The Mechanism of Accumulation of Vanadium by Ascidians: Some Progress towards an Understanding of this Unusual Phenomenon

Author: Michibata, Hitoshi

[REVIEW]

The Mechanism of Accumulation of Vanadium by Ascidians: Some Progress towards an Understanding of this Unusual Phenomenon

Hitoshi Michibata

Mukaishima Marine Biological Laboratory, Faculty of Science and Laboratory of Marine Molecular Biology, Graduate School of Science, Hiroshima University, Mukaishima-cho, Hiroshima 722, Japan

ABSTRACT—Since the discovery of vanadium in the blood cells (coelomic cells) of an ascidian by Henze in 1911, this unusual phenomenon has attracted the interest of many investigators. We started our studies by examining the vanadium contents of several tissues from 20 ascidian species, collected not only from Japanese waters but also from the Mediterranean since about 18 years ago, using an extremely sensitive method, namely, neutron-activation analysis. We found the highest concentration of vanadium, 350 mM, in the blood cells of *Ascidia gemmata* which belongs to the suborder Phlebobranchia. This concentration of vanadium is 10^7 times higher than that in seawater. Among the approximately ten types of blood cells, the signet ring cells were revealed to be the true vanadocytes by a combination of cell fractionation and neutron-activation analysis. Of the vanadium in these vanadocytes, 97.6% was in the +3 oxidation state while the rest was in the +4 oxidation state. The contents of the vanadocytes in *A. gemmata* had a low pH of 2.4 and these cells contained the highest levels of vanadium. These observations suggested the possibility that protons, concentrated by a H^+-ATPase, might be linked energetically to the accumulation of vanadium. Antibodies raised against a vacuolar-type H^+-ATPase were found to react with the vacuolar membranes of signet ring cells and the addition of bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPases, inhibited the uptake of protons by the vacuoles of signet ring cells, with resultant neutralization of the contents of the vacuoles. A monoclonal antibody, S4D5, prepared for the purpose of identifying signet ring cells, reacted with the signet ring cells not only of *A. sydneiensis samea*, which had been used as the antigen, but also with those of other species. During embryogenesis, a vanadocyte-specific antigen, recognized by this monoclonal antibody, appeared for the first time in the body wall at the same time as the significant accumulation of vanadium became apparent. Characterization of vanadium-binding proteins extracted from the blood cells of vanadium-rich ascidians is in progress and shows to help us determine the way in which ascidians selectively accumulate high levels of vanadium from seawater. The unusual phenomenon whereby some ascidians accumulate vanadium to levels more than ten million times higher than those in seawater has attracted the interest of researchers in various fields. Studies of ascidians with this unusual physiological property may help us to clarify not only how ascidians but also other organisms accumulate transition metals, as well as the physiological roles of these metals.

INTRODUCTION

From the end of 19th century through the first decades of this century, numerous advances were made in many scientific fields. In the field of analytical chemistry, the techniques for emission spectrometry and colorimetric analysis became established, and analyses of the elementary composition of living organisms were popular. A German chemist, Martin Henze (Fig. 1) was one of the pioneers of such bioinorganic chemistry. In 1902, he was employed at the Stazione Zoologica di Napoli, which had been founded by Dr. Anton Dohrn in 1874 and is now one of the oldest marine biological laboratory in the world, when the Department of Physiological Chemistry was established. In 1911, Henze discovered high levels of vanadium in the blood cells (coelomic cells) of an ascidian, known as the sea squirt (Henze, 1911).

After Henze's interesting discovery, many investigators, including not only analytical chemists but also physiologists, bioinorganic chemists and chemists of natural products, joined in attempts to characterize the vanadium in living organisms,

in part because of the initial interest in its very presence in ascidians and in part because of the considerable interest in its possible involvement in as an oxygen-carrier in addition to iron and copper. However, studies on the mechanism of the accumulation and the physiological significance of vanadium made very slow progress since the problems required interdisciplinary cooperation to be solved.

Nevertheless, levels of vanadium in various ascidians were determined by many analytical chemists, who often were little interested in the phylogeny of the ascidians that they were studying. Furthermore, as discussed below, the blood cell that contains high levels of vanadium was misidentified initially as the morula cell (Webb, 1939; Endean, 1960; Kalk, 1963a, b; Kustin et al., 1976), since the color of the morula cell was thought to be that of a vanadium complex. A tunichrome that was proposed to participate in the reduction of vanadium was reported to be present in morula cells and not in signet ring cells (Robinson et al., 1984). The problems to be resolved had been approached in a one-sided manner only by chemists, and the absence of cooperation with biologists hampered appropriate interdisciplinary studies.

Concerning the accumulation of vanadium by ascidians, although many reviews have been published (Goodbody, 1974; Biggs and Swinehart, 1976; Kustin et al., 1983; Boyd and Kustin, 1985; Michibata, 1989, 1993; Michibata and Sakurai, 1990; Wever and Kustin, 1990; Smith et al., 1995; Kustin and Robinson, 1995), we intend to focus on the stream of this field in the present paper.

Fig. 1. Dr. Friedrich Wolfgang Martin Henze (1873~1956) who first found out high levels of vanadium in the blood cells (coelomic cells) of an ascidian in 1911 at Stazione Zoologica di Napoli.

DETERMINATION OF THE VANADIUM CONTENTS OF SEVERAL TISSUES

During the past several decades, the vanadium in ascidians has been analyzed by a variety of analytical methods, such as colorimetry, emission spectrometry and atomic absorption spectrometry (Cantacuzène 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). These techniques vary widely in sensitivity and in the precision with which the quantification of vanadium can be performed. Moreover, data were reported in terms of dry weight, wet weight, ash weight, inorganic dry weight, or amount of protein. Thus, early data could not be compared directly, presenting problems to those attempting to pursue physiological studies of the accumulation of vanadium by ascidians.

To bring some order to the field, we decided to re-determine the contents of vanadium in several tissues of ascidians by neutron-activation analysis, which is an extremely sensitive method for the quantification of vanadium. We collected many species of ascidians that belonged to two of three suborders, Phlebobranchia and Stolidobranchia, mainly from the waters around Japan and the Mediterranean. Specimens were dissected into eight samples for analysis, namely, blood cells (coelomic cells), plasma, tunic, mantle (muscle), branchial basket, stomach, hepatopancreas, and gonad. The samples were dried and weighed and then they were mineralized at 500°C and submitted to neutron-activation analysis in the nuclear reactor at the Institute for Atomic Energy, Rikkyo University, Yokosuka, Japan. Some of the stable vanadium in each sample was converted to the
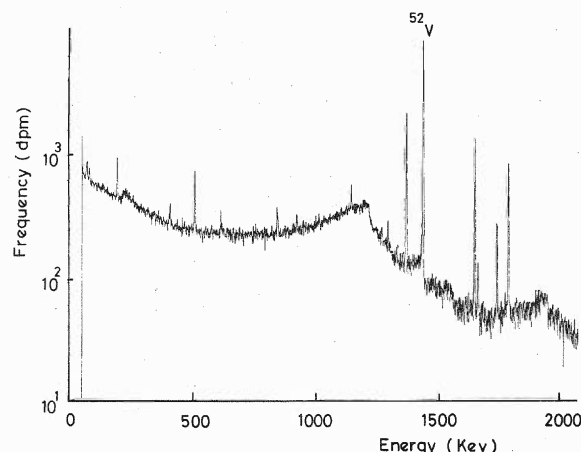

Fig. 2. γ-ray spectrogram of the blood cells of Ascidia ahodori. Some of the stable vanadium contained in the samples were activated to radioactive nuclides, ^{52}V, which emitted γ-ray at 1,434 keV after irradiation of thermal neutrons in the TRIGA MARK II nuclear reactor at Rikkyo University.

Table 1. Concentrations of vanadium in 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.

radioactive nuclide, ^{52}V, which emits γ-rays at 1,434 keV (Fig. 2). Since the frequency of γ-rays emitted depends on the level of stable vanadium in the sample, the original amount of vanadium in the tissues could be calculated (Michibata, 1984; Michibata et al., 1986).

The data that we obtained are summarized in Table 1. Although vanadium was detectable in samples from almost all species examined, the ascidian species belonging to the suborder Phlebobranchia apparently contained higher levels of vanadium than those belonging to the Stolidobranchia. We also confirmed that blood cells especially contained the highest amounts of vanadium among the tissues examined. Levels of iron and manganese, determined simultaneously, did not vary much among the members of the two suborders. Webb (1939) 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 (Michibata et al., 1986). Furthermore, the highest concentration of 350 mM vanadium was found in the blood cells of Ascidia gemmata belonging to the suborder Phlebobranchia (Michibata et al., 1991a), whose concentration corresponds 10^7 times higher than that in seawater (Cole et al., 1983; Collier, 1984).

