High Accumulation of Elements in the Kidney of the Marine Bivalve *Cyclosunetta menstrualis*

Toshiaki Ishii,*¹ Kunio Ikuta,*² Tsuguo Otake,*³ Masako Hara,*³
Masafumi Ishikawa,*¹ and Taku Koyanagi*¹

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Distribution of eighteen elements in the soft part of the marine bivalve *Cyclosunetta menstrualis* was examined by means of inductively coupled plasma atomic emission spectroscopy, atomic absorption spectrophotometry, and electron probe X-ray microanalysis. The highest concentration was found in the kidney for Mg, Ca, Sr, P, Mn, Fe, Co, Ni, Zn, Cd, Sn and Pb. Especially, the manganese concentration in the kidney was $6820 \pm 1760 \mu\text{g/g}$ wet weight, which accounted for more than 90 percent of the total manganese in the soft part.

Extracellular granules of various sizes were observed in the lumen of the kidney tubules by light and electron microscopy. The scanning electron micrograph indicated that large sized granules (10–200 μm) were composed of numerous fine (0.1–1.0 μm) and small (1.0–10 μm) sized granules. Many spherical substances and black concentric layers were observed inside the large granules. The granules contained extremely high levels of Mg, Ca, P, Mn, Fe, Zn *etc.* For example, the concentrations of manganese and zinc in the dried granules were $44200 \pm 15600 \mu\text{g/g}$ and $22800 \pm 1600 \mu\text{g/g}$, respectively. It was concluded that the high accumulation of certain elements in the kidney resulted from the existence of metal-containing granules.

Bioaccumulators of specific elements have been used to monitor the levels of pollutant metals in the marine environment. High accumulation might suggest a relation with the special detoxication ability or the existence of characteristic intracellular ligands which the accumulators possess in their bodies. It has been reported that distributions of heavy metals are not uniform among the tissues and in the cells of marine organisms. For example, the concentration of copper in the branchiae of the polychaete *Melinna palmata* represents 30–40% of the total body burden, although these organs account for only 3–4% of the dry body weight.¹⁾ The concentration of cadmium in the hepatopancreas of the lobster *Panulirus japonicus* is two thousand times higher than that in the abdominal muscle.²⁾ Reports on the subcellular localization of heavy metals describe that concretions in the kidney epithelial cells of the bay scallop *Argopecten irradians* contain large amounts of calcium, phosphorus, magnesium, manganese and zinc,³⁾ and that the concentric layers of intracellular granules in the hepatopancreas of the shore crab *Carcinus maenas*

contain calcium, phosphorus, magnesium and lead.⁴⁾

In this report, the distribution of eighteen elements in the soft part of the marine bivalve *Cyclosunetta menstrualis* was examined by means of inductively coupled plasma atomic emission spectroscopy (ICP-AES), atomic absorption spectrophotometry (AAS) and electron probe X-ray microanalysis.

In addition, the kidney was examined morphologically by an optical, a scanning and a transmission electron microscope in order to clarify causes of the high accumulation of elements.

Material and Methods

Material

Cyclosunetta menstrualis, 67–73 mm in shell length and 68–87 g in whole body weight, were collected from the offshore area of Ibaraki Prefecture (Japan) from October 1983 to July 1984. Twenty five individuals were dissected into various tissues such as adductor muscles, kidneys, mantles, gills, foot, *etc.*

*¹ Division of Marine Radioecology, National Institute of Radiological Sciences, Isozaki 3609, Nakaminato 311-12, Japan (石井紀明, 石川昌史, 小柳 卓: 放射線医学総合研究所海洋放射生態学研究部).

*² Faculty of Agriculture, Miyazaki University, Kumano, Miyazaki 889-21, Japan (生田國雄: 宮崎大学農学部).

*³ Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164, Japan (大竹二雄, 原 政子: 東京大学海洋研究所).

Determination of Elements by ICP-AES and AAS

The dissected tissues were dried at 70°C and digested with nitric and perchloric acids. The digests were diluted with 0.5 N nitric acid to a volume of 100 ml. High purity inorganic compounds (sodium, potassium, calcium, magnesium and phosphorus) were purchased from Johnson Matthey Chemicals Ltd. and Spex Industries Inc. for matrix matching. Water used in this experiment was purified by a Barnstead NANOpure D-2794 four-module system.

