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Spectrophotometric Studies on Hydroxamic Acid-Borate and Aluminate Complex

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Borate was found to prevent or reverse remarkably the inhibition of urease activity by hydroxamic acids. Based on these facts, formation of hydroxamic acid-borate complex was studied spectrophotometrically.

1. All the metal ions tested except boric ion showed little protective effect against hydroxamate-inhibition of urease activity.

2. Characteristic absorption maximum of benzohydroxamic acid-borate complex was observed at 265 nm in the pH range from 8 to 11. The investigation of pH effect on the ultraviolet absorption of the complex suggests that keto form of hydroxamic acid-borate complex is tautomerized to imidol-borate complex around neutral pH, which is dissociated to the imidol anion and borate in a more alkaline pH range than 11. Aluminate is assumed spectrophotometrically to form the similar complex with benzohydroxamic acid, though it did not prevent the inhibition of urease activity by hydroxamic acid.

3. Based upon these observations, we proposed new spectrophotometric methods of determination of benzohydroxamic acid, borate and aluminate.

It has been well established that hydroxamic acids are the most potent and strictly specific inhibitors of urease activity (urea amidohydrolase, EC 3.5.1.5) of plant and bacterial origin.[2)] We have already presented the evidence that -CONHOH is the group which is absolutely necessary in the chemical structure for the inhibition of urease activity.[2a)] Through the studies on the mechanism of hydroxamate inhibition, boric ion was found to prevent completely the inhibition of urease activity by caprylohydroxamic acid. The ion was found not only to prevent, but also to recover the inhibition by benzohydroxamic acid.[3)] These observations strongly indicate that hydroxamic acids, in general, form non-inhibitory complexes with boric ion under physiological conditions.

We have already reported that hydroxamic acid-borate complex has a characteristic absorption maximum at 265 nm.[3)] We investigated in our present study the stability of the complexes by measuring the change in ultraviolet (UV) absorption spectrum in consideration of protective effect of borate on hydroxamate inhibition of urease activity. Aluminium ion belonging to the same Group III as boron was also found to form a complex with hydroxamic acid through the spectrophotometric study. Based on this spectrophotometric properties of the complex, we proposed the new determination method of aromatic hydroxamic acid in the presence of excess borate and in addition that of borate or aluminium ion in the presence of excess benzohydroxamic acid.

Material and Method

Sword bean powder was stirred for one hour with 5 vol. of 0.1M phosphate buffer (pH 6.7). The suspension was centrifuged at 10000 × *g* for 15 min and the supernatant after filtration was used as a crude enzyme

1) Location: *3190 Gofuku, Toyama.*

2) *a*) K. Kobashi, J. Hase, and K. Uehara, *Biochim. Biophys. Acta*, **65**, 380 (1962); *b*) W.N. Fishbein and P.P. Carbone, *J. Biol. Chem.*, **240**, 2407 (1965); *c*) J. Hase and K. Kobashi, *J. Biochem.* (Tokyo), **62**, 293 (1967); *d*) R.L. Blakeley, J.A. Hinds, H.E. Kunze, E.C. Webb, and B. Zerner, *Biochemistry*, **8**, 1991 (1969); *e*) K. Kobashi, K. Kumaki, and J. Hase, *Biochim. Biophys. Acta*, **227**, 429 (1971); *f*) K. Kobashi, J. Hase, and T. Komai, *Biochem. Biophys. Res. Commun.*, **23**, 34 (1966).

3) J. Hase, K. Kobashi, and K. Kumaki, *Chem. Pharm. Bull.* (Tokyo), **15**, 534 (1967).

preparation (81.5 units/mg of protein). Urease activity was measured colorimetrically according to the method of van Slyke.[4]

Benzohydroxamic acid (mp 131°) and caprylohydroxamic acid (mp 64°) were synthesized by the method of Hauser, *et al.*[5] and Inoue, *et al.*[6] respectively. All other chemicals were of analytical reagent grade. Solutions were prepared in all-glass distilled water.

All the spectrophotometric measurements were carried out with a Hitachi spectrophotometer Model 139 and pH measurements with a Hitachi-Horiba pH meter Model F-5.

