Molecular biological approaches to the accumulation and reduction of vanadium by ascidians

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Abstract

About 90 years ago, Henze discovered high levels of vanadium in the blood (coelomic) cells of an ascidian collected from the Bay of Naples. His discovery attracted the interdisciplinary attention of chemists, physiologists, and biochemists. Two decades ago, we quantified the vanadium levels in several ascidian tissues definitively using neutron-activation analysis and revealed that some species in the family Ascidiidae accumulate vanadium at concentrations in excess of 350 mM, corresponding to about 10^7 times that found in seawater. Vanadium accumulated is reduced to the +3 oxidation state via the +4 oxidation state and stored in vacuoles of vanadocytes (vanadium-containing blood cells) where high levels of protons and sulfate are also contained. To investigate this unusual phenomenon, we isolated several proteins and genes that are expressed in vanadocytes. To date, three types of vanadium-binding protein, designated as Vanabins, have been isolated, with molecular masses of 12.5, 15, and 16 kDa, along with the cDNAs encoding these proteins. In addition, four types of enzyme related to the pentose phosphate pathway that produces NADPH were revealed to be located in vanadocytes. The pentose phosphate pathway participates in the reduction of vanadium(V) to vanadium(IV). The cDNA for each of the vacuolar-type H^+-ATPase (V-ATPase) *A*, *B*, *C,* and *D* subunits, which are located on the vacuolar membranes of vanadocytes, has been isolated and analyzed. V-ATPase generates a proton-motive force, and is thought to provide the energy for vanadium accumulation.

To clarify the entire mechanism involved in the accumulation and reduction, much more genes and proteins expressed in the blood cells need to be systematically identified. Thus, we have performed an expressed sequence tag (EST) analysis of blood cells and have established the functional assay system to elucidate the functions of genes and proteins obtained from ascidian blood cells.

1. Introduction

About 90 years ago, a German physiological chemist, Martin Henze, discovered high levels of vanadium in the blood (coelomic) cells of an ascidian, known as the sea squirt, collected from the Bay of Naples [1]. His discovery attracted the interdisciplinary attention of chemists, physiologists, and biochemists, in part because of 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, and in part because of the strong interest in the extraordinarily high levels of vanadium never before reported in other organisms [2-8]. Much of the interest developed because vanadium was found in ascidians, which phylogenically belong to the Chordata. After Henze's finding [1], many analytical chemists looked for vanadium in many species of ascidians. Since a variety of analytical methods has been applied, including colorimetry, emission spectrometry and atomic absorption spectrometry, and the data were reported in terms of dry weight, wet weight, ash weight, inorganic dry weight or amount of protein, early data could not be compared directly. 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.

The data obtained [9-11] 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. Levels of iron and manganese, determined simultaneously, did not vary much among the members of the two suborders. Webb [12] first proposed the hypothesis that ascidians are animals that represent a transition form between users of

vanadium and users of iron and that the relative concentrations of vanadium and iron reflect phylogeny. This hypothesis was based on earlier reports that species in the suborders Phlebobranchia and Aplousobranchia contained high levels of vanadium, whereas the evolutionarily more advanced species in the Stolidobranchia contained smaller quantities of vanadium but retained large quantities of iron. However, we found little difference among levels of iron in specimens from the two suborders and only the vanadium content varied substantially depending on the suborder [10].

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 [11]. This concentration is 10^7 times higher than that in seawater [13, 14]. The mechanism of vanadium accumulation and reduction by ascidians revealed up to date is schematically representated in Fig. 1.

2. Identification of vanadocytes

Ascidian blood cells can be classified into nine to eleven different types, which are grouped into six categories on the basis of their morphology: hemoblasts, lymphocytes, leukocytes, vacuolated cells, pigment cells, and nephrocytes [15]. The vacuolated cells can be further divided into at least four different types: morula cells, signet ring cells, compartment cells, and small compartment cells.

For many years, morula cells had been thought to be the so-called vanadocytes, or vanadium-containing cells [12, 16-18]. These cells have a pale green color that resembles the color of an aqueous solution of vanadium, and their dense granules, which can be observed under an electron microscope after fixation with osmium tetroxide, were assumed to be vanadium deposits. At the end of the 1970's, scanning transmission electron microscopes with energy dispersive x-ray detectors became available, and it was demonstrated that the characteristic x-ray due to vanadium was detected not from morula cells, but from granular amoebocytes, signet ring cells and type-II compartment cells

[19-24].

Identification of the true vanadocytes became a matter of the highest priority for those concerned with the mechanism of accumulation of vanadium by ascidians. Using density-gradient centrifugation to isolate specific types of blood cell, and thermal neutron-activation analysis to quantify vanadium in isolated subpopulations of blood cells, we showed that vanadium is accumulated in signet ring cells in *Ascidia ahodori* [25]. 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 [11, 26-27].

In *Phallusia mammillata*, analysis with the chelating reagent 2,2'-bipyridine, which is known to complex with vanadium in the +3 oxidation state, revealed that blood cells, including signet ring cells, vacuolated amoebocytes, granular amoebocytes and type-II compartment cells, were stained brown, indicating the existence of vanadium [28]. We also found evidence of vanadium in the signet ring cells of *Phallusia nigra* by transmission x-ray microscopy at the SR center of Ritsumeikan University, Kyoto, Japan [29]. However, it is impossible to obtain direct evidence of vanadium localization in vanadocytes using these methods. In addition, more convincing evidence is required to clarify whether any other cell type(s) accumulate vanadium, and where the vanadium is localized.

