  Bull. Chem. Soc. Jpn., 56, 1382—1387 (1983) [Vol. 56, No. 5

Determination of a Small Amount of Biological Constituent by Use of Chemiluminescence. I. The Flow-injection Analysis of Protein

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(Received October 14, 1982)

A new method in which luminol-hydrogen peroxide luminescent system is used has been proposed for the determination of the presence of protein. Since the catalytic activity of copper(II) for the chemiluminescent reaction between luminol and hydrogen peroxide decreased when copper(II) interacted with polypeptide linkage, this phenomenon was applied to the determination of protein. Determination of protein was carried out by a flow-injection method. The effects of reagent concentration, flow-rate, and reaction time on the analytical value were examined and the conditions for the determination of protein were established. Similar calibration curves were obtained for human serum albumin, bovine serum albumin, bovine serum α-globulin, and bovine serum γ-globulin. According to the present flow-injection method using chemiluminescent reaction, a small amount of protein could be conveniently and economically determined over a wide range of concentration, 7×10^{-4}—7×10^{-2} g dm^{-3}, with the detection limit of 0.2 μg and at the rate of about 30 samples per hour. The present method was applicable to the determination of protein in serum.

It is well known that the chemiluminescence (CL) emitted by 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) in the presence of hydrogen peroxide(H_2O_2) is remarkably enhanced by a catalyst such as Co(II), Co(III), Cr(III), Cu(II), Fe(II), Fe(III), Hg(II), Mn(II), Ni(II), Os(IV), Os(VIII), and their complex compounds.[1] This has been used for the determination of a small amount of H_2O_2 or metal ion.[2-4] Since the catalytic activity of copper(II) for the CL reaction between luminol and H_2O_2 was found to decrease with the amount of coexisting protein, a new method for the determination of protein based on this phenomenon was examined. Judging from the facts that the catalytic activity of a copper(II) complex is maximum when two of the four coordination sites are occupied by a ligand, while it is not observed when all four coordination sites are occupied by a ligand,[5] and that copper(II) in the biuret reaction reacts with four nitrogen atoms of a polypeptide chain to form a violet complex,[6] the decrease of the catalytic activity of a copper(II) complex in the present study seems to be attributed to the formation of a copper(II)–polypeptide complex, that is, the occupation of four coordination sites by a protein molecule.

Various methods such as a Kjeldahl method, a biuret method, a UV method, a Lowry method, a dyestuff combination method, and a fluorometric(thiamine) method have been reported for the determination of protein.[7] Though both a Kjeldahl method and a biuret method are known as standard methods, the former is difficult to operate and the latter is less sensitive. A UV method and a Lowry method are sensitive but they are unsuitable for the determination of protein in a unknown sample because the analytical values obtained by them vary by the kind of protein. A dyestuff combination method is also unsuitable for the determination of protein having different origins because the reaction between dyestuff and protein is too complicated. These methods can estimate protein amounts more than 1 μg, whereas a thiamine method based on fluorescence can estimate 0.1—1.0 μg protein.[8] However the fluorometric method requires a complicated procedure and has to be applied under rigid conditions. The CL method for the determination of protein is characterized by the following: 1) Highly sensitive, 2) determinable over a wide range of protein concentration, 3) easy to operate, 4) inexpensive apparatus and reagent, and 5) it is easy to set up the flow-injection system. This will make the CL method available for the determination of a trace amount of protein in various samples.

Experimental

Reagent. A luminol solution was prepared as 1.0×10^{-3} mol dm^{-3} by dissolving luminol in the buffer solution (pH 10.15) containing 0.1 mol of potassium hydroxide and of boric acid in 1 dm^{-3}. A H_2O_2 solution was prepared (2.5×10^{-3} mol dm^{-3}) by diluting a 0.3 wt% H_2O_2 solution with water.