CELL SEPARATION BY DENSITY-GRADIENT CENTRIFUGATION

Our analytical study confirmed that ascidian blood cells contain the highest amounts of vanadium among the tissues examined. In general, ascidians have nine to eleven different types of blood cells that can be grouped into six categories on the basis of their morphology: hemoblasts, lymphocytes, leucocytes, 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. The morula cells were thought for many years to be the so-called vanadocytes (Webb, 1939; Endean, 1960; Kalk, 1963a, b; Kustin et al., 1976). The pale-green color of morula cells resembles that of a vanadium complex dissolved in aqueous solution and the dense granules in morula cells that can be observed under the electron microscope after fixation with osmium tetroxide were assumed to be deposits of vanadium. However, at the end of the 1970's, with the increasing availability of scanning transmission electron microscopes equipped with an energy disperse X-ray detector, it became possible to address the question of whether or not morula cells are the vanadocytes with greater confidence. An Italian group was the first to demonstrate that the characteristic X-ray due to vanadium was not detected from morula cells but from granular amoebocytes, signet ring cells and compartment cells and, moreover, that vanadium was selectively concentrated in the vacuolar membranes of these cells, with vanadium granules being present inside the vacuoles (Botte et al., 1979b; Scippa et al., 1982, 1985; Rowley, 1982). Robinson et al. (1984) criticized these results, stating that metals must have been lost during the fixation and dehydration procedures required for such X-ray microanalysis and that it is unlikely that each type of blood cells would lose metals to the same extent. Identification of the true vanadocytes became a matter of the highest priority to those concerned with the mechanism of accumulation of vanadium by ascidians.

To put end to the controversy about the identification of the true vanadocytes, we attempted to settle the problem not by X-ray microanalysis but by a combination of density gradient centrifugation, for the isolation of specific types of blood cells and neutron-activation analysis, for the quantification of the vanadium contents of the isolated subpopulations of blood cells (Michibata et al., 1987). Ascidia ahodori, one of the vanadium-rich ascidians, was used as the material for this analysis. Blood cells were separated from the serum by centrifugation and then loaded onto a discontinuous gradient that consisted of four different concentrations of Ficoll in artificial seawater, prepared without Ca^{2+} and Mg^{2+} to prevent

clotting, and the gradient was centrifuged at 100 x g. The blood cells were partitioned into four discrete layers and the subpopulation recovered from the each layer was submitted to neutron-activation analysis. The subpopulation of cells in layer 4, where signet ring cells were dominant, contained the highest level of vanadium. The pattern of distribution of vanadium coincided with that of signet ring cells but not with that of morula cells or that of compartment cells, as shown in Fig. 3. These results proved that the signet ring cells were the true vanadocytes (Fig. 4). The same experiment was repeated with other ascidians, namely, *A. sydneiensis samea* and *A. gemmata*, and again signet ring cells were revealed to be the true vanadocytes (Michibata *et al.*, 1990, 1991a; Hirata and Michibata, 1991).

CHEMICAL SPECIES OF VANADIUM

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 (cf. Chasteen, 1981, 1983; Kustin *et al.*, 1983; Boas and Pessoa, 1987). An E vs. pH diagram and redox potentials are shown in Fig. 5. Vanadium ions in the +3 oxidation state [V(III)] are usually unstable towards air oxidation, and V(III) ions are hydrolyzed to $V(OH)^{2+}$ which tend to dimerize to $(VOV)^{4+}$ at pH 2.2 and over. In neutral and alkaline conditions, simple soluble V(III) compounds without any strong ligands 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 $[(VO)_2(OH)_2]^{2+}$ are formed. At neutral and alkaline pH, vanadyl ions are polymerized to $[VO(OH)_3]_n^-$ (cf. Boas and Pessoa, 1987).
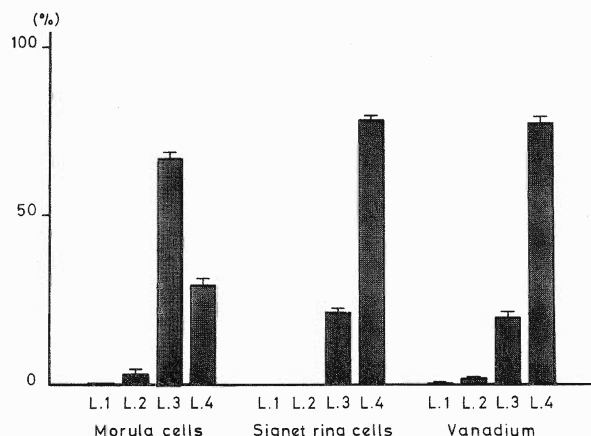
Fig. 3. Distribution patterns of subpopulations of blood cells and content of vanadium after density-gradient centrifugation. Histograms revealed that the pattern of distribution of vanadium coincided with that of signet ring cells but not with that of morula cells or that of compartment cells. Namely, signet ring cells were identified as vanadium-containing blood cells (vanadocytes) in ascidians (Michibata *et al.*, 1987). L.1: layer 1, L.2: layer 2, L.3: layer 3, and L.4: layer 4 from the top. to bottom of discontinuous density-gradient.
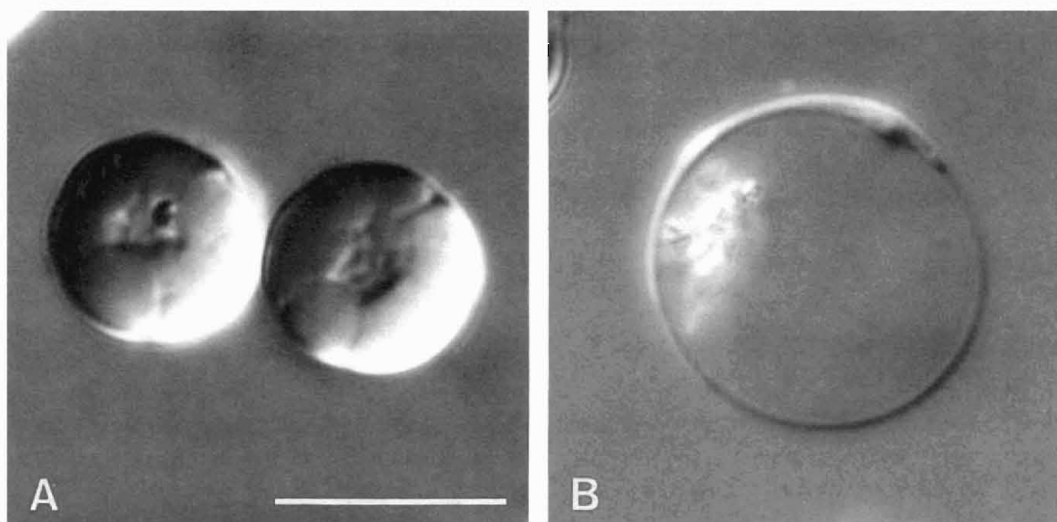
Fig. 4. Morula cells (A), misidentified initially as vanadocytes, and signet ring cell (B), identified newly as vanadocyte, in the ascidian, *Ascidia ahodori*. Scale bar indicates 10 μm.
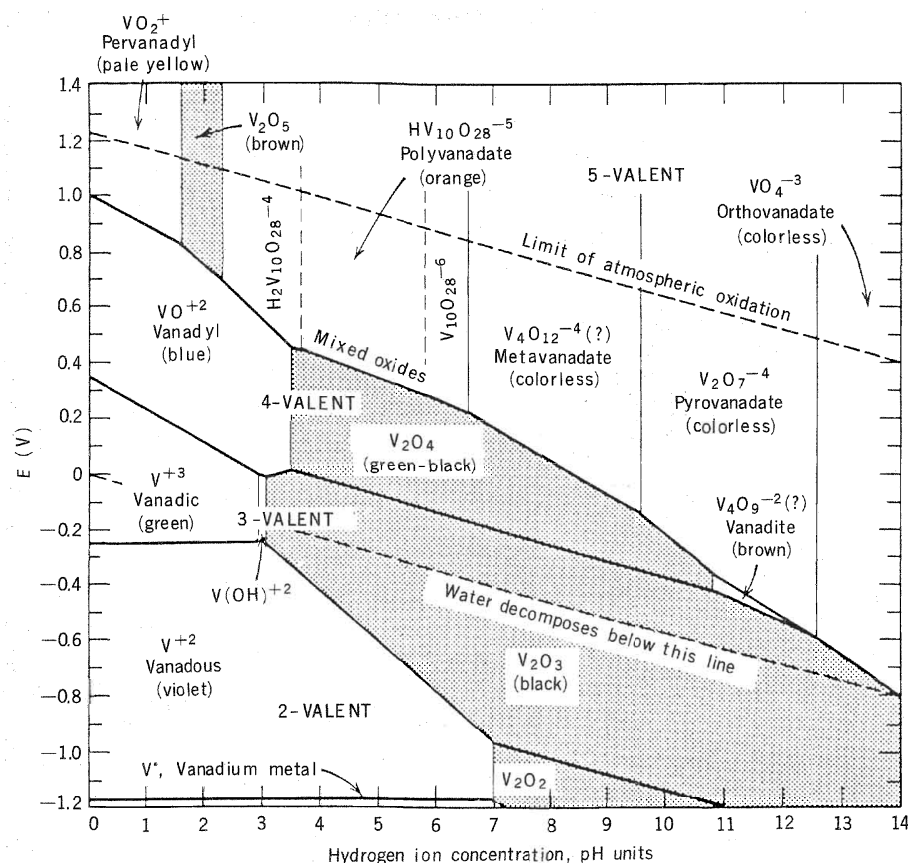
Fig. 5. Redox potential *versus* pH diagram for the vanadium-water system. Vanadium is a multivalent transition metal belonging to the first transition series in the periodic table. The chemical species of vanadium are strongly influenced by the oxidation/reducing properties of the metallic center and by pH conditions. Vanadium ions are, therefore, limited to only the +3, +4 and +5 oxidation states under physiological conditions. Although vanadium ions in the +3 oxidation state are unstable at physiological pH and in the presence of oxygen, yet they are the predominant in ascidian blood cells (vanadocytes) under extremely low pH conditions. The chemical species in the meshed area are in precipitated (cf. Boas and Pessoa, 1987).