The concentrations of Na, K, Mg, Ca, Sr, S, P, Mn, Fe, Cu, Zn, Al and Sn were measured using a Shimadzu ICPQ-1012W inductively coupled plasma atomic emission spectrometer. The spectrometer is equipped with a Paschen-Runge mount polychromator which has a Rowland circle of 100 cm. The reciprocal linear dispersion of 0.37 nm/mm at 180.5 nm is provided by a master holographic grating with 2700 grooves/mm. Operating conditions were almost the same as those described previously.²⁾

Cobalt, nickel, cadmium, lead, and rubidium were analyzed using a Perkin-Elmer Model 5000 atomic absorption spectrophotometer equipped with an HGA-400 programmer and an AS-40 auto sampler. The ICP-AES could not be used to determine these elements because of poor detection limits.

Preparation of Specimens for Elemental Analyses by Electron Probe X-ray Microanalyzers (EPMA)

For the analysis by EPMA with an energy dispersive X-ray detector (EDX), small pieces (approximately 1 × 1 mm) of the kidney of *C. menstrualis* were fixed for 2 h in cold fixative which contained 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Then, they were post-fixed in 2% osmium tetroxide. Specimens were dehydrated through graded ethanols and embedded in Epon 812. Sections of 0.1 µm thickness were placed on an aluminum, a molybdenum and a titanium grid. Elemental analysis (point analysis) was performed by a Hitachi H-600/H-6010 electron microscope with a Kevex 7000Q energy dispersive X-ray analyzer.

For the analysis by EPMA with a wavelength dispersive X-ray detector (WDX), freeze-dried sections of the kidney were cut at 20 µm in thickness with a Lipshaw Model 1900 cryostat microtome. Linear scanning analysis was carried out

using a Hitachi X-650 scanning electron microscope.

Observation of the Kidney by Microscopes

Specimens for light microscopy were prepared by decalcifying in 1% formic acid for 2 or 3 days because granules in the kidney were exceedingly hard. Sections of 5 µm thickness were stained with hematoxylin and eosin.

Specimens for transmission electron microscopy were fixed and embedded by the same procedure as that of EPMA with EDX, as mentioned above. Ultra-thin sections, stained with uranyl acetate and lead citrate, were examined in a JEOL JEM 100-CX electron microscope.

Separation of Granules from the Kidney

The size of extracellular granules in the lumen of the kidney tubules ranged from 0.1 to 200 µm. One large sized granule was generally present in one lumen, though numerous fine and small sized granules were observed in the same lumen. Most of the small (1–10 µm) and the large (10–200 µm) granules were separated from the soft tissue of the kidney by shaking or simple homogenizing in seawater. Granules collected on Nuclepore filters (pore size 1.0 µm) were dried at 110°C. A large number of fine granules (0.1–1.0 µm) might have been lost by decantation to remove cell debris or filtration.

Results and Discussion

Distribution of Elements in the Soft Part

The concentrations of elements in various tissues of *C. menstrualis* are shown in Table 1. The highest concentration is found in the kidney for Mg, Ca, P, Sr, Mn, Fe, Zn, Co, Ni, Cd, Sn, and Pb. Especially, the manganese is concentrated in the kidney about six thousand times higher than in the foot. More than 90% of the total soft part burden of manganese is accumulated in the kidney which accounts for only 2% of the total weight of the soft part. The concentration factor of manganese by the kidney is 2×10^8 when calculated by using the concentration of manganese in coastal water off Ibaraki Prefecture (2.94 µg/l*).

The major elements are usually considered to be distributed uniformly among the soft tissues of marine organisms. In the case of this species, however, the concentrations of magnesium, calcium and phosphorus in the kidney are more

* This manganese concentration which we measured by neutron activation analysis was cited in the Annual Report of National Institute of Radiological Sciences NIRS-AR-17.

