# Scanning X-ray Microscopy of Living and Freeze-Dried Blood Cells in Two Vanadium-Rich Ascidian Species, Phallusia mammillata and Ascidia sydneiensis samea

Authors: Ueki, Tatsuya, Takemoto, Kuniko, Fayard, Barbara, Salomé, Murielle, Yamamoto, Akitsugu, et al.

Source: Zoological Science, 19(1) : 27-35

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.27

ZOOLOGICAL SCIENCE **19**: 27–35 (2002)


# Scanning X-ray Microscopy of Living and Freeze-Dried Blood Cells in Two Vanadium-Rich Ascidian Species, *Phallusia mammillata* and *Ascidia sydneiensis samea*

Tatsuya Ueki[1], Kuniko Takemoto[2], Barbara Fayard[3], Murielle Salomé[3], Akitsugu Yamamoto[4], Hiroshi Kihara[2], Jean Susini[3], Silvia Scippa[5], Taro Uyama[1] and Hitoshi Michibata[1*]

[1]*Marine Biological Laboratory, Graduate School of Science, Hiroshima University, Hiroshima 722-0073, Japan*
[2]*Department of Physics, Kansai Medical University, Uyamahigashi 18-89, Hirakata, Osaka 573-1136, Japan*
[3]*European Synchrotron Radiation Facility (ESRF), BP220, 38043 Grenoble cedex, France*
[4]*Department of Physiology-I, Kansai Medical University, Fumizono 10-15, Moriguchi, Osaka 570-8506, Japan*
[5]*Department of Genetics, General and Molecular Biology, Faculty of Science, University of Naples, via Mezzocannone 8, 80134 Naples, Italy and Stazione Zoologica 'Anton Dohrn', Villa Comunale I 80121 Naples, Italy*

**ABSTRACT**—Some ascidians (sea squirts) accumulate the transitional metal vanadium in their blood cells at concentrations of up to 350 mM, about $10^7$ times its concentration found in seawater. There are approximately 10 different types of blood cell in ascidians. The identity of the true vanadium-containing blood cell (vanadocyte) is controversial and little is known about the subcellular distribution of vanadium. A scanning x-ray microscope installed at the ID21 beamline of the European Synchrotron Radiation Facility to visualize vanadium in ascidian blood cells. Without fixation, freezing or staining realized the visualization of vanadium localized in living signet ring cells and vacuolated amoebocytes of two vanadium-rich ascidian species, *Phallusia mammillata* and *Ascidia sydneiensis samea*. A combination of transmission and fluorescence images of signet ring cells suggested that in both species the vacuoles contain vanadium.

**Key words**: vanadium, ascidians, vanadocytes, X-Ray microscopy, synchrotron radiation

## INTRODUCTION

Ninety years ago, Henze discovered extremely high levels of vanadium in the blood cells (coelomic cells) of a Mediterranean ascidian, *Phallusia mammillata*, at Statione Zoologica 'Anton Dohrn', Naples, Italy (Henze, 1911). This discovery has attracted the interdisciplinary attention of chemists, physiologists, and biochemists, because of the strong interest in the extraordinarily high levels of vanadium never before reported in other organisms (for Review, see Kustin 1998; Michibata, 1993, 1996; Michibata and Kanamori, 1998; Michibata and Sakurai, 1990; Michibata *et al*., 1998, 2001). Using thermal neutron-activation analysis, we determined the concentration of metals in several different species of ascidians. In the family Ascidiidae, several species accumulate vanadium in blood cells at concentrations of up to 350 mM, corresponding to about $10^7$ times the vanadium concentration of seawater (Michibata *et al*., 1986, 1991).

Ascidian blood cells are classified into about ten different types based on morphology. One cell type, vacuolated cells, can be further divided into at least four different types: morula cells, signet ring cells, compartment cells and vacuolated amoebocytes (Wright, 1981). For many years, morula cells were thought to be the vanadocytes, or vanadium-containing cells (Webb, 1939; Endean, 1960; Kalk, 1963a, b). 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 charac-

* Corresponding author: Tel. +81-848-44-1143;
FAX. +81-848-44-5914.
E-mail: hmichi@sci.hiroshima-u.ac.jp