OXIDATION STATE OF VANADIUM IN ASCIDIANS

It is believed that vanadium is dissolved in the +5 oxidation state in seawater (McLeod *et al.*, 1975) but this possibility remains to be confirmed (Sugimura *et al.*, 1978). With regard to the oxidation state of vanadium in ascidian blood cells, 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. Such studies 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 (Carlson, 1975; Tullius *et al.*, 1980; Dingley *et al.*, 1981; Frank *et al.*, 1986; Lee *et al.*, 1988; Brand *et al.*, 1989).

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 cells, 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 (Hirata and Michibata, 1991). Only the +4 oxidation state of vanadium is detectable by ESR spectrometry. As shown in Fig. 6, weak signals due to the +4 oxidation state were recorded when the subpopulation of vanadocytes was submitted to ESR spectrometry at 77K under nitrogen (a). Then, when the sample was lysed by thawing and submitted again to ESR spectrometry at 77K in a reducing atmosphere, the heights of peaks in the ESR spectrum increased (b). Bubbling of oxygen gas into the lysate dramatically increased peak height, with a 7-fold increase 2 hr later (c) and a 13-fold increase 24 hr later (d). However, addition of hydrogen peroxide to the lysate caused the peaks that had been due to oxo-vanadium to disappear. These results indicate that the oxidation state of vanadium in vanadocytes is predominantly the +3 oxidation state, with a small amount

of vanadium being in the +4 oxidation state. The ratio of chemical species of vanadium in the two states is 97.6:2.4. No other organisms apart from a polychaeta, *Pseudopotamilla occelata* (Ishii *et al.*, 1993), are known at present to contain high levels of vanadium in the +3 oxidation state, the most reduced chemical species of vanadium.
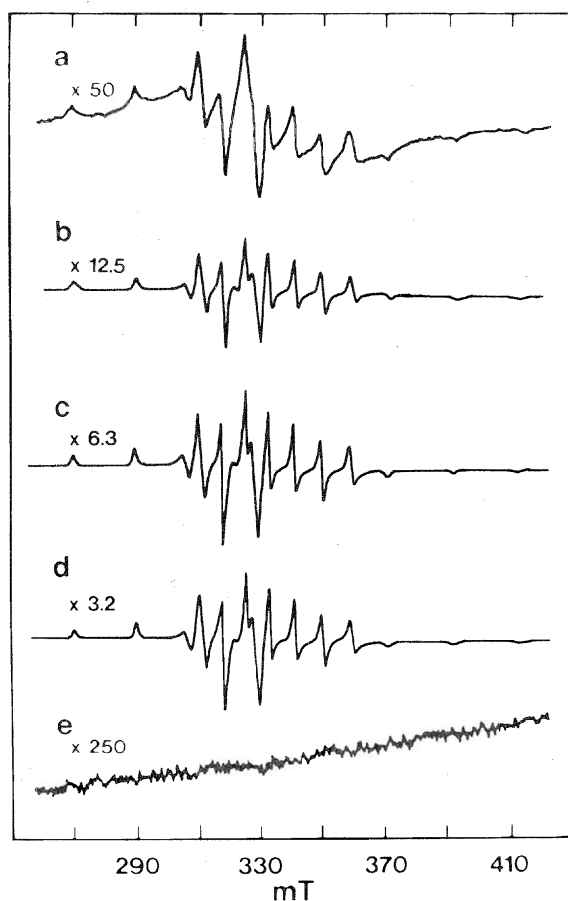

Fig. 6. ESR (electron spin resonance) spectrometry of vanadium. Vanadium ions in the +4 oxidation state are paramagnetic and give a blue solution of oxo-ions, VO^{2+} (vanadyl ions), under moderately acidic conditions. Only the +4 oxidation state of vanadium is detectable by ESR spectrometry . Weak signals due to the +4 oxidation state of vanadium were observed in the intact vanadocytes (a), indicating that the vanadocytes contain a small amount of vanadium in the +4 oxidation state. Lysing the sample increased the signal intensity (b) and bubbling of oxygen gas into the lysate dramatically increased the signals, with a 7-fold 2 hr later (c) and a 13-fold 24 hr later (d), meaning that vanadium in the +3 oxidation state was oxidized to that in the +4 oxidation state. Addition of hydrogen peroxide to the lysate caused the signal to disappear (e), showing that vanadium in the +4 oxidation state was further oxidized to that in the +5 oxidation state. That is to say, it was revealed that vanadium contained initially in the vanadocytes is predominantly in the +3 oxidation state and based on the data, 97.6% of vanadium was calculated to be in the +3 oxidation state in vanadocytes (Hirata and Michibata, 1991). ×50 means magnifications of signal intensity and mT does milli tesra (= $gs^{-2}A^{-1}$).

COMPOUNDS THAT REDUCE VANADIUM

Henze (1911, 1932) was the first to report the coexistence of sulfate with vanadium in ascidian blood cells. Vanadium was thought to bind to a nitrogenous compound and sulfate to form a complex, designated haemovanadin, that acted as a respiration pigment in ascidian blood cells (Califano and Boeri, 1950; Webb, 1956). Bielig *et al.* (1966) suggested that haemovanadin could reduce vanadium. However, the chemical structure of haemovanadin was not determined even though model compounds were proposed. Kustin's group proposed that haemovanadin was actually an artifact formed by air-oxidation and disputed the possibility that it might be involved in respiration (Macara *et al.*, 1979a). They isolated a low-molecular compound that they thought might be involved in the reduction of vanadium from the blood cells of *Ascidia nigra* and *Ciona intestinalis* and named it tunichrome (Macara *et al.*, 1979b, c). Tunichrome was subsequently revealed to be a compound composed of three pyrogallol moieties by Nakanishi and his co-workers (Bruening *et al.*, 1985). However, since it was demonstrated that tunichrome had no special binding sites for vanadium, since it seemed unlikely that tunichrome could reduce vanadium in the +5 oxidation state to that in the +3 oxidation state (Bulls *et al.*, 1990), and since tunichrome was absent from the vanadium-containing blood cells, namely, the signet ring cells (Michibata *et al.*, 1988, 1990), the possibility could be ruled out that tunichrome is involved in the reduction of vanadium in ascidian blood cells. Recently, Ryan *et al.* (1992) observed the reduction of vanadium in the +5 and +4 oxidation states by a tunichrome, designated Mm-1, in buffer solution at pH 7 *in vitro*. It is, however, unclear whether such a reaction could occur in ascidian blood cells.

SULFATE IN VANADOCYTE

A considerable amount of sulfate has always been found in association with 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), suggesting that sulfate might be involved in the biological function and/or the accumulation and reduction of vanadium. However, Frank *et al.* (1987) 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, we determined the ratio of the level of sulfate to that of vanadium in the blood cells of the ascidian *Ascidia gemmata* by Raman spectroscopy, as shown in Fig. 7. The ratio obtained was approximately 1.5, as would be predicted if sulfate ions were present as the counter ions of vanadium ions in the +3 oxidation state. We also found evidence that an aliphatic sulfonic acid was present in the blood cells (Kanamori and Michibata, 1994).
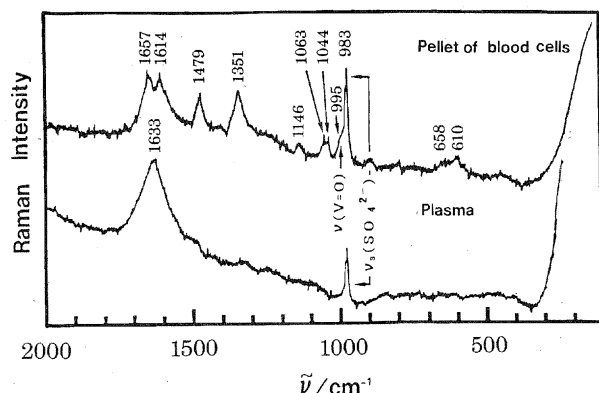
Fig. 7. Raman spectra of the blood cells of *Ascidia gemmata*.
High levels of sulfate and/or sulfur compounds have found in association with vanadium in ascidian blood cells. Raman spectrometry can determine noninvasively the amounts of sulfate and vanadium in the vanadocytes. A band at 983 cm^{-1} and a shoulder at 995 cm^{-1} are derived from SO_4^{2-} symmetric stretching vibration and V=O stretching vibration, respectively. Based on these intensities of the peaks, the concentration ratio of SO_4^{2-} and V^{3+} was calculated to be 1.47 (Kanamori and Michibata, 1994).

