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The Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. VI. The Flow-Injection Analysis of Protein Using a 1,10-Phenanthroline-Hydrogen Peroxide System

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Since the catalytic activity of copper(II) for the chemiluminescent reaction between 1,10-phenanthroline and hydrogen peroxide decreased in the presence of protein, this phenomenon was applied to the determination of protein. The determination of protein was carried out by a flow-injection method, and the conditions for the determination of protein were established. The catalytic activity of copper(II) was enhanced by using an amino acid instead of the conventional biuret reagent. Similar calibration curves were obtained for human serum albumin, bovine serum albumin, human serum γ-globulin, bovine serum γ-globulin, and ovalbumin. According to the present flow-injection method using a chemiluminescent reaction, a small amount of protein could be conveniently and economically determined over a wide range of concentrations, 5.0×10^{-6} g dm^{-3}—1.0×10^{-1} g dm^{-3} at the rate of about 20 samples per hour, with the detection limit of 250 pg as the injected amount and the coefficient of variation of 5.9% (n=9). The sensitivity of the present method was about 40 times that of the previous luminol-hydrogen peroxide system.

In the previous paper,[1] several proteins in the concentration range of 2.0×10^{-4} g dm^{-3}—1.0×10^{-1} g dm^{-3} were determined by the use of the fact that the catalytic activity of copper(II) for the chemiluminescent (CL) reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) and hydrogen peroxide (H_2O_2) decreased in the presence of proteins. Though the method was sensitive, non-labeled, and applicable to the detection of an amino acid as well as protein,[2] its sensitivity was not yet satisfactory for the determination of a trace amount of a constituent in a human serum sample.

According to the authors' knowledge about the reaction between copper(II) and protein at high temperatures, a more sensitive method for the copper(II) determination was necessary for the determination of a smaller amount of protein than that in the previous paper. With this in mind, the CL reaction between 1,10-phenanthroline(phen) and H_2O_2, which had previously been used for the determination of ultratraces of copper(II),[3] was applied to the determination of a protein in the present paper. The present method showed a wider linear dynamic range and a smaller detection limit than those in the previous papers. The catalytic activity of copper(II) in a phen–H_2O_2 system was enhanced in the presence of an amino acid in opposition to that in a luminol–H_2O_2 system. On the basis of this phenomenon, bovine serum albumin (BSA) could be determined more sensitively in the presence of an amino acid.

Since the present method was expected to be useful for the development of a sensitive, rapid, and inexpensive detector by which a trace amount of protein could be determined without labeling it, the optimum conditions for the determination of protein, the improvement of the detection limit, and so on were examined.

Experimental

Reagents. All the reagents used were of a commercially available special grade. Ion exchange water was distilled for use. A 1.0×10^{-7} mol dm^{-3} copper(II) solution (biuret reagent) was prepared by diluting a 2.0×10^{-2} mol dm^{-3} copper(II) stock solution[4] with a buffer solution (pH 10.19) consisting of 0.1 mol dm^{-3} boric acid and 0.1 mol dm^{-3} potassium hydroxide. A phen solution containing ethylhexadecyldimethylammonium bromide (EHDAB), tetraethylenepentamine (TEPA), and sodium hydroxide (NaOH), and a H_2O_2 solution were prepared daily. The sample solution of BSA and bovine serum γ-globulin (BγG) from Sigma Chemical Co., and human serum albumin (HSA) and human serum γ-globulin (HγG) from ICN Pharmaceuticals, Inc., and control serum (Q-PAC-Chemistry Control Serum I) from HYLAND were prepared by diluting them in buffer solution. All α-amino acids were from Kyowa Hakko Co., Ltd.

Apparatus and Procedure. A schematic diagram of the apparatus used for the present flow-injection analysis is shown in Fig. 1. All the tubes and connectors used were made of Teflon except for the flow cell. Each solution of phen(a) and H_2O_2(b), held at 25 °C by means of a thermostat(n) (Netsudenshi Kogyo Co., Ltd.), and copper(II) (c), held at room temperature, was fed by means of 5.0×10^5 Pa gauge pressure of nitrogen gas; the flow rates were all adjusted to 1.0 cm^3 min^{-1} by the use of a needle valve (h) (GILMONT). A buffer solution(d) was fed at the flow rate of 1.0 cm^3 min^{-1} by means of a pump(i) (Pharmacia High-precision Pump P-500).