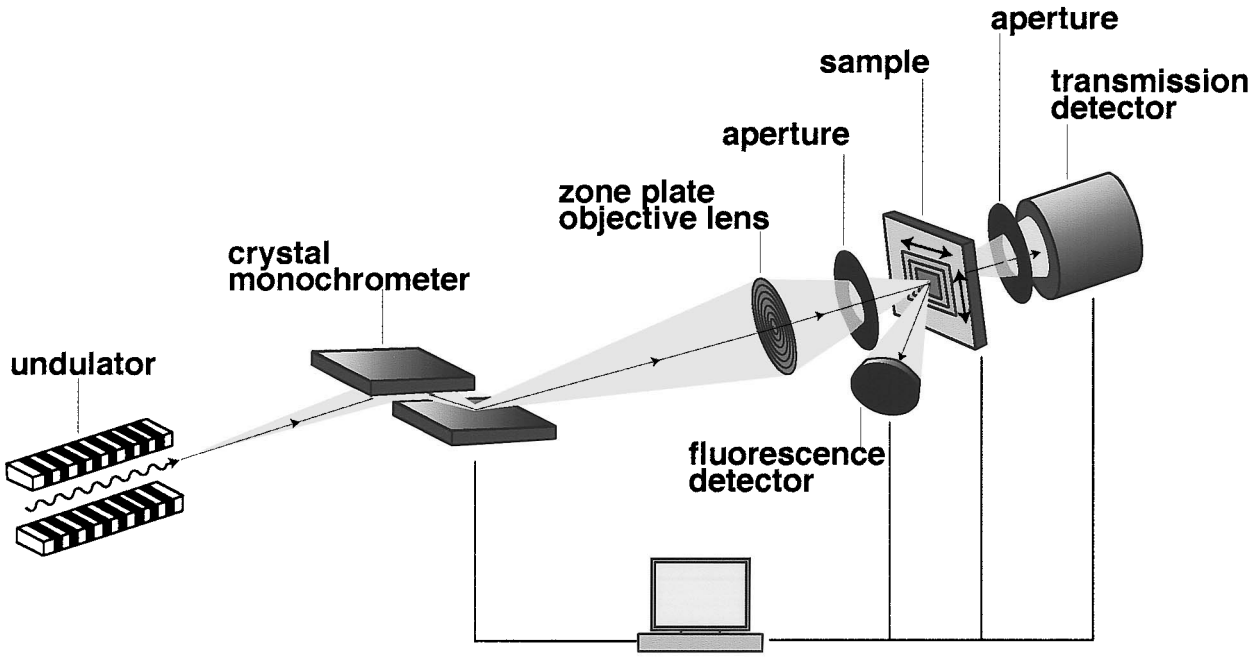


**Fig. 1.** Diagram of the scanning x-ray microscope installed at the European Synchrotron Radiation Facility ID21 beamline. The x-ray beam from the undulator at the storage ring is monochromated by a fixed exit double crystal monochromator and focused on the sample by a zone plate objective lens and a small pinhole aperture. The beam size is approximately 0.5 μm×0.5 μm at the sample, and the sample is scanned using a piezo driven flexure giving a scan area of 100×100 $\mu m^2$. A photodiode detector detects the transmitted x-ray and a germanium detector located near the sample detects the emitted x-ray fluorescence.

teristic x-ray due to vanadium was detected not from morula cells, but from vacuolated and granular amoebocytes, signet ring cells and type-II compartment cells (Botte *et al*., 1979; Scippa *et al*., 1982a, 1982b, 1985, 1988; Rowley, 1982). 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* (Michibata *et al*., 1987). 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, bivacuolated cells and type-II compartment cells, were stained brown, indicating the existence of vanadium (Nette *et al*., 1999). 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 (Takemoto *et al*., 2000). 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) ID 21 beamline (Fig. 1). This microscope is dedicated to x-ray imaging and spectromicroscopy in the 0.2 to 7 keV range, in both absorption and fluorescence modes (Susini *et al*., 1996, 2000). This energy range covers the K-absorption edges of medium-light elements (*e.g.*, Al, Si, P, S, Cl, K, Ca, Sc, Ti, V, Cr, Mn) and provides spatial resolution of about 0.5 μm. Because x-ray microscopy in this energy range is useful for observing hydrated specimens up to 10 μm thick, we used this technology to observe vanadium in living ascidian blood cells.

We here report the observation of blood cells from *Phallusia mammillata* and *Ascidia sydneiensis samea*. These species contain vanadium ions in their blood cells at concentrations of 19 mM and 13 mM, respectively (Michibata *et al*., 1986). We describe results from freeze-dried and wet living specimens of both species.

## MATERIALS AND METHODS

### Blood cell preparation

Adults of two species of ascidians, *Phallusia mammillata* and *Ascidia sydneiensis samea*, were collected at the Gulf of Naples, Italy, and at the Gulf of Yamada, Iwate, Japan, respectively. Blood was extracted and mixed with F12 medium (ICN Biomedicals, Inc., USA) containing 0.5 M NaCl. Blood cells were isolated by centrifugation at 300×g for 10 min at 4°C, resuspended in the same medium and stored at 4°C. Some batches of blood-cell suspensions were mixed with low-melting-point agarose gel at a final concentration of 0.5% and kept at 4°C until use. The blood cells remained alive and healthy in these conditions for at least one