EXTREMELY LOW pH IN VANADOCYTES

Henze (1911, 1912, 1913, 1932) was the first to report that a homogenate of ascidian blood cells is extremely acidic. Almost all subsequent investigations have been supported his observation. Recently, however, Kustin's group disputed the earlier reports and claimed that the previously used methods gave spurious results when applied to ascidian blood cells because the interior of blood cells that contained high levels of vanadium and iron was probably a strongly reducing environment (Dingley *et al.*, 1982; Agudelo *et al.*, 1983). In their view, the possibility remained that it was the intracellular redox potential, and not the pH, that had been measured. They reported that the intracellular pH was neutral on the basis of measurements made by a new technique, which involved the transmembrane equilibrium of ^{14}C-labeled methylamine. Hawkins *et al.* (1983) and Brand *et al.* (1987) also reported nearly neutral values for the pH of the interior of ascidian blood cells, which they determined noninvasively from the chemical shift of ^{31}P-NMR. However, Frank *et al.* (1986) demonstrated

that the interior of the blood cells of *Ascidia ceratodes* has a pH of 1.8, basing their results on the new finding that the ESR line width accurately reflects the intracellular pH. Thus, the reported pH inside ascidian blood cells has excited considerable controversy, as summarized in Table 2.

We consider that the main reason for the extreme variations is that the measurements of pH were made with entire populations of blood cells and not with the subpopulation of vanadocytes specifically. Thus, one or two specific types of blood cells might have a highly acidic solution within their vacuoles, in which vanadium would be present in a reduced state. With this possibility in mind, we designed an experiment in which we combined the separation of each type of blood cells, measurement of pH with a microelectrode under anaerobic conditions, to avoid air-oxidation, and ESR spectrometry as a noninvasive method for the measurement of pH to confirm the results obtained with the microelectrode (Michibata *et al.*, 1991a). Three species of vanadium-rich ascidians, *Ascidia gemmata*, *A. ahodori*, and *A. sydneiensis samea*, were used as the materials. Blood cells drawn each species were fractionated by density-gradient centrifugation, as described above. A comparison of the patterns of distribution of each type of blood cells, the measured pH values after conversion to concentrations of protons ([H$^+$]) and levels of vanadium in each layer of cells are shown in Fig. 8. It is clear from Fig. 8 that the patterns of distribution of protons and vanadium were similar. Thus, the signet ring cells contained high levels of vanadium and had a low intracellular pH in all three species. In *A. sydneiensis samea* one other type of blood cells in addition to signet ring cells was also found to be acidic. These cells were large and spherical, measuring 30 to 80 μm in diameter and having a single fluid-filled vacuole. This type of cell is probably analogous to the so-called nephrocyte, as judged by the criteria proposed by Wright (1981). However, we propose to call these cells as "giant cells" until their function is revealed. Giant cells have the lowest specific gravity of all the blood cells, they contain no vanadium but have a very low pH of 1.48 (Michibata *et al.*, 1991a) The contents and function of these cells remain to be clarified.

ESR spectrometry was also used for noninvasive measurements of the intracellular acidity of blood cells (Michibata *et al.*, 1991a). The method is based on the

Table 2. Reported pH inside ascidian blood cells

| pH	Ascidian species	Analytical methods
1 N sufuric acid	*Phallusia mammillata*	titration (Henze, 1911)
1.8 N acid	*P. mammillata*	titration (Webb, 1939)
0.4 N acid	*Pyura stolonifera*	titration (Endean, 1955a)
pH 1.5	*Eudistoma ritteri*	electrode (Levine, 1961)
pH 7.2	*Ascidia nigra*	^{14}C-methylamine (Dingley *et al.*, 1982)
pH 7.19	*Boltenia ovifera*	^{14}C-methylamine (Agudelo *et al.*, 1983)
pH 6.4	*Pyura stolonifera*	^{31}P-NMR (Hawkins *et al.*, 1983)
pH 1.8	*Ascidia ceratodes*	ESR (Frank *et al.*, 1986)
pH 6.5	*Phallusia julinea*	electrode under N$_2$ (Brand *et al.*, 1987)
pH 6.98	*Ascidia ceratodes*	^{14}C-dimethyloxazolidine (Lee *et al.*, 1990)
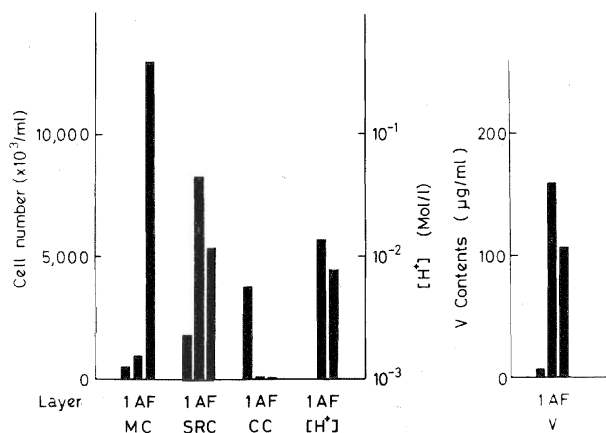
Fig. 8. Determination of acidic blood cells of *Ascidia gemmata*. Histograms depict the number of each type of blood cells and the concentrations of H$^+$ and vanadium in three different layers (layers 1, A and F; cf. Michibata *et al.*, 1991a) of blood cells which were fractionated by density-gradient centrifugation in Percoll. MC, morula cells; SRC, signet ring cells; CC, compartment cells; [H$^+$], concentration of protons; V, vanadium

Table 3. Correlation between concentrations of vanadium and pH values in ascidian blood cells

Species	Conc. of vanadium	pH
Ascidia gemmata	350mM	1.86
A. ahodori	60mM	2.67
A. sydneiensis samea	13mM	4.20

phenomenon that the ESR line width due to oxo-vanadium [VO(IV)] ions increases (cf. Frank *et al.*,1986) almost linearly from pH 1.4 to pH 2.3, as shown in Fig. 9. We confirmed that the low pH values obtained with a microelectrode were not artifacts when we applied this method to the measurement of the pH of the contents of signet ring cells from *A. gemmata*. We note, to avoid any misunderstanding, that the acidic solution is assumed to be contained in the vacuole of each
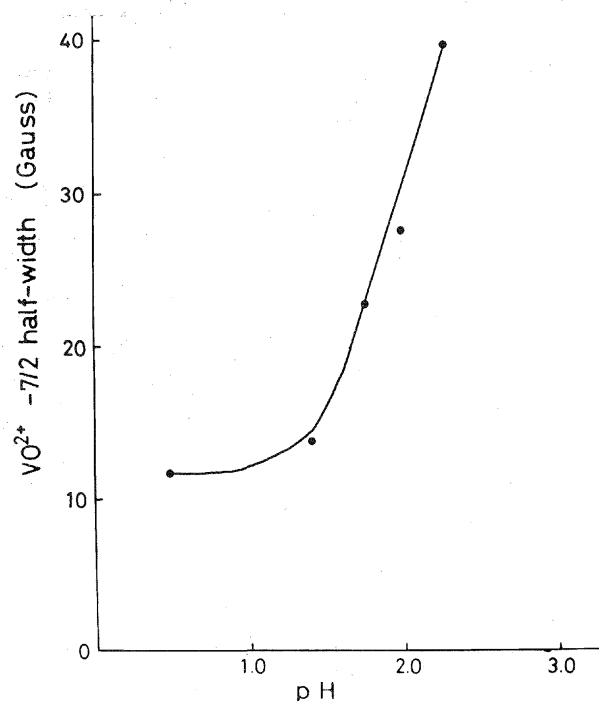
signet ring cells. However, most of each signet ring cell is, in fact, occupied by the vacuole itself, so the contents of the vacuole are almost equivalent to the contents of the cell.

ENERGETICS OF THE ACCUMULATION OF VANADIUM

Our comparison of pH values and levels of vanadium in the signet ring cells of three different species, as shown in Table 3, 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 H$^+$-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 (cf. Forgac, 1989, 1992; Nelson, 1992).

Therefore, we examined the signet ring cells of the ascidian *Ascidia sydneiensis samea* for presence of H$^+$-ATPase (Uyama *et al.*, 1994). The vacuolar-type H$^+$-ATPase is composed of several subunits, and subunits of 72 kDa and 57 kDa have been reported to be common to all eukaryotes examined. Antibodies prepared against the 72 kDa and 57 kDa subunits of a vacuolar-type H$^+$-ATPase from bovine chromaffin granules did indeed react with the vacuolar membranes of signet ring cells. Immunoblotting analysis confirmed that the antibodies reacted with specific antigens in ascidian blood cells. Furthermore, addition of bafilomycin A$_1$, a specific inhibitor of vacuolar-type H$^+$-ATPases (Bowman *et al.*, 1988), inhibited the pumping function of the vacuoles of signet ring cells, with resultant neutralization of the contents of the vacuoles, as shown in Fig. 10.

The acidification of vacuolar compartments in eukaryotic cells has been shown to have a number of important functions in neuronal and endocrine systems (Moriyama *et al.*, 1992) and to be important for the degradation of proteins (Yoshimori *et al.*, 1991). There appears to be a strong possibility that the activity of the enzyme is linked to the accumulation of vanadium in signet ring cells. We are trying to obtain direct evidence for such an association.

Fig. 9. Calibration curve for pH values estimated by the half-width of (-7/2)// line given by ESR spectrometry. pH values in the blood cells of vanadium-rich ascidians are noninvasively measurable over the range from 1.4 to 2.3 (Michibata *et al.*, 1991a).