Result

Effect of Metal Ion on the Inhibition of Urease Activity by Hydroxamic Acid

We examined the effect of various metal ions on urease-inhibition by benzohydroxamic acid under the experimental conditions where 100 times molar excess amount of metal ion as much as benzohydroxamic acid (1.8×10^{-6} M) was added. Only boric ion prevented remarkably the inhibition by the hydroxamic acid, and other metal ions such as Fe^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Al^{3+} did not prevent it. Little protective effect of these metal ions may be due to their lower solubilities and chelating activities at neutral pH range than boric acid.

The inhibition by benzohydroxamic acid was markedly recovered by the addition of boric ion within several minutes (Fig. 1). This restoring effect of boric ion suggests that the ion binds the benzohydroxamic acid in the enzyme-inhibitor complex to form a non-inhibitory hydroxamic acid-borate complex. The inhibition by caprylohydroxamic acid was not recovered by the addition of boric ion, though the proceeding of inhibition reaction

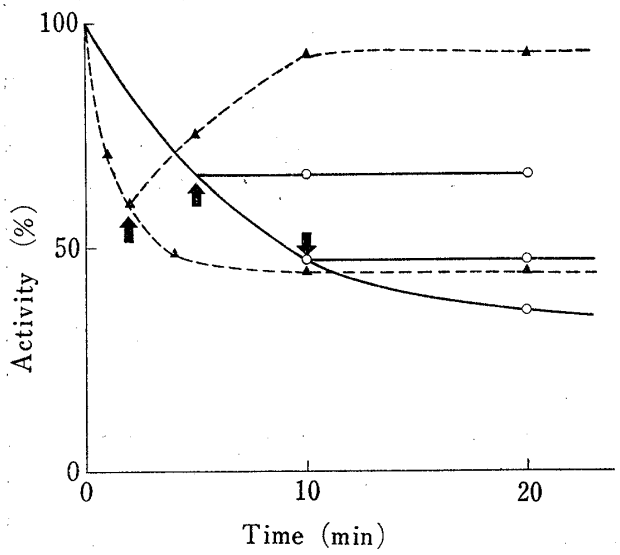

Fig. 1. Effect of Boric Acid on Urease-inhibition by Caprylo- and Benzohydroxamic Acid

The percentage of inhibition was measured under the following conditions. A mixture of 140 units of crude urease in 0.3 ml of 0.1 M phosphate buffer (pH 7.7) and of 2 n moles of caprylo- (—○—) or 1.2 n moles of benzohydr oxamic acid (---▲---) in 0.3 ml of the same buffer was preincubated at 37°. 500 times molar excess of borate as much as the hydroxamic acid was added at the times indicated by the arrows. The urease activities at different intervals of preincubation were measured colorimetrically by adding 5.0 ml of 3.0% urea solution in 0.1 M phosphate buffer (pH 6.7) and one drop of alcoholic solution of 0.1% phenol red.

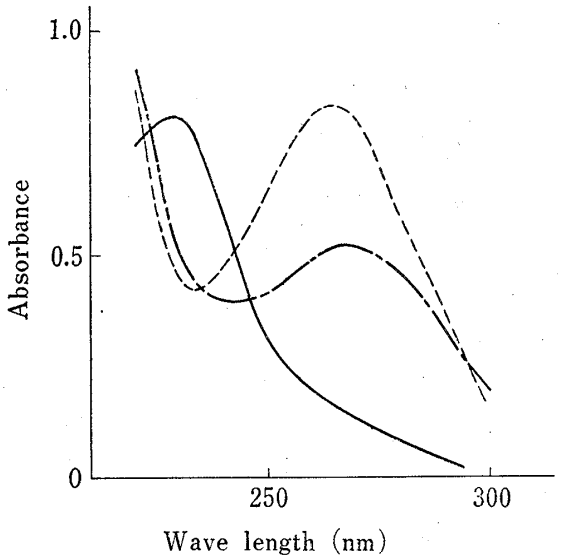

Fig. 2. UV Absorption Spectra of Benzohydroxamic Acid

UV absorption spectrum of 10^{-4} M benzohydroxamic acid was measured in 0.1 M phosphate buffer, pH 8.0 (——), pH 10.0 (—·—·—) or 0.1 M borate buffer, pH 8.0 (-----).

