Determination of Trace Metals Forming Large Molecular Complexes in Natural Waters as Estimated by Ultrafiltration/Liquid Chromatography/Atomic Spectroscopy

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(Received May 31, 1989)

Concentration of trace metals forming complexes with large organic molecules in natural waters have been determined. Natural water samples (sea water and lake water) were filtered through a membrane filter (pore size: 0.45 μm) just after sampling, and concentrated by ultrafiltration. The concentrated natural water samples were fractionated by high performance gel filtration chromatography, and metal ion concentration in each fraction was determined by inductively coupled plasma atomic emission spectrometry and/or by graphite furnace atomic absorption spectrometry. Concentration of zinc forming large molecular complexes (LMC) in sea water was 0.07 ng mL^{-1}, and that in lake water was 0.06 ng mL^{-1}. Copper, iron, vanadium, and molybdenum forming LMC in natural waters were also determined. The present results suggest the existences of LMC-forming metal ions, some of which may be metalloenzymes dissolved in natural waters.

In the early stage, the investigations of trace elements in natural waters mostly dealt with the measurements of their total concentrations. Recently it has been, however, recognized that the chemical behaviors of each trace element in environmental systems depend on its dissolved state.[1,2] There have been two basic approaches to the identification of the chemical species of trace elements in natural water. One approach has been to develop some models applicable to the marine environment, and this has been done on the basis of our knowledge of the thermodynamic properties of elements. Many investigators have speculated the major forms of trace elements from the calculations of chemical equilibria of ion concentrations in sea water.[3,4] In such studies, however, metal complexes in organic forms have been usually ignored.

The second approach has been to develop experimental techniques which can separate the various chemical forms of trace elements in natural waters. Furthermore, in these studies, sensitive analytical methods or preconcentration techniques are required in order to identify or determine the different chemical species, since even the total concentration of each element is mostly not more than the ppb (ng mL^{-1})-level. There have been several reports employing this second approach. Florence et al. reported that only a part of copper, lead, cadmium, or zinc in sea water was removed with the chelating resin (Chelex-100).[5] Sugimura et al. reported that most of vanadium, iron, cobalt, copper, and cadmium in sea water existed as the organic forms, where they used Amberite XAD-2 resin for separation.[6] The present authors have identified alkaline phosphatase as one of chemical species for zinc in natural waters using its specific enzymatic activity, after separation with gel filtration chromatography.[7,8] The presence of carboxypeptidase (a zinc enzyme) and nitrate reductase (a molybdenum/iron enzyme) in sea water have also been suggested.[9,10]

In this paper, trace metal ions forming large molecular complexes (hereafter abbreviated as LMC) in natural waters are determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) and/or graphite furnace atomic absorption spectrometry (GFAAS) after preconcentration with ultrafiltration and separation with high performance liquid chromatography (HPLC) using a porous gel column.

Experimental

Chemicals. All chemicals used in the present study were of analytical reagent grade. A glass-fiber filter (Whatman GF/C; pore size: 10 μm; diameter: 47 mm) and a membrane filter (Toyo Kagaku Sangyo Co., TM-2; pore size: 0.45 μm, diameter: 47 mm; equivalent to Millipore HA-type membrane filter) were used for filtration of natural water samples. An ultrafilter (Type UK-10; diameter: 62 mm; equivalent to Amicon UM-10), purchased from Toyo Kagaku Sangyo Co., was used to concentrate molecules larger than 10000 g mol^{-1} (M.W.).