PREPARATION OF MONOCLONAL ANTIBODIES AGAINST ASCIDIAN BLOOD CELLS

The identification of vanadocytes has been a subject of controversy, in part because of difficulties associated with morphological discrimination between several types of blood cells and in part because of our inadequate knowledge of cell
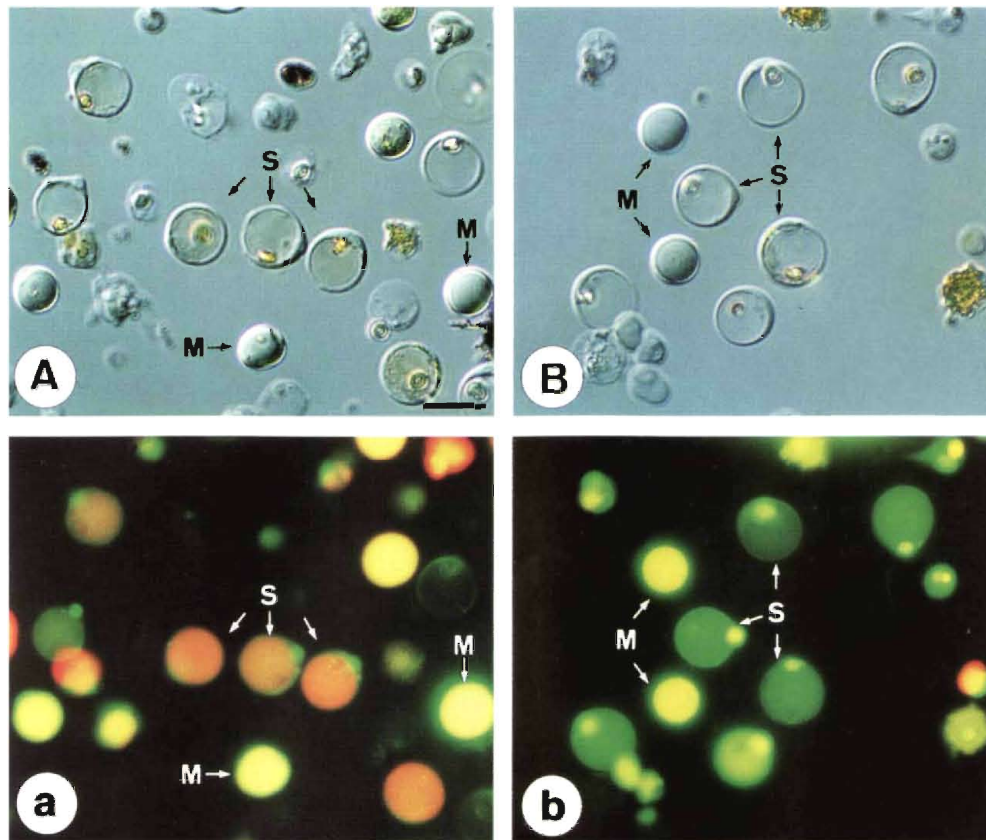
Fig. 10. Acidity of vacuoles of vanadocytes and inhibition of the acidification by bafilomycin A_1. Signet ring cells (vanadocytes) were revealed to emit a brilliant vermilion indicating acidic pH after incubation of blood cells of *Ascidia sydneiensis samea* with 2 μM acridine orange for 1 hr but the other types of blood cells did not indicate acidic pH. However, addition of 1 μM bafilomycin A_1, a specific inhibitor of vacuolar H^+-ATPase, caused vanadocytes neutralized (showing green fluorescence) with resultant inhibiting pump function of the H^+-ATPase. No changes in color of autonomous fluorescence emitted from morula cells were found with or without bafilomycin A_1 (Uyama *et al.*, 1994). S, signet ring cells (vanadocytes); M, morula cells. Scale bar indicates 10 μm.

lineages from the so-called stem cells to the peripheral cells. For example, Scippa *et al.* (1988) reported that the vacuolated and granular amoebocytes and a variety of compartment cells in *Phallusia mammillata* could be considered to be vanadocytes, in addition to the signet ring cells.

The establishment of reliable cell markers for the recognition of different types of blood cells seems, therefore, to be a necessary step if we are to clarify not only the function but also the lineage of each type of cell. 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 obtained, S4D5, was shown to react specifically with the vanadocytes not only from *A. sydneiensis samea* but also from two additional species, *A. gemmata* and *A. ahodori*. Immunoblotting analysis showed that this antibody recognized a single polypeptide of about 45 kDa from all three species. Recently, an other monoclonal antibody, C2A4, was prepared and it reacted specifically with the vacuolar amoebocytes and recognized a single band of a protein of about 200 kDa (Kaneko *et al.*, 1995), as shown in Fig. 11.

There are several reports on the localization of blood cells

in ascidian (Kalk, 1963a; Smith, 1970a; Ermak, 1975, 1976). According to these earlier reports, haematogenic activity is observed in three main areas of ascidians, 1) in the connective tissues around alimentary canal, 2) in the pharyngeal wall and transverse vessels of the branchial basket, and 3) in discrete nodules located in the body wall. Employing these monoclonal antibodies and the autonomous fluorescence emitted by each type of cell as cell markers (Wuchiyama and Michibata, 1995), we tried to identify the haematopoietic sites of vanadocytes. We found vanadocytes in the connective tissues around the alimentary canal, separate from morula cells, compartment cells and amoebocytes. It seems that the precursor cells of vanadocytes are formed in the connective tissues, while other types of blood cells are formed in the other sites (Kaneko *et al.*, 1995).

ACCUMULATION OF VANADIUM DURING EMBRYOGENESIS

Monoclonal antibodies are also useful tools with which to determine the time at which the accumulation of vanadium starts during embryogenesis. Since the amount of vanadium stored in embryos is beneath the limits of detection of
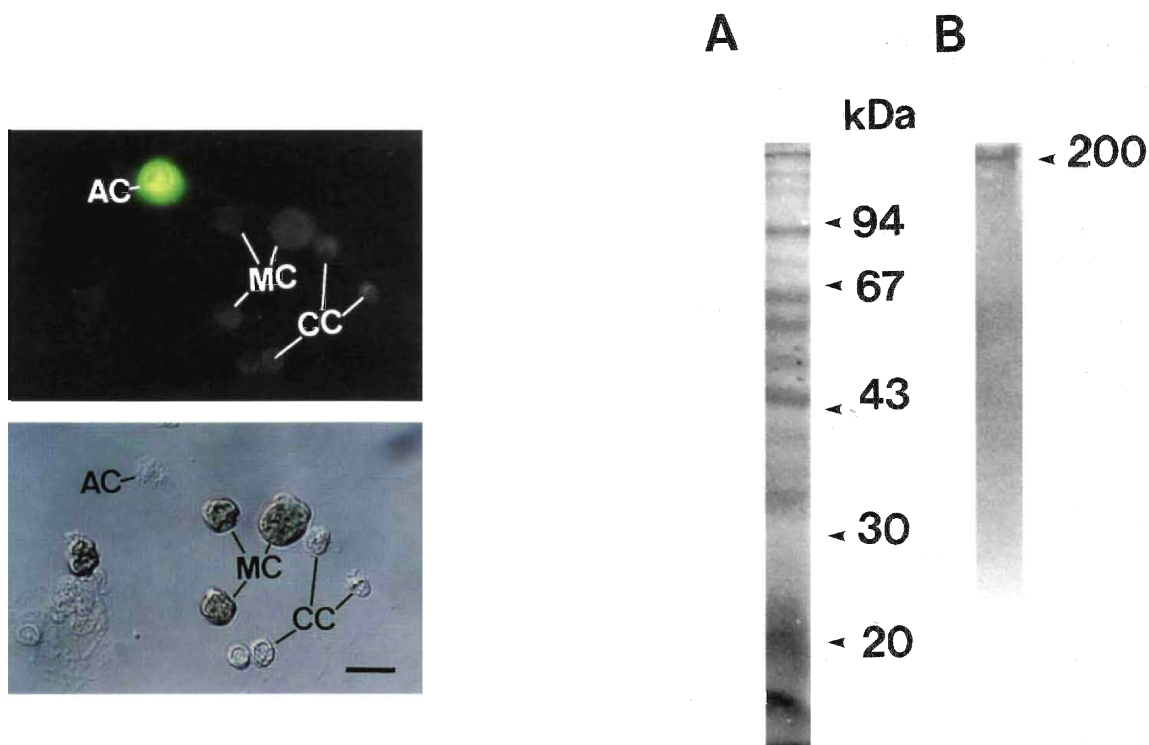
Fig. 11. A monoclonal antibody, C2A4, reacted specifically with the vacuolar amoebocytes among several types of blood cells of *Ascidia sydneiensis samea* (the left figures) and the antigen recognized by the monoclonal antibody was subjected to immunoblot analysis (the right figure). AC, vacuolar amoebocytes; MC, morula cells; CC, compartment cells. Scale bar indicates 10 μm. The antibody recognized a single band of a protein about 200 kDa, after treatment with endoglycosidase F (Kaneko *et al.*, 1995).
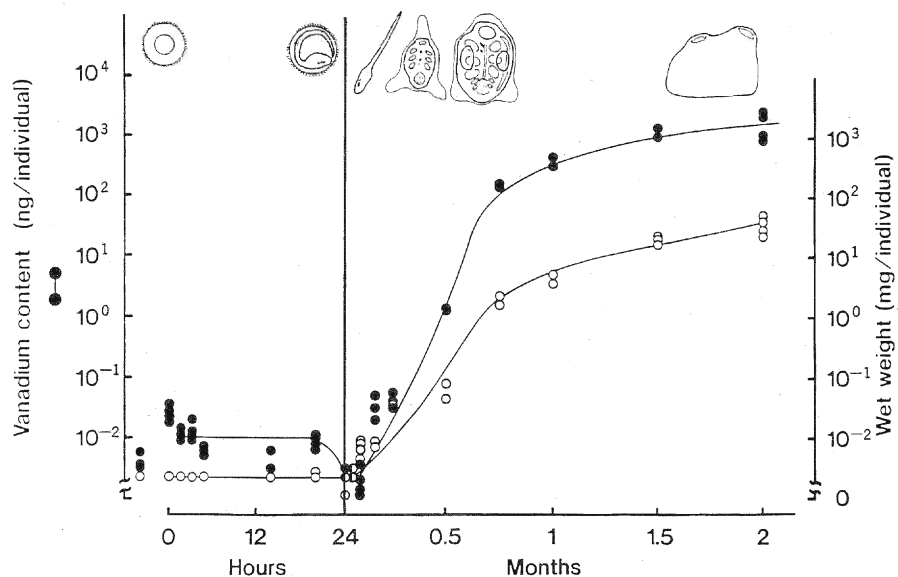

