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

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A flow-injection analysis method of protein by direct injection has been established by use of the luminol-hydrogen peroxide luminescence system. The determination of protein is based on the measurement of the decreasing catalytic activity of copper(II) for the chemiluminescent reaction between luminol and hydrogen peroxide. The present flow-injection analysis in which a protein solution is directly injected is quite different from the previous one in which a protein solution is injected after having been previously allowed to react with copper(II) outside the flow-through system. The optimum conditions for the determination of protein were determined with regard to reagent concentration, flow rate, reaction time, and reaction temperature. The present method is simple, rapid, and applicable to the determination of 2×10^{-4}—1×10^{-1} g dm^{-3} of protein with 40 ng of detection limit at a rate of about 30 samples per hour. The present method was also applied to the determination of the ratio of albumin to globulin in a sample. The present flow-injection system is expected to be a sensitive detector for the determination of small amounts of protein.

A new method for the determination of protein, in which use was made of the decreasing catalytic activity for the chemiluminescence (CL) reaction between 5-amino-2, 3-dihydro-1, 4-phthalazinedione(luminol) and hydrogen peroxide(H_2O_2) when copper(II) (Cu(II)) interacted with polypeptide linkage, was reported by the authors in the previous paper[1]. The method was more sensitive and applicable to a wider range of protein concentration than is available with the conventional methods. However the protein in a sample solution had to be made to react with Cu(II) at 30 °C for 50 min before its injection into a flow-injection system.

Here the flow-injection system in the previous paper has been modified to enable a protein solution to inject directly into the flow-injection system. In the previous paper,[2] protein was also determined by use of a cobalt(III) complex. However Cu(II) is used in the present work as a catalyst for the CL reaction because Cu(II) shows faster reaction with protein and smaller difference between CL responses of individual protein compared with those of cobalt(III). In the present method, the protein solution is directly injected into the flow-injection system without any pretreatment and the analytical results are obtained simply and rapidly because the reaction between Cu(II) and protein proceeds in the flow-path.

Experimental

Reagents. By diluting with the buffer solution (pH 10.15) consisting of boric acid and potassium hydroxide presented in the previous paper,[1] a 5.0×10^{-4} mol dm^{-3} luminol solution and a 2.0×10^{-6} mol dm^{-3} Cu(II) catalyst solution from a 2.0×10^{-2} mol dm^{-3} Cu(II) stock solution[1] were prepared. A 5.0×10^{-4} mol dm^{-3} H_2O_2 solution was prepared by diluting a 0.3 wt% H_2O_2 solution with water. The reagents used were of commercially available special grade.

Human serum albumin(HSA) and bovine serum α-globulin from ICN Pharmaceuticals, Inc., and human serum γ-globulin, bovine serum albumin(BSA), and bovine serum γ-globulin from Sigma Chemical Co., and control serum(Q-PAK-Chemistry Control Serum I) from HYLAND were used. All these proteins were dissolved in the buffer solution and diluted with it.

Ion exchange water was distilled for use.

Apparatus and Procedure. The 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. Each solution of luminol(a), H_2O_2(b), Cu(II) (c), and a buffer(d) was fed by means of 2.3×10^5 Pa gauge pressure of nitrogen gas; the flow rate was adjusted to 10 cm^3 min^{-1} by a needle valve. A 0.4 cm^3 of protein sample solution was injected into the buffer solution line through the sampling loop(k) and the six-way cook(j). The protein sample solution was first mixed with a Cu(II) catalyst solution and passed through 1.6 m of tube held at 0 °C in the cooling bath (n) *via* a 1.0 m of tube held at 95 °C in the heating bath(m). Then the solution was mixed with a H_2O_2 solution and a luminol solution in that order, and passed through the flow cell(o). The distances between T and S or U were 35 and 10 cm, respectively. The same flow cell as in the previous paper was used. The CL intensity of the solution passing through the cell was measured by a photomultiplier (p) (Hamamatsu TV Co. Ltd., R928), amplified with a amplifier(g) (Horiba Ltd., OPE-402), and recorded on a recorder(r) (Yokogawa Electric Works, Ltd., 3046). The CL intensity of the solution containing no sample was recorded