4) D.D. van Slyke and R.M. Arichibald, *J. Biol. Chem.*, **154**, 623 (1944).
5) C.R. Hauser and W.B. Renfrow, Jr., *Org. Syn.*, **19**, 15 (1935).
6) Y. Inoue and H. Yukawa, *J. Agr. Chem.* (Japan), **16**, 504 (1940).

was stopped at the time when the ion was added. The latter case indicates that the boric ion binds only free caprylohydrocamic acid in the reaction mixture to form an inactive complex, but does not bind the hydroxamic acid in the enzyme-inhibitor complex, because caprylohydroxamic acid forms more stable and undissociable complex with the urease molecule.[2f)]

Spectrophotometric Properties of Benzohydroxamic Acid-Borate Complex

Benzohydroxamic acid-borate complex has a characteristic high absorption maximum at 265 nm[3)] and the molar ratio of hydroxamic acid to boric acid in the complex was found to be two[3)] in an alcoholic solution and to be one[7)] in an aqueous solution. From the resemblance of absorption spectrum and maximum of the complex with those of benzaldoxime and acetophenon oxime, it was speculated that boric acid binds an imidol tautomer of hydroxamic acid.[3)]

Benzohydroxamic acid in 0.1 M phosphate buffer has an absorption maximum at 230 nm at pH 8.0 and that at 265 nm at pH 10 (Fig. 2). This change in absorption maximum is considered to be caused by ionization of hydroxamic acid. Therefore, we investigated the effect of pH on absorbance at 265 nm of the compound. As is shown in Fig. 3, the absorbance started to increase at pH 7.5 and reached the maximum at pH 10, and became almost constant in a more alkaline medium. The inflection point (pH 8.8) coincided well with the pK_a of the hydroxamic acid. Therefore, the absorption at 265 nm was evidently attributable to the ionic form of benzohydroxamic acid.

The benzohydroxamic acid in 0.1 M borate buffer, pH 8.0, has an absorption maximum at 265 nm and showed a resembled absorption spectrum to that of ionic form of the acid, although the absorbance at 265 nm in a borate buffer was 1.6 times higher than that of the ionic form (Fig. 2). Effect of pH on absorbance at 265 nm of the hydroxamic acid in 0.1 M borate buffer was shown in Fig. 3. The absorbance started to increase at pH 5.8 and reached the maximum at pH 7.8 and thereafter became constant until pH 11. Then it decreased with the increase of pH and became constant in the range of pH 13 and higher, where the absorbance was the same value as that of the hydroxamic acid in 0.1 M phosphate buffer. The absorbance at 265 nm of benzohydroxamic acid-borate complex in the range of pH 7.8—11 was observed to be remarkably high. Therefore, benzohydroxamic acid was determined in the concentration between 1×10^{-5} and 5×10^{-5} M[3)] by measuring the difference of absorbance at 265 nm at pH 8.0 in the presence and in the absence of borate. The sensitivity of this

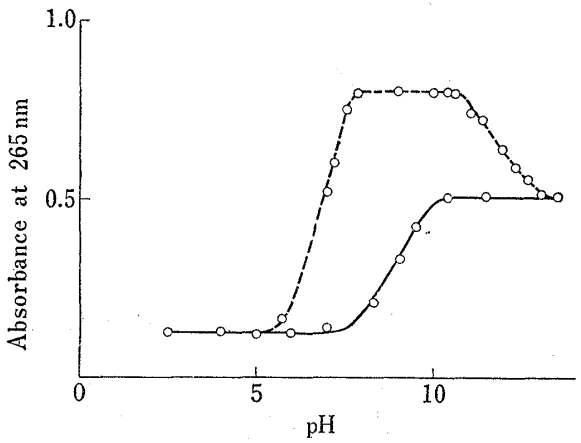

Fig. 3. Effect of pH on Absorbance at 265 nm of Benzohydroxamic Acid

Absorbance at 265 nm of 10^{-4} M benzohydroxamic acid was measured at various pHs in 0.1 M phosphate buffer (——), or in 0.1 M borate buffer (------).