Table 1. The concentrations of major and trace elements in various soft tissues of *C. mensturalis* ($\mu\text{g/g}$ wet weight)

	Mantle	Gill	Foot	Digestive diverticulum	Kidney	Siphon	Adductor muscle	Gonad	Others	Whole soft tissue
Element concentrations* ¹										
Na	4350 $\pm$ 240	5370 $\pm$ 180	2040 $\pm$ 330	2300 $\pm$ 90	4560 $\pm$ 230	4010 $\pm$ 670	2790 $\pm$ 560	2030 $\pm$ 180	2400 $\pm$ 1510	3450 $\pm$ 550* ²
K	2000 $\pm$ 130	2360 $\pm$ 380	3210 $\pm$ 180	2570 $\pm$ 150	2950 $\pm$ 160	2530 $\pm$ 200	2000 $\pm$ 610	2420 $\pm$ 90	1880 $\pm$ 340	2030 $\pm$ 370
Mg	1520 $\pm$ 10	1090 $\pm$ 660	410 $\pm$ 70	430 $\pm$ 10	4160 $\pm$ 2020	690 $\pm$ 90	510 $\pm$ 100	350 $\pm$ 90	460 $\pm$ 240	820 $\pm$ 80
Ca	790 $\pm$ 230	530 $\pm$ 100	200 $\pm$ 60	1110 $\pm$ 60	15420 $\pm$ 7360	390 $\pm$ 90	310 $\pm$ 30	120 $\pm$ 40	960 $\pm$ 310	1000 $\pm$ 330
S	5070 $\pm$ 250	4290 $\pm$ 750	4290 $\pm$ 370	3300 $\pm$ 500	4090 $\pm$ 260	4460 $\pm$ 510	2640 $\pm$ 640	2220 $\pm$ 180	2460 $\pm$ 1040	3910 $\pm$ 350
P	770 $\pm$ 40	1210 $\pm$ 240	1470 $\pm$ 190	1550 $\pm$ 160	14560 $\pm$ 5790	1350 $\pm$ 120	1160 $\pm$ 300	1490 $\pm$ 210	950 $\pm$ 490	1300 $\pm$ 230
Mn	3.9 $\pm$ 1.5	9.0 $\pm$ 3.6	1.1 $\pm$ 0.2	2.1 $\pm$ 1.0	6820 $\pm$ 1760	5.1 $\pm$ 3.3	5.1 $\pm$ 3.2	1.4 $\pm$ 0.8	12.8 $\pm$ 2.3	121 $\pm$ 39
Fe	27.0 $\pm$ 2.7	36.9 $\pm$ 9.2	16.3 $\pm$ 1.6	86.8 $\pm$ 51.8	690 $\pm$ 160	77.3 $\pm$ 39.5	12.8 $\pm$ 3.4	28.6 $\pm$ 9.8	61.5 $\pm$ 18.0	61 $\pm$ 24
Cu	4.5 $\pm$ 1.6	35.4 $\pm$ 8.1	2.6 $\pm$ 0.5	57.1 $\pm$ 7.7	29 $\pm$ 8	7.2 $\pm$ 1.4	3.0 $\pm$ 2.0	5.9 $\pm$ 1.6	5.8 $\pm$ 3.6	7 $\pm$ 2
Zn	12.5 $\pm$ 1.0	20.5 $\pm$ 5.2	14.9 $\pm$ 1.7	23.5 $\pm$ 2.9	2370 $\pm$ 530	10.2 $\pm$ 1.7	15.7 $\pm$ 4.4	4.8 $\pm$ 2.1	28.2 $\pm$ 23.9	68 $\pm$ 36
Al	8.2 $\pm$ 3.4	7.2 $\pm$ 3.2	1.8 $\pm$ 0.2	69.3 $\pm$ 42.8	49 $\pm$ 29	35.3 $\pm$ 25.3	2.6 $\pm$ 1.6	2.6 $\pm$ 1.1	13.5 $\pm$ 4.7	10 $\pm$ 5
Sr	4.8 $\pm$ 1.2	4.9 $\pm$ 0.9	1.6 $\pm$ 0.2	5.4 $\pm$ 0.5	310 $\pm$ 130	3.7 $\pm$ 0.7	2.9 $\pm$ 0.3	1.4 $\pm$ 1.0	7.4 $\pm$ 5.0	10 $\pm$ 4
Co	0.27 $\pm$ 0.01	0.49 $\pm$ 0.12	0.13 $\pm$ 0.01	0.31 $\pm$ 0.03	10.2 $\pm$ 1.3	0.29 $\pm$ 0.06	0.56 $\pm$ 0.16	0.10 $\pm$ 0.08	0.24 $\pm$ 0.17	0.49 $\pm$ 0.30
Ni	0.82 $\pm$ 0.04	0.89 $\pm$ 0.06	0.36 $\pm$ 0.03	0.61 $\pm$ 0.04	18.0 $\pm$ 4.2	0.86 $\pm$ 0.18	2.01 $\pm$ 0.44	0.23 $\pm$ 0.07	0.73 $\pm$ 0.43	0.99 $\pm$ 0.22
Cd	0.25 $\pm$ 0.01	0.21 $\pm$ 0.01	0.19 $\pm$ 0.07	0.38 $\pm$ 0.40	2.1 $\pm$ 0.4	0.19 $\pm$ 0.18	0.05 $\pm$ 0.03	0.04 $\pm$ 0.02	0.39 $\pm$ 0.16	0.25 $\pm$ 0.10
Sn	1.13 $\pm$ 0.12	1.32 $\pm$ 1.07	0.72 $\pm$ 0.39	0.75 $\pm$ 0.52	8.7 $\pm$ 3.9	0.66 $\pm$ 0.48	0.69 $\pm$ 0.11	0.24 $\pm$ 0.23	0.81 $\pm$ 0.47	0.80 $\pm$ 0.34
Pb	0.32 $\pm$ 0.05	0.48 $\pm$ 0.11	0.20 $\pm$ 0.05	0.22 $\pm$ 0.03	18.0 $\pm$ 2.0	0.30 $\pm$ 0.08	0.20 $\pm$ 0.05	0.21 $\pm$ 0.13	0.27 $\pm$ 0.18	0.23 $\pm$ 0.15
Rb	0.13 $\pm$ 0.06	0.05 $\pm$ 0.01	0.42 $\pm$ 0.29	0.33 $\pm$ 0.16	0.2 $\pm$ 0.1	0.12 $\pm$ 0.09	0.10 $\pm$ 0.05	0.10 $\pm$ 0.02	0.21 $\pm$ 0.12	0.23 $\pm$ 0.11
Weight percent (%) ^{*3}										
	17.8 $\pm$ 2.9	2.7 $\pm$ 0.7	36.5 $\pm$ 8.7	10.4 $\pm$ 1.6	2.0 $\pm$ 0.4	2.7 $\pm$ 0.6	9.8 $\pm$ 1.4	9.6 $\pm$ 2.9	8.5 $\pm$ 3.5	100.0