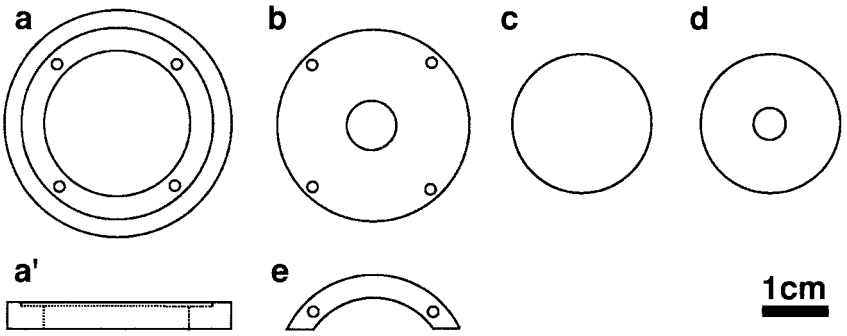


**Fig. 2.** A sample holder and films used for the observation of blood cells by x-ray microscopy. (a) Stainless steel sample holder. (a') Sample holder viewed from the side. (b) Stainless-steel support with a hole, 8 mm in diameter, at the center. (c) Polyethylene terephthalate (PET) film with a diameter of 22 mm and thickness of 23 μm. (d) Polyimide film with a diameter of 22 mm and thickness of 10 μm. A hole 6 mm in diameter was made at the center of the polyimide film. Freeze-dried blood cells mounted on a copper mesh were attached to a stainless steel support (b) with glue, and fixed to the sample holder (a) with two parts (e) and four screws. Wet living blood cells were placed between two PET films (c) with the polyimide film (d) as a spacer. The three films were attached with silicon grease, placed between two stainless steel supports (b), and fixed to the sample holder (a).

week.

To observe freeze-dried specimens by electron microscopy, blood cell suspensions were placed on a copper mesh covered by plastic film with small holes (Micro Grid 150-B, Ohken Shoji, Japan), and left for about 10 min. The mesh was then dipped in isopentane at –150°C and vacuum-dried in a freeze-drying apparatus (Spectrolab, Inc.) for at least 4 hours at $5 \times 10^{-1}$ mbar and -65°C. The dried samples were stored at room temperature until observation. The copper mesh was attached to the sample holder (Fig. 2) before observation by x-ray microscopy.

To observe wet, living blood cells, one drop of a blood cell suspension in solution or in gel was placed between two 23-μm polyethylene terephthalate (PET) films along with a 10-μm donut-shaped spacer of polyimide film (Fig. 2). The three films were attached together by silicon grease, immediately placed in a stainless-steel sample holder (Fig. 2) and observed with the x-ray microscope.

Prior to x-ray microscopy, several major blood cell types can be identified by their morphological characteristics using light microscopy. Signet ring cells are spherical cells with a large fluid-filled vacuole surrounded by a small amount of cytoplasm. Compartment cells are spherical and have one large vacuole and several small vacuoles, in which granules are apparent. Morula cells contain several greenish globules and resemble morula-stage embryos, while vacuolar amoebocytes have several pseudopods and small vacuoles (Wright, 1981; Michibata *et al*., 1990; Wuchiyama *et al*., 1995).

**X-ray microscopy**

The scanning transmission x-ray microscope used in this study is operated at the ID21 x-ray microscopy beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France; http://www.esrf.fr/). An outline of the optical units is shown in Fig. 1. The x-ray beam has a fixed exit double crystal monochromator, and two Si<111> crystals provide a monochromatic beam. A Fresnel zone plate was used to focus the x-ray beam down to a microprobe size of 0.5×0.5 $\mu m^2$. 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

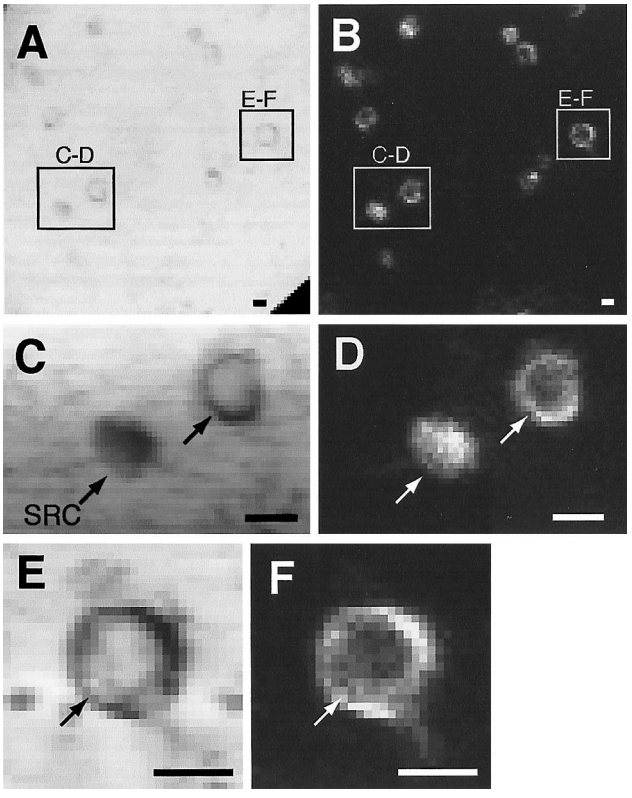


**Fig. 3.** Freeze-dried *Phallusia mammillata* blood cells as shown by x-ray microscopy in transmission (A, C, E) and fluorescence mode for vanadium concentration (B, D, F). A and B show the same field of view; C and D, and E and F are taken from the fields indicated by boxes in A and B. Transmission and fluorescence images were taken by scanning cells with a 5.500 keV x-ray at 2 μm×2 μm resolution for 100 ms per pixel for A and B, or at 0.5 μm×0.5 μm resolution for longer periods for C and F. Vanadium was located in signet ring cells (SRC, shown by arrows) and in some case at the periphery of the cell. Each scale bar =5 μm.

control for the normalization of signals. To obtain diffraction phase contrast images of blood cells, we placed a 50-μm aperture just in front of the photodiode detector.