A copper(II) solution was prepared as a 2.0×10^{-2} mol dm^{-3} concentration by dissolving copper(II) sulfate pentahydrate 5.0 g, potassium sodium tartrate 45.0 g, sodium hydroxide 8.0 g, and potassium iodide 5.0 g in that order and by bringing the mixture to 1 dm^{-3} with water.[9] Each catalyst solution, which contained copper(II), hematin(ICN Pharmaceuticals, Inc.), hexacyanoferrate(III), or osmium(VIII) oxide, was prepared by diluting with the above-mentioned buffer solution. The reagents used were of commercially available special grade. Human serum albumin and bovine serum α-globulin from ICN Pharmaceuticals, Inc., and bovine serum albumin(BSA) and bovine serum γ-globulin from SIGMA CHEMICAL Co., and control serum(Q-PAK-Chemistry Control Serum II) from HYLAND were used.

Ion exchange water was distilled for use.

Apparatus and Procedure. Both a luminol solution which had been left standing overnight in a dark place and a buffer solution were degassed by stirring under reduced pressure and were then used. The schematic diagram of the apparatus which was set up for flow-injection analysis is shown in Fig. 1. A Teflon tube(1 mm i.d.) and a connector were used. A buffer solution(a), a H_2O_2 solution(b), and a luminol solution(c) were delivered at the flow rate of 2.5 cm^3 min^{-1} with the corresponding pumps: (p_1), (p_2), (p_3)-(PERISTA MINI PUMP SJ-1211). A buffer solution and

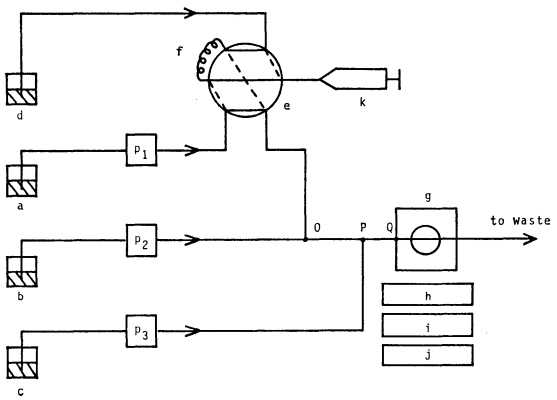

Fig. 1. Flow-injection system.
a: Buffer solution, b: hydrogen peroxide solution, c: luminol solution, d: sample solution, e: six-way cook, f: sampling loop, g: flow cell, h: photomultiplier, i: amplifier, j: recorder, k: cylinge, and P_1, P_2, P_3: pump.

a H_2O_2 solution were first mixed and the resulting mixture was combined with a luminol solution; the distances between P and O or Q were 35 and 10 cm, respectively. A sample solution containing protein was diluted with a buffer solution and to it a copper(II) solution was added so as to become 5.0×10^{-6} mol dm^{-3}; it was held at 30 °C for 50 min. A definite amount(0.4 cm^3) of the sample solution thus obtained was injected into the line of a buffer solution. If the catalytic activity of a metal salt catalyst had to be examined, the catalyst solution alone was injected into the line of a buffer solution instead of the above-mentioned sample solution. A poly(vinyl chloride) tube(45 cm$\times$0.8 mm i.d.) was spirally wound and was then used as the cell (g). The CL intensity of the solution passing through the cell was measured by a photomultiplier(h) (Hamamatsu TV Co. Ltd., R928), amplified with a amplifier(i)(Horiba Ltd., OPE-402), and recorded on a recorder(j)(Yokogawa Electric Works, Ltd., 3046). The net CL intensity of a sample solution was obtained by subtracting the background (the CL intensity of the solution containing no sample) from the observed intensity. The ratio of CL intensity of a protein sample solution to that of a 5.0×10^{-6} mol dm^{-3} copper(II) solution was used for the presentation of data. According to the present apparatus, measurement could be done at the rate of about 30 samples per hour.

Results and Discussion

Determination of Analysis Conditions. The effect of H_2O_2 concentration and of luminol concentration on the CL intensity are shown in Figs. 2 and 3, respectively. As can be seen from Fig. 2, the CL intensity increased with an increasing concentration of H_2O_2 and was accompanied by increasing noise. The CL intensity in Fig. 3 was found to be maximum when the luminol concentration was the same (1.0×10^{-3} mol dm^{-3}) as the H_2O_2 concentration. Therefore 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2 were chosen as their optimum concentrations in the following experiment.