A 50 mm^3 portion of a protein sample solution was injected into the buffer solution line through the sampling loop(k) and the six-way cock(j). The protein sample solution was first mixed with a copper(II) catalyst solution and then passed through a reaction tube(o) (0.40 m), held at 95 °C, and a cooling tube(p) (0.64 m), held at 0 °C, in that order. Then the reacting solution was further mixed with

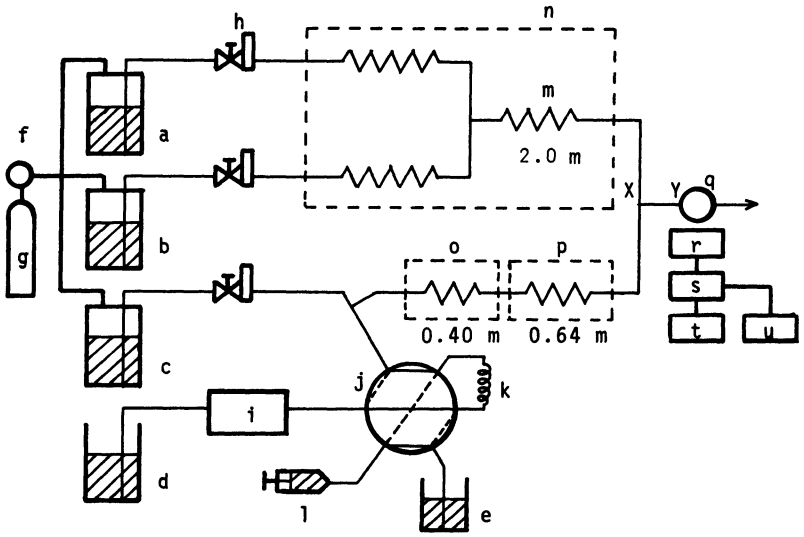

Fig. 1. Schematic diagram of the flow-injection system.
a: Phen solution, b: H_2O_2 solution, c: copper(II) catalyst solution, d: buffer solution, e: sample solution, f: regulator, g: N_2 cylinder, h: flowmeter equipped with a needle valve, i: pump, j: six-way cock, k: sampling loop, l: syringe, m: mixing coil, n: thermostat, o: reaction tube, p: cooling tube, q: flow cell, r: photomultiplier, s: amplifier, t: recorder, and u: integrator.

the phen-H_2O_2 solution, which had been thoroughly premixed by means of a 2.0 m mixing coil(m); the solution thus obtained was passed through a flow cell(q) (25 cm×0.8 mm i.d. poly(vinyl chloride) tube). The CL intensity of the solution passing through the cell was measured by means of a photomultiplier(r) (Hamamatsu TV Co., Ltd., R928), amplified with an amplifier(s) (Horiba Ltd., OPE-214), and recorded on a recorder(t) (Yokogawa Electric Works, Ltd., 3046), followed by the measurement of the peak area by the use of an integrator(u) (Shimadzu Chromatopac E1A or C-R2A). The distance between X and Y was 3 cm.

The CL intensity of a protein-free sample was recorded as a base line, while that of a protein-containing sample was recorded as a negative peak because the CL intensity was weakened due to the formation of a complex compound between copper(II) and protein.

Results and Discussion

Determination of Analysis Conditions. BSA was used as a model protein for the determination of the analysis conditions. The length of the reaction tube and the cooling tube and the reaction temperature were the same as in the previous paper. The flowing paths of both phen and H_2O_2 and the mixing coil were passed through a thermostat at 25 °C to keep them from the effect of the room temperature. A 2.0 m mixing coil was experimentally selected on the basis of its maximum CL intensity. The relationships between the peak area obtained by injecting a definite amount of BSA and phen, EHDAB, TEPA, NaOH, H_2O_2, and copper(II) solutions are shown in Figs. 2—7. From these results, the following optimum concentrations for the maximum peak area were chosen: 5.0×10^{-5} mol dm^{-3} phen; 4.0×10^{-5} mol dm^{-3} EHDAB; 2.0×10^{-7} mol dm^{-3} TEPA; 1.0×10^{-1} mol dm^{-3} NaOH; 5% H_2O_2; and 1.0×10^{-7} mol dm^{-3} copper(II).