## RESULTS

An x-ray beam with an energy of 5.500 keV was used to obtain good yields of vanadium transmission and fluorescence. At this energy, vanadium ions at the +3, +4, and +5 redox states are detected simultaneously.

### Freeze-dried specimens of *Phallusia mammillata* and *Ascidia sydneiensis samea*

Specimens were freeze-dried by a method that is frequently used at the ID21 beamline to observe cells such as cultured mammalian cells or yeast cells. *P. mammillata* blood cells were collected, attached to a copper grid with small plastic films and freeze-dried. Because sodium salts accumulate on the grid after freeze-drying, it was difficult to identify blood cell types. We identified several signet ring cells and observed them by x-ray microscopy. A resolution of up to 0.5×0.5 $\mu m^2$ was achieved, which is comparable to the beam size of the x-ray (Fig. 3). Vanadium was detected in all of the signet ring cells examined. In some cells, vanadium was localized at the periphery; this seemed to be caused by a leakage of vacuole contents during the freeze-drying procedure.

*A. sydneiensis samea* blood cells were similarly prepared and observed by x-ray microscopy (Fig. 4). In this species, we observed a uniform distribution of vanadium in signet ring cells in many samples, but we could not exclude the possibility of artifacts such as vanadium redistribution or leakage during freeze-drying. The giant cells did not contain vanadium, which is consistent with results obtained from density-gradient centrifugation and atomic absorption spectrophotometry (Michibata *et al.*, 1991).

### Living specimens of *Phallusia mammillata* and *Ascidia sydneiensis samea*

To obtain images without subjecting cells to freezing or fixation, *P. 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. Despite the presence of high concentrations of sodium chloride and low-melting-point agarose in these media, cells were readily observed. Blood cells attached to the plastic film immediately, but sometimes became detached during observation because the sample holder was set perpendicularly. Cells suspended in gel did not detach.

We first used a normal photodiode detector (Fig. 5A-C) to observe vanadium-containing signet ring cells. Because we were unable to obtain transmission images of blood cells that did not contain vanadium, we attached a 50-μm-diameter aperture to the photodiode detector, and succeeded in visualizing all types of blood cell (Fig. 5D-I and Fig. 6A-C). All subsequent transmission images were taken using this configuration. Signet ring cells, morula cells, compartment cells (Fig. 5E, 6B) and a vacuolated amoebocyte (Fig. 5H) 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

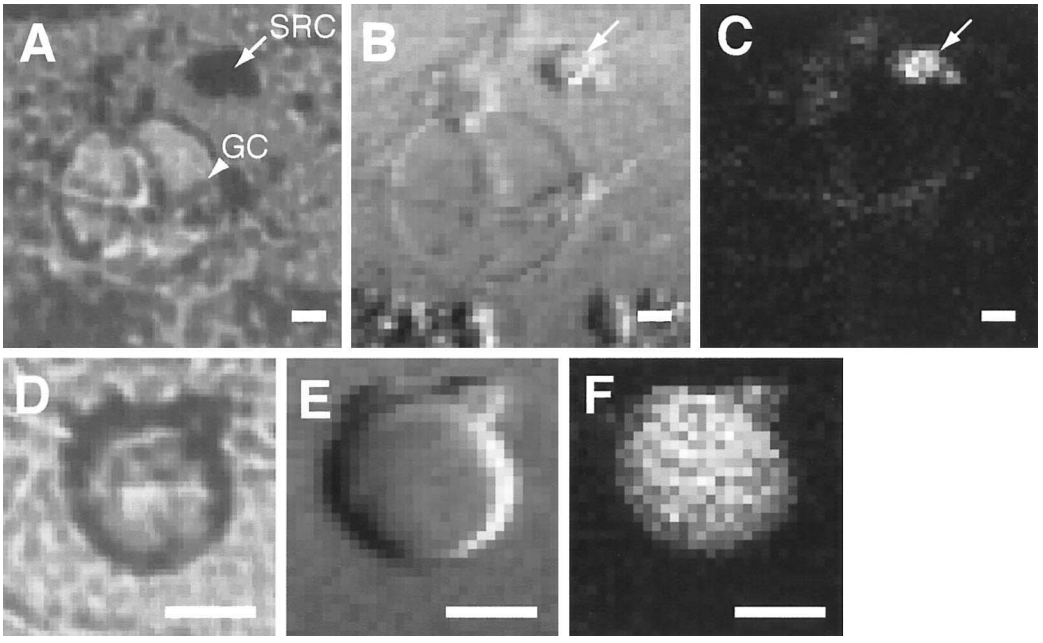


**Fig. 4.** Freeze-dried *Ascidia sydneiensis samea* blood cells observed by differential interference contrast optical microscopy (A, D), x-ray microscopy in transmission mode (B, E) and in the fluorescence mode for vanadium (C, F). A to C and D to F show the same field of view, respectively. Transmission and fluorescence images were taken by scanning cells at 0.5 μm×0.5 μm resolution with a 5.500 keV x-ray. Vanadium is located in signet ring cells (SRC, shown by arrows) but not in the giant cell (GC). Each scale bar =5 μm.