Standard proteins for molecular weight calibration in HPLC were as follows: Ferritin (indicator protein, from Boehringer-Mannheim Co., M.W.: 450000);[11] catalase (Bovine liver, from Sigma, M.W.: 250000);[12] alkaline phosphatase (*E. coli*, from Sigma, M.W.: 89000);[13] carbonic anhydrase (Bovine erythrocytes, from Sigma, M.W.: 31000);[14] and cytochrome-c (indicator protein, from Boehringer-Mannheim Co., M.W.: 125000).[15] Distilled water used was further purified with a sub-boiling distiller from Daiken Sekiei Co.[16]

Instruments. Ultrafiltration was carried out with an Amicon Diaflo Cell (Type 202) and an Amicon Sample Reservoir (Type RG-5, made of glass fiber). A Shimadzu LC-3A high performance liquid chromatography with an SPD-2A spectrophotometric detector was used for HPLC-

separation together with a fraction collector (Toyo Kagaku Sangyo Co., Signal Fracon SF-60L). Metal contents were determined with an ICP-AES instrument (Jarrell-Ash Plasma Atom Comp Mk II) and/or an atomic absorption spectrophotometer (Shimadzu Model AA-640-13) equipped with a graphite furnace atomizer (Model GFA-3).

Water Sampling and Pre-Concentration. Surface sea water was collected off the coast of Misaki (Kanagawa prefecture, Japan) on September 16, 1981. Lake water was collected in Takahamairi Bay of Lake Kasumigaura on July 20, 1981.[8] Both samples were filtered with a membrane filter immediately after sampling, and stored in a plastic container at 0 °C.

Four liters of filtered water was concentrated to ca. 4 mL (concentration factor of 1000 times) using ultrafiltration (UF) technique. During this procedure, the sample was kept at around 4 °C in a thermostatic bath. All the analyses were performed no later than 6 months after sampling. All the equipments and filters were cleaned and sterilized by soaking in 7 M nitric acid (1 M=1 mol dm^{-3}) and/or 40% ethanol aqueous solution.

Table 1. Operating Conditions for ICP-AES and GFAAS

ICP atomic emission spectrometry	
Instrument:	Jarrell-Ash Plasma Atomcomp Mk II
RF power:	1.1 kW
Gas flow rate:	
coolant	argon 19 L min^{-1}
auxiliary	argon 0.4 L min^{-1}
carrier	argon 1.0 L min^{-1}
Observation height:	17 mm above load coil
Graphite furnace atomic absorption sperctrometry	
Instrument:	Shimadzu AA-640-13 with Shimadzu GFA-3 graphite furnace atomizer
Wavelength:	213.8 nm
Sample volume:	10 μL
Temperature program:	
drying	150 °C, 40 s, ramp mode
ashing	600 °C, 30 s, ramp mode
atomizing	1700 °C, 5 s, step mode

Table 2. Detection Limits for Various Elements in ICP-AES

Element	Wavelength[a]		Detection limit[b]
	nm		ng mL^{-1}
Al	308.2	I	19
V	292.4	II	3.9
Cr	205.2	II[c]	6.3
Mn	257.6	II	0.3
Fe	259.9	II	11
Co	228.6	II	7.5
Ni	231.6	II[c]	14
Cu	324.7	I	2.7
Zn	213.8	I[c]	6.0
Mo	202.0	II	7.5
Cd	228.8	I[c]	3.6
Pd	220.3	II	33

a) I and II represent atomic and ionic lines, respectively. b) The detection limits were estimated as the analyte concentration corresponding to three times of standard deviations of background signals at each wavelength. c) Second order lines were used.

Separation with HPLC. Two hundred microliters of the concentrated sample were injected into HPLC, equipped with a Shimadzu aqueous porous gel column W-71 (7.9 mm i.d.×30 cm long). As a carrier, 16 mM of dipotassium hydrogenphosphate-nitric acid buffer (pH 7.30) was used with the flow rate of 1.0 mL min^{-1}. The effluent was collected with a fraction collector every 0.8 min, and then 10 μL of concentrated nitric acid was added to each fraction to prevent precipitation of metal species. Since about 2 mL of sample solution was required in the ICP-AES measurement, the procedures described above were repeated 3 times. Thus 2.4 mL of each sample fraction was available for the ICP-AES measurement.