Though the CL intensity increased with an increasing flow rate, all the flow rates were set at 2.5 cm^3 min^{-1} because of the capacity of the pumps. The high sensitivity was attained by adding a H_2O_2 solution to a buffer solution, followed by mixing the mixed solution with a luminol solution.

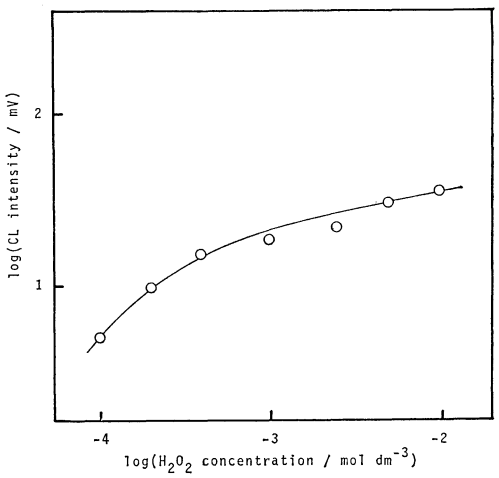

Fig. 2. Effect of H_2O_2 concentration on CL intensity. Conditions: 1.0×10^{-4} mol dm^{-3} luminol and 1.0×10^{-5} mol dm^{-3} $K_3[Fe(CN)_6]$.

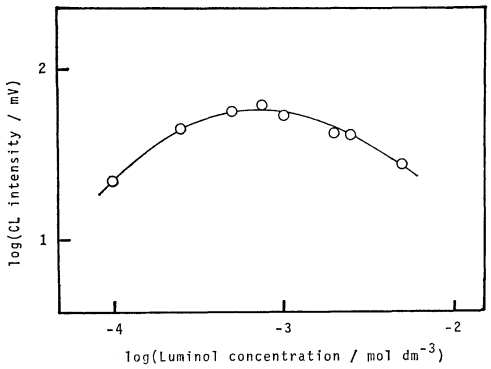

Fig. 3. Effect of luminol concentration on CL intensity. Conditions: 1.0×10^{-3} mol dm^{-3} H_2O_2 and 1.0×10^{-5} mol dm^{-3} $K_3[Fe(CN)_6]$.

Selection of Chemiluminescent Catalyst. We examined the catalytic activity of copper(II) sulfate, and of osmium(VIII) oxide and hematin, which are compounds of Os and Fe belonging to the eighth group transition elements in the periodic table and had been known to show high catalytic activity for the CL reaction.[10] The relationship between catalyst concentration and CL intensity was obtained by means of the apparatus shown in Fig. 1 (Fig. 4). Chemiluminescent response was observed for 1×10^{-7}—5×10^{-5} mol dm^{-3} copper(II) sulfate, 2×10^{-9}—2×10^{-6} mol dm^{-3} osmium(VIII) oxide, and 5×10^{-10}—5×10^{-6} mol dm^{-3} hematin, respectively.

By using a definite amount of 5.0×10^{-6} mol dm^{-3} copper(II) sulfate, 7.9×10^{-7} mol dm^{-3} osmium(VIII) oxide, and 4.0×10^{-7} mol dm^{-3} hematin solutions, which were slightly higher concentrations than those at each middle point in the above-mentioned catalyst concentrations, three calibration curves were obtained for BSA (Fig. 5). When a hematin solution or copper(II) sulfate solution was added to a protein solution, their catalytic activities or CL intensity decreased with time and finally stabilized after 2 h at room tem-