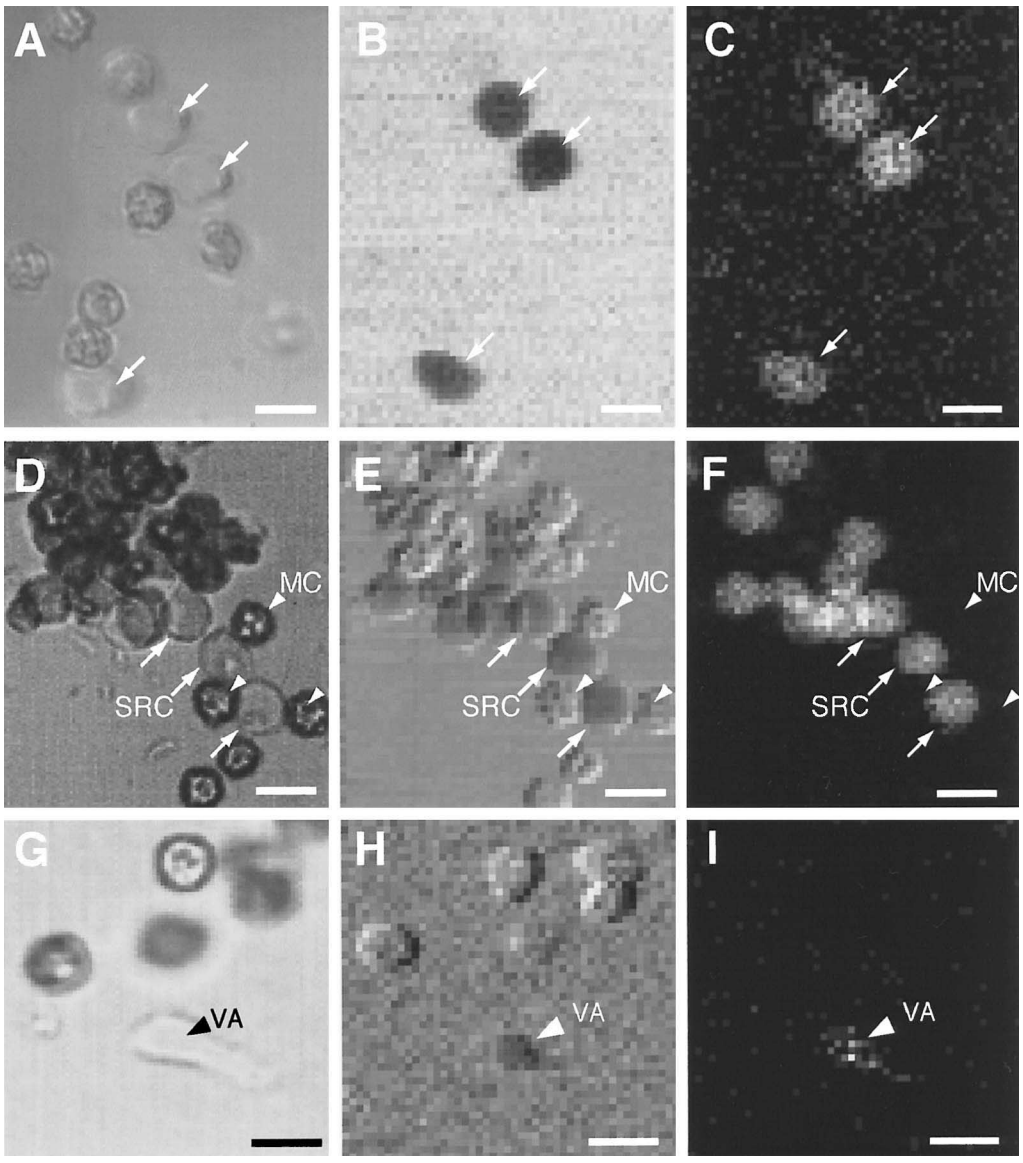


**Fig. 5.** *Phallusia mammillata* blood cells observed by differential interference contrast optical microscopy (A, D, G), by x-ray microscopy in transmission mode (B, E, H), and in the fluorescence mode for vanadium (C, F, I). Photographs A to C are from the same field of view. Photographs D to F are from another field, and G to I from yet another. Transmission and fluorescence images were taken by scanning cells with a 5.500 keV x-ray at a 1 μm×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 G-I), but not in morula cells (MC, shown by arrowheads in D-F). Each scale bar =10 μm.

showed that the signet ring cells (Fig. 5F, 6C) and vacuolated amoebocytes (Fig. 5I) 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 (Scippa *et al.*, 1985, 1988). The fluorescence images in Fig. 5F, however, clearly demonstrate that vanadium is distributed uniformly in the vacuole of signet ring cells. When superimposed, the fluorescent images (Fig. 5F) are slightly smaller than the image from the optical microscope (Fig. 5D) and the x-ray transmission image (Fig. 5E). 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. 6). Consequently, we concluded that vanadium accumulates in signet ring cells and vacuolated