*¹ Mean and standard deviation of five groups. Each group is composed of five individuals.*² Mean and standard deviation of twenty individuals. Whole soft tissue samples are different from samples which are used to examine the distribution of elements.*³ The weight percent of each tissue to the total soft tissues.

Table 2. The concentrations of elements in five granule samples*¹ isolated from the kidney of *C. menstrualis* ($\mu\text{g/g}$ dry weight)

	No. 1	No. 2	No. 3	No. 4	No. 5	Mean $\pm$ s.d.
Na	5420	6110	5840	5730	4290	5480 $\pm$ 630
K	1210	1590	2110	2330	1030	1650 $\pm$ 500
Mg	31500	30900	20000	37400	32600	30500 $\pm$ 5700
Ca	133000	134000	108000	149000	131000	131000 $\pm$ 13000
Sr	3050	3070	3150	—	3120	3100 $\pm$ 40
(Ba)* ²	110	130	190	75	96	120 $\pm$ 39
P	124000	118000	106000	128000	119000	119000 $\pm$ 7000
(Cr)* ²	26	27	—	41	27	30 $\pm$ 6
Mn	44400	20000	68200	49500	39000	44200 $\pm$ 15600
Fe	4770	4720	12100	5540	4430	6310 $\pm$ 2920
Co	53	59	—	20	49	45 $\pm$ 15
Ni	110	95	—	69	140	104 $\pm$ 26
Cu	92	200	—	59	98	112 $\pm$ 53
Zn	23200	24300	24500	20600	21200	22800 $\pm$ 1600
Cd	13	—	—	—	—	—
Al	450	410	230	550	440	420 $\pm$ 100
Pb	270	290	360	320	280	300 $\pm$ 30
(Ag)* ²	12	17	—	11	12	13 $\pm$ 2
Total	371690	343920	350680	399250	356810	365180

*¹ Each granule sample is isolated from different individuals.*² These elements are determined to obtain more detailed elemental composition.

than ten times higher than those in the foot, gonad, etc.

