanadium commences during embryogenesis, eggs and embryos were subjected to neutron-activation analysis. The levels of vanadium began to increase during embryogenesis and the amount in

larvae reached 2.3 micro g/individual, which was about 600,000 times higher than the amount in unfertilized eggs (Michibata *et al*., 1992). Furthermore, a vanadocyte-specific antigen first appeared in the body wall at the same time as the first significant accumulation of vanadium (Uyama *et al*., 1993).

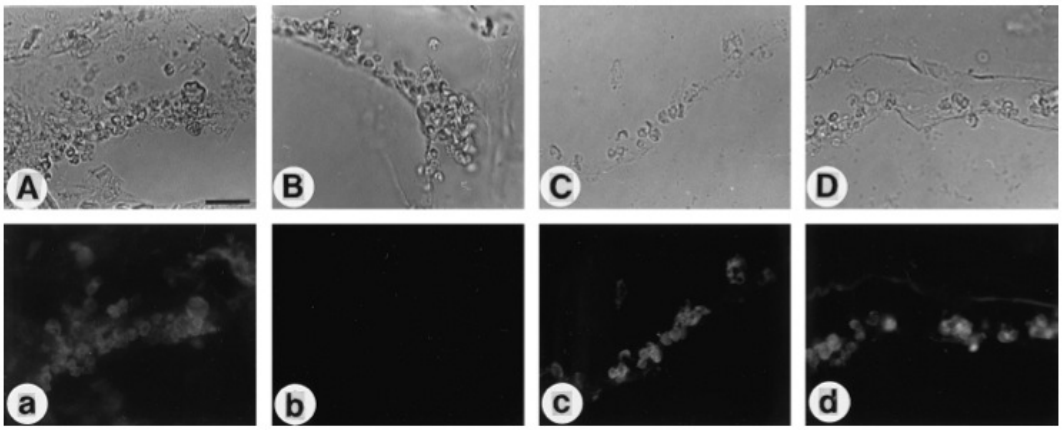

Fig.6. Occurrence of the antigen in coelomic cells that seem to be the presumptive vanadocytes. A cross-section of a 2-week-old juvenile incubated with S4D5 monoclonal antibody was observed under Nomarski differential-interference (A) and fluorescence microscopes (a). Scale bar = 50 micro meter. Another cross-section of the same sample incubated with non-immune mouse serum as a negative control was observed in the same manner as above (B and b). Specimens of a 3-week-old juvenile (C and c) and a 1.5 month juvenile (D and d) were observed after treatment with the monoclonal antibody.

A vanadocyte-specific antigen, recognized by S4D5 monoclonal antibody, first appears in the body wall of a 2-week-old juvenile after metamorphosis. The antigen is evident in the cytoplasm of vanadocytes 3 weeks and one and a half months after metamorphosis. Vanadocytes recognized by S4D5 monoclonal antibody are clearly identified morphologically.

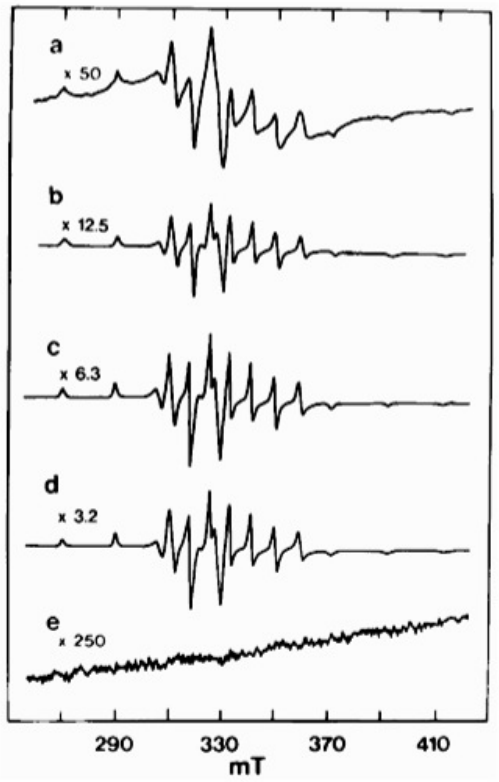

Fig. 7. ESR spectra of *Ascidia gemmata* at 77K. (a) Living cells, which were washed twice with 5 mL ASW and measured under a nitrogen atmosphere. (b) Washed cells lysed by a freeze-thaw cycle under a nitrogen atmosphere. (c) The same lysate oxidized with blowing oxygen gas: the intensity of the spectrum increased 7-fold after 2 hours at room temperature. (d) The same sample 24 hours later: the intensity of the spectrum increased 13-fold. (e) Hydrogen peroxide added to the lysate: the spectrum due to vanadium(+4) has vanished.