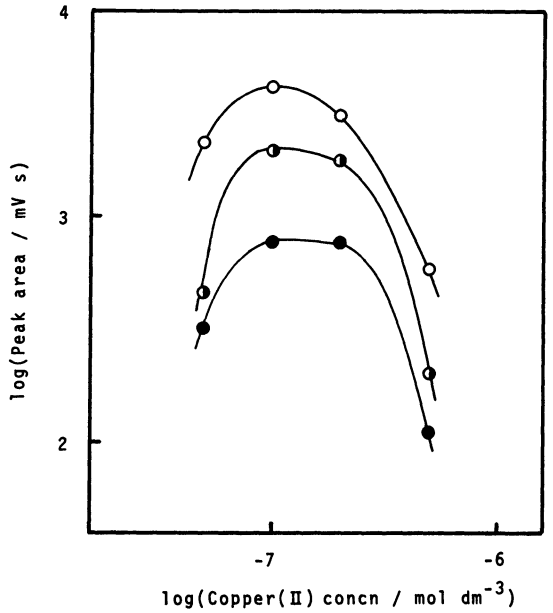

Fig. 2. Relationship between copper(II) concn and peak area.
○: 1.0×10^{-2} g dm^{-3}-, ◑: 5.0×10^{-3} g dm^{-3}-, and ●: 1.0×10^{-3} g dm^{-3}-BSA.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, and 5% H_2O_2.

Determination of Various Proteins. The calibration curves of BSA and BγG were obtained under the above-mentioned conditions (Fig. 8). As can be seen from Fig. 8, each linear response was obtained over a wide range of concentrations, and 2×10^{-5}—1×10^{-1} g dm^{-3} BSA and 2×10^{-5}—5×10^{-2} g dm^{-3} BγG could

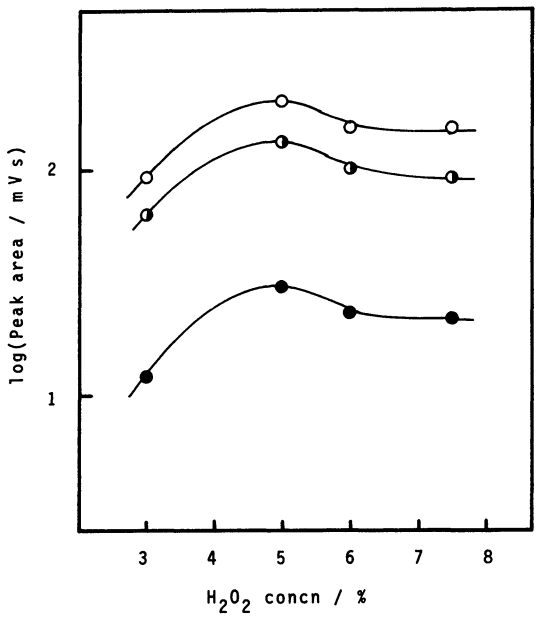

Fig. 3. Relationship between H_2O_2 concn and peak area.
O: 1.0×10^{-2} g dm^{-3}-, ◑: 5.0×10^{-3} g dm^{-3}-, and ●: 1.0×10^{-3} g dm^{-3}-BSA.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, and 1.0×10^{-7} mol dm^{-3} copper(II).