We measured trace element concentrations in the kidney for ten species of Japanese marine bivalves which were collected off the Pacific coast. For example, the concentrations of manganese and zinc for *Meretrix lamarcki* were 1.5 and 18 $\mu\text{g/g}$ wet weight, respectively. Those for *Gomphina melanaegis* were 0.82 and 66 $\mu\text{g/g}$ wet weight, respectively. These two clams belong to the same family, Veneridae, as *C. menstrualis* and inhabit the same unpolluted sandy area (2–10 m in water depth) off the coast of Ibaraki Prefecture. The other eight species also did not show high concentrations of manganese and zinc. Furthermore, the high accumulation of trace elements including manganese, zinc, strontium, cobalt, and nickel has not been previously reported for marine organisms in Japan.

It was concluded that *C. menstrualis* has the characteristic ability to accumulate elements at much higher concentrations than other Japanese marine organisms. We hope *C. menstrualis* can be used as an effective indicator for monitoring the levels of stable heavy metals and/or evaluating radiation dose from artificial radionuclides in the marine environment.

Concentrations of Elements in the Granules

The concentrations of elements in the granules

are shown in Table 2. Significantly high concentrations of calcium, phosphorus, magnesium, manganese, and zinc are found in the dried granules. This suggests that high levels of these elements in the kidney (see Table 1) are attributable to the existence of the metal-containing granules. Meanwhile, the total amount of metals and phosphorus in the dried granules is only about 40 percent (see Table 2). Therefore, it is considered that metals exist in various chemical forms such as chloride, sulphide, carbonate or oxide in addition to phosphate. Furthermore, we suspect that water (e.g. the water of crystallization) and organic substances occupy a small part of the weight of the granules. Mason and Nott⁽⁵⁾ report in the review that intracellular granules isolated from the soft tissues of some gastropods contain PO_4^{3-} and CO_3^{2-} as the major anions and polysaccharide, protein and lipid as the organic components. Ghiretti et al.⁽⁶⁾ indicate that large amounts of carbon, oxygen, hydrogen and phosphorus are contained in the nephroliths of the fan shell *Pinna nobilis*, and part of the nephrolith body is formed by organic materials.

In the case of *C. menstrualis*, chemical forms (including organometallic compounds) of metals in the granules are not known though the principal constituent of them is thought to be calcium phosphate. Therefore, we are examining the

Fig. 1. X-ray spectrum obtained by EPMA with EDX for a fine sized granule in the lumen of the kidney tubule of *C. menstrualis*.

elemental composition of the granules in detail.

The manganese concentration in the dried kidney was calculated to be 25,700 $\mu\text{g/g}$ from the dry/wet weight ratio ($26.5 \pm 3.7\%$). The total weight of the granules (average Mn concentration 44,200 $\mu\text{g/g}$ dry weight) was estimated to occupy about 50 percent of the dry matter of the kidney, on the assumption that the contribution of the kidney tissues excluding granules to the total manganese was negligibly small or at most 10%.

Marine bivalves have calcium-rich shells which are composed of a large quantity of calcium carbonate and a small quantity of organic compounds such as protein and polysaccharide. Concentrations of some elements in the shells of *C. menstrualis* are as follows: 449,000 $\mu\text{gCa/g}$, 7.6 $\mu\text{gP/g}$, 2.2 $\mu\text{gMn/g}$, 1.7 $\mu\text{gFe/g}$ and 1.5 $\mu\text{gZn/g}$ (dry weight). This means that the concentrations of phosphorus, manganese, iron and zinc in the shells were much lower than those in the granules. We are planning to study the difference in mechanisms of metal deposition between the two types of calcium compounds.

Elemental Analyses by EPMA

The X-ray spectrum (Fig. 1) obtained by EPMA with EDX for a fine granule (approximately 0.2

Fig. 2. Linear scanning analysis by EPMA with WDX for sectioned large sized granules. Fig. 2-A: calcium, -B: phosphorus, -C: manganese.

Fig. 3. Light micrograph of a cross section of the kidney indicates that concentrically structured granules (G) with various shapes exist in the lumen (L) of the kidney tubules. Scale bar represents 100 μm .

