Determination of Ultratrace Zinc by Enzymatic Activity of Carbonic Anhydrase. II. Use of Carbonate Hydro-lyase Activity

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A method for the determination of trace zinc has been investigated using hydro-lyase activity of carbonic anhydrase. It was proved theoretically and experimentally that the activation rate of the apo-carbonic anhydrase with zinc is proportional to the amount of zinc present in the solution. Hydro-lyase activity was measured electrochemically using sodium hydrogencarbonate as a substrate. The limit of detection was 0.89 ng/ml or 40 pg of zinc, lower than that obtained by the esterase activity method. Application of the present method to the determination of zinc in real samples has been examined in order to evaluate its analytical feasibility.

At the present time, enzymes show high potentiality for utilization in analytical chemistry in terms of their selectivity for substrates and high sensitivity originating from biochemical amplification.[1,2] Actually, the availability of enzyme electrode or enzyme colorimeter is well recognized, especially in clinical and agricultual fields.[3,4] Of the enzymes, metalloenzymes have specific metal ions located at their active centers, which play essential roles in enzymatic activities. The specificity of enzymatic activity to certain metal ions of metalloenzymes indicates the possibility of determination of trace metal ions. Only a few workers have studied the determination of zinc and copper by enzymatic activity of metalloenzymes, such as aminopeptidase,[5] alkaline phosphatase,[6] and polyphenoloxidase (tyrosinase).[7,8]

In a previous paper, we reported on the determination of trace zinc using the esterase activity of carbonic anhydrase, the recovery of the esterase activity of apo-enzyme (a zinc-free enzyme) being monitored by colorimetric technique.[9] The carbonic anhydrase method has a high selectivity to zinc, and is useful for determining trace zinc ion in natural samples such as fruit juices. On the other hand, carbonic anhydrase has another function, *i.e.*, hydration-dehydration catalysis of carbon dioxide (hereafter referred to as "hydro-lyase activity"), which is more essential in biological systems and has a higher turn-over rate than its esterase activity.

In the present investigation, the hydro-lyase activity of carbonic anhydrase was used for the determination of zinc in combination with the electrochemical detection method. We have found an almost linear relationship between the recovery of enzymatic activity and the amount of zinc. Since this method is more sensitive and convenient than the previous one, it was applied to the determination of zinc in natural water.

Theoretical Consideration

Carbonic anhydrase (CA) has one zinc atom at its active center, the atom being essential for its enzymatic activity (All symbols which follow are described in the Appendix.). Under the conditions $[E_a]_0 \gg [Zn]_0$, the equation between metal ion and apo-CA concentration is written as follws:[9]

$$[E] = K[E_a]_0[Zn]_0/(1+K[Zn]_0). \quad (1)$$

Since the relation $K[E_a]_0 \gg 1$ can be assumed, we obtain

$$[E] \simeq [Zn]_0. \quad (2)$$

CA catalyzes the following hydration-dehydration equilibrium,

$$H_2O + CO_2 \underset{A}{\rightleftharpoons} H_2CO_3 \underset{B}{\rightleftharpoons} H^+ + HCO_3^-, \quad (3)$$

where A is the rate-determining step. Thus, Eq. 3 can be written as

$$H_2O + CO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H^+ + HCO_3^-. \quad (4)$$

When sodium hydrogencarbonate is used as a substrate, the non-enzymatic reaction rate can be expressed as the decrease of hydrogen ion concentration:

$$(d[H^+]/dt)_{non} = k_1[H_2O][CO_2] - k_{-1}[HCO_3^-][H^+]. \quad (5)$$

The reaction rate for the enzymatic process can also be expressed as

$$(d[H^+]/dt)_{enz} = [E](V'_m[HCO_3^-]/K'_m - V_m[CO_2]/K_m)/(1+[HCO_3^-]/K'_m+[CO_2]/K_m). \quad (6)$$