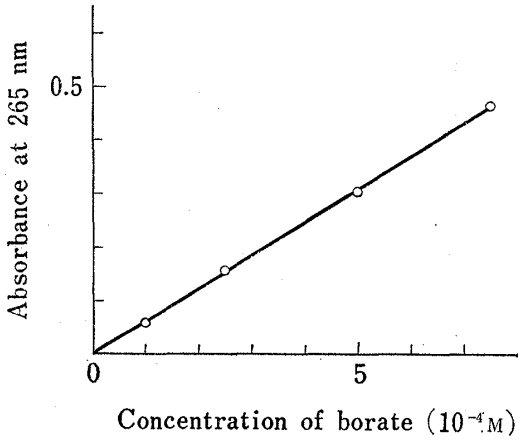

Fig. 4. Calibration Curve of Borate

Absorbance at 265 nm of 10^{-3} M benzohydroxamic acid was measured in the presence of different concentrations of borate in 0.1 M phosphate buffer, pH 8.0.

7) A.R. Fields, B.M. Daye, and R. Christian Jr., *Talanta*, **13**, 929 (1966).

method was ten times higher than that of the determination method of Lipmann and Tuttle.[8] Based on this observation, boric ion was also determined with ease in the presence of excess amount of benzohydroxamic acid (Fig. 4).

Spectrophotometric Properties of Benzohydroxamic Acid-Aluminate Complex

In order to know the chelation ability of aluminium ion, we measured the absorption spectrum of the solution containing 1×10^{-4}M benzohydroxamic acid and 1×10^{-2}M aluminium potassium sulfate at pH 3.3. The spectrum was observed to be almost consistent with that of benzohydroxamic anion in 0.1M phosphate buffer, pH 10 as is shown in Fig. 2. The characteristic absorption maximum at 265 nm at pH 3.3 was considered to be due to the formation of hydroxamic acid-aluminate complex, because benzohydroxamic acid does not dissociate at pH 3.3 at all.

Effect of pH on this absorption maximum is shown in Fig. 5. The absorbance at 265 nm started to increase sharply at pH 2, but was unable to determine at more alkaline pH region than 4, because the solution became turbidly by the precipitation of aluminium hydroxide (curve A in Fig. 5).

Curve B was obtained under the condition where 1×10^{-3}M benzohydroxamic acid was added to 5×10^{-5}M aluminium potassium sulfate solution in a final concentration, and then the pH of the mixture was adjusted. Aluminium hydroxide was not precipitated under these conditions. Therefore, a linear increase of absorbance with the increase of pH was caused probably by the increase of formation of benzohydroxamic acid-aluminate complex. When more sodium hydroxide was added to the turbid mixture of benzohydroxamic acid and aluminium potassium sulfate (curve A), the turbidity was dissolved at pH 12, where the absorbance

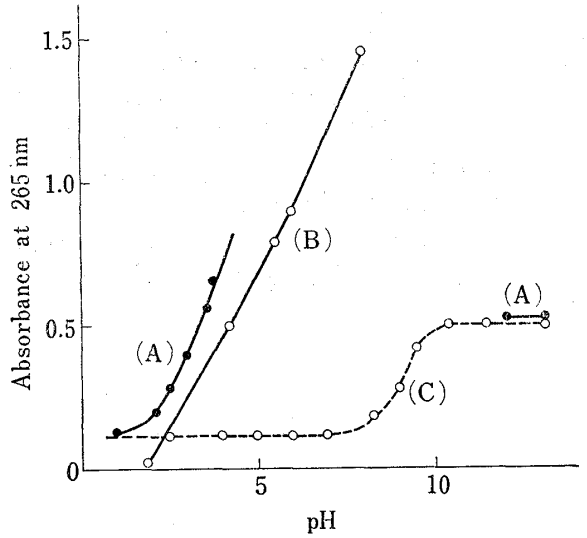

Fig. 5. Effect of pH on Absorbance at 265 nm of Benzohydroxamic Acid in the Presence of Aluminium Ion

Absorbance at 265 nm was measured of the solution under the following conditions.
curve A (—●—): The pH of the solution containing 10^{-2} M aluminium potassium sulfate and 10^{-4} M benzohydroxamic acid was adjusted by HCl or by NaOH.
curve B (—○—): 10^{-3} M benzohydroxamic acid was added to 5×10^{-5} M aluminium potassium sulfate solution and the pH of the solution was adjusted. The absorbance of 10^{-3} M benzohydroxamic acid was subtracted as a blank.
curve C (---○---): The absorbance of 10^{-4} M benzohydroxamic acid solution in absence of aluminium ion.