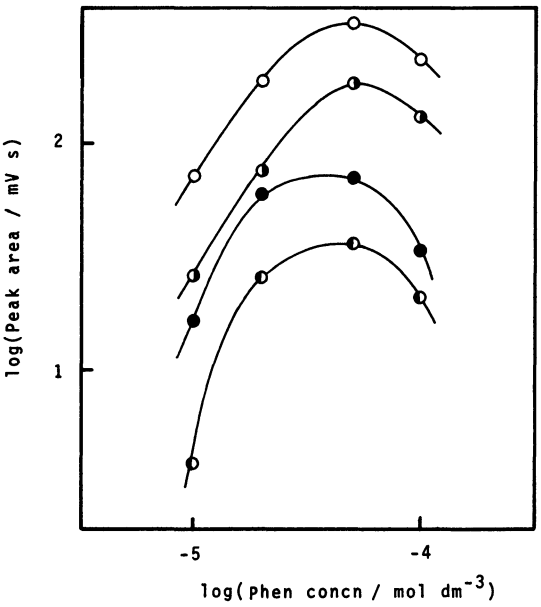

Fig. 4. Relationship between phen concn and peak area.
O: 1.0×10^{-2} g dm^{-3}-, ◑: 5.0×10^{-3} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, and ◐: 5.0×10^{-4} g dm^{-3}-BSA.
Conditions: 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

be determined. The detection limit (S/N=2) of the present method (a biuret reagent containing copper(II) and tartrate) was 1 ng BSA; this corresponded to one-tenth that in the previous paper. The coefficient of variation was 4.9% for ten measurements of 1×10^{-3} g dm^{-3} BSA.

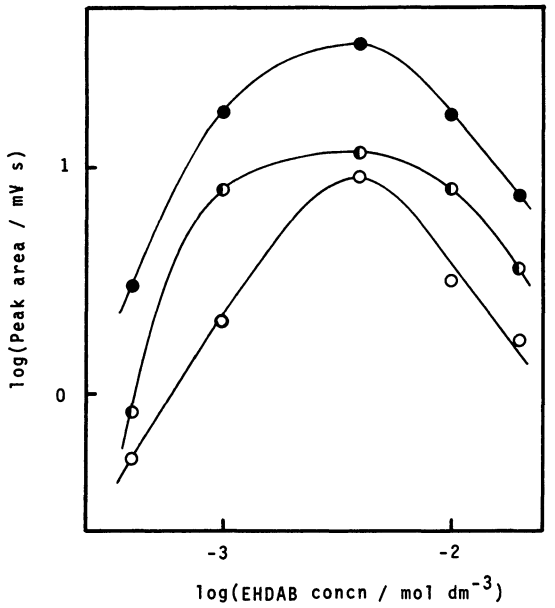

Fig. 5. Relationship between EHDAB concn and peak area.
●: 1.0×10^{-3} g dm^{-3}-, ◐: 5.0×10^{-4} g dm^{-3}-, and O: 1.0×10^{-4} g dm^{-3}-BSA.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

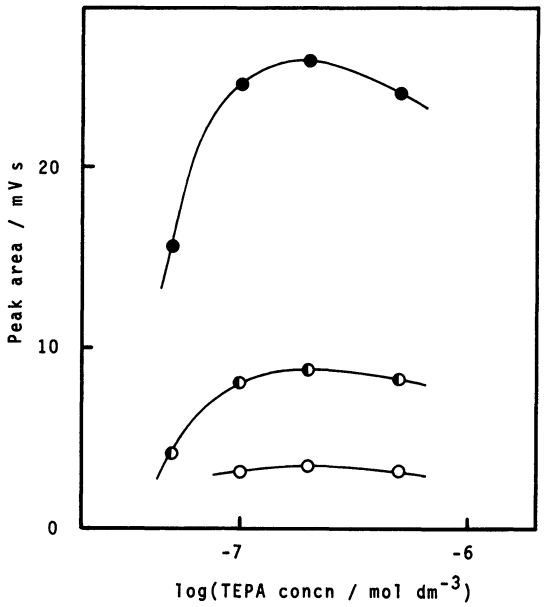

Fig. 6. Relationship between TEPA concn and peak area.
●: 1.0×10^{-3} g dm^{-3}-, ◐: 5.0×10^{-4} g dm^{-3}-, and O: 1.0×10^{-4} g dm^{-3}-BSA.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

A similar sensitivity was also obtained for HSA, HγG, and ovalbumin. Furthermore, the rapid formation of a very stable complex compound between copper(II) and protein by heating was suggested because the calibration curves were still linear, even at the limit of the detection of BSA and BγG. Therefore, the