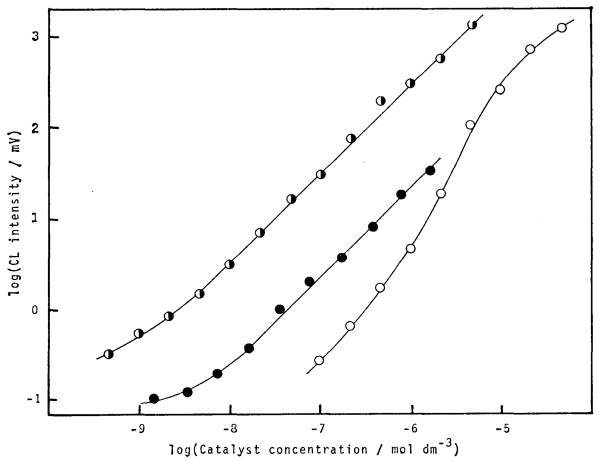

Fig. 4. Relationship between catalyst concentration and CL intensity.
○: $CuSO_4$, ●: OsO_4, and ◑: hematin. Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

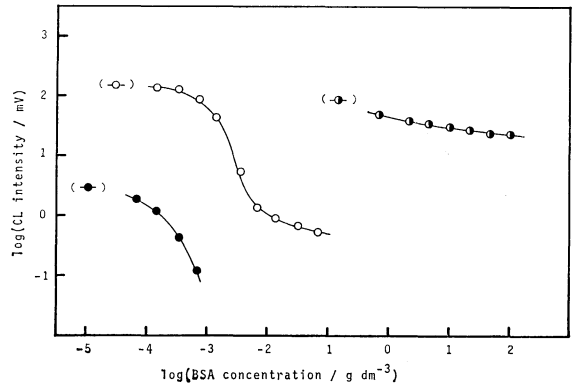

Fig. 5. Calibration curve of BSA for each catalyst.
○: $CuSO_4$, ●: OsO_4, ◑: hematin, and (○): BSA free. Conditions: 5.0×10^{-6} mol dm^{-3} $CuSO_4$, 7.9×10^{-7} mol dm^{-3} OsO_4, 4.0×10^{-7} mol dm^{-3} hematin, 1.0×10^{-3} mol dm^{-3} luminol, and 2.5×10^{-3} mol dm^{-3} H_2O_2.

perature. In a similar experiment using osmium(VIII) oxide, the catalytic activity or CL intensity did not stabilize until after about 24 h. Judging from the detection limit of protein, concentration range suitable for the determination of protein, lowering of CL intensity by a definite amount of protein, and time necessary for the stabilization of CL intensity, copper(II) sulfate was most recommendable as a chemiluminescent catalyst for the present study.

The CL intensities in the presence of copper(II) sulfate alone or of copper(II) sulfate-tartaric acid are shown in Fig. 6. The catalytic activity of the former was a little smaller than that of the latter and its difference was remarkable at high concentration of copper(II), 1×10^{-5}—5×10^{-5} mol dm^{-3}, where the formation of copper(II) hydroxide was observed after allowing the copper(II) solution to stand alone for several hours. Therefore, the above difference seems to be related to the formation of copper(II) hydroxide. The catalytic activity of copper(II) solution was ob-

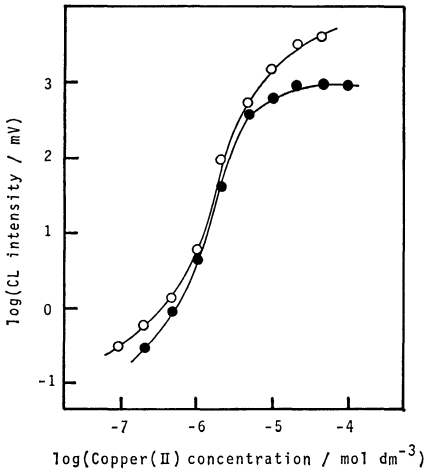

Fig. 6. Effect of tartaric acid on the CL intensity in the presence of copper(II) catalyst.
○: Copper(II)+tartaric acid and ●: copper(II) alone. Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