The total reaction rate, $d[H^+]/dt$, is given by the summation of Eqs. 5 and 6. Under the conditions in which the concentration of total carbonate (*i.e.*, $[HCO_3^-]+[CO_2]$) and the initial pH are constant, V'_m, V_m, $[HCO_3^-]$, and $[CO_2]$ are represented as the functions of $[H^+]$. We then have

$$d[H^+]/dt = [E]f([H^+]) + g([H^+]), \quad (7)$$

where $f([H^+])=(d[H^+]/dt)_{enz}/[E]$, and $g([H^+])=(d[H^+]/dt)_{non}$. Here we define τ and τ_0 as the time required for the pH change from pH_A to pH_B, when CA is added and not added, respectively. Then τ and τ_0 can be expressed as follows:

$$\tau = \int_{[H^+]_A}^{[H^+]_B} (dt/d[H^+])d[H^+] = \int_{[H^+]_A}^{[H^+]_B} [1/\{[E]f([H^+])+g([H^+])\}]d[H^+], \quad (8)$$

$$\tau_0 = \int_{[H^+]_A}^{[H^+]_B} \{1/g([H^+])\}d[H^+]. \quad (9)$$

Since the condition $[E]\,f([H^+]) \gg g([H^+])$ is easily satisfied because of the high turn-over rate of carbonic anhydrase, we obtain

$$1/\tau = [E]/\int_{[H^+]_A}^{[H^+]_B} d[H^+]/f([H^+]) + \int_{[H^+]_A}^{[H^+]_B} \{g([H^+])/f^2([H^+])\}d[H^+]/\left\{\int_{[H^+]_A}^{[H^+]_B} d[H^+]/f([H^+])\right\}^2. \quad (10)$$

When we take $1/\int_{[H^+]_B}^{[H^+]_A} d[H^+]/f([H^+])$ as c, and the second term as c', we get

$$1/\tau = c[E] + c', \quad (11)$$

which shows that a plot of $1/\tau$ against [E] is linear. When $[H^+]_B$ is nearly equal to $[H^+]_A$, c' could be approximated to $1/\tau_0$. After all, the following expression is derived from Eqs. 2 and 11:

$$1/\tau = c[Zn]_0 + 1/\tau_0. \quad (12)$$

Namely, the amount of zinc in the solutions can be determined from the plot of $1/\tau$ *vs.* $[Zn]_0$.

Experimental

Chemicals. Bovine carbonic anhydrase C-7500 (EC 4. 2. 1. 1, abbreviation BCA, Sigma Chem. Co.) was used. Buffer (barbital sodium–HCl, abbreviation Veronal) was purified by means of solvent extraction using a 0.001% dithizone–chloroform solution.[11] The substrate, sodium hydrogencarbonate (Wako Pure Chemicals) was of pH measurement grade. Sodium salts of analytical grade were used as anion standards, and metal chlorides (except $Pb(NO_3)_2$ and $FeSO_4(NH_4)_2SO_4$) of analytical grade were used as metal ion standards, both purchased from Wako Pure Chemicals. The distilled water was prepared by a Daiken Sekiei subboiling distiller, zinc content not exceeding 20 pg/ml.[12]

Instruments. A Toa pH meter HM-5B with a glass electrode GS-125C, a pH stat HSM-10A, and a recorder EPR-200A, were used for the measurement of hydro-lyase activity of carbonic anhydrase. A Hitachi 170-50 atomic absorption spectrophotometer with a Jarrel-Ash FLA-100 carbon rod atomizer was used to determine the zinc content in the sample solutions. UV absorption was measured with Shimadzu UV-210A spectrophotometer. All the pH measurements and ultrafiltration were performed in a Thomas Scientific bath circulator TRL-111SP.

Preparation of the Apo-enzyme. A zinc-free enzyme (apo-enzyme) was prepared by a modified method of Hunt *et al.*,[13] which also differs from the previous method.[9] The procedure is as follows: 30 mg BCA was dissolved in 5 ml of 25 mM Veronal buffer (pH 7.0) containing 50 mM 2,6-pyridinedicarboxylic acid (referred to as PDA solution) (1 M=1 mol dm^{-3}). The enzyme was placed in an Amicon Diaflo cell (type 12), and the PDA solution placed in the reservoir was allowed to run through the Amicon UM-10 ultrafiltration membrane for several hours. Zinc concentration of the effluent was monitored by atomic absorption spectrometry. After zinc concentration of the effluent was as low as the original PDA solution and constant, sub-boiling-distilled water was allowed to flow through the ultrafiltration membrane until the UV absorbance of the effluent at 271 nm (due to PDA) was negligible. All the ultrafiltration was done at 0.5 °C. The residue in the cell (apo-BCA solution) was then taken out and filled up to 3 ml as a "stock reagent," and zinc and protein concentrations were measured by atomic absorption and micro-biuret methods,[14] respectively.