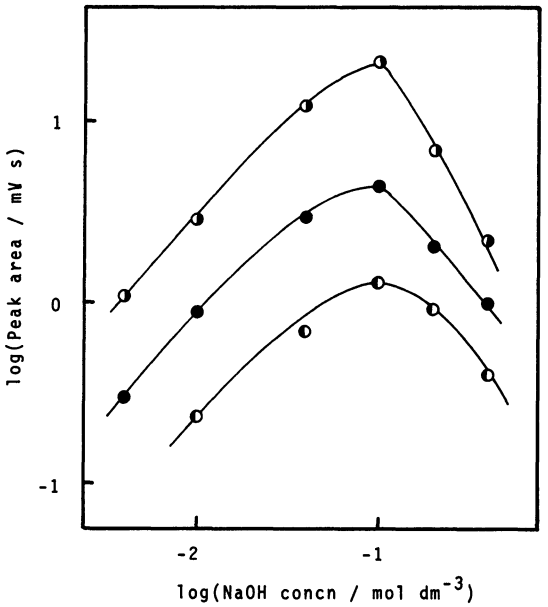

Fig. 7. Relationship between NaOH concn and peak area.
◑: 4.0×10^{-3} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, and ◐: 4.0×10^{-4} g dm^{-3}-BSA.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

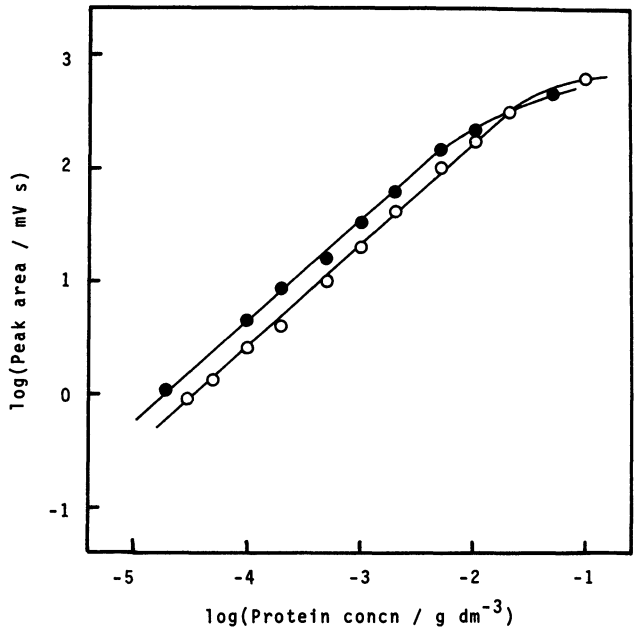

Fig. 8. Calibration curves of proteins.
○: BSA and ●: BγG.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