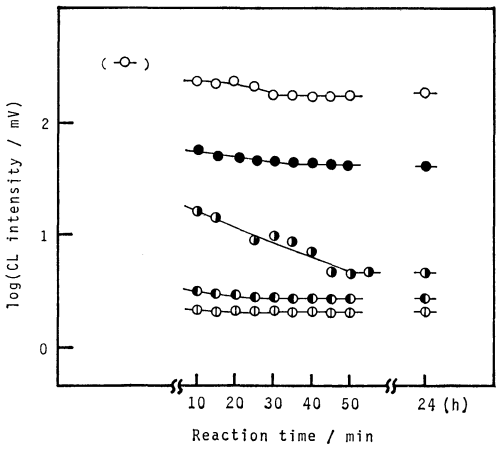

Fig. 7. Relationship between reaction time and CL intensity.
○: 1.4×10^{-3} g dm^{-3}-, ●: 3.5×10^{-3} g dm^{-3}-, ◑: 7.0×10^{-3} g dm^{-3}-, ◐: 1.4×10^{-2} g dm^{-3}-, ⓘ: 3.5×10^{-2} g dm^{-3}- BSA, and (○): BSA free. Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

tained with good reproducibility in the presence of tartaric acid without producing the copper(II) hydroxide. A small amount of potassium iodide was also added to the copper(II) solution to prevent it from undergoing autoreduction.[11] The calibration curve for copper(II) sulfate-tartaric acid was not linear but sigmoidal. The reason for this remains unclear.

Determination of Protein. The relationship between CL intensity and elapsed time after mixing of copper(II) with each BSA solution of 1.4×10^{-3}, 3.5×10^{-3}, 7.0×10^{-3}, 1.4×10^{-2}, and 3.5×10^{-2} g dm^{-3} was examined at 30 °C; the result was shown in Fig. 7. CL intensity decreased with time after mixing, but no lowing of CL intensity was observed in 50 min—24 h after mixing. It was, therefore, necessary for the determination of protein to hold the mixed solution

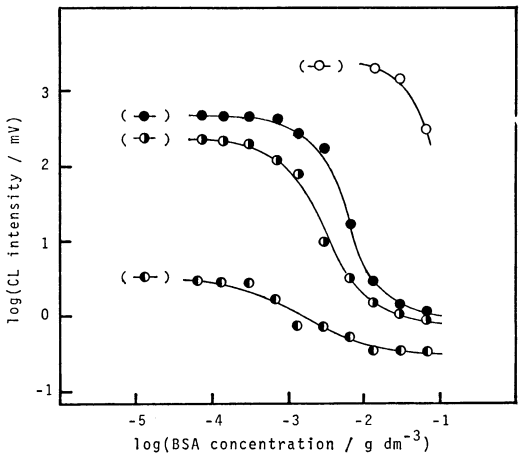

Fig. 8. Effect of copper(II) concentration on the calibration curve of BSA.
○: 5.0×10^{-5} mol dm^{-3}-, ●: 1.0×10^{-5} mol dm^{-3}-, ◑: 5.0×10^{-6} mol dm^{-3}-, ◐: 1.0×10^{-6} mol dm^{-3}-copper(II), and (○): BSA free.
Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

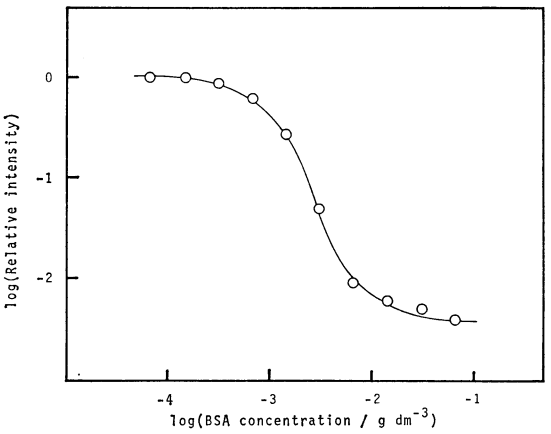

Fig. 9. Calibration curve of BSA by the present method.
Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

of copper(II) and protein at 30 °C for 50 min.