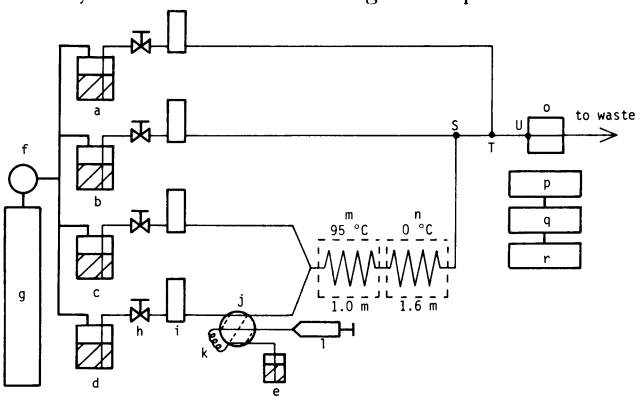

Fig. 1. Schematic diagram of the flow-injection system.
a: Luminol solution, b: H_2O_2 solution, c: Cu(II) catalyst solution, d: buffer solution, e: sample solution, f: regulator, g: N_2 bomb, h: needle valve, i: flow meter, j: six-way cock, k: sampling loop, l: cylinge, m: heating bath, n: cooling bath, o: flow cell, p: photomultiplier, g: amplifier, and r: recorder.

as a base line while the CL intensity of the solution containing protein was recorded as a negative peak because the catalytic activity of Cu(II) decreased because of the complex formation between Cu(II) and protein. The amount of protein was estimated from the peak area.

Results and Discussion

Determination of Analysis Conditions. Bovine serum albumin was used as a model protein.

The CL intensity was measured by using 0.5, 1.0, 2.0, and 3.0 m of tube held at 95 °C in the heating bath, but no difference between CL intensities was observed in all the tubes except the first one. Then a 1.0 m of tube was used for the heating bath in the following experiment.

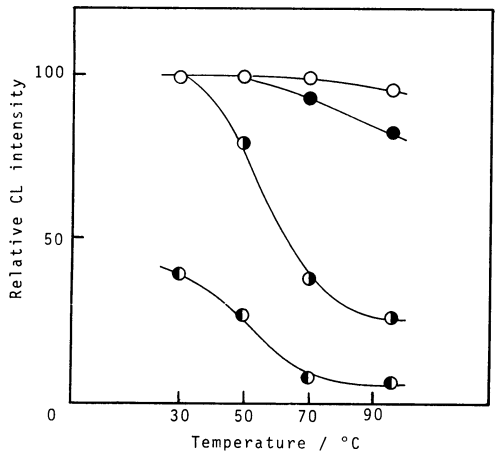

Fig. 2. Effect of heating bath temperature on relative CL intensity.[a)]
○: 2.0×10^{-4} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, ◑: 1.0×10^{-2} g dm^{-3}-, and ◐: 1.0×10^{-1} g dm^{-3}-BSA.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol, 5.0×10^{-4} mol dm^{-3} H_2O_2, and 2.0×10^{-6} mol dm^{-3} Cu(II).
a) The CL intensity of the solution containing protein to the CL intensity of the solution containing no protein.