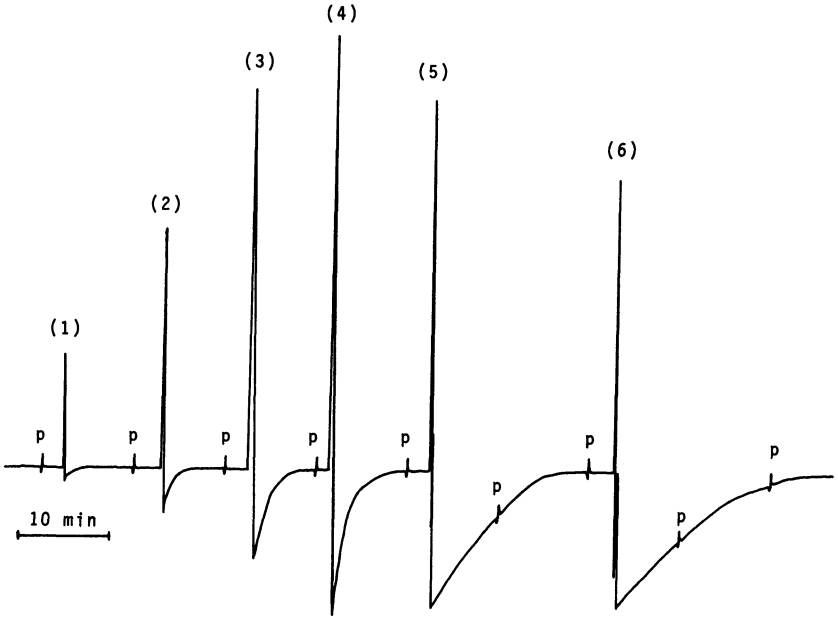

Fig. 9. Signals of L-aspartic acid as a model of an amino acid.
(1): 1.0×10^{-6} mol dm^{-3}-, (2): 1.0×10^{-5} mol dm^{-3}-, (3): 1.0×10^{-4} mol dm^{-3}-, (4): 1.0×10^{-3} mol dm^{-3}-, (5): 1.0×10^{-2} mol dm^{-3}-, (6): 2.0×10^{-2} mol dm^{-3}- Asp, and p: periodical shock peak accompanying with a reciprocation of the pump piston.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

feasibility for the determination of a much smaller amount of copper(II) was expected to improve the sensitivity of protein determination.

Copper(II) Catalyst Containing α-Amino Acid. The present method was applied to the determination of α-amino acid; the signals of L-aspartic acid (Asp) as a model are shown in Fig. 9. The shape of the signals was quite different from that in the luminol–H_2O_2 system; examination showed a first positive peak after the injection of an amino acid solution, followed by a second negative peak based on the formation of a copper(II)–Asp complex compound. The

Table 1. The Relative Responses of Various Amino Acids (5.0×10^{-5} mol dm^{-3}) Based on the Response of L-Aspartic Acid

α-Amino acid	Positive peak height	Positive peak area	Negative peak area	Formation constant*) $\log K_1K_2$
Asp	1.00	1.00	1.00	15.35[a)]
Gly	0.96	0.81	0.67	15.67
Pro	0.91	0.85	0.61	16.58[b)]
Ala	0.90	0.75	0.64	15.52
His	0.85	0.84	3.50	18.53
Glu	0.83	0.74	0.51	14.16
Lys	0.75	0.65	0.28	13.90
Arg	0.67	0.57	0.31	13.76

*) Temp: 25°C, Method: glass electrode, almost the same ionic strength. (The Chemical Society, "Stability Constant," Special Publication No. 17, London (1964)). a) 30°C. b) 20°C.

peak height corresponding to the positive catalytic activity increased with an increase in the Asp concentration and then decreased through the maximum value at 1×10^{-3} mol dm^{-3} Asp, while the peak area corresponding to the negative catalytic activity increased with an increase in the Asp concentration. These phenomena were also observed for such other amino acids as L-glutamic acid(Glu), L-glycine (Gly), L-alanine (Ala), L-proline (Pro), L-lysine hydrochloride (Lys), L-arginine hydrochloride (Arg), and L-histisine hydrochloride (His), and for ethylenediamine and 2,2′-bipyridine. However, a negative peak alone was obtained in the presence of such a ligand as triethylenetetramine, ethylenediaminetetraacetic acid, and diethylenetriaminepentaacetic acid, which contained more than four donor atoms and which formed a stable copper(II) complex compound. A negative peak alone was also obtained, even in the presence of ethylenediamine, when the mole ratio of ethylenediamine to copper(II) was very large ([ethylenediamine]/[copper(II)]=10^6). These results suggest that the final response rate has a close relation with the compositional structure of a copper(II) complex compound. A detailed investigation of the positive and negative peaks is now in progress. The response of each amino acid relative to that of Asp is shown in Table 1. Though the negative peak area of His was abnormally large, this seemed to be attributable to a larger formation constant of a copper(II)–His complex compound.

Since the positive cytalytic activity of copper(II) was much more enhanced in the presence of an amino acid than in that of a tartrate, while the negative catalytic activity alone of copper(II) was observed in the presence of protein, an attempt was made to determine microamounts of protein by using an amino acid instead of a tartrate contained in a biuret reagent. The relationship between the Arg concentration and the negative peak area was obtained by adding definite amounts of BSA to a solution containing a definite amount of copper(II) and various amounts of Arg as a coexisting amino acid (Fig. 10).