Determination of Metal Concentration. The concentration of metal elements in fractionated samples were determined simultaneously by ICP-AES. The matrices of the standard solution for ICP-AES were matched with that of the HPLC carrier (16 mM phosphate buffer, pH 7.30). The concentration of zinc was also determined by GFAAS. The operating conditions for ICP-AES and GFAAS are summarized in Table 1. Analytical lines used in ICP-AES are shown in Table 2.

Results

UF/HPLC/ICP-AES Chromatograms of Metalloenzymes. To evaluate the present UF/HPLC/ICP-AES system, metalloenzymes such as catalase, Bovine carbonic anhydrase, and *E. coli* alkaline phosphatase were analyzed. Catalase is an iron-containing metalloenzyme, and carbonic anhydrase and alkaline phosphatase are zinc-containing metalloenzymes. In Fig. 1, the chromatogram of zinc in alkaline phosphatase is shown as one of the examples. Respective peaks for iron and zinc corresponding to those for UV absorbance at 280 nm were observed in the chromatograms. At the present condition, more than 95% of the metal contents for metalloenzymes in the original dilute sample were recovered in the fractions of their peaks after UF/HPLC/ICP-AES analysis.

Chromatograms of Natural Waters. Figure 2 shows the chromatogram for copper in sea water obtained by the UF/HPLC/ICP-AES method. The measurements were duplicated in order to test the reproducibility of the present method. The fraction eluted earlier than ca. 10 min were considered as those containing LMC's of copper (M.W.>10000). The chromatographic patterns of the duplicates had some differences. The small deviations in the analytical values may be ascribed mostly to errors in the ICP-AES measurements. From the peak areas in both of the chromatograms, however, it was calculated that 100±10 ng L^{-1} of copper was dissolved in the form of LMCs, which gave good reproducibility.

Figures 3 shows the chromatogram for zinc in lake water obtained by the UF/HPLC/GFAAS method along with that by the UV absorption method. According to calculation from the peak areas, about 60 ng L^{-1} of zinc in the form of LMC (LMC-Zn) was

present in natural lake water.

Figure 4 also shows the chromatograms for copper and iron in lake water observed by the UF/HPLC/ICP-AES method. It was found from the chromatographic peak areas that 200 ng L^{-1} of iron and 50 ng L^{-1} of copper were dissolved as LMCs in lake water. The chromatographic patterns for iron and copper are clearly different from each other, which shows the difference of the dissolved states of those elements. It should be noted here that the elution peak for iron appeared only at about 5.5 min, which corresponds to some large molecule. On the other hand, three clear peaks were observed for copper, and one of them was consistent with that for iron.

The chromatogram for zinc in sea water by the UF/HPLC/GFAAS method is also shown in Fig. 5. LMC-Zn in the sea water was around 90 ng L^{-1}.

The analytical results for LMC-forming metal ions in natural waters examined are summarized in Table 3. The concentration level of LMC-Fe in sea water was too low to be determined by the present method. Total values for lake water shown in Table 3 were estimated by ICP-AES without preconcentration, and those for sea water were determined by the gallium coprecipitation/ICP-AES technique.[17]

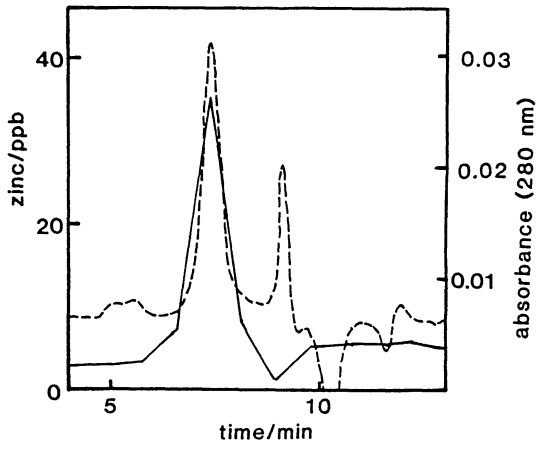

Fig. 1. HPLC chromatogram for *E. coli* alkaline phosphtase.
Sample: ultrafiltration-concentrated (×1000) sample of 0.1 mg L^{-1} *E. coli* alkaline phosphatase, sample volume: 200 μL. ——: zinc concentration determined by ICP-AES at 218.3 nm; ----: UV absorbance at 280 nm.