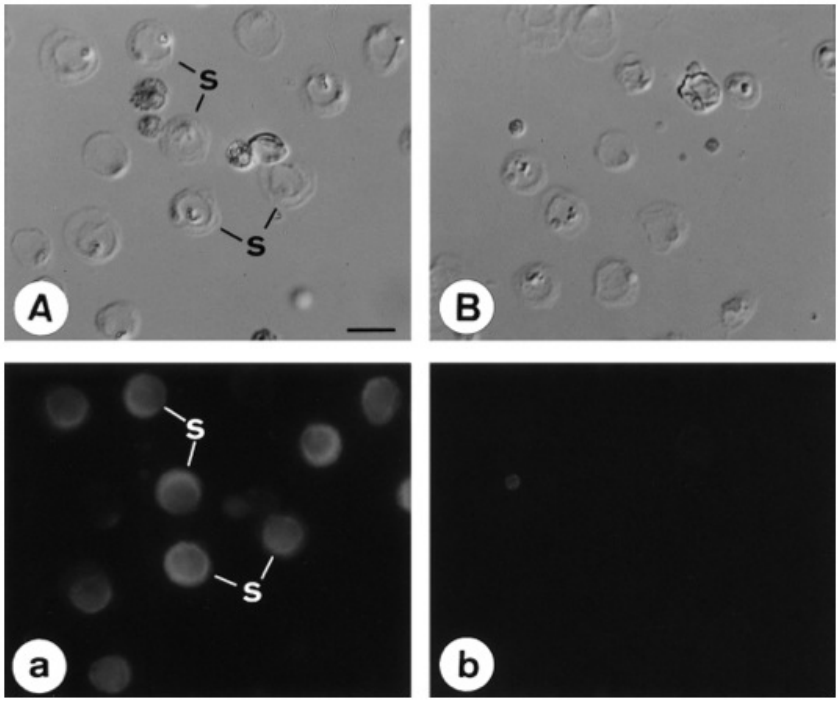

Fig. 8. Immunocytological detection of G6PDH in the vanadocytes of the vanadium-rich ascidian, *Ascidian sydneiensis samea*. The blood cells observed in panels (A) and (a) were reacted with anti-G6PDH antibody, while those in panels (B) and (b) were reacted with preimmune rabbit serum as a negative control. The upper (A and B) and lower (a and b) panels are visualized by Nomarski differential-interference and fluorescence microscopy, respectively. Anti-G6PDH antibody and fluorescence with FITC recognizes vanadocytes (signet ring cells) exclusively. No immunoreactivity is observed in the other types of blood cells. s, vanadocytes (signet ring cells). Scale bar = 10 micro meter.