The calibration curves of BSA shown in Fig. 11 were obtained by adding various amounts of BSA to

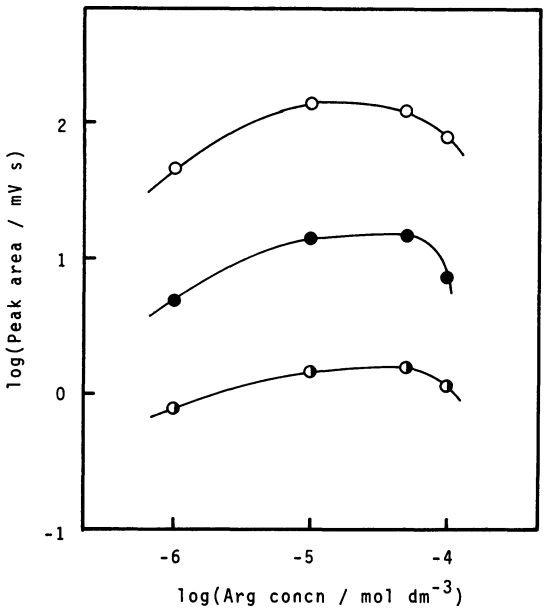

Fig. 10. Relationship between Arg concn and peak area.
○: 1.0×10^{-2} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, and ◑: 1.0×10^{-4} g dm^{-3}-BSA.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-7} mol dm^{-3} copper(II), and 5% H_2O_2.

a copper(II)–Arg solution, a copper(II)–biuret reagent solution, and a copper(II)–Asp solution, and by then measuring their peak areas. As can be seen from Fig. 11, the present modified method enabled us to determine 5.0×10^{-6}—1.0×10^{-1} g dm^{-3} BSA and showed the detection limit of 250 pg BSA. This value was one-fourth that in a biuret reagent and about one-fortieth that in the luminol–H_2O_2 CL system reported in the previous paper. The coefficient of variation obtained for nine measurements of 1×10^{-4} g dm^{-3} BSA was 5.9%. The other proteins, such as Bγ G, and HSA also showed a sensitivity similar to that in BSA (Fig. 12).

Though the present method was developed as a detector for high performance liquid chromatography, it was also applied to a human serum sample. First

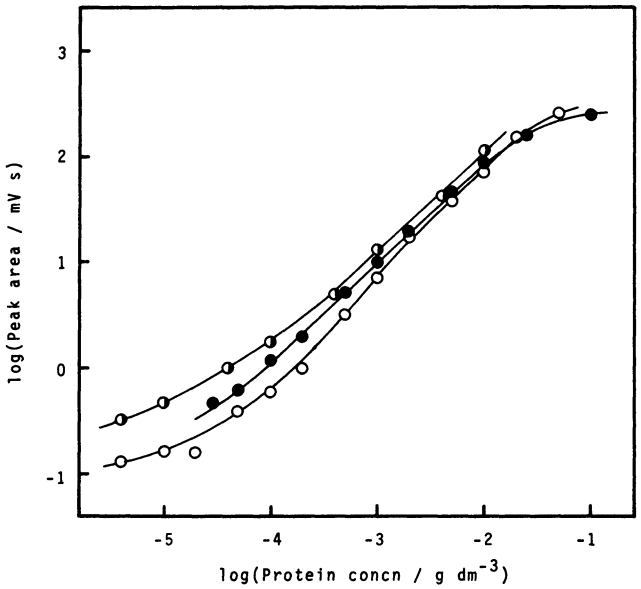

Fig. 11. Calibration curves of BSA in copper(II) catalyst solution.
◑: copper(II)–Arg soln, ●: copper(II)–biuret reagent soln, and ○: copper(II)–Arg soln.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 5% H_2O_2, 1.0×10^{-7} mol dm^{-3} copper(II), and 8.0×10^{-7} tartrate or 5.0×10^{-5} mol dm^{-3}-amino acids.