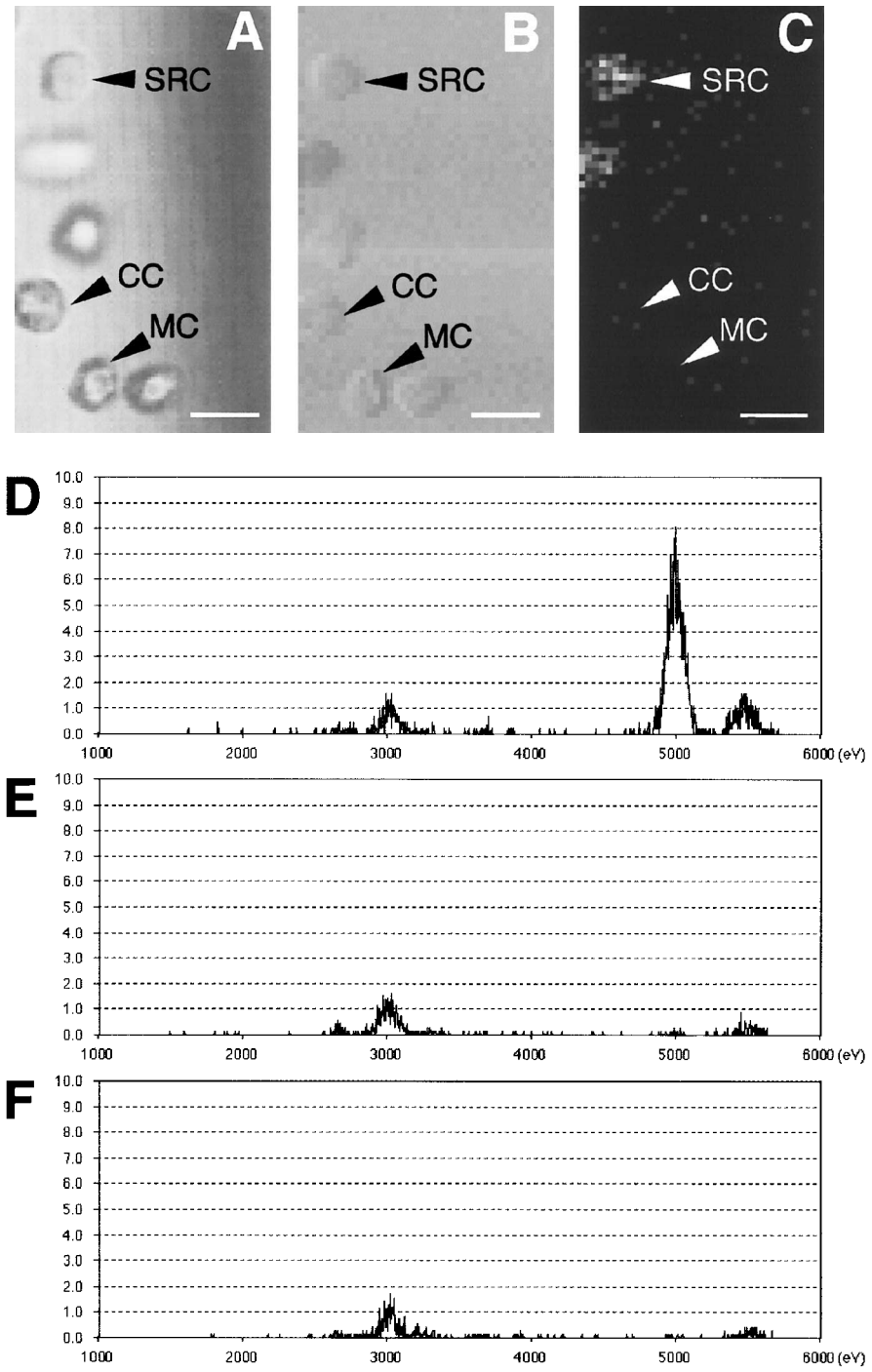


**Fig. 6.** *Phallusia mammillata* blood cells observed by x-ray microscopy and fluorescence spectra emitted from the blood cells. (A) Blood cells of *P. mammillata* viewed with a differential interference contrast optical microscope, (B) x-ray images taken at 5.500 keV in transmission mode, and (C) by fluorescence from vanadium. Transmission and fluorescence images are taken by scanning cells at 1 μm×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).

amoebocytes. We did not examine the granular amoebocytes and type-II compartment cells, reported by Scippa and colleagues to contain vanadium, because they are relatively rare in this species.