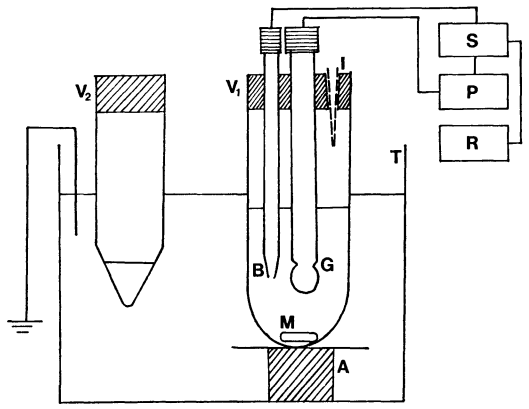

Fig. 1. Reaction vessel for the present method.
V_1: Reaction vessel, V_2: incubation vessel, T: thermostatic bath, P: pH meter, S: pH stat, R: recorder, A: acrobat stirrer, M: magnetic spinner, G: glass electrode, B: buret (0.1 M NaOH is contained), I: inlet of reagent.

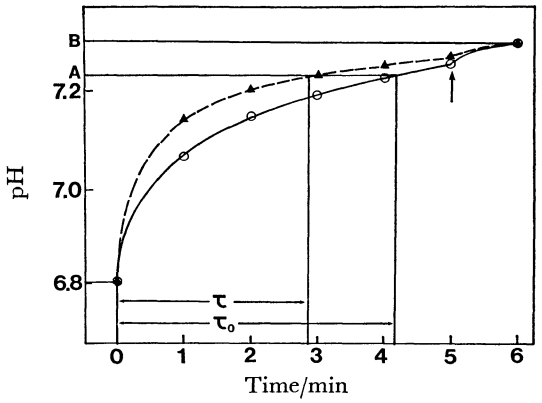

Fig. 2. Time dependence of pH change.
○——○: Without BCA, ▲----▲: with 5 nmol apo-BCA and 230 pg zinc ion, ——→: excess holo-BCA is added, A: $pH_{90\%}$, B: pH_{eq}.

Measurement of Enzymatic Activity. There are some conventional methods for measuring the hydro-lyase activity of BCA,[15,16] where carbon dioxide or sodium hydrogencarbonate is usually used as a substrate, but these are not suitable for zinc-determination. We have used sodium hydrogencarbonate as a substrate, working out a new method to determine zinc. The reaction vessel for this method is shown in Fig. 1. Reaction temperature was maintained at 0.5±0.2 °C.

In order to accelerate the reaction between the apo-enzyme and zinc in the sample, 20 μl of 200 μM apo-BCA solution was pre-incubated with a mixture of 100 μl of the Veronal buffer (pH 6.8) and 100 μl of the sample for 5 min. The mixture was added to the measurement media of 20 ml of the Veronal buffer (pH 6.8). After stirring for 1 min (t=6 min) the substrate solution, 100 μl of 0.65 M sodium hydrogencarbonate, was added to the reaction vessel. The pH change of the mixture resulting from the enzymatic reaction was recorded (Fig. 2). After equilibrium was reached (*ca.* 5 min after mixing the substrate), $[H^+]_{90\%}$ was calculated by the equation

$$[H^+]_{90\%} = \{[H^+]_0 + 9[H^+]_{eq}\}/10. \quad (13)$$

τ is defined as the time required to reach $[H^+]_{90\%}$ (Fig. 2).

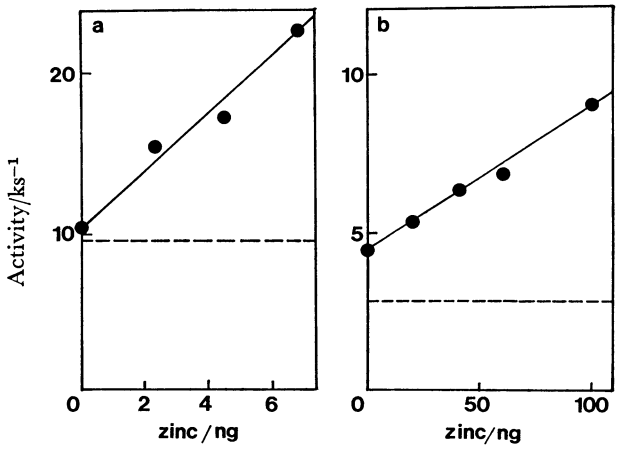

Fig. 3. Calibration curve for zinc measured by hydro-lyase activity of Bovine carbonic anhydrase.
a) Pre-incubation was done. 4.1 ng of apo-BCA and zinc sample were mixed in a separate vessel for 5 min, and added into the reaction vessel. b) No pre-incubation was done. 18 ng of apo-BCA and zinc sample were added directly into the reaction vessel, and kept 5 min before measurement.