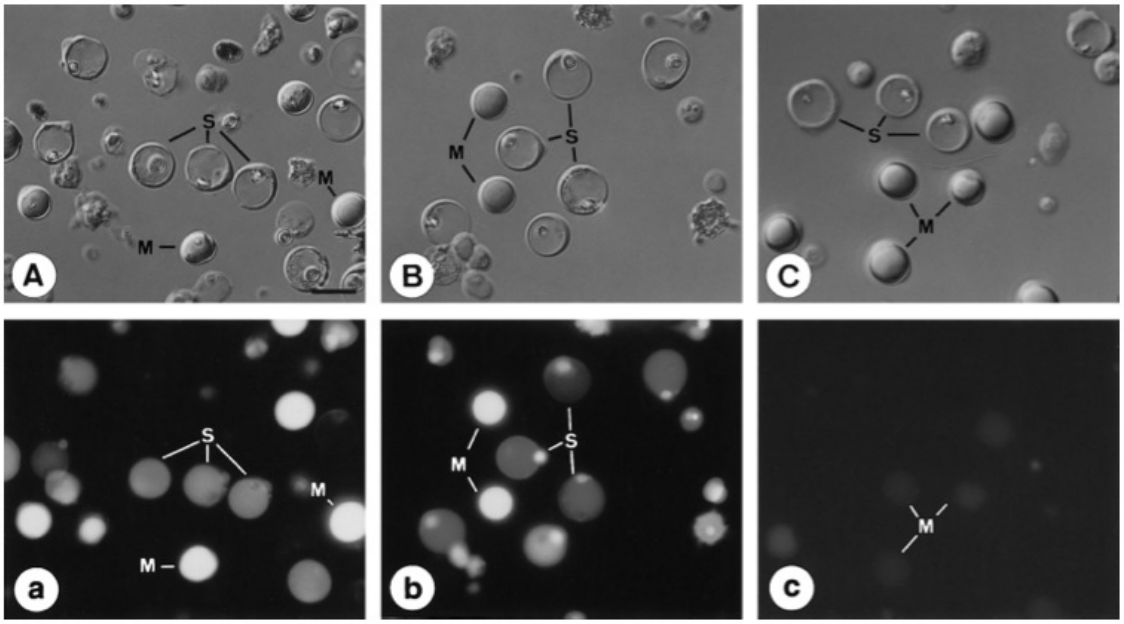

Figure 9. Acidity of vanadocyte vacuoles and inhibition of the acidification by bafilomycin A_1. After incubating *Ascidia sydneiensis samea* blood cells with 2 ?M acridine orange for 1 hr, the signet ring cells (vanadocytes) emitted a brilliant vermilion indicating an acidic pH. None of the other types of blood cells had an acidic pH. However, the addition of 1 micro M bafilomycin A_1, a specific inhibitor of vacuolar H^+-ATPase, neutralized the vanadocytes (showing green fluorescence) and inhibited the H^+-ATPase pump. Bafilomycin A_1 did not change the color of autonomous fluorescence emitted from morula cells (Uyama *et al*, 1994). S, Signet ring cells (vanadocytes); M, morula cells. Scale bar indicates 10 micro meter.

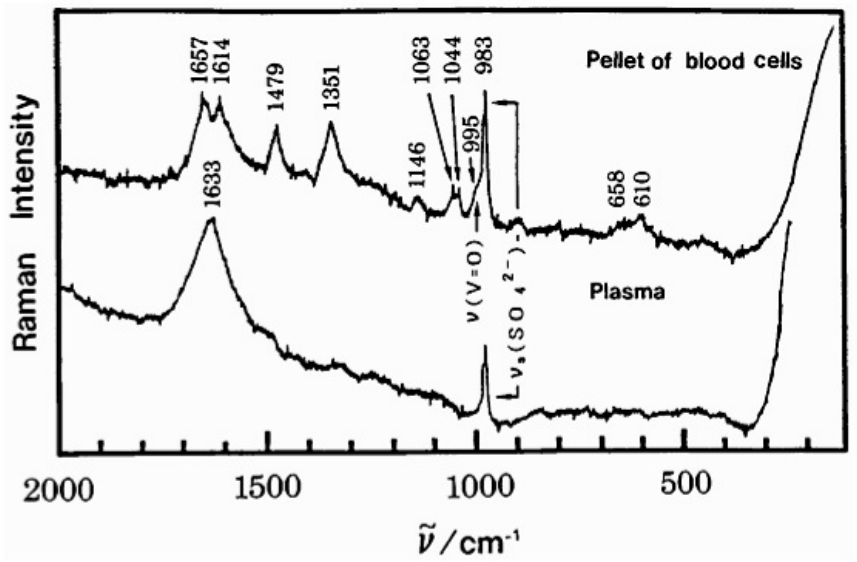

Fig. 10. Raman spectra of the blood cells of *Ascidia gemmata*. High levels of sulfate or sulfur compounds are associated with vanadium in ascidian blood cells. Raman spectrometry can determine the amounts of sulfate and vanadium in the vanadocytes noninvasively. The band at 983 cm^{-1} and the shoulder at 995 cm^{-1} are derived from SO_4^{2-} symmetric stretching vibration and V=O stretching vibration, respectively. Based on the intensities of these peaks, the ratio of SO_4^{2-} to V^{3+} was calculated to be 1.47 (Kanamori and Michibata, 1994).