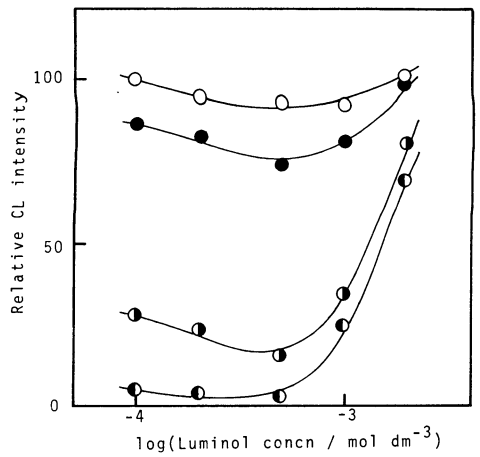

Fig. 3. Effect of luminol concn on relative CL intensity.[a)]
○: 2.0×10^{-4} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, ◑: 1.0×10^{-2} g dm^{-3}-, and ◐: 1.0×10^{-1} g dm^{-3}-BSA.
Conditions: 5.0×10^{-4} mol dm^{-3} H_2O_2 and 2.0×10^{-6} mol dm^{-3} Cu(II).
a) The CL intensity of the solution containing protein to the CL intensity of the solution containing no protein.

The effect of the temperature of the heating bath on the CL intensity is shown in Fig. 2. Each plot represents the CL intensity in the presence of BSA against the CL intensity 100 of a base line. Then the temperature of the heating bath was set at 95 °C because the CL intensity decreased with a rise of temperature.

The tube held at 0 °C in the cooling bath played two important roles: cooling the hot effluent so as not to affect the CL intensity and charging the back pressure so as not to produce a foam inside the tube in the heating bath. 1.6 m tube length was chosen to make the diffusion of the samples as small as possible.

The effect of the concentration of luminol, H_2O_2, and Cu(II) solutions on the CL intensity are shown in Figs. 3, 4, and 5. From these results, the optimum

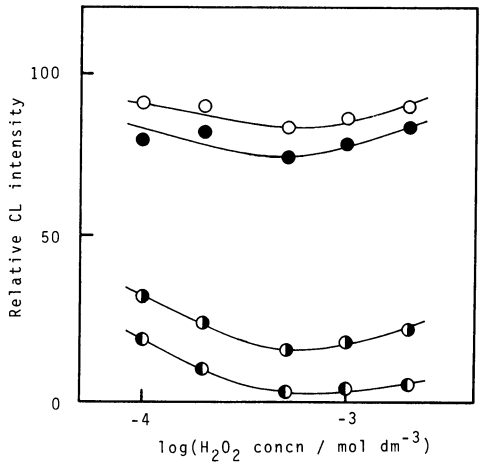

Fig. 4. Effect of H_2O_2 concn on relative CL intensity.[a)]
○: 5.0×10^{-4} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, ◑: 1.0×10^{-2} g dm^{-3}-, and ◐: 1.0×10^{-1} g dm^{-3}-BSA.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol and 2.0×10^{-6} mol dm^{-3} Cu(II).
a) The CL intensity of the solution containing protein to the CL intensity of the solution containing no protein.

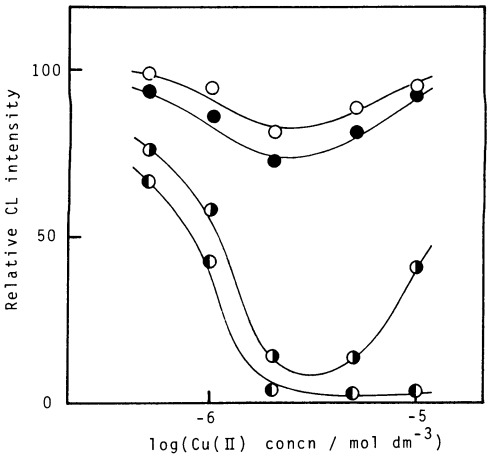

Fig. 5. Effect of Cu(II) concn on relative CL intensity.[a)]
○: 5.0×10^{-4} g dm^{-3}-, ●: 1.0×10^{-3} g dm^{-3}-, ◑: 1.0×10^{-2} g dm^{-3}-, and ◐: 1.0×10^{-1} g dm^{-3}-BSA.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol and 5.0×10^{-4} mol dm^{-3} H_2O_2.
a) The CL intensity of the solution containing protein to the CL intensity of the solution containing no protein.

concentrations corresponding to maximum decreases in the CL intensity were as follows: luminol solution 5.0×10^{-4} mol dm^{-3}; H_2O_2 solution 5.0×10^{-4} mol dm^{-3}; and Cu(II) catalyst solution 2.0×10^{-6} mol dm^{-3}.