Fig. 12. Accumulation of vanadium during embryogenesis of the ascidian, *Ascidia sydneiensis samea*. To determine the time at which the accumulation of vanadium commences during embryogenesis, eggs and embryos were submitted for neutron-activation analysis. The levels of vanadium began to increase and the amount in larvae reached 2.3 μg/individual, which was about 600,000 times higher than the amount in unfertilized eggs (Michibata *et al.*, 1992). Furthermore, a vanadocyte-specific antigen, first became apparent in the body wall at the same time as the first significant accumulation of vanadium (Uyama *et al.*, 1993).

conventional analytical methods, such as atomic absorption spectrometry, there have been no reports of 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. Within two months, the amount accumulated in larvae was about 600,000 times greater than that in the unfertilized eggs of *A. sydneiensis samea* (Michibata *et al.*, 1992), as shown in Fig. 12. A vanadocyte-specific antigen, recognized by a monoclonal antibody specific to the signet ring cells, first became apparent in the body wall at the same time as the first significant accumulation of vanadium (Uyama *et al.*, 1993).

VANADIUM-BINDING PROTEINS

With respect to the pathway for the accumulation of vanadium from seawater, it seems likely that some proteins might participate, even though the results reported to date seem to indicate that vanadium is present in a free, noncomplexed form or is associated with low-molecular-weight components in ascidians. We are now trying to extract proteins that bind to or associate with vanadium in ascidians, assuming that there should be at least three types of proteins, namely, vanadium-transfer, vanadium-receptor and vanadium-channel proteins, as shown in Fig. 13.

The route for the accumulation of vanadium ions from seawater in the blood system has not yet been revealed. The uptake of vanadium ions was studied with radioactive vanadium ions (^{48}V). Previous studies were commonly designed to clarify the direct uptake of vanadium ions from the surrounding seawater and were, therefore, limited to an examination of how much vanadium was incorporated into some tissues (Goldberg *et al.*, 1951; Bielig *et al.*, 1963; Dingley *et al.*, 1981; Michibata *et al.*, 1991b) except a few reports (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 the low-molecular-weight substances rather than proteins (cf. Kustin and Robinson, 1995).

In general, heavy metal ions incorporated into the tissues of living organisms are known to bind to macromolecules such as proteins. We have, therefore, searched for vanadium-binding proteins in the blood cells of ascidians. Using a combination of SDS-PAGE and flameless atomic absorption spectrometry, we succeeded in isolating at least four different types of vanadium-binding proteins. Monoclonal antibodies against some of these proteins have been prepared as part of our efforts to determine whether the proteins are associated with vanadium-containing blood cells (Wuchiyama *et al.*, unpublished data).

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 (1955a, b, c, 1960) and Smith (1970a, b) proposed that the cellulose of the tunic might be produced by vanadocytes. Carlisle (1968) 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 (Stoecker, 1978; Rowley, 1983). However, most of the proposals put forward do not seem to be supported by sufficient evidence.

Recently, we observed an unexpected phenomenon of great interest. We found that the number of vanadocytes increased when ascidians were immersed in a solution that contained 10 mM or 20 mM NH$_4$Cl (Hayashi *et al.*, 1996). The increase in size of the population of signet ring cells might be interpreted as a self-defense response. Of course, the reason why the number of signet ring cells increased in response to NH$_4$Cl is still a matter of conjecture. However, attempts to characterize this phenomenon can be expected to promote more information about the unusual accumulation of vanadium by one class of marine organisms.
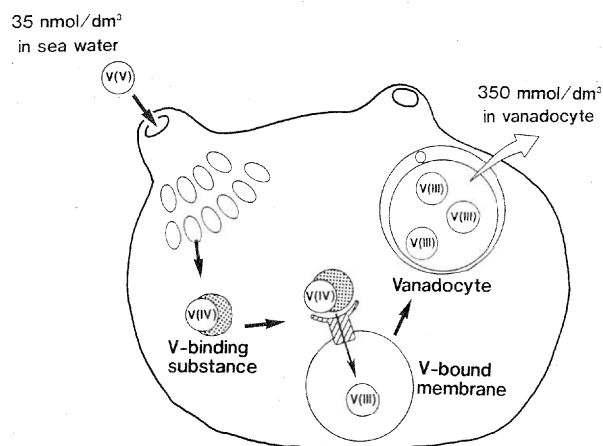

Fig. 13. Schematic representation of the pathway of accumulation of vanadium by ascidians. Vanadium in the +5 oxidation state dissolved in seawater is thought to be incorporated into the interior of the body via branchial baskets, where vanadium is reduced to the +4 oxidation state. Vanadium in the +4 oxidation state is further reduced to the +3 oxidation state and stored in the vacuole of vanadocyte. Some proteins, namely, vanadium transfer, vanadium receptor and vanadium channel proteins, are thought to be involved in the accumulation of vanadium during the course.

REFERENCES

Agudelo MI, Kustin K, McLeod GC (1983) The intracellular pH of the blood cells of the tunicate *Boltenia ovifera*. Comp Biochem Physiol 75A: 211–214

Anderson DH, Swinehart JH (1991) The distribution of vanadium and sulfur in the blood cells, and the nature of vanadium in the blood cells and plasma of the ascidian, *Ascidia ceratodes*. Comp Biochem Physiol 99A: 585–592

Bell MV, Pirie BJS, McPhail DB, Goodman BA, Falk-Petersen I-B, Sargent JR (1982) Contents of vanadium and sulphur in the blood cells of *Ascidia mentula* and *Ascidiella aspersa*. J Mar Biol Ass UK 62: 709–716

Bertrand D (1950) Survey of contemporary knowledge of biogeochemistry. 2. The biogeochemistry of vanadium. Bull Am Museum Natl History 94: 403–455

Bielig H-J, Bayer E, Califano L, Wirth L (1954) The vanadium containing blood pigment. II. Hemovanadin, a sulfate complex of trivalent vanadium. Publ Staz Zool Napoli 25: 26–66

Bielig H-J, Joste E, Pfleger K, Rummel W, Seifen E (1961a) Aufnahme und Verteilung von Vanadin bei der Tunicate *Phallusia mammillata* Cuvier. Hoppe-Seyler's Z Physiol Chem 325: 122–131

Bielig H-J, Joste E, Pfleger K, Rummel W (1961b) Sulfataufnahme bei *Phallusia mammillata* Cuvier. Verteilung und Schicksal von Sulfat- und Aminosäure-Schwefel im Blut (Untersuchungen über Hämovanadin, VI). Hoppe-Seyler's Z Physiol Chem 325: 132–145

Bielig H-J, Pfleger K, Rummel W, Seifen E (1961c) Sulfataufnahme bei *Ciona intestinalis* L. und deren Beeinflussung durch Vanadin. Hoppe-Seyler's Z Physiol Chem 327: 35–40

Bielig H-J, Pfleger K, Rummel W, de Vincentiis M (1963) Beginning of the accumulation of vanadium during the early development of the ascidian *Phallusia mammillata* Cuvier. Nature 197: 1223–1224

Bielig H-J, Bayer E, Dell HD, Robins G, Möllinger H, Rüdiger W (1966) Chemistry of Haemovanadium. Protides Biol Fluids 14: 197–204

Biggs WR, Swinehart JH (1976) Vanadium in selected biological systems. In "Metal Ions in Biological Systems Vol 6" Ed by Sigel H, Marcel Dekker Inc, New York, pp 141–196

Boas LV, Pessoa JC (1987) Vanadium. In "Comprehensive Coordination Chemistry Vol 2" Pergamon Press, Oxford, pp 453–583

Boeri E (1952) The determination of hemovanadin and its oxidation potential. Arch Biochem Biophys 37: 449–456

Boeri E, Ehrenberg A (1954) On the nature of vanadium in vanadocytes hemolysate from ascidians. Arch Biochem Biophys 50: 404–416

Botte L, Scippa S, de Vincentiis M (1979a) Content and ultrastructural localization of transitional metals in ascidian ovary. Dev Growth Differ 21: 483–491

Botte LS, Scippa S, de Vincentiis M (1979b) Ultrastructural localization of vanadium in the blood cells of Ascidiacea. Experientia 35: 1228–1230

Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of

inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci USA 85: 7972–7976

Boyd DW, Kustin K (1985) Vanadium: A versatile biochemical effector with an elusive biological function. Adv Inorg Biochem 6: 311–365

Brand SG, Hawkins CJ, Parry DL (1987) Acidity and vanadium coordination in vanadocytes. Inorg Chem 26: 627–629

Brand SG, Hawkins CJ, Marshall AT, Nette GW, Parry DL (1989) Vanadium chemistry of ascidians. Comp Biochem Physiol 93B: 425–436

Bruening RC, Oltz EM, Furukawa J, Nakanishi K, Kustin K (1985) Isolation and structure of tunichrome B-1, a reducing blood pigment from the tunicate *Ascidia nigra* L. J Am Chem Soc 107: 5298–5300

Bulls AR, Pippin CG, Hahn FE, Raymond KN (1990) Synthesis and characterization of a series of vanadium-tunichrome B1 analogues. Crystal structure of a tris (catecholamide) complex of vanadium. J Am Chem Soc 112: 2627–2632

Califano L, Boeri E (1950) Studies on haemovanadin. III. Some physiological properties of haemovanadin, the vanadium compound of the blood of *Phallusia mammillata* Cuv. J Exp Zool 27: 253–256

Carlisle DB (1968) Vanadium and other metals in ascidians. Proc Royal Soc B 171: 31–42

Cantacuzène J, Tchekirian A (1932) Sur la présence de vanadium chez certains tuniciers. Compt Rend Acad Sci Paris 195: 846–849

Carlson RMK (1975) Nuclear magnetic resonance spectrum of living tunicate blood cells and the structure of the native vanadium chromogen. Proc Natl Acad Sci USA 72: 2217–2221

Chasteen ND (1981) Vanadyl(IV) EPR spin probes. Inorganic and biochemical aspects. Biol Magnetic Resonance 3: 53–119

Chasteen ND (1983) The biochemistry of vanadium. Structure Bonding 53: 105–138

Ciereszko LS, Ciereszko EM, Harris ER, Lane CA (1963) Vanadium content of some tunicates. Comp Biochem Physiol 8: 137–140

Cole PC, Eckert JM, Williams KL (1983) The determination of dissolved and particular vanadium in sea water by X-ray fluorescence spectrometry. Anal Chim Acta 153: 61–67

Collier RW (1984) Particulate and dissolved vanadium in the North Pacific Ocean. Nature 309: 441–444

Danskin GP (1978) Accumulation of heavy metals by some solitary tunicates. Can J Zool 56: 547–551

Dingley AL, Kustin K, Macara IG, McLeod GC (1981) Accumulation of vanadium by tunicate blood cells occurs via a specific anion transport system. Biochim Biophys Acta 649: 493–502

Dingley AL, Kustin K, Macara IG, McLeod GC, Roberts MF (1982) Vanadium-containing tunicate blood cells are not highly acidic. Biochim Biophys Acta 720: 384–389

Endean R (1955a) Studies of the blood and tests of some Australian ascidians. I. The blood of *Pyura stolonifera* (Heller). Austr J Mar Freshwat Res 6: 35–59

Endean R (1955b) Studies of the blood and tests of some Australian ascidians. II. The test of *Pyura stolonifera* (Heller). Austr J Mar Freshwat Res 6: 139–156

Endean R (1955c) Studies of the blood and tests of some Australian ascidians. III. The formation of the test of *Pyura stolonifera* (Heller). Austr J Mar Freshwat Res 6: 157–164

Endean R (1960) The blood cells of the ascidian, *Phallusia mammillata*. Quart J Microscop Sci 101: 177–197

Ermak TH (1975) Autoradiographic demonstration of blood cell renewal in *Styela clava* (Urochordata: Ascidiacea). Experientia 31: 837–839

Ermak TH (1976) The hematogenic tissues of tunicate. In "Phylogeny of Thymus and Bone Marrow-Bursa Cells" Ed by Wright RK, Cooper EL, Elsevier/ North-Holland Biomed Press, The Netherlands, pp 45–56

Forgac M (1989) Structure and function of the vacuolar class of ATP-driven proton pumps. Physiol Rev 69: 765–796

Forgac M (1992) Structure, function and regulation of the coated vesicle V-ATPase. J Exp Biol 172: 155–169

Frank P, Carlson RMK, Hodgson KO (1986) Vanadyl ion EPR as a non-invasive probe of pH in intact vanadocytes from Ascidia ceratodes. Inorg Chem 25: 470–478

Frank P, Hedman B, Carlson RK, Tyson TA, Row AL, Hodgson KO (1987) A large reservoir of sulfate and sulfonate resides within plasma cells from Ascidia ceratodes, revealed by X-ray absorption near-edge structure spectroscopy. Biochemistry 26: 4975–4979

Frank P, Hedman B, Carlson RMK, Hodgson KO (1994) Interaction of vanadium and sulfate in blood cells from the tunicate Ascidia ceratodes: Observations using X-ray absorption edge structure and EPR spectroscopies. Inorg Chem 33: 3794–3803

Frank P, Kustin K, Robinson WE, Linebaugh L, Hodgson KO (1995) Nature and ligation of vanadium within whole blood cells and Henze solution from the tunicate Ascidia ceratodes, as investigated by using X-ray absorption spectrometry. Inorg Chem 34: 5942–5949

Goldberg ED, McBlair W, Taylor KM (1951) The uptake of vanadium by tunicates. Biol Bull 101: 84–94

Goodbody I (1974) The physiology of ascidians. Adv Mar Biol 12: 1–149

Hawkins CJ, Parry DL, Pierce C (1980a) Chemistry of the blood of the ascidian Podoclavella moluccensis. Biol Bull 159: 669–680

Hawkins CJ, Merefield PM, Parry DL, Biggs WR, Swinehart JH (1980b) Comparative study of the blood plasma of the ascidians Pyura stolonifera and Ascidia ceratodes. Biol Bull 159: 656–668

Hawkins CJ, Kott P, Pary DL, Swinehart JH (1983) Vanadium content and oxidation state related to ascidian phylogeny. Comp Biochem Physiol 76B: 555–558

Hayashi M, Nose Y, Uyama T, Moriyama Y, Michibata H (1996) Rapid increases in number of vanadocytes in the vanadium-rich ascidian, Ascidia sydneiensis samea, upon treatment of live animals with NH$_4$Cl. J Exp Zool 275: 1–7

Henze M (1911) Untersuchungen über das Blut der Ascidien. I. Mitteilung. Die Vanadiumverbindung der Blutkörperchen. Hoppe-Seyler's Z Physiol Chem 72: 494–501

Henze M (1912) Untersuchungen über das Blut der Ascidien. II. Mitteilung. Hoppe-Seyler's Z Physiol Chem 79: 215–228

Henze M (1913) Über das Vorkommen freier Schwefelsäure im Mantel von Ascidia mentula. Hoppe-Seyler's Z Physiol Chem 88: 345–346

Henze M (1932) Über das Vanadiumchromogen des Ascidienblutes. Hoppe-Seyler's Z Physiol Chem 213: 125–135

Hirata J, Michibata H (1991) Valency of vanadium in the vanadocytes of Ascidia gemmata separated by density-gradient centrifugation. J Exp Zool 257: 160–165

Ishii T, Nakai I, Numako C, Okoshi K, Otake T (1993) Discovery of a new vanadium accumulator, the fan worm Pseudopotamilla occelata. Naturwissenschaften 80: 268–270

Kalk M (1963a) Intracellular sites of activity in the histogenesis of tunicate vanadocytes. Quart J Microscop Sci 104: 483–493

Kalk M (1963b) Absorption of vanadium by tunicates. Nature 198: 1010–1011

Kanamori K, Michibata H (1994) Raman spectroscopic study of the vanadium and sulphate in blood cell homogenates of the ascidian, Ascidia gemmata. J Mar Biol Ass UK 74: 279–286

Kaneko A, Uyama T, Moriyama Y, Michibata H (1995) Localization, with monoclonal antibodies and by detection of autonomous fluorescence, of blood cells in the tissues of the vanadium-rich ascidian, Ascidia sydneiensis samea. Zool Sci 12: 733–739

Kobayashi S (1935) On the presence of vanadium in certain Pacific ascidians. Sci Rep Tohoku Univ 4th Ser 18: 185–193

Kustin K, Levine DS, McLeod GC, Curby WA (1976) The blood of Ascidia nigra: Blood cell frequency distribution, morphology, and the distribution and valence of vanadium in living blood cells. Biol Bull 150: 426–441

Kustin K, McLeod GC, Gilbert TR, Briggs 4th LBR (1983) Vanadium and other metal ions in the physiological ecology of marine organisms. Structure Bonding 53: 139–160

Kustin K, Robinson WE (1995) Vanadium transport in animal systems. In "Metal Ions in Biological Systems Vol 31" Ed by Sigel H, Sigel A, Marcel Dekker Inc, New York, pp 511–542

Lane DJW, Wilkes SL (1988) Localization of vanadium, sulphur and bromine within the vanadocytes of Ascidia mentula Müller: A quantitative electron probe X-ray microanalytical study. Acta Zool (Stockh) 69: 135–145

Lee S, Kustin K, Robinson WE, Frankel RB, Spartalian K (1988) Magnetic properties of tunicate blood cells. I. Ascidia nigra. Inorg Biochem 33: 183–192