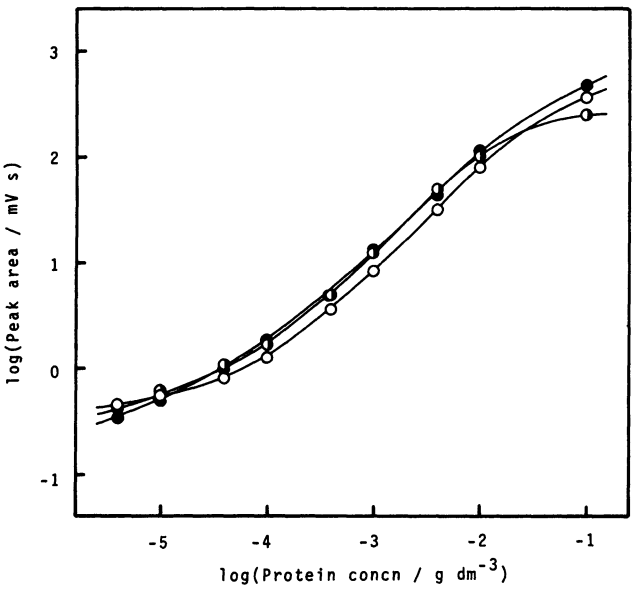

Fig. 12. Calibration curves of various proteins.
○: HSA, ●: BSA, and ◑: BγG.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 5% H_2O_2, 1.0×10^{-7} mol dm^{-3} copper(II), and 5.0×10^{-5} mol dm^{-3} Arg.

of all, the effect of the coexisting constituents in a human serum sample on the determination of HSA by the present modified method was examined. Various amounts of HSA were estimated in either the presence or absence of a definite amount of control serum (diluted to a volume of 10^5 times with a buffer solution), and the relationship between HSA concentration and

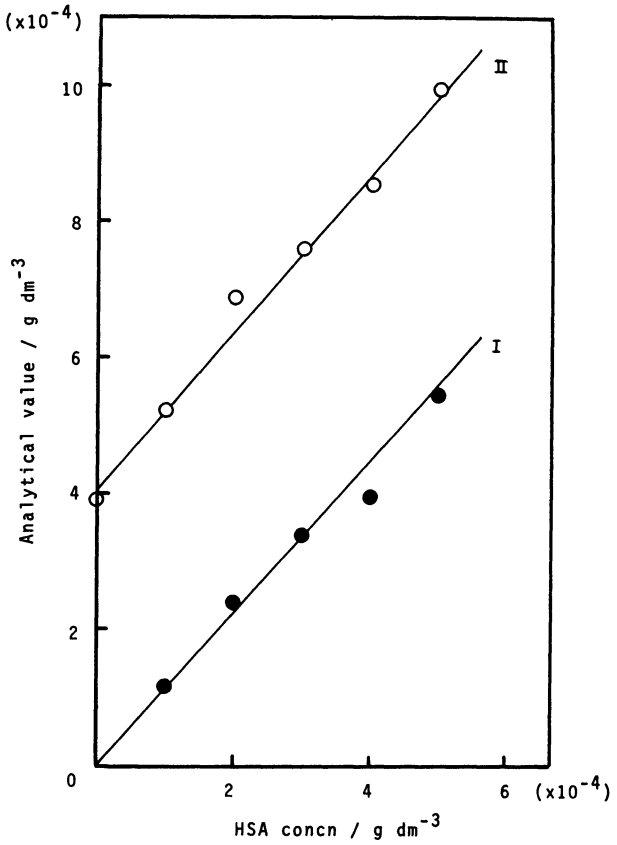

Fig. 13. Relationship between HSA concn and analytical value.
●: HSA and ○: HSA+control serum.
Conditions: 5.0×10^{-5} mol dm^{-3} phen, 4.0×10^{-3} mol dm^{-3} EHDAB, 2.0×10^{-7} mol dm^{-3} TEPA, 1.0×10^{-1} mol dm^{-3} NaOH, 5% H_2O_2, 1.0×10^{-7} mol dm^{-3} copper(II), and 5.0×10^{-5} mol dm^{-3} Arg.

the analytical value was obtained by the use of the calibration curve shown in Fig. 12. All the analytical values of the ordinate in Fig. 13 were represented as the amount of HSA. The difference between the two straight lines, I and II, in the analytical value was approximately equal to the amount of protein in the added control serum. This means that the present method was applicable to the sensitive determination of protein in a serum sample without being interfered with by coexisting constituents, when a sample solution is diluted to a volume of 10^5 times with a buffer solution and then measured.

The present method may be expected to be useful for the development of a sensitive, rapid, and inexpensive detector by which a trace amount of protein can be determined without labeling it.

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