What can provide direct evidence for the location of vanadium, however, is the scanning x-ray microscope installed at the European Synchrotron Radiation Facility's (ESRF). As we have reported in detail the outline and results of the scanning x-ray microscope in our new paper [30], we describe them in brief here. A Fresnel zone plate was used to focus the x-ray beam down to a microprobe size of 0.5 x 0.5 micro m^2. The transmitted x-ray was detected by a photodiode detector and x-ray fluorescence emitted from the sample was analyzed using a high-energy resolution germanium solid state detector. The beam energy was set to 5.500 keV to ensure good fluorescence yield for vanadium (K-edge energy at 5.470 keV). Each sample was scanned by piezo-driven flexure and both the transmission signal and the fluorescence spectrum were recorded to produce a set of images. The multichannel fluorescence detector was calibrated with

standards. The theoretical peak of x-ray fluorescence for each element is as follows: sulfur, 2.308 keV; chloride, 2.622 keV; argon, 2.957 keV; potassium, 3.313 keV; and vanadium, 4.952 keV. Argon is naturally abundant in air, and was used as a control for the normalization of signals. To obtain diffraction phase contrast images of blood cells, we placed a 50-micro m aperture just in front of the photodiode detector.

To obtain images without subjecting cells to freezing or fixation, *Phallusia mammillata* blood cells were suspended in a liquid or gel medium, sealed between two plastic films with a thin spacer film and observed by x-ray microscopy (Fig. 2). Signet ring cells, morula cells, compartment cells and a vacuolated amoebocyte were clearly identified by the x-ray transmission detector. The vanadium image obtained by simultaneously integrating the fluorescence signal in only the vanadium window clearly showed that the signet ring cells and vacuolated amoebocytes contained vanadium, but the morula cells and compartment cells did not.

The nucleus and cytoplasm of signet ring cells are found in the cell periphery, because a large fluid-filled vacuole occupies the cell. Based on observations with a scanning transmission electron microscope equipped with an energy-dispersive x-ray detector, Scippa and colleagues suggested that vanadium is selectively concentrated in the vacuolar membranes of signet ring cells, based on electron microscopic images of cross-sectioned specimens [22-23]. The fluorescence images in Fig. 2C, however, clearly demonstrate that vanadium is distributed uniformly in the vacuole of signet ring cells. If vanadium is localized in the vacuolar membrane, bright fluorescence image around the membrane would be observed in a ring. When superimposed, the fluorescent images are slightly smaller than the image from the optical microscope (Fig. 2A) and the x-ray transmission image (Fig. 2B). This size difference may indicate that vanadium is distributed mainly in the vacuole, not in the peripheral cytoplasm.

X-ray fluorescence energy spectra covering vanadium, potassium, chloride, and argon were recorded from the three types of blood cell. They clearly showed an intense, 4.952 keV vanadium signal that was emitted only from signet ring

cells (Fig. 3). Consequently, we concluded that vanadium accumulates in signet ring cells and vacuolated amoebocytes. We did not examine the granular amoebocytes and type-II compartment cells, reported by Scippa and colleagues [23] to contain vanadium, because they are relatively rare in this species. This study has provided definitive evidence that the true vanadocytes are signet ring cells. We also showed that in *P. mammillata*, vacuolated amoebocytes contain vanadium, although they appear to contain less than signet ring cells (Fig. 2D-2F).

3. 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. One of them is difficulty in morphological discrimination between several types of blood cells and the other is our little knowledge of the cell lineage from the so-called stem cells to the peripheral cells. We prepared monoclonal antibodies, which we hoped might serve as powerful tools for solving these problems, using a homogenate of the subpopulation of signet ring cells from *Ascidia sydneiensis samea* as the antigen [31]. 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 [32]. S8E4 monoclonal antibody, also specific to vanadocytes, recognized a 100 kDa antigen in the cytoplasm, which was identified as glycogen phosphorylase [33]. 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 [34]. V2C3 monoclonal antibody reacts with a 130 kDa polypeptide in the vacuolar membranes of vanadocytes (unpublished data).

4. Oxidation state of vanadium in ascidians

Vanadium is a multivalent transition metal. Vanadium ions under ordinary aqueous conditions are limited to the oxidation states, +2, +3, +4 and +5 and to only the +3, +4 and +5 oxidation states under physiological conditions [35-38]. Vanadium ions in the +3 oxidation state [V(III)] are usually unstable towards air or moisture, and V(III) ions are hydrolyzed to $V(OH)^{2+}$ at and above pH 2.2 and over. In neutral and alkaline solutions, chemical species with vanadium in the +3 oxidation state have never been reported. As described below, vanadium ions in the blood cells of ascidians are stable under the strongly acidic conditions in these cells. It is of interest to coordination chemists to determine whether any ligands participate in the stabilization of vanadium ions in the +3 oxidation state in the ascidian blood cells.

Vanadium ions in the +5 oxidation state give a pale yellow solution that is due to the presence of VO_2^+ under strongly acidic conditions and a colorless solution that is due to the presence of VO_4^{3-} under strongly alkaline conditions. Vanadium ions in the +4 oxidation state are paramagnetic and give a blue solution of oxo-ions, VO^{2+} (vanadyl ions), under moderately acidic conditions. Above pH 4, the chemical species, $VO(OH)^+$ or $[(VO)_2(OH)_2]^{2+}$
is formed. At neutral pH the insoluble hydroxide $VO(OH)_2$ is formed and alkaline pH, vanadyl species becomes $[VO(OH)_3]_n^-$ [38]. Vanadium ions in the +4 oxidation state are principal oxidation state at which the trace levels is found in living organisms including ascidians.