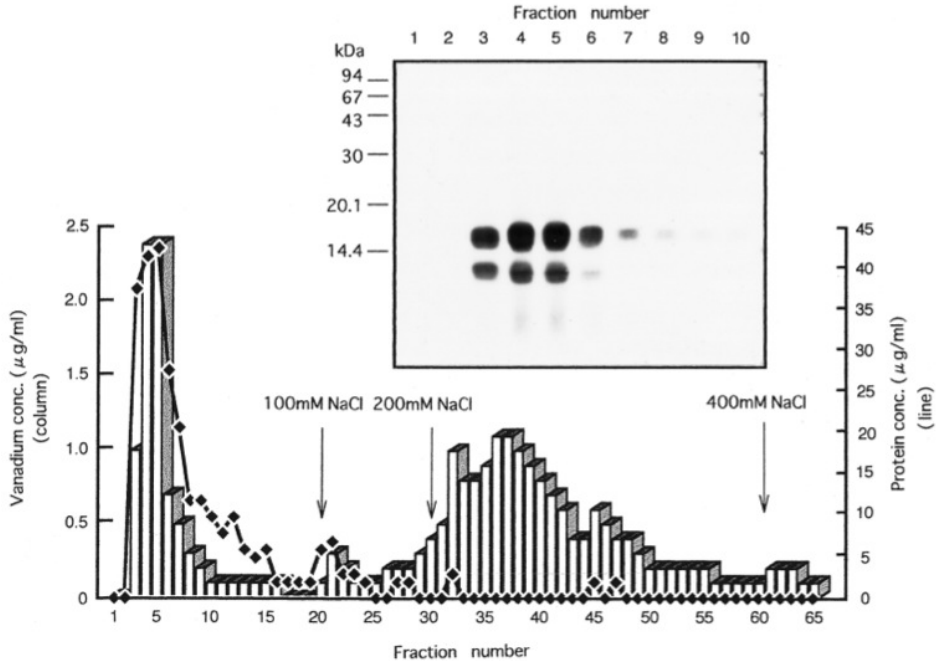

Fig. 11. Isolation of vanadium-associated proteins (VAPs) from the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea.* When the supernatant from blood cells is applied to a DEAE-Sephacel anion-exchange column, one major peak (Peak 1) containing both proteins and vanadium is obtained in fractions 3-9. A second major peak (Peak 2) containing only vanadium is found in fractions 30-35, followed by 200 to 400 mM NaCl. The Peak 1 fractions contained 25 micro g/mL protein and 0.95 micro g/mL vanadium. The Peak 2 fractions contained an inorganic vanadium species. The insert is SDS-PAGE, showing that Peak 1 contains three proteins whose molecular weights are estimated to be 12.5, 15, and 16 kDa.

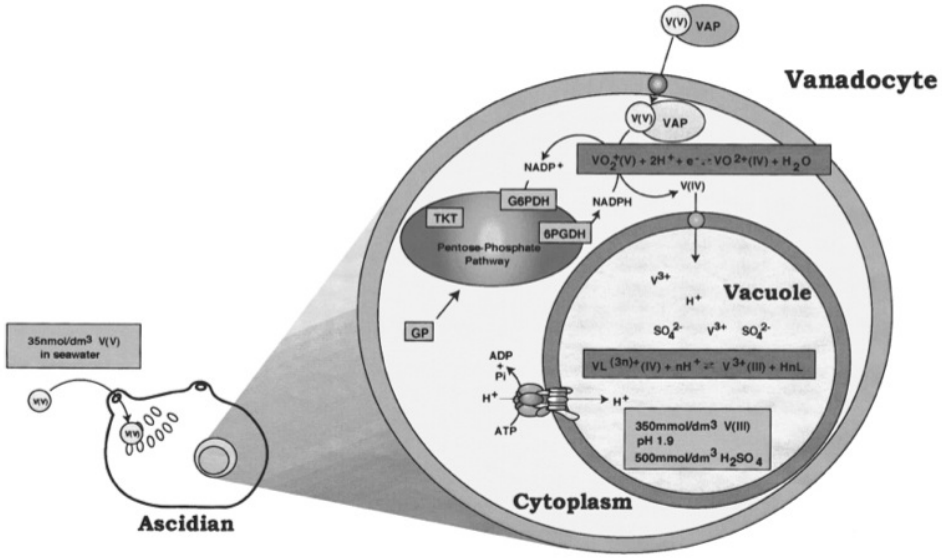

Fig. 12. Schematic representation of the ascidian vanadium accumulation pathway. Vanadium in the +5 oxidation state dissolved in seawater is thought to be incorporated into the interior of the body via the branchial baskets, where vanadium is reduced to the +4 oxidation state. Vanadium in the +4 oxidation state is further reduced to the +3 oxidation state and stored in the vanadocyte vacuoles. Some proteins, specifically vanadium transfer, vanadium receptor, and vanadium channel proteins, are thought to be involved in the accumulation of vanadium.