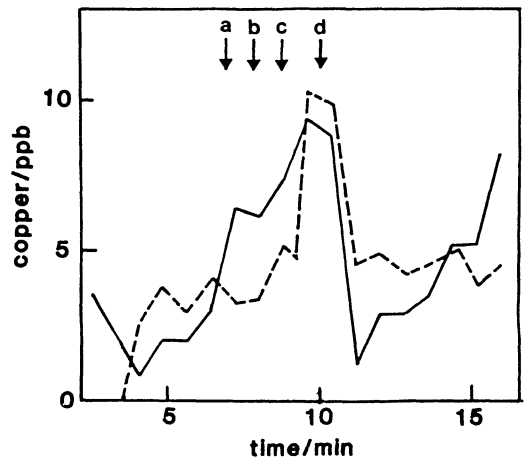

Fig. 2. HPLC chromatograms for concentrated sea water detected by ICP-AES at 324.7 nm.
Sample: concentrated (×1200) sea water collected off Misaki on September 16, 1981. ——: measurement No. 1; ----: measurement No. 2. Elution times for standard proteins: a: ferritin (M.W.: 450000); b: *E. coli* alkaline phosphatase (M.W.: 89000); c: Bovine carbonic anhydrase (M.W.: 31000); d: cytochrome c (M.W.: 12500).

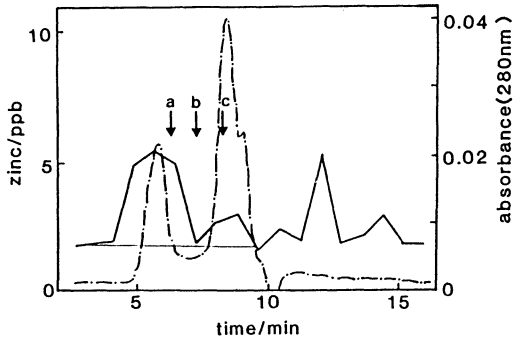

Fig. 3. HPLC chromatograms for concentrated lake water.
Sample: concentrated (×930) lake water collected from Lake Kasumigaura (Sta. 2) on July 20, 1981. ——: zinc concentration determined by ICP-AES at 213.8 nm; —·—: UV absorbance at 280 nm. Elution times for standard proteins: a: ferritin; b: *E. coli* alkaline phosphatase; c: Bovine carbonic anhydrase.

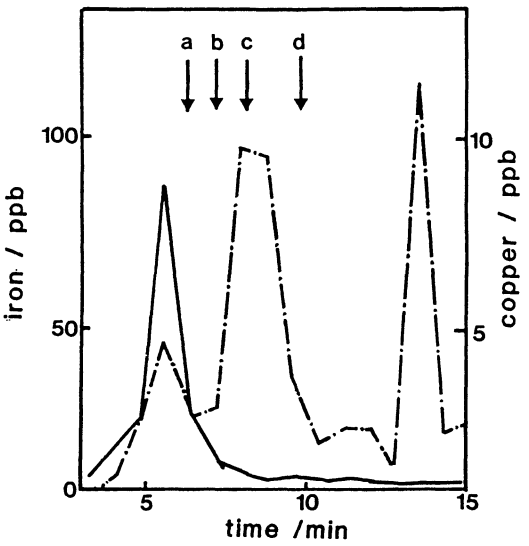

Fig. 4. HPLC chromatograms for concentrated lake water.
Sample: concentrated (× 930) lake water collected from Lake Kasumigaura (Sta. 2) on July 20, 1981. ——: iron concentration determinde by ICP-AES at 259.9 nm; —·—: copper concentration determined by ICP-AES at 324.7 nm. Elution times for standard proteins: a: ferritin; b: *E. coli* alkaline phosphatase; c: Bovine carbonic anhydrase; d: cytochrome c.