Henze [1] was the first to suggest the existence of vanadium in the +5 oxidation state. Later, many workers reported the +3 oxidation state of vanadium. More recently, studies using noninvasive physical methods, including ESR, XAS, NMR, and SQUID, indicated that the vanadium ions in ascidian blood cells were predominantly in the +3 oxidation state, with a small amount being in the +4 oxidation state [39-44]. These results were,

however, derived not from the vanadocytes but from the entire population of blood cells. Thus, some questions remained to be answered. In particular, does vanadium exist in two oxidation states in one type of blood cell, or is each state formed in a different cell type? After separation of the various types of blood cells of *A. gemmata*, we made noninvasive ESR measurements of the oxidation state of vanadium in the fractionated blood cells under a reducing atmosphere [27]. Consequently, it was revealed that vanadium in vanadocytes is predominantly in the +3 oxidation state, with a small amount being in the +4 oxidation state. The ratio of vanadium(III) to vanadium(IV) was 97.6:2.4 [27].

5. Vanadium reducing agents

Reducing agents must, therefore, 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 [45], glutathione, H_2S, NADPH, dithiothreitol [46], and thiols such as cysteine [47].

As described above, it has been 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 [32]. 6-PGDH is the third enzyme in the pentose phosphate pathway. Glucose-6-phosphate dehydrogenase (G6PDH: EC1.1.1.49), the first enzyme in the pentose phosphate pathway, was also localized immunocytologically and enzymatic activity in the cytoplasm of vanadocytes was confirmed [48]. These two enzymes are known to produce 2 mols of NADPH in the pentose phosphate pathway. In addition, transketolase (TKT: EC2.2.1.1), a rate-limiting enzyme in the non-oxidative pathway, has already been cloned and revealed to be exclusively expressed in the vanadocytes [49]. 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* [50-53]. These

observations suggest that NADPH conjugates the reduction of vanadium(V) to vanadium(IV) in the vanadocytes of ascidians. We have, in fact, found that vanadium(V) species are reduced to vanadium(IV) directly by NADPH in the presence of EDTA [54] (Fig. 4). Moreover, we discovered that cysteine methyl ester can reduce vanadium(IV) to vanadium(III) in the presence of aminopolycarboxylate in water [55], although it should be confirmed whether these phenomena occur in vanadocytes.

6. Low pH and energetics of the accumulation of vanadium

Henze [1], 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 [1, 56-58]. 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. From microelectrode measurements of blood cell lysate, and non-invasive ESR measurements on intact cells under anaerobic conditions, we found a correlation between the concentration of vanadium(III) ions and the pH within the vacuole [11], 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 [11].

Our comparison of pH values and levels of vanadium in the signet ring cells of three different species suggested that there might be a close correlation between a higher level of vanadium and lower pH, namely, a higher concentration of protons. It is well known that vacuolar-type H^+-ATPases (V-ATPases) can generate a proton-motive force by hydrolyzing ATP. This enzyme plays a role in pH homeostasis in various intracellular organelles, including clathrin-coated vesicles, endosomes, lysosomes,

Golgi-derived vesicles, multivesicular bodies and chromaffin granules that belong to the central vacuolar system [59-61].

Therefore, we examined the signet ring cells of the ascidian *Ascidia sydneiensis samea* for presence of V-ATPase [62]. V-ATPase is composed of several subunits, and subunits of *A* and *B* have been reported to be common to all eukaryotes examined. Immunocytological studies, using antibodies against subunits *A* and *B* of V-ATPase developed from bovine chromaffin granules, show that V-ATPases are localized in the vacuolar membranes of vanadocytes [62]. A specific V-ATPase inhibitor, bafilomycin A_1 [63], inhibits the proton pump in the vanadocyte vacuoles, neutralizing the vacuoles' contents [62]. Therefore, one definite function of V-ATPase 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-ATPase, 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 [64]. We have proposed that two mechanisms may be responsible for the proton accumulation in vanadocytes. One is the hydrolysis of the water molecules coordinating with the vanadium(III) ions. In our recent study, we showed that an extremely low pH could be achieved by hydrolysis of the water molecules coordinating with vanadium(III) ions. The other mechanism involves the extremely tight coupling of ATP hydrolysis and proton pumping by V-ATPase in the vanadocytes. As a first step to assess the latter possibility, we isolated and analyzed 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 [65]. So far, we have not found any evidence of other isoforms of subunits *A* or *B* in the V-ATPase from vanadocytes. 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 [66]. Therefore, we isolated a cDNA encoding subunit

C of V-ATPase from vanadocytes. By expressing the ascidian cDNA for subunit *C* under the control of a galactose-inducible promoter, the pH-sensitive phenotype of the corresponding *vma5* mutant of a budding yeast was successfully rescued [67].

7. Sulfate in vanadocytes

A considerable amount of sulfate has always been found in association with vanadium in ascidian blood cells [19-23, 42, 47, 58, 68-77], suggesting that sulfate might be involved in the biological function and/or the accumulation and reduction of vanadium. Frank *et al.* [47] suggested the existence of a non-sulfate sulfur compound, such as an aliphatic sulfonic acid, in ascidian blood cells.

As the first step towards an analysis of the possible correlation between the accumulation and/or reduction of vanadium and sulfate, Raman spectroscopy can be used to detect sulfate ion selectively in ascidian blood cells because sulfate ion gives a very intense Raman band at the diagnostic position, 983 cm^{-1}. We observed fairly good Raman spectrum of the blood cell lysate from Ascidia gemmata, which has the highest concentration of vanadium(III) among ascidians [78]. Vanadium(III) ions in the blood cells were converted to vanadyl(IV) ions by air-oxidation prior to Raman measurements so as to facilitate detection based on V=O stretching vibration. From analysis of the band intensities due to $V{=}O^{2+}$ and SO_4^{2-} ions, we estimated the content ratio of sulfate to vanadium to be approximately 1.5, as would be predicted if sulfate ions were present as the counter ions of vanadium(III). We also found evidence that an aliphatic sulfonic acid was present in the blood cells [78]. Carlson [39] reported a similar value of the content ratio for *Ascidia ceratodes*, but lower values were obtained by Bell *et al.* [72] and Frank *et al.* [42]. Recently, we have isolated ascidian homologues of sulfate-ion channels from ascidian blood cells (unpublished data). At present, we have not yet got any clue to clarify the role of high levels of sulfate in vanadocytes.