Lee S, Nakanishi K, Kustin K (1990) The intracellular pH of tunicate blood cells: Ascidia ceratodes whole blood, morula cells, vacuoles and cytoplasm. Biochim Biophys Acta 1033: 311–317

Levine EP (1961) Occurrence of titanium, vanadium, chromium, and sufuric acid in the ascidian Eudistoma ritteri. Science 133: 1352–1353

Lybing S (1953) The valence of vanadium in hemolysates of blood from Ascidia obliqua Alder. Arkiv Kemi 6: 261–269

Macara IG, McLeod GC, Kustin K (1979a) Vanadium in tunicates: oxygen-binding studies. Comp Biochem Physiol 62A: 821–826

Macara IG, McLeod GC, Kustin K (1979b) Tunichromes and metal ion accumulation in tunicate blood cells. Comp Biochem Physiol 63B: 299–302

Macara IG, McLeod GC, Kustin K (1979c) Isolation, properties and structural studies on a compound from tunicate blood cells that may be involved in vanadium accumulation. Biochem J 181: 457–465

McLeod GC, Ladd KV, Kustin K, Toppen DL (1975) Extraction of vanadium(V) from seawater by tunicate: A revision of concepts. Limnol Oceanograph 20: 491–493

Michibata H (1984) Comparative study on amounts of trace elements in the solitary ascidians, Ciona intestinalis and Ciona robusta. Comp Biochem Physiol 78A: 285–288

Michibata H (1989) New aspects of accumulation and reduction of vanadium ions in ascidians, based on concerted investigation for both a chemical and biological viewpoint. Zool Sci 6: 639–681

Michibata H (1993) The mechanism of accumulation of high levels of vanadium by ascidians from seawater: Biophysical approaches to a remarkable phenomenon. Adv Biophys 29: 103–131

Michibata H, Sakurai H (1990) Vanadium in ascidians. In" Vanadium in Biological Systems" Ed by Chasteen ND, Kluwer Acad Publ, Dortrecht, pp 153–171

Michibata H, Terada T, Anada N, Yamakawa K, Numakunai T (1986) The accumulation and distribution of vanadium, iron, and manganese in some solitary ascidians. Biol Bull 171: 672–681

Michibata H, Hirata J, Uesaka M, Numakunai T, Sakurai H (1987) Separation of vanadocytes: Determination and characterization of vanadium ion in the separated blood cells of the ascidian, Ascidia ahodori. J Exp Zool 244: 33–38

Michibata H, Hirata J, Terada T, Sakurai H (1988) Autonomous fluorescence of ascidian blood cells with special reference to identification of vanadocytes. Experientia 44: 906–907

Michibata H, Uyama T, Hirata J (1990) Vanadium containing cells (vanadocytes) show no fluorescence due to the tunichrome in the ascidian Ascidia sydneiensis samea. Zool Sci 7: 55–61

Michibata H, Iwata, Y, Hirata, J (1991a) Isolation of highly acidic and vanadium-containing blood cells from among several types of blood cell from Ascidiidae species by density gradient centrifugation. J Exp Zool 257: 306–313

Michibata H, Seki Y, Hirata J, Kawamura M, Iwai K, Iwata R, Ido T (1991b) Uptake of [48]V-labeled vanadium by subpopulations of

blood cells in the ascidian, *Ascidia gemmata*. Zool Sci 8: 447–452

Michibata H, Uchiyama J, Seki Y, Numakunai T, Uyama T (1992) Accumulation of vanadium during embryogenesis in the vanadium-rich ascidian *Ascidia gemmata*. Biol Trace Element Res 34: 219–223

Moriyama Y, Maeda M, Futai M (1992) The role of V-ATPase in neuronal and endocrine system. J Exp Biol 172: 171–178

Nelson N (1992) The vacuolar H^+-ATPase - one of the most fundamental ion pumps in nature. J Exp Biol 172: 19–27

Noddack I, Noddack W (1939) Die Häufigkeiten der Schwermetalle in Meerestieren. Arkiv Zool 32A: 1–35

Pirie BJS, Bell MV (1984) The localization of inorganic elements, particularly vanadium and sulphur, in haemolymph from the ascidians *Ascidia mentula* (Müller) and *Ascidiella aspersa* (Müller). J Exp Mar Biol Ecol 74: 187–194

Robinson WE, Agudelo MI, Kustin K (1984) Tunichrome content in the blood cells of the tunicate, *Ascidia callosa* Stimpson, as an indicator of vanadium distribution. Comp Biochem Physiol 78A: 667–673

Roman DA, Molina J, Rivera L (1988) Inorganic aspects of the blood chemistry of ascidians. Ionic composition, and Ti, V, and Fe in the blood plasma of *Pyura chilensis* and *Ascidia dispar*. Biol Bull 175: 154–166

Rowley AF (1982) The blood cells of *Ciona intestinalis*: An electron probe X-ray microanalytical study. J Mar Biol Ass UK 62: 607–620

Rowley AF (1983) Preliminary investigations on the possible antimicrobial properties of tunicate blood cell vanadium. J Exp Zool 227: 319–322

Rummel W, Bielig H-J, Forth W, Pfleger K, Rudiger W, Seifen E (1966) Absorption and accumulation of vanadium by tunicates. Protides Biol Fluids 14: 205–210

Ryan DE, Ghatlia ND, McDermott AE, Turro NJ, Nakanishi K (1992) Reactivity of tunichromes: Reduction of vanadium(V) and vanadium(IV) to vanadium(III) at neutral pH. J Am Chem Soc 114: 9659–9660

Scippa S, Botte L, de Vincentiis M (1982) Ultrastructure and X-ray microanalysis of blood cells of *Ascidia malaca*. Acta Zool (Stockh) 63: 121–131

Scippa S, Botte L, Zierold K, de Vincentiis M (1985) X-ray microanalytical studies on cryofixed blood cells of the ascidian *Phallusia mammillata*. I. Elemental composition of morula cells. Cell Tissue Res 239: 459–461

Scippa S, Zierold K, de Vincentiis M (1988) X-ray microanalytical studies on cryofixed blood cells of the ascidian *Phallusia mammillata*. II. Elemental composition of the various blood cell types. J Submicroscop Cytol Pathol 20: 719–730

Smith MJ (1970a) The blood cells and tunic of ascidian *Halocynthia aurantium* (Pallas). I. Hematology, tunic morphology, and partition of cells between blood and tunic. Biol Bull 138: 345–378

Smith MJ (1970b) The blood cells and tunic of ascidian *Halocynthia aurantium* (Pallas). II. The histochemistry of the blood cells and tunic. Biol Bull 138: 379–388

Smith MJ, Ryan DE, Nakanishi K, Frank P, Hodgson, KO (1995) Vanadium in ascidians and the chemistry of tunichromes. In "Metal Ions in Biological Systems" Ed by Sigel H, Sigel A, Marcel Dekker Inc, New York, pp 423–490

Stoecker D (1978) Resistance of a tunicate to fouling. Biol Bull 155: 615–626

Sugimura Y, Suzuki Y, Miyake Y (1978) Chemical forms of minor metallic elements in the ocean. J Oceanogr Soc Japan 34: 93–96

Swinehart JH, Biggs WR, Halko DJ, Schroeder NC (1974) The vanadium and selected metal contents of some ascidians. Biol Bull 146: 302–312

Tullius TD, Gillum WO, Carlson RMK, Hodgson KO (1980) Structural study of the vanadium complex in living ascidian blood cells by X-ray absorption spectrometry. J Am Chem Soc 102: 5670–5676

Uyama T, Nishikata T, Satoh N, Michibata H (1991) Monoclonal antibody specific to signet ring cells, the vanadocytes of the tunicate, *Ascidia sydneiensis samea*. J Exp Zool 259: 196–201

Uyama T, Uchiyama J, Nishikata T, Satoh N, Michibata H (1993) The accumulation of vanadium and manifestation of an antigen recognized by a monoclonal antibody specific to vanadocytes during embryogenesis in the vanadium-rich ascidian, *Ascidia sydneiensis samea*. J Exp Zool 265: 29–34

Uyama T, Moriyama Y, Futai M, Michibata H (1994) Immunological detection of a vacuolar-type H^+-ATPase in the vanadocytes of the ascidian *Ascidia sydneiensis samea*. J Exp Zool 270: 148–154

Vinogradov AP (1934) Distribution of vanadium in organisms. Compt Rend Acad Sci URSS 3: 454–459

Webb DA (1939) Observations on the blood of certain ascidians, with special reference to the biochemistry of vanadium. J Exp Biol 16: 499–523

Webb DA (1956) The blood of tunicates and the biochemistry of vanadium. Publ Staz Zool Napoli 28: 273–288

Wever R, Kustin K (1990) Vanadium: A biologically relevant element. Adv Inorg Chem 35: 81–115

Wright RK (1981) Urochordata. In "Invertebrate Blood Cells Vol 2" Ed by Ratcliffe NA, Rowley AF, Academic Press, London, pp 565–626

Wuchiyama J, Michibata H (1995) Classification, based on autonomous fluorescence, of the blood cells of several ascidians that contain high levels of vanadium. Acta Zool (Stockholm) 76: 51–55

Yoshimori T, Yamamoto A, Moriyama Y, Futai M, Tashiro Y (1991) Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J Biol Chem 266: 17707–17712