Next, we observed *A. sydneiensis samea*. In this species, we previously reported that the vanadium concentra-

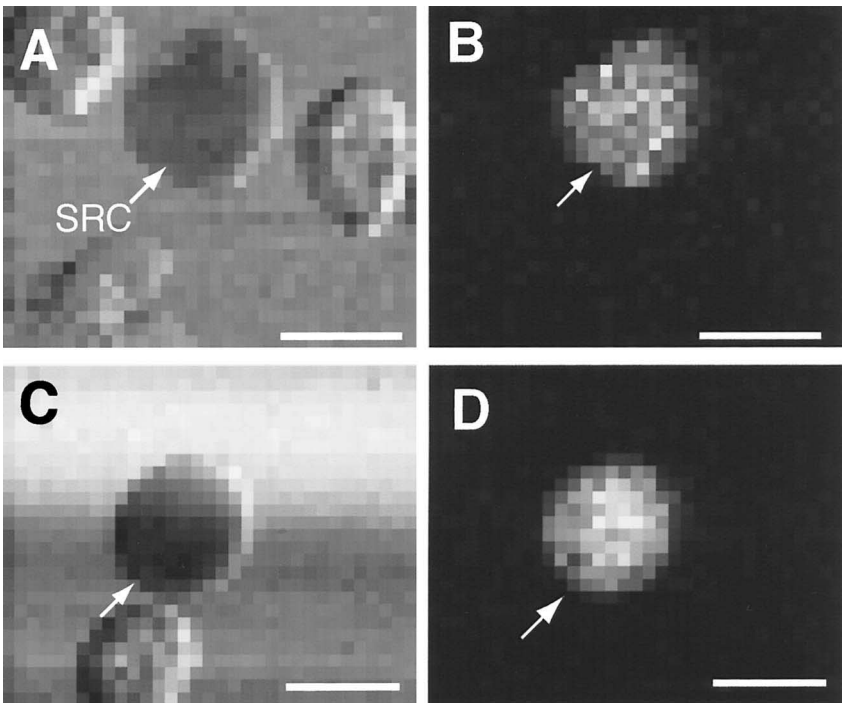


**Fig. 7.** *Ascidia sydneiensis samea* blood cells observed by x-ray microscopy in transmission mode (A, C) and in the fluorescence mode for vanadium (B, D). Transmission and fluorescence images were taken by scanning cells at 0.74 μm×0.74 μm resolution with a 5.500 keV x-ray for 500 ms per pixel. A and B show the same field of view; C and D another field. Vanadium is located in signet ring cells (SRC, shown by arrows) but not in the other cells. Each scale bar =10 μm.

tion in blood cells is 13 mM (Michibata *et al*., 1987) and that signet ring cells are the only blood cells that contain vanadium (Michibata *et al*., 1991). As shown in Fig. 7, when blood cells were imaged with transmission and fluorescence modes by x-ray at 5.500 keV, only signet ring cells contained vanadium. Although we did not find vanadium in any other cells, it is possible that there are very rare type(s) of blood cell that contain vanadium. Like *P. mammillata*, the vanadium in *A. sydneiensis samea* was uniformly distributed in the vacuole.

## DISCUSSION

In this study, we successfully visualized vanadium in ascidian blood cells using a scanning x-ray microscope at the European Synchrotron Radiation Facility (ESRF). Both freeze-dried and living blood-cell specimens were examined by scanning x-ray microscopy. To observe blood cells and detect cellular vanadium, we first used a freeze-drying method to prepare blood cell specimens from two vanadium-accumulating ascidians. Then, we used wet living specimens that were untreated. In samples prepared with both methods, we observed a uniform distribution of vanadium in signet ring cells and vacuolar amoebocytes. In some freeze-dried specimens, however, vanadium was distributed only in the peripheral region of signet ring cells. This was probably due to puncture of cells during freeze-drying and consequently we preferred to use wet living cells.

This study has provided critical evidence that the true vanadocytes are signet ring cells. As shown in Figs. 5, 6 and 7, signet ring cells from the two ascidian species were clearly shown to contain vanadium. The vanadium was uniformly distributed in the vacuoles of these cells in both species. The intensity of fluorescence from vanadium cannot be compared quantitatively because it is limited by exposure time. We also showed that in *P. mammillata*, vacuolated amoebocytes contain vanadium, although they appear to contain less than signet ring cells. We observed several vanadium-containing vacuolated amoebocytes, but most disappeared during observation. Many signet ring cells also disappeared during observation. These two types of cells may be fragile to x-ray irradiation because of their high vanadium concentrations. For that reason we were unable to achieve a higher spatial resolution of the samples and could not use x-ray microspectroscopy. The ID21 x-ray microscope can analyze images at higher spatial resolutions by reducing the pixel size and increasing the exposure time per pixel. We may be able to overcome the problem of radiation damage by using a cryofixing technique.

Physical methods have contributed to the characterization of chemical species of vanadium contained in ascidian blood cells. Noninvasive physical methods such as electron spin resonance (ESR) spectrometry and x-ray absorption spectrometry (XAS) revealed that the vanadium in ascidian blood cells is predominantly in the +3 oxidation state (Carlson, 1975; Tullius *et al*., 1980; Dingley *et al*., 1981; Frank *et*

*al*., 1986; Lee *et al*., 1988; Hirata and Michibata, 1991). Furthermore, XAS and Raman spectrometry revealed that vanadium ions are stable under extremely acidic and sulphurous conditions, such as the aliphatic sulfonic acid environment found in the vacuole (Frank *et al*., 1987; Michibata *et al*., 1991; Kanamori and Michibata, 1994). Synchrotron radiation is expected to clarify whether any ligands participate in the stabilization of vanadium ions in the +3 oxidation state.