Table 3. Analytical Results for Total and Speciated Metal Contents in Lake and Sea Waters

Element	Concentration/ng mL^{-1}					
	Lake water[a]			Sea water[b]		
	Total[c]	LMC[d]	AP[f]	Total[e]	LMC[d]	AP[f]
Zn	(3.7)	0.06	0.0006	2.3	0.07	0.00002
Cu	2.7	0.05		0.7	0.1	
Fe	(9.3)	0.2		3.2	ND[g]	
V	ND[g]	0.025		—[h]	0.14	
Mo	(3.5)	0.009		—[h]	0.35	

a) Freshwater sampled from Lake Kasumigaura on July 20, 1981. b) Sea water sampled off Misaki on September 16, 1981. c) Determined by ICP-AES; figures in parentheses are more than standard deviations of background signals but below three times of them. d) Concentration of LMC-forming metal ion determined by ICP-AES. e) Determined by Ga-coprecipitation and ICP-AES. f) Concentration of zinc in alkaline phosphatase. g) Not detected. h) Not determined.

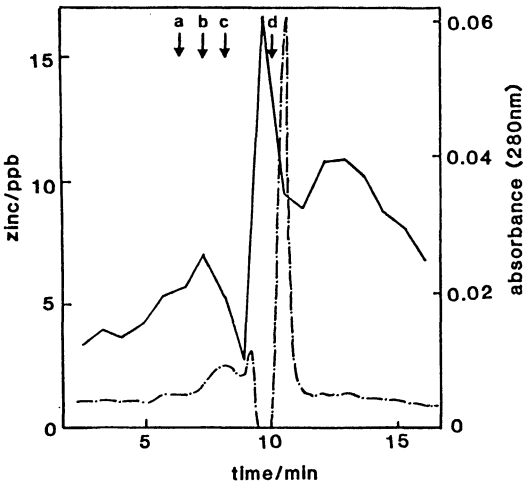

Fig. 5. HPLC chromatograms for concentrated sea water.
Sample: concentrated (×1200) sea water collected off Misaki on September 16, 1981. ——: zinc concentration determined by ICP-AES at 213.8 nm; — · —: UV absorption at 280 nm. Elution times for standard proteins: a: ferritin; b: *E. coli* alkaline phosphatase; c: Bovine carbonic anhydrase; d: cytochrome c.

Discussion

Concentration Techniques. There are a number of concentration techniques for metal ions in the solution samples, and they can be classified into (i) exclusion of solvents from the solution, and (ii) separation of solutes from the solution by changing the form of the solutes. Ultrafiltration (UF), reverse osmosis extraction (RO), rotary evaporation (RE), and freeze-drying (FD) are included in the first category: Coprecipitation, salting out, and adsorption on resins belong to the second one. In the RO, RE, and FD techniques, ionic strengths of metal ions in solution are generally increased during the concentration process. In the course of concentration of solutes such as metal complexes, it is required that the physical and chemical forms of solutes and the ionic strength of solutions should remain constant, and also that the chemical equilibria should not be changed. In addition, a high recovery ratio and low contamination are required.

The ultrafiltration technique satisfies most of the conditions described above. The only problem of ultrafiltration is the recovery of solutes in concentration. Actually adsorption on the filter sometimes caused less effective concentration. In the case of the concentration factor of not more than 1000 times for the lake water samples, the recovery was more than 90% (more than 95%, in the case of very clean samples such as dilute metalloenzyme standard solutions). In the case of the factor over 1000 times, however, the recovery was seriously deteriorated, because insolubilized organic compounds started to be adsorbed on filter surface. The practical limit of concentration for the lake water was therefore 1000 times, while that for the sea water used in the present study (containing less organic compounds than the lake water) was ca. 5000 times. This limitation will, of course, vary after the composition of the natural water.