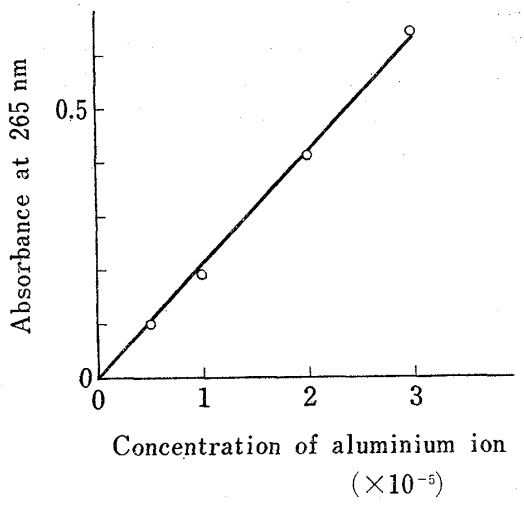

Fig. 6. Calibrations Curve of Aluminium Ion

Absorbance at 265 nm of 10^{-3} M benzohydroxamic acid was measured in the presence of diferent concentrations of aluminium ion in 0.1 M Tris-HCl buffer, buffer, pH 7.8.

8) F. Lipmann and L.C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).

at 265 nm was the same value as that of benzohydroxamic acid in absence of aluminium ion (curve C). This observation suggested that the complex was completly dissociated at pH 12.

Benzohydroxamic acid-aluminate complex was found to have a high absorption maximum at 265 nm. Aluminium ion was able to be quantitatively determined with high sensitivity in the concentration between 5×10^{-6}M and 3×10^{-5}M (Fig. 6).

Discussion

Hydroxamic acid-borate complex is highly dissociable and could not been isolated from the solution. However, the complex was isolated[3] from the alcoholic solution as white needles decomposed at 258°, according to the method of preparation of mannitol-borate complex.[9] From the analysis of the complex, the molar ratio of benzohydroxamic acid to borate was found to be two.[3] The result does not necessarily mean the complex in an aqueous solution to be identical with that isolated from the ethanol solution. Green[10] speculated spectrophotometrically and tirimetrically the 1:1 complex formation of isonicotinohydroxamic acid with boric acid. Fields, *et al.*[7] found that the acids carrying no substitution on nitrogen formed 1:1 complexes in an aqueous solution with boric acid by measuring the hydrogen ion concentrations according to the following equation.

$$RCONHOH + H_3BO_3 \text{ —— } (1{:}1 \text{ complex})^- + H^+$$

As shown in Fig. 3, the absorbance at 265 nm of benzohydroxamic acid solution was found to be proportional to the amount of anionic form of the compound. According to Exner,[11] most hydroxamic acid is present in a keto form and little in an imidol form in an acidic or a neutral medium. Ionization of the compound proceeds to A and B form from the keto and to B and C form from the imidol form, and the rate of ionization to B form is predominantly higher than those to other forms. Therefore, most hydroxamic anion is present in B form, little in A and C form in an alkaline medium. This Exner's explanation and our spectrophotometric study reveal that a characteristic absorption maximum of benzohydroxamic acid was due to its imidol anion.

We speculated in our previous report[3] that boric acid formed the complex with benzohydroxamic acid in an imidol form of tautomer, because of its resemblance in UV spectrum with oximes. In consideration of our present results, Exner's report[11] and the co-ordination