It remains to be resolved at which step of the cell lineage vanadium begins to accumulate. However, we must first obtain a clearer understanding of cell lineages in these organisms, from the so-called stem cells to the peripheral cells. Moreover, the physiological roles of vanadium accumulation in ascidians remain unclear. Recently, novel vanadium-binding proteins were extracted from an ascidian (Kanda *et al*., 1997). We are currently investigating these proteins and genes encoding some metal-transporting proteins, which are also found in signet ring cells (unpublished data).

## ACKNOWLEDGMENTS

We thank the European Synchrotron Radiation Facility for the use of the ID21 beamline. This work was supported in part by a Grant-In-Aid (13874118) to H. M. from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant for T. Ueki. by the Scientist Exchanges of Bilateral Programs between the Japan Society for the Promotion of Science (JSPS) and the National Research Council of Italy (CNR). We greatly appreciate Mr. K. Morita and the other staff at Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, Iwate, Japan, and the Fishery Department of Stazione Zoologica 'Anton Dohrn', Naples, Italy, for providing adult ascidians. We also thank Prof. R. De Santis at Stazione Zoologica 'Anton Dohrn', and Prof. M. de Vincentiis, Dr. G. Nette, Dr. M. Cammarano and Dr. A. De Cadia at the University of Naples for helpful discussions and technical assistance.

## REFERENCES

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

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

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

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

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

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

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

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

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

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

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 Assoc UK 74: 279–286

Kanda T, Nose T, Wuchiyama J, Uyama T, Moriyama Y, Michibata H (1997) Identification of a vanadium-associated protein from the vanadium-rich ascidian, *Ascidia sydneiensis samea*. Zool Sci 14: 37–42

Kustin K (1998) Perspectives on Vanadium Biochemistry. In "Vanadium Compounds, Chemistry, Biochemistry, and Therapeutic Applications" Ed by AS Tracey, DC Crans, Am Chem Soc Syp Ser 711: 170–185

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

Michibata H (1993) The mechanism of accumulation of high levels of vanadium by ascidians from seawater: Biophysical approaches to a remarkable phenomenon. In "Advances in Biophysics", Ed by E Setsuro, Japan Sci Soc Press, Tokyo & Elsevier, Limerick, Adv Biophys 29: 105–133

Michibata H (1996) The mechanism of accumulation of vanadium by ascidians: Some progress towards an understanding of this unusual phenomenon. Zool Sci 13: 489–502

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 224: 33–38

Michibata H, Iwata Y, Hirata J (1991) 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, Kanamori K (1998) Selective accumulation of vanadium by ascidians from sea water. In "Vanadiums in the Environment. Part 1: Chemistry and Biochemistry", Ed by JO Nriagu, John Wiley & Sons, Inc., New York, pp 217–249

Michibata H, Sakurai H (1990) Vanadium in ascidians. In "Vanadium in Biological systems, Physiology and Biochemistry", Ed by ND Chasteen, Kluwer Academic Publishers, Netherlands, pp 153–171

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

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

Michibata H, Uyama T, Kanamori K (1998) The accumulation mechanism of vanadium by ascidians — An interdisciplinary study between biology and chamistry on extraordinary high levels and reduced form of vanadium in vanadocytes. In "Vanadium Compounds, Chemistry, Biochemistry, and Therapeutic Applications" Ed by AS Tracey, DC Crans, Am Chem Soc Syp Ser 711: 248–258

Michibata H, Uyama T, Ueki T, Kanamori K (2001) The mechanism of accumulation and reduction of vanadium by ascidians. In "The Biology of Ascidians" Ed by H Sawada, H Yokosawa, CC Lambert, Springer-Verlag, Tokyo, pp 363–373

Nette G, Scippa S, Genovese M, de Vincentiis M (1999) Cytochemical localization of vanadium(III) in blood cells of ascidian *Phallusia mammillata* Cuvier, and its relevance to hematic cell

lineage determination. Comp Biochem Biophys Part C 122: 231–237

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

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

Scippa S, Botte L, de Vincentiis M (1982b) X-ray microanalysis of cryofixed blood cells of *Phallusia mammillata*. Acta Embryol Morphol Exper ns 3: 22–23

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 Submicrosc Cytol Pathol 20: 719–730

Susini J, Barrett R (1996) The X-ray microscopy facility project at the ESRF. In "X-Ray Microscopy and Spectromicroscopy", Ed by J Thieme, G Schmahl, E Umbach, Springer Verlag, Wuerzburg, Germany, pp I45–I54

Susini J, Barrett R, Kaulich B, Oestreich S, Salomé M (2000) The X-ray microscopy facility at the ESRF: a status report. In "X-Ray Microscopy. Proceeding of the Sixth International Conference", Ed by W Meyer-Ilse, T Warwick, D Attwood, American Institute of Physics, Melville, New York, pp 19–26

Takemoto K, Yamamoto A, Michibata H, Uyama T, Ueki T, Ikeda S, Kihara H (2000) Observation of blood cells of the ascidian, *Phallusia nigra*, with X-ray microscope. Mem SR Center Ritsumeikan Univ 2000: 71–77

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

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

Wright RK (1981) Urochordata. In "Invertebrate Blood Cells", Ed by NA Ratcliffe, AF Rowley, 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 76: 51–55

(Received October 15, 2001/ Accepted November 15, 2001)