8. Vanadium-binding proteins

Some vanadium-binding molecules are expected to participate in the pathway for the accumulation of vanadium from seawater. Therefore, we have searched for vanadium-binding proteins in ascidian blood cells. Using a combination of anion-exchange columns to separate proteins contained in the blood cells and flameless atomic absorption spectrometry to determine vanadium content in each fraction, we succeeded in identifying a fraction containing vanadium-associated proteins (VAPs), which was estimated to associate with vanadium in an approximate ratio of 1:16. SDS-PAGE revealed that the fraction contained three peptides whose molecular weights were estimated to be 12.5, 15, and 16 kDa [79]. 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 [80].

Recently, we isolated the cDNA encoding the 12.5 and 15 kDa VAP (manuscript in preparation). These cDNAs encoded about 120 amino-acid proteins in which the content of cysteine residues is very high. A plasmid encoding a fusion protein of a maltose-binding protein (MBP) and each of the two VAPs was transformed into *E. coli* strain BL21. The fusion proteins purified by an amylose resin column chromatography were analyzed by equilibrium dialysis experiments to have an affinity with vanadium ions. The 12.5 kDa and 15 kDa VAPs bound to roughly 20 molar equivalent of vanadium ions in the +4 or +5 oxidation state, respectively. We have, therefore, designated VAP as Vanabin.

9. Hunting genes and proteins

Our ultimate goal is to clarify the entire mechanism involved in the accumulation and reduction of vanadium in ascidian vanadocytes. To attain this goal, we have a plan to obtain many more genes and proteins expressed in the blood cells systematically, employing an expressed sequence tag (EST) analysis and a metal-chelating column coupled with

iminodiacetic acid to which vanadium(IV) was immobilized.

Up to the present, 300 EST clones from a blood cell library have been isolated (Yamaguchi *et al.*, submitted). The amino acid sequences were then compared with protein sequences registered in the SwissProt database. A similarity search revealed that 127 of the clones (42.3%) were known genes, and 173 of the clones (57.7%) did not have any similarity to genes registered in the SwissProt database. Sixteen of the sequences were found to be similar to gene products that are related to the transport or redox of metals, such as Ca, Cu, Fe, Na, and Zn. In addition, two sequences were obtained for V-ATPase subunits previously identified in our laboratory [65]. These two subunits may participate in maintaining the high acidity within the vanadocyte vacuoles. Among them, the most frequently appearing cDNA clones were those encoding the ferritin H-subunit. The similarity search using the program BLASTX showed that the ascidian ferritin H-subunit is similar to some mammalian ferritin H-subunits. In the human ferritin H-subunit, eight residues, Glu-27, -61, -62, -64, -107, Gln-58, -141, and His-65, are considered to be iron-binding sites [81]. These residues were conserved in the ascidian ferritin H-subunit, with the exceptions of Gln-58, Glu-64, and His-65. His-118, a vanadium-binding site in mammalian ferritin H-subunits, was also conserved in the ascidian ferritin H-subunit [82]. Genes encoding several metal transporters and metalloproteins, such as Na^+/K^+-ATPase, transferrin and ceruloplasmin, were also identified in this study.

Employing the vanadium-chelating column, several proteins have been hunted up to date. Among them, 4.5 kDa protein sequence almost matched with that obtained by EST analysis of a cDNA library derived from vanadocytes. The predicted protein was a novel protein composed of 43 amino acids, including 4 histidine residues (unpublished data). Further, several other vanadium-associated proteins have been extracted from the coelomic serum, a main component of which is 14 kDa protein having basic amino acid-rich in N-terminal amino acids (unpublished data). Analysis of

full sequences of the protein and examination of their metal binding activity are under progress.

10. Functional assay of genes obtained

To elucidate the functions of genes and proteins obtained from ascidian blood cells by the above described methods, it is necessary to establish the functional assay system. Now, we are examining whether the cultured CHO-K1 cell line which is overexpressed the fusion protein of ascidian Nramp accumulates vanadium. Nramp (natural resistance-associated macrophage protein) family is known to transport several heavy metals including Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+} and is highly conserved among mammals, nematodes, yeast, and bacteria [83]. As a first step to search for vanadium transporter in ascidian blood cells, we isolated Nramp homologue from the blood cells of the vanadium-rich ascidian, *Ascidia sydneiensis samea* and showed that ascidian Nramp homologue, named AsNramp, was expressed in the vanadocytes exclusively by *in situ* hybridization. To examine whether AsNramp acts as a proton-coupled vanadium transporter in ascidians, we constructed a plasmid expressing a fusion protein of AsNramp and a green fluorescent protein (GFP) under the control of cytomegalovirus (CMV) promoter. We have preliminarily found that the cultured CHO-K1 cells overexpressed the fusion protein specifically uptake vanadium (unpublished data).

11. Physiological roles of vanadium in ascidians

Although the unusual phenomenon whereby some ascidians accumulate vanadium to levels more than ten million times higher than those in seawater has attracted researchers in various fields, the physiological roles of vanadium remain to be explained. Endean [16, 84-85] and Smith [86-87] proposed that the cellulose of the tunic might be produced by vanadocytes. Carlisle [88] suggested that

vanadium-containing vanadocytes might reversibly trap oxygen under conditions of low oxygen tension. The hypothesis has also been proposed that vanadium in ascidians acts to protect them against fouling or as an antimicrobial agent [89-90]. However, most of the proposals put forward do not seem to be supported by sufficient evidence. Therefore, we have not yet obtained any clue to resolve the physiological roles of vanadium in ascidians. Attempts to characterize this phenomenon can be expected to promote more information about the unusual accumulation of vanadium by one class of marine organisms.