By ultrafiltration, some small-molecular weight metal complexes (SMC's) were still remaining in the concentrated sample, whose concentration could be no less than that in the original sample. It may mislead the estimation of the concentration of large-molecular weight complexes (LMC's). Thus we applied high performance gel filtration technique to separate LMC's and SMC's. In addition, the present technique is useful to characterize LMC's by their molecular weights. For example, molecular weights of major LMC-Zn were about 30000 and 90000 (Fig. 5). As shown in the figure, the peaks of UV-absorbance (280 nm) and those of zinc concentration were not corresponded. It suggested that only small portion of UV-absorbing compounds were complexed with zinc; zinc ion itself does not absorb UV light at 280 nm.

Conventional (low-pressure) gel filtration might be useful for the present purpose. High performance gel filtration is, however, superior to conventional

one, because the former requires shorter analytical time than the latter. Longer run time may cause denaturalization of the organic compounds and/or dissociation of the complexes.

Evaluation of the Present Analytical System. In Fig. 1, a predominant peak for zinc concentration and that for absorbance at 280 nm can be observed at the same elution time. Other smaller peaks are seen in the chromatogram with UV-absorbance, no corresponding peak appearing in the chromatogram with zinc concentration. They may be considered as the peaks of denaturalized proteins in the sample. The results for standard metalloenzymes provided high recovery ratio (>95%) of the metal ions in the present system. When only alkaline phosphatase was injected, neither peaks of iron nor copper were found, and no other peaks than iron were found when catalase was injected. Thus the present technique can be a promising one to determine LMC in natural waters.

Determination of LMC in Natural Waters. The concentrations of LMC-forming zinc, iron, copper, vanadium, and molybdenum are summarized in Table 3. These were obtained by simultaneous multielement determination of such metals in chromatographic fractions, which provided the chromatograms similar to those in Fig. 4. As can be seen in Table 2, the detection limits for these elements in the ICP-AES measurement were low enough at the concentration level of their organic-formed species.

On the other hand, LMC of aluminum, chromium, manganese, cobalt, nickel, cadmium, and lead could not be determined, maybe due to the poor detectability of the present analytical method and low concentrations of these elements in natural waters. Either an increase in the concentration factor of the sample, preconcentration of the effluents of HPLC prior to detection, or improved sensitivity of the detection methods would be required to determine many kinds of LMC-forming metal elements.

LMC and Metalloenzymes. In Table 3 the value of zinc combining with alkaline phosphatase is shown, which was estimated from the enzymatic activity using the specific enzymatic activity of *E. coli* alkaline phosphatase (Boehringer-Mannheim Co., No. 15429).[7,8] The zinc content as alkaline phosphatase (AP-Zn) was corresponding to 0.03—1% of total LMC-Zn, 1—20×10^{-5} of total Zn in natural water. Although its concentration corresponded to only an extremely small part of total dissolved zinc, AP-Zn in natural waters plays quite a significant role in the cycles of nutrient elements in natural water.[18]

These facts suggest that LMC-Zn contains not only nonspecific zinc-containing proteins, but also possibly other zinc-containing metalloenzymes such as carboxypeptidase and carbonic anhydrase, although enzyme assay other than alkaline phosphatase was not carried out. Further researches on the speciation of individual complexes such as metalloenzymes and characterization of trace elements dissolved in natural waters would complement to elucidate LMC's in organic complexes of metal ions[6] and complexing capacity of natural waters.[19,20]

The present authors express their sincere thanks to Dr. Keiichiro Fuwa and Dr. Akira Otsuki in the National Institute for Environmental Studies for their kind help in sampling and valuable discussion.

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