Fig. 4. Light micrograph of the internal structure of a large sized granule. Scale bar represents 50 μm .

Fig. 5. Transmission electron micrograph of the kidney tubule, showing a small sized granule (G), lumen (L), and microvilli (MV). Scale bar represents 2 μm .

Fig. 6. Ultrastructure of the lumen of the kidney tubule, showing fine sized granules (G) and microvilli (MV). Scale bar represents 1 μm .

Fig. 7. Scanning electron micrographs for two large sized granules Fig. 7-A: a smaller granule, -B: a larger granule.

μm in diameter) indicates that large amounts of calcium, manganese, iron and zinc are localized in the granule. The results of Table 2 and Fig. 1 suggest that the main metal composition of fine granules is similar to that of larger ones. Probably, fine granules develop into larger ones without any change in the elemental composition.

Though a high level of phosphorus is contained in the granule, the peak would be interfered by a characteristic X-ray ($M\alpha$) of osmium. No significant peaks of calcium, manganese, iron or zinc were observed in the spectra of the nucleus, mitochondria, cytoplasm in kidney cells.

Fig. 2-A, B, C shows the result of linear scanning analyses by EPMA with WDX for sectioned granules. Calcium, phosphorus and manganese are distributed uniformly in the granules.

As discussed later, a concentric structure is observed inside the granules (Fig. 4). Concentric layers are thought to have no relation to the metals being studied. But additional studies on the relationship between concentric layers and metals need to be conducted though we think layers are composed of organic substances.

Observation of the Kidney Tissue

A pair of kidneys is located beside the posterior adductor muscle of *C. menstrualis*. Distinction of the kidney from other tissues is very easy because of its dark brown color.

Large extracellular granules with various shapes are observed in the lumens of tightly packed and twisted blind tubules in the kidney (Fig. 3). Several concentric layers are recognized in most granules when decalcified in a 1% formic acid solution.

Intracellular granules abundant in heavy metals are often present in various tissues of molluscs, but there are few reports about metal-containing extracellular granules. Ghiretti *et al.*⁶⁾ report that a metal-rich stone appears in the renal cavity surrounded by a monolayer of secreting cells of *P. nobilis*. Mauri and Orlando⁷⁾ report that the wedge shell *Donax trunculus* can form extracellular renal concretions when reared under higher manganese levels than those in natural seawater.

Small round substances of various colors (mainly yellow) are observed in a large sized granule, as shown in Fig. 4. It is suggested that one large granule is an aggregate of many fine and small ones. The proximal layers have a naturally dark brown color and are not stained with hematoxylin and eosin. The eosinophilic

distal layers indicate the presence of organic substances. Some black concentric layers and a white area similar to interstice are also observed. The relationship between the formation of the concentric structure and physiological functions of the kidney is under investigation. However, it is possible that the white area resulted from an artefact which occurred during fixation, decalcifying or sectioning.

In a transmission electron micrograph (Fig. 5) a small granule is found at the center of the lumen surrounded with microvilli. Many particles are observed inside the granule.

Electron-dense spherical granules are observed beside microvilli, as shown in Fig. 6. But no substances with a high electron density are present in the cytoplasm of kidney cells.

A scanning electron micrograph (Fig. 7) indicates that the large sized granules are composed of numerous fine and small sized ones. It is estimated that spaces between the fine granules were gradually packed by adhesion with new deposits like phosphate or organic substances while the fine granules basically keep the original spherical shape. For example, Fig. 7-B (arrow) indicates that the spherical shape appear to change into an uncertain one. But it is suspected that some interspaces containing liquid interstitial materials remain inside the granules.

These morphological aspects suggest that the spherical fine granules appear extracellularly beside microvilli and develop into larger granules while moving to the center of the lumen of the kidney tubules.

Further experiments are intensively in progress in our laboratory to examine the reasons why the metal-containing extracellular granules appear in the lumen of the kidney tubules of *C. menstrualis*. If the granules could be formed actively through biological processes, studies on their physiological roles might be very necessary to estimate the purpose for the granule formation. On the other hand, if the granules could be formed passively by abiotic pathways, the quantities and physico-chemical forms of elements when excreted from microvilli to the lumen will be important subjects in clarifying the mechanisms of metal deposition.

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