12. Conclusion

Some ascidians especially belonging to the suborder Phlebobranchia accumulate vanadium to levels more than ten million times higher than those in seawater. Vanadium dissolved in the +5 oxidation state (V(V)) in seawater is incorporated into the interior of the vanadocytes, where vanadium is bound with Vanabin (vanadium binding protein) and reduced to the +4 oxidation state (V(IV)) with NADPH produced by the pentose phosphate pathway. Vanadium(IV) bound with Vanabin is transferred to an unknown protein on vacuolar membrane, where vanadium(IV) is trapped with metal-binding domains of a metal ATPase on the surface of vacuolar membrane and stored in the vanadocyte vacuoles having both high levels of H^+ and SO_4^{2-}. Vanadium(IV) is further reduced to the +3 oxidation state by unknown reductant(s). The pathway of vanadium accumulation and mechanism of vanadium reduction are schematically representated in Fig. 5. The physiological roles of vanadium remain to be explained.

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

Species	Tunic	Mantle	Branchial	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	0.003
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. Vanadium contents in each tissue were quantitatively determined by a neutron activation analysis [10] and that in *A. gemmata* was determined by an ESR (electron spin resonance) spectrometry [11].

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

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

pH values were measured by both a microelectrode and ESR (electron spin resonance) under anaerobic conditions and converted into $[H^+]$ [11]. On the other hand, vacuoles of signet ring cells were revealed to emit a brilliant vermilion indicating acidic pH after incubation with 2 μM acridine orange for 1 hr but neither the cytoplasm of signet ring cells nor the other types of blood cells indicated acidic pH. While, the addition of 1 μM bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, caused the vacuolar contents neutralized (showing green color) with resultant inhibiting pump function of the H^+-ATPase [62]. Taking those results into consideration, it is inevitable to interpret that the acidity of the blood cells result from the vacuoles of signet ring cells.

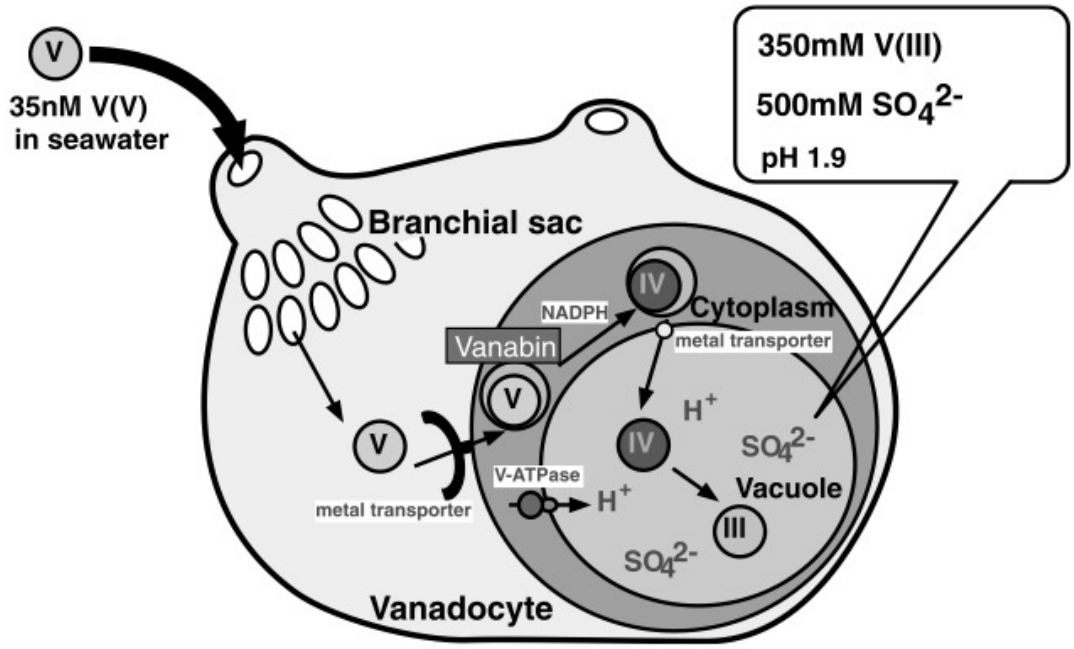

Fig. 1. Schematic representation of vanadium accumulation and reduction by ascidians.

The concentration of vanadium dissolved in sea water is only 35 nM in the +5 oxidation state. While, the highest concentration of vanadium in ascidian blood cells attains up to 350 mM. In addition 500 mM of sulfate is contained. The contents of vacuoles are maintained in an extremely low pH of 1.9 by H^+-ATPases. Although no direct determination of the pH within vacuoles has done, it is inevitable to interpret that the acidity of the blood cells result from the vacuoles of signet ring cells based on the following reasons. That is to say, vacuoles of signet ring cells were revealed to emit a brilliant vermilion indicating acidic pH after incubation with 2 μM acridine orange for 1 hr but neither the cytoplasm of signet ring cells nor the other types of blood cells indicated acidic pH. While, the addition of 1 μM bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, caused the vacuolar contents neutralized (showing green color) with resultant inhibiting pump function of the H^+-ATPase [62]. Taking those results into consideration, it is inevitable to interpret that the acidity of the blood cells result from the vacuoles of signet ring cells. Under the environment, almost all vanadium accumulated is reduced to V(III) via V(IV). Recently, we have

found out vanadium binding proteins, designated Vanabins and cloned cDNAs. The pentose phosphate pathway, which produces NADPH, has been disclosed to localize in the cytoplasm and by *in vitro* experiments NADPH has been revealed to reduce V(V) to V(IV). A metal-ATPase might be involved in vanadium transport has been found in vacuolar membrane.