Figure 8 showed calibration curves of BSA which were obtained by use of 1.0×10^{-6}, 5.0×10^{-6}, 1.0×10^{-5}, and 5.0×10^{-5} mol dm^{-3} of copper(II) sulfate. The detection limit of protein concentration and the decrease of CL intensity for a definite amount of protein suggested that a 5.0×10^{-6} mol dm^{-3} copper(II) sulfate solution be used in the present study.

The calibration curve for BSA was obtained under the same conditions as specified above (Fig. 9), and it was usable for the determination of protein in the concentration range of 7×10^{-4}—7×10^{-2} g dm^{-3}. The decrease of CL intensity in the presence of protein is attributed to the lowing of the catalytic activity of copper(II) based on the formation of a copper(II)–polypeptide complex. This is understandable from the following facts: 1) The catalytic activity of a copper(II) complex is maximum when two of the four coordination sites are occupied by a ligand, while it is

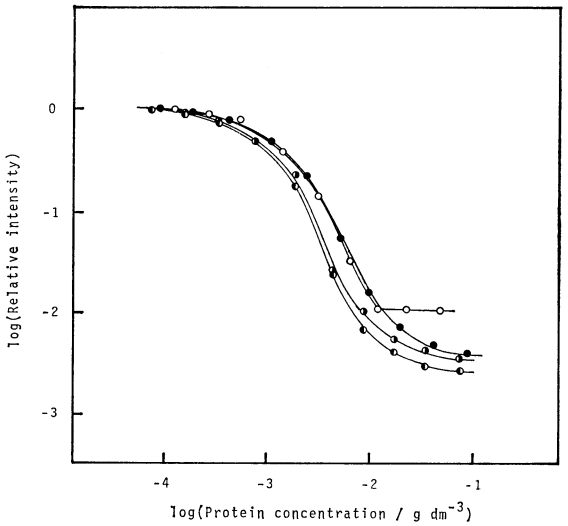

Fig. 10. Calibration curves of various proteins.
○: Bovine serum α-globulin, ●: human serum albumin, ◑: bovine serum albumin, and ◑: bovine serum γ-globulin.
Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

not observed when all four coordination sites are occupied by a ligand,[5] 2) copper(II) in the biuret reaction reacts with four nitrogen atoms of a polypeptide chain to form a violet complex.[6] The present method makes use of the formation of copper(II)–polypeptide complex and estimates the amount of protein indirectly by measuring the catalytic activity of copper(II) for CL; it will be suitable for the determination of protein in a biological fluid sample in which no constituent except protein shows biuret reaction.[12]

The calibration curves for human serum albumin, bovine serum α-globulin, and bovine serum γ-globulin were prepared in a way similar to that for BSA; they are compared in Fig. 10. The constant CL intensity in the concentration range of 1.2×10^{-2}—6.0×10^{-2} g dm^{-3} bovine serum α-globulin may be attributed to insolubility of protein because suspended substances were observed at protein concentrations above 1.0×10^{-1} g dm^{-3}. Furthermore there were some differences between CL intensities of individual proteins. Assuming that the decrease of CL intensity is based on the decrease of catalytic activity by the formation of copper(II)–polypeptide complex, the differences between CL intensities of individual proteins will markedly depend on the total amount of the peptide linkage in a definite weight of protein.

According to the present method, 7×10^{-4}—7×10^{-2} g dm^{-3} protein could be determined with a detection limit of 0.2 μg. The present method is shown in Table 1 together with some typical conventional methods and with their determinable amounts of protein. The present method was 10—100 times as sensitive as in the conventional methods, except for a thiamine(fluorometric) method, and was applicable to the determination of a wide concentration range of protein. Furthermore the present method is characterized by easy operation based on a flow-injection system and by

TABLE 1. RANGE OF PROTEIN DETERMINABLE BY VARIOUS METHODS

Method	Determinable range (μg)	Ref.
Present	0.2—20	
Micro biuret	26—530	13
Kjeldahl	30—100	14
UV	20—100	15
Lowry	5—100	16
Dyestuff combination	1—10	17
Thiamine	0.1—1.0	8

TABLE 2. REPRODUCIBILITY OF THE PRESENT METHOD

BSA concentration/g dm^{-3}	CV value/%
0	7.7
7.0×10^{-4}	7.9
7.0×10^{-3}	6.5
7.0×10^{-2}	3.7

inexpensive reagent and apparatus. According to a thiamine(fluorometric) method, 0.1—1.0 μg of protein can be determined but it requires a complicated procedure and has to be applied under rigid conditions.