Results

Calibration Curve for Zinc. The calibration curve for zinc under the conditions described (see Experimental), is shown in Fig. 3a. The detection limit is 0.89 ng/ml or 40 pg of zinc. The detection limit and dynamic range of the method are dependent on the amount of apo-BCA, the volume of the sample-apo-BCA mixture, and the incubation time in the vessel.

By this method, the dimension of enzymatic activity value is time^{-1} (*i.e.*, reciprocal of τ). When activity becomes high, τ-value becomes smaller, and $1/\tau$ value varies widely. The upper limit of detection is restricted by the deviation of $1/\tau$ value.

Interference of Coexistent Metal Ions. The interferences of the concomitant metal ions under the conditions $\sum_i [M_i^{2+}]_0 \gtrsim [E_a]_0$ are summarized in Table 1. Only Co^{2+} at a concentration equal to that of zinc ion increases the hydro-lyase activity by *ca.* 50%. Other metal ions might interfere with the enzymatic activity, particularly Hg^{2+}. When $\sum_i [M_i^{2+}]_0 \ll [E_a]_0$, the influence of concomitant metal ions is small.

Interference of Coexistent Anions. The interference of the concomitant anions in the present method is given in Table 2. Excess amounts of cyanide and perchlorate ions (100 times more than $[Zn^{2+}]$,) significantly interfere with the analytical results as in the esterase method. On the other hand, thiocyanate and nitrite ions at high level (1000 times in excess of $[Zn^{2+}]$) appear to activate enzymatic activity. If interference of the concomitants occurs. the standard addition method should be employed.

For the determination of zinc in sea water, we studied the influence of 0.5 M NaCl in order to estimate the ppb-level of zinc. No apparent inhibition was observed, the calibration curve for zinc being the same as that without NaCl.

Table 1. Effect of metal ions on hydro-lyase activity of carbonic anhydrase[a)]

Coexistent metal ions	Concentration of coexistent metal ions/ppb				
	10000	1000	100	10	1
Hg^{2+}		10	50	74	96
Co^{2+}			150		
Cu^{2+}	3	16	101		
Fe^{2+}	74	104	101		
Ni^{2+}	45	99	97		
Mn^{2+}	91	96	98		
Cd^{2+}	6	30	100		
Pb^{2+}	22	112	105		

a) The ratio (%) of the enzymatic activities measured with and without coexistent metal ions. Zn^{2+}: 35 pmol (100 ppb) apo-BCA: 140 pmol, incubation time: 5 min.

Table 2. Effect of anions on hydro-lyase activity of BCA[a)]

Anions	Concentration/mM	Activity/%[b)]
CN^-	100	45
	10	57
SCN^-	100	990
	10	228
	1	107
NO_2^-	100	290
	10	126
ClO_4^-	100	43
I^-	100	68
Br^-	100	82
Cl^-	100	106
F^-	100	89
CH_3COO^-	100	90
NO_3^-	100	84

a) Zn^{2+}: 1.5 μM (100 ppb), apo-BCA: 6.0 μM, incubation time: 5 min. b) Ratio (%) of the enzymatic activities measured with and without the anions.

Table 3. Determination of zinc in real samples

Sample	Zinc concentration/(ng/ml)	
	Present method[a)]	Atomic absorption
City water	155	157
Orange juice	131	124

a) apo-BCA: 200 pmol, sample: 20 μl, incubation time: 5 min.

Determination of Zinc in Real Samples. Analytical results for orange juice and city water (Both filtrated with a membrane filter; pore size 0.2 μm) are given in Table 3, together with the amount of zinc in the samples determined by flame atomic absorption spectrometry. The analytical values are in line with those obtained by atomic absorption spectrometry.

Discussion

Consideration for Incubation. The formation reac-

tion between zinc and apo-BCA is

$$E_a + Zn \underset{k_f}{\longrightarrow} E. \quad (14)$$

The k_f value estimated by Henken *et al.*[17] is *ca.* 10^4 $M^{-1}\,s^{-1}$. When $[E_a]_0 \gg [Zn]_0$, the concentration of holo-BCA ([E]) obtained after the incubation for t s is

$$[E] = [Zn]_0\{1-\exp(k_f[E_a]_0 t)\}. \quad (15)$$

By this equation, 1 min is enough to complete the reaction of forming holo-BCA, since $[E_a]_0$ is *ca.* 5 μM by the esterase method.