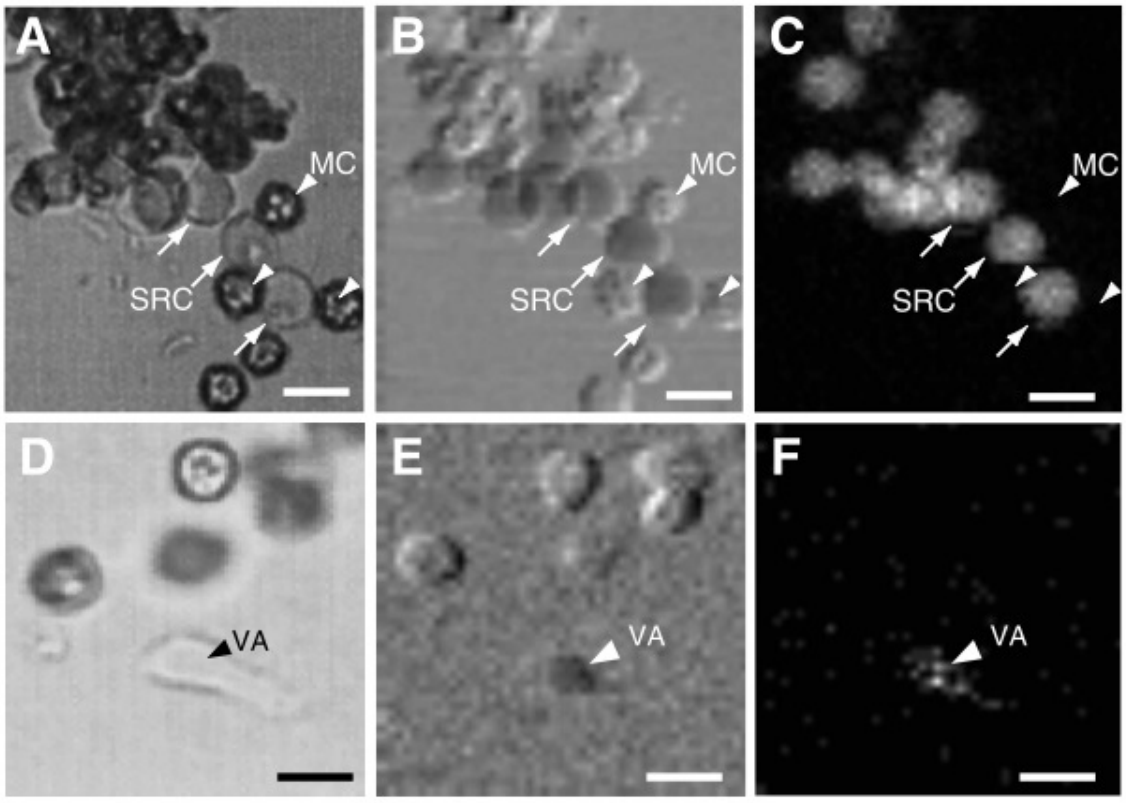

Fig. 2. *Phallusia mammillata* blood cells observed by differential interference contrast optical microscopy (A, D), by x-ray microscopy in transmission mode (B, E), and in the fluorescence mode for vanadium (C, F). Photographs A to C are from the same field of view. Photographs D to F are from another field. Transmission and fluorescence images were taken by scanning cells with a 5.500 keV x-ray at a 1 μm x 1 μm resolution for 500 ms per pixel. Vanadium is accumulated in signet ring cells (SRC, shown by arrows) and in a vacuolated amoebocyte (VA, shown by arrowheads in D-F), but not in morula cells (MC, shown by arrowheads in A-C). Each scale bar = 10 μm. See [30].