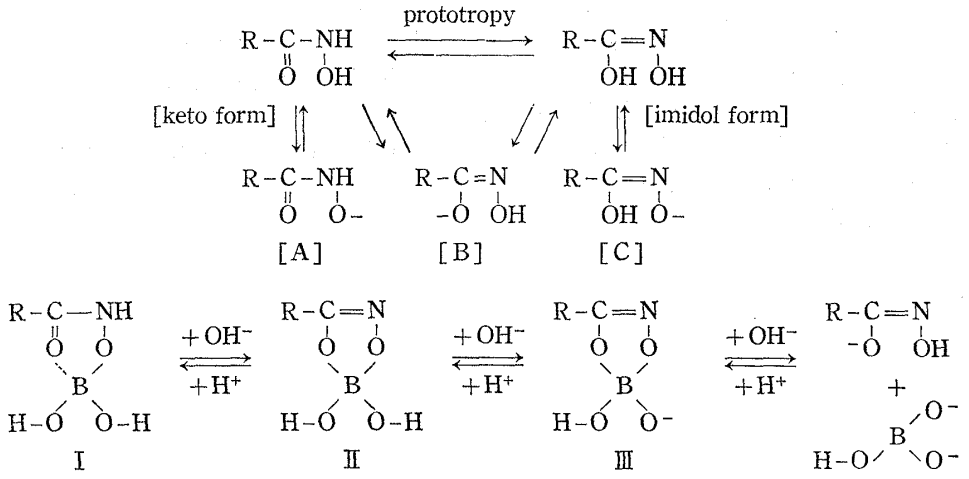

Fig. 7. Tautomerism and Ionization of Hydroxamic Acid

9) J.J. Fox and A.J.H. Gange, *J. Chem. Soc.*, **99**, 1075 (1911).
10) A.L. Green, *J. Org. Chem.*, **1956**, 2566.
11) O. Exner, *Dansk. Tidsskr. Farm.*, **42**, 145 (1968).

number of boron, possible mechanism of ionization and borate-complex formation of benzohydroxamic acid were illustrated in Fig. 7.

The increase in absorbance at 265 nm of benzohydroxamic acid in the presence of borate (Fig. 2) was observed in a pH region where hydroxamic acid does not dissociate. The increase is considered to be not due to the ionization of hydroxamic acid, but to the formation of imidol-borate complex (II) which is tautomerized from keto-borate complex (I) by the addition of alkali as illustrated in Fig. 7. The imidol-borate complex (II) and its monoprotic anion (III)[7] are stable from pH 8 to 11, because the absorbance at 265 nm was constant. The imidol-borate complex (III) is dissociated to the imidol anion and borate anion by further addition of alkali, because the absorbance at 265 nm is gradually decreased at pH 11 and higher, and finally reaches at pH 13 to the same value as the absorbance of benzohydroxamic anion at the same pH in absence of borate. We have no spectrophotometric evidence for the formation of keto-borate complex (I), but marked protective effect of boric acid on urease inhibition by hydroxamic acid was also observed even at pH 5.6. This results suggested that hydroxamic acid-borate complex existed in a lower pH region where no characteristic absorption was observed.

Aluminate, one of the elements belonging to the same group III as boron, is assumed spectrophotometrically to form a similar complex with benzohydroxamic acid.

Aliphatic hydroxamic acids are also assumed to form the corresponding complexes above described with boric acid, from the observation of the protective effect of borate on urease-inhibition by the hydroxamic acids. However, the change of their absorption spectra in the presence of borate could not be observed and in addition the complex could not be isolated by the similar way to the case of benzohydroxamic acid.

It is clear from the protective effect of borate on urease-inhibition that hydroxamic acid-borate complex is non-inhibitory on urease activity. The inhibition by benzohydroxamic acid was completely reversed by the addition of borate, the fact of which shows the urease-benzohydroxamic acid complex to be dissociable. On the contrary, the inhibition of caprylohydroxamic acid was not reversed by the addition of borate. These observations reveal that the mode of inhibition by aromatic hydroxamic acid is distinguished from that by aliphatic inhibitor.

A large number of hydroxamic acid derivatives have been described in the literature[12,13] as the reagents for metal ions. However, the investegations hitherto have been done to see if a variety of hydroxamic acid could be used as colorimetric or gavimetric reagents for metal ions. Through the spectrophotometric study of benzohydroxamic acid-borate or aluminate complex, we suggested a new method of determination of borate and aluminate. The spectrophotometric measurement is easier and more simple in the procedure than the curcumin method for borate.[14,15] The practical application of this method for various natural samples is limited, because the benzohydroxamic acid does not always specifically form the complex with borate or aluminate, and thus the contaminating metal ion may interfere the spectrophotometric determination.

12) W.W. Brandt, *Record of Chem. Progress*, **21**, 159 (1960).
13) V.C. Bass and J.H. Toe, *Talanta*, **13**, 735 (1966).
14) M. Miyamoto, *Bunseki Kagaku*, **11**, 635 (1962).
15) M. Miyamoto, *Bunseki Kagaku*, **12**, 115 (1963).