In the present system, the amount of apo-enzyme needed for analysis is less than that for the esterase method. This is due to the higher activity of BCA in the hydro-lyase reaction than that in the esterase reaction. The use of a less amount of apo-BCA reduces background activity which is due to the contaminant zinc in apo-enzyme. The incubation time in the analytical procedure should be prolonged as can be estimated from Eq. 15. The sample and apo-enzyme were pre-incubated before detection, using a separate vessel so as to increase the net value of $[E_a]_0$ in Eq. 15. The analytical time could be reduced to *ca.* 1% of that in the direct analysis (without pre-incubation (*ca.* 5 min).

No pre-incubation was required for the determination of zinc in high concentration, because τ in Eq. 8 became too small. The calibration curve for zinc in the case of without pre-incubation is shown in Fig. 3b. Here Eq. 2 does not hold, while holo-BCA is approximately proportional to zinc content if incubation time is kept constant (Eq. 15). Employing these two methods (with and without pre-incubation) properly according to the zinc concentration, we could get a wide dynamic range from 40 pg to 100 ng for zinc.

Interference of Coexistent Metal Ions. Several transition metal ions such as copper(II), iron(II), cadmium(II), mercury(II), cobalt(II), and zinc(II) can coordinate to the active center of apo-BCA. Only cobalt-BCA and zinc-BCA have hydro-lyase and esterase activities.[18] Let us assume the following equilibrium between metal ions (M_i^{2+}) and the apo-BCA in solution,

$$E_a + M_i^{2+} \underset{K_i}{\rightleftharpoons} M_i^{2+}\text{-}E_a, \quad (16)$$

$$K_i = [M_i^{2+}\text{-}E_a]/[E_a][M_i^{2+}]. \quad (17)$$

Metal ions interfere with the reaction by sharing the coordination sites of the active center in apo-BCA, inhibiting the association of zinc(II) and apo-BCA. When $\sum_i [M_i^{2+}]_0 \ll [E_a]_0$, the influence of concomitant metal ions is small. Conversely, when $\sum_i [M_i^{2+}]_0 \simeq [E_a]_0$, the influence of metal ions becomes appreciable according to the K_i values which are specific to each concomitant metal ion, M_i^{2+}, as shown by

$$[E] \simeq K_{Zn}[Zn]/\sum_i K_i[M_i^{2+}]. \quad (18)$$

The degree of interference due to the concomitant metal ions is given in Table 1. Generally, interference of metal ions, except mercury(II) ion (for K_{Hg} is not small) and cobalt(II) ion can be neglected when $[M_i^{2+}]_0 < [E_a]_0$. The level of mercury(II) ion or cobalt(II) ion contained in most of the real samples, such as biological samples and sea water, is much lower than that of zinc(II) ion,[19,20] so that the interferences of metal(II) ions can be ignored.

Interference of Coexistent Anions. The effects of the coexisting anions (X^-) on the activity of carbonic anhydrase[21] are based on the fact that the holo-BCA forms 1:1 complexes with monovalent anions which have no enzymatic activity.

$$Zn\text{-}BCA + X_j^- \underset{K_j}{\rightleftharpoons} BCA\text{-}Zn\text{-}X_j^-, \quad (19)$$

where

$$K_j = [BCA\text{-}Zn\text{-}X_j^-]/[Zn\text{-}BCA][X_j^-]. \quad (20)$$

From Eq. 16, we get

$$\alpha = [BCA\text{-}Zn\text{-}X_j^-]/[BCA\text{-}Zn] = [X_j^-]K_j. \quad (21)$$

As compared to the esterase method, samples are more diluted with buffer in the present method, indicating the lowering in α-value (*i.e.*, the interference rate of anions,) and reduction of anion effects.

Application to Zinc Determination in Real Samples. The results shown in Table 2 indicate that the present method can be applied to the determination of zinc in water. The fact that the concentrations of zinc analyzed by the present method are close to those obtained by atomic absorption spectrometry in most of the samples shows that zinc in such samples is dissolved as the reactive form with apo-BCA (exchangeable zinc.) Since the present method is applicable to samples with high salinity, the zinc at the proper concentration in sea water could be directly analyzed by this method.

In the esterase activity method, non-specific esterases in real samples might interfere with the results.[9] By contrast, hydro-lyase activity is specific to carbonic anhydrase, so only its contamination in samples such as serums, should be taken into account. Precision in the present method would thus be better than that in the previous method.

Conclusion

The hydro-lyase method shows promise for determining trace zinc. Carbonic anhydrase has the following characteristics.