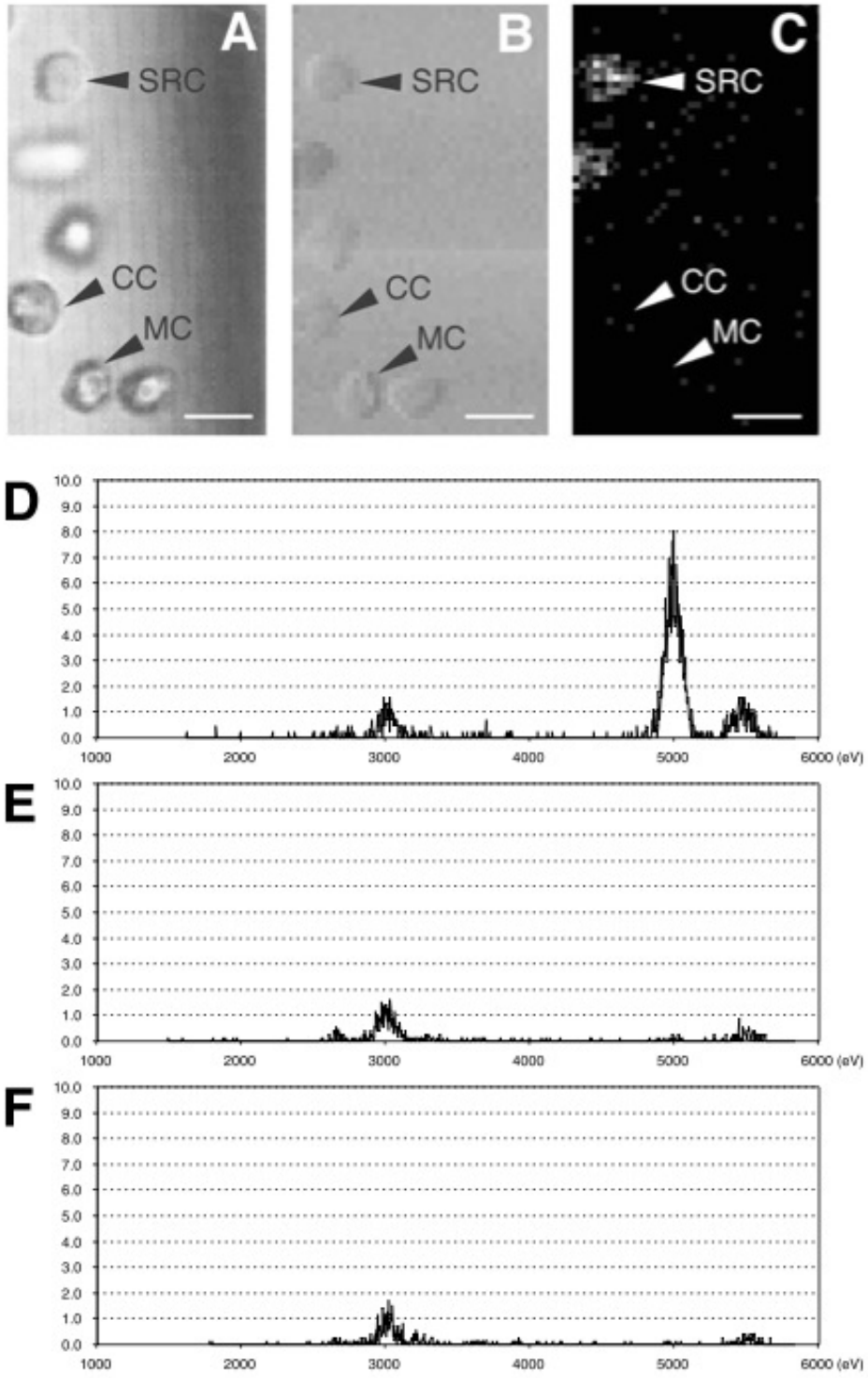

Fig. 3. *Phallusia mammillata* blood cells observed by x-ray microscopy and fluorescence spectra emitted from the blood cells. (A) Blood cells of *P. mammillata* viewed with a differential interference contrast optical microscope, (B) x-ray images taken at 5.500 keV in transmission mode, and (C) by fluorescence from vanadium. Transmission and fluorescence images

are taken by scanning cells at 1 μm x 1 μm resolution for 100 ms per pixel. Vanadium is accumulated in signet ring cell (SRC) but is not seen in morula cells (MC) or compartment cells (CC). Each scale bar = 10 μm. X-ray fluorescence spectra are taken from (D) a signet ring cell, (E) a morula cell, and (F) a compartment cell, shown by arrowheads (A-C), by scanning each cell independently for 800 ms per pixel. The horizontal axes of the spectra correspond to the energy (eV) of fluorescence. The vertical axes indicate the relative intensity of fluorescence when the argon peak from each cell was set as one. Note that 4.952 keV vanadium fluorescence is only detected in the signet ring cell (D). See [30].