As can be seen from Fig. 10, the difference in analytical data based on the difference of CL intensity was about 50% for the two kinds of protein in which the difference was maximum. This value seems reasonable if we take the corresponding values of a dyestuff combination method(about 200%) and of a Lowry method(about 20%) into consideration.

The coefficient of variation (CV) of the present method for the determination of protein is shown in Table 2. The CV values increased with a decreasing concentration of protein. Since the fluctuating flow offered by a pump seriously affected the precision of the present determination, an improvement of the apparatus was still necessary for the minimization of this CV value.

Application to Serum Analysis. In order to determine the amount of protein in serum by the present method, a serum sample is diluted to 10^3—10^5 times its volume. The effect of iron salt, chloride, and urea contained in the serum on the CL intensity was examined by use of iron(III) chloride hexahydrate, potassium chloride, and urea. Fe(III) 2.0×10^{-8} mol dm^{-3}, Cl^- 1.0×10^{-4} mol dm^{-3}, and $(NH_2)_2CO$ 1.0×10^{-5} mol dm^{-3} corresponding to the concentration at 10^3 times' dilution of a serum sample did not influence the CL intensity at all.

Then different amounts of BSA were added to a definite amount of control serum(final concentration 1.2×10^{-3} g dm^{-3}), and the total amount of protein was estimated by use of the calibration curve of BSA (Fig. 11). The analytical value of the ordinate indicated the protein content in a sample solution, which was expressed as BSA. The difference between straight lines I and II just corresponded to the added amount of control serum. This result showed that the present method was applicable to the determination of protein in a serum sample without being interfered with by

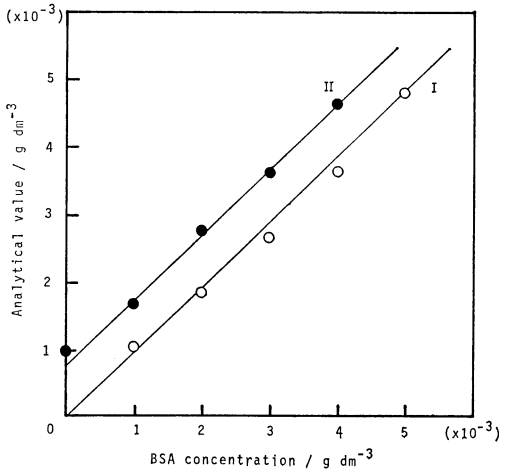

Fig. 11. Relationship between BSA concentration and analytical value.
○: BSA and ●: BSA+control serum.
Conditions: 1.2×10^{-3} g dm^{-3} control serum, 1.0×10^{-3} mol dm^{-3} luminol, and 2.5×10^{-3} mol dm^{-3} H_2O_2.

coexisting constituents. Here 0.01 μl of control serum was diluted to 10^5 times volume and analyzed.

On the basis of the above-mentioned investigation, a method for the determination of a small amount of protein was newly established. The present method by means of a flow-injection system is characterized by the following: 1) Protein amounts are determinable in the protein concentration range of 7×10^{-4}—7×10^{-2} g dm^{-3} with detection limit of 0.2 μg; 2) the method is 10—100 times more sensitive than the conventional methods except the fluorometric one; 3) it is easy to operate; 4) it is inexpensive in the apparatus and the reagent; 5) about 30 samples per hour can be measured. The present method is applicable to the determination of protein in a serum sample and is expected to promote the field of highly sensitive detection of protein amounts.

The authors wish to thank Mr. Masakatsu Imaki (Horiba Ltd.,) for providing the apparatus necessary for the present study.

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