1) Reversible dissociation of zinc from protein; zinc-free apo-enzyme has no activities.

2) High specificity to zinc ion as a central metal ion; other than zinc ion, only cobalt(II) ion reactivates apo-enzyme.

3) High biochemical amplification; hydro-lyase activity of carbonic anhydrase has maximum turn-over rate.

4) Linearity between metal concentration and enzyme activity; *i.e.*, only one zinc ion per enzyme.

5) Easy enzymatic assay.

6) An enzyme easily available.

These characteristics, especially 2) and 4), indicate the superiority of carbonic anhydrase over other zinc metalloenzymes for zinc determination.

The present method is superior in selectivity and

sensitivity to the conventional colorimetric method such as the dithizone method.[22] It is possible to determine zinc in a concentrated salt solution, such as sea water, which is one of the most difficult subjects in furnace atomic absorption spectrometry.

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Appendix

E: Holoenzyme,
E_a: Apo-enzyme,
M_i^{z+}: Metal ion,
X_j^-: Monovalent anion,
K: Stability constant between enzyme protein and zinc,
K_i: Stability constant between enzyme protein and metal ions (M_i^{z+}),
K_j: Stability constant between holoenzyme and monovalent anion (X_j^-),
k_0: Molecular activity of enzyme,
k_1, k_{-1}: Rate constant,
k_f: Formation rate constant,
t: Reaction time,
V_m: Maximum reaction rate of hydration reaction,
V_m': Maximum reaction rate of dehydration reaction,
K_m: Michaelis constant of hydration reaction,
K_m': Michaelis constant of dehydration reaction,
$[]_0$: Concentration at the condition of initial stage,
$[]_{eq}$: Concentration at an equilibrium stage,
$()_{non}$: Value when no enzyme is added,
$()_{enz}$: Value when enzyme is added.

References

1) G. G. Guilbault, "Enzymatic Methods of Analysis," Pergamon Press, Oxford (1970).
2) M. M. Fishman and H. F. Schiff, *Anal. Chem.*, **48**, 322R (1980).
3) L. D. Boweres and P. W. Carr, *Anal. Chem.*, **48**, 544A (1976).
4) Y. Umezawa, *Kagaku No Ryoiki*, **31**, 20 (1977).
5) P. Lehky and E. A. Stein, *Anal. Chim. Acta*, **49**, 366 (1969).
6) A. Townshend, and A. Vaughan, *Talanta*, **17**, 289 (1970).
7) J. V. Stone and A. Townshend, *J. Chem. Soc., Dalton Proc.*, **1973**, 495.
8) B. Mattiasson, H. Nilsson, and B. Olsson, *J. Appl. Biochem.*, **1**, 377 (1979).
9) K. Kobayashi, K. Fujiwara, H. Haraguchi, and K. Fuwa, *Bull. Chem. Soc. Jpn.*, **52**, 1932 (1979).
10) J. C. Kernohan, *Biophys. Biochim. Acta*, **81**, 345 (1964).
11) B. G. Malmström, *Arch. Biochim. Biophys.*, **46**, 345 (1953).
12) T. Harada, K. Fujiwara, and K. Fuwa, *Bunseki Kagaku*, **26**, 877 (1977).
13) J. B. Hunt, M. -J. Rhee, and C. B. Storm, *Anal. Biochem.*, **79**, 614 (1977).
14) R. F. Itzhaki and D. M. Gill, *Anal. Biochem.*, **9**, 401 (1964).
15) E. R. Waygook, "Methods in Enzymology," ed by S. P. Colowick and N. O. Kaplan, Academic Press, New York (1955), Vol. 2, pp. 836—846.
16) K. M. Wilbur and N. G. Anderson, *J. Biol. Chem.*, **176**, 147 (1948).
17) R. W. Henkens and I. M. Sturtevant, *J. Am. Chem. Soc.*, **90**, 669 (1968).
18) J. E. Coleman, *Biochemistry*, **4**, 2644 (1965).
19) E. J. Underwood, "Trace Elements in Human and Animal Nutrition," 3rd ed, Academic Press, New York (1971).
20) S. Okabe, I. Hirota, and K. Shimizu, "Kaiyo No Kagaku," Tokai Daigaku Shuppan-kai, Tokyo (1980), pp. 11—14.
21) Y. Pocker and J. T. Stone, *Biochem. J.*, **40**, 319 (1946).
22) E. B. Sandell, "Colorimetric Determination of Traces of Metals," 3rd ed, Interscience, New York (1959).