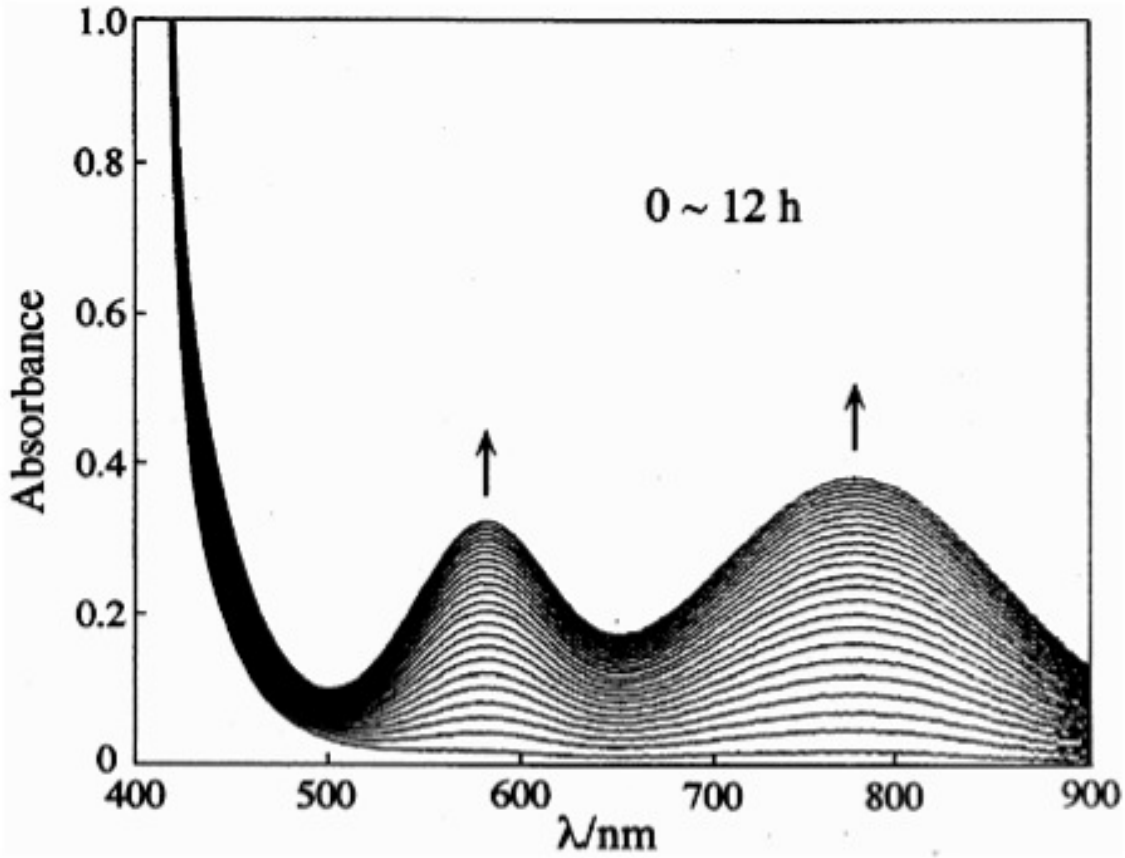

Fig. 4. Successive absorption spectra observed at 30 mim intervals for 12 h under anaerobic conditions. A Tris-HCl buffer solution at pH 7.0 containing 20mM of Na3[VO2(EDTA)] and 200 mM of NADPH was used. See [54].

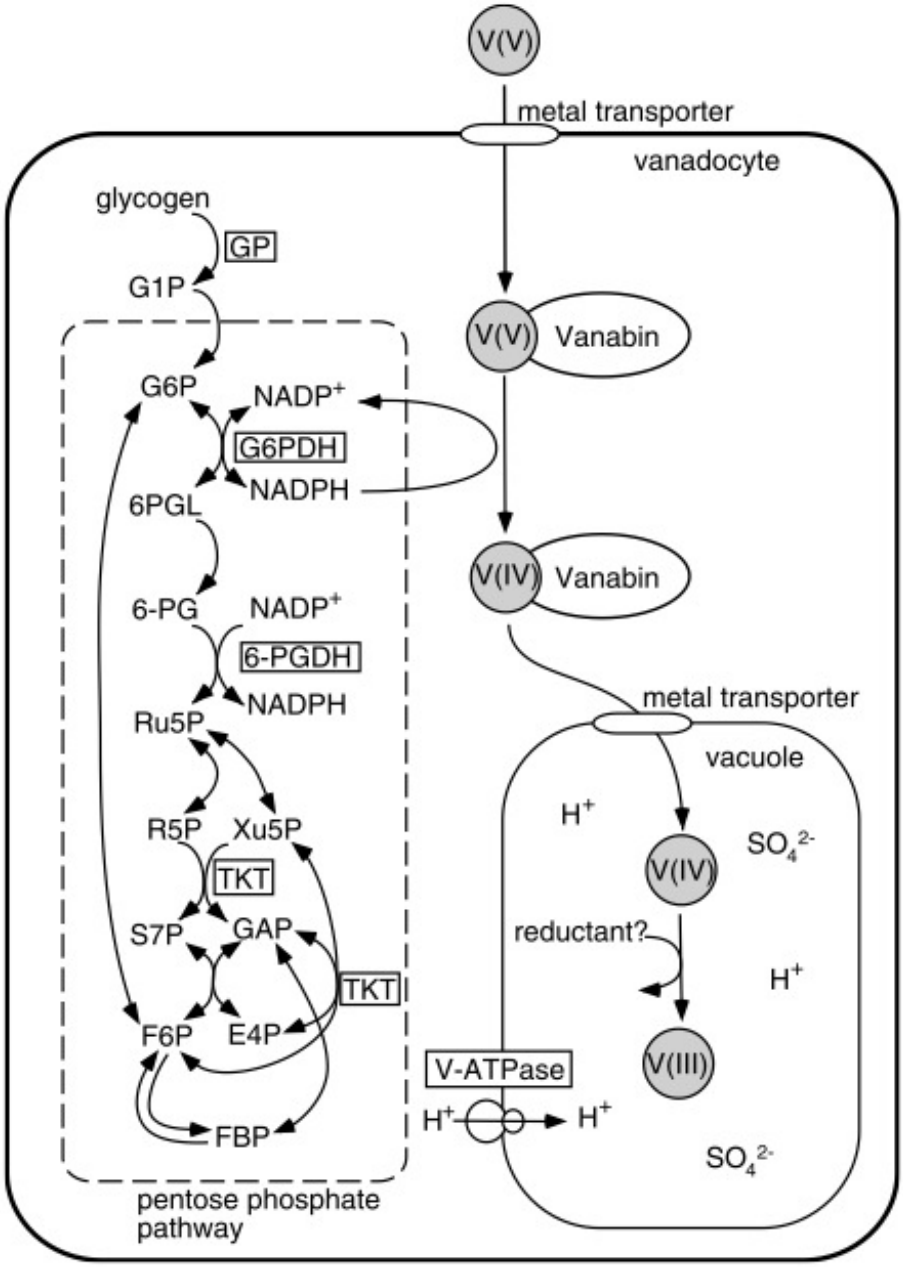

Fig. 5. Schematic representation of the pathway ofvanadium accumulationand mechanism of vanadium reduction. Vanadium in the +5 oxidation state dissolved in seawater is thought to be incorporated into the interior of the vanadocytes, where vanadium is bound with Vanabin (vanadium binding protein) and reduced to the +4 oxidation state with NADPH produced by the pentose phosphate pathway. Vanadium in the +4 oxidation state is stored in the vanadocyte vacuoles and further reduced to the +3 oxidation state. Some proteins, specifically vanadium transporters, vanadium receptors, and vanadium channel proteins, are thought to be involved in the accumulation of

vanadium. Vanabin, vanadium binding protein; TKT, transketolase, GP, glycogen phosphorylase; G6PDH, glucose-6-phosphate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase, $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; V-ATPase, vacuolar-type H^+-ATPase.