Determination of Protein. The calibration curve of BSA obtained under the above-mentioned conditions is shown in Fig. 6. As can be seen from it, 1×10^{-4}—1×10^{-1} g dm^{-3} protein gave the CL response which corresponded to the peak area of the negative peak recorded in the addition of BSA solution. The decrease of the CL intensity in the presence of protein is attributed to the lowering of the catalytic activity of Cu(II) based on the formation of a Cu(II)-polypeptide complex. The fact that the ratio of the change of peak area to the change of protein concentration is rather small seemed to be based on the diffusion of a sample and the dilution of a sample solution by additions of reagent solutions. The flow chart obtained by injecting each 1.0×10^{-4}, 5.0×10^{-3}, and 5.0×10^{-2} g dm^{-3} BSA and that obtained at a blank experiment are shown in Fig. 7.

The CL responses of HSA, human serum γ-globulin, bovine serum α-globulin, and bovine serum γ-globulin by the present method were examined in the manner used in the preparation of the calibration curve of BSA (Fig. 8). Approximately similar responses were obtained for them. This suggests that the present flow-injection system will serve as a promising detector for the determination of protein.

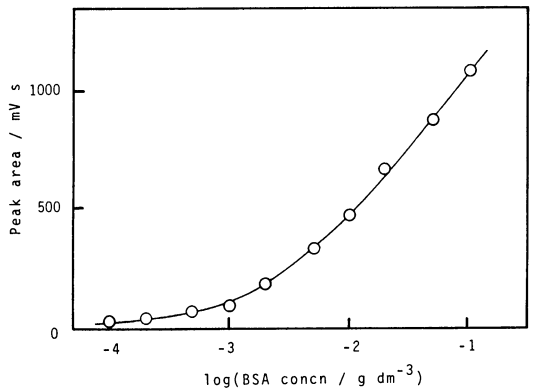

Fig. 6. Calibration curve of BSA by the present method.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol, 5.0×10^{-4} mol dm^{-3} H_2O_2, and 2.0×10^{-6} mol dm^{-3} Cu(II).

Precision was not so good in the previous paper because each solution was delivered by means of a peristaltic pump, accompanied by fluctuating flow, while precision in the present work was drastically improved by means of the utilization of a gas pressure without any fluctuations in flow. Therefore the present method makes it possible to estimate a small amount of protein with the concentration range of 2×10^{-4}—1×10^{-1} g dm^{-3} and the detection limit of about 40 ng. The coefficients of variation of the present method for 10 analyses of 5.0×10^{-3} and 5.0×10^{-2} g dm^{-3} protein solutions were both 4.1%.

Estimation of the Ratio of Albumin to Globulin. The ratio of albumin to globulin(A/G) has been looked upon as an important parameter for diagnosis of the abnormal metabolism of protein in human body. Here the A/G ratio was estimated to examine whether or not the present method could be applied to a mixed protein sample.

To examine the effect of the coexisting constituents in a control serum on the CL intensity when a control serum was diluted to 2000 times of volume with a buffer solution and measured, the analytical values against HSA or a definite amount of control serum (containing 2.3×10^{-2} g dm^{-3} of protein) plus HSA were obtained by use of the calibration curve shown in Fig. 8. All the analytical values of the ordinate in Fig. 9 were represented as the amount of HSA. The difference between straight lines I and II in the analytical value was approximately equal to the protein amount of the added control serum. This means that the constituents other than protein in the serum do not significantly affect the CL intensity when a sample solution is diluted to 2000 times of volume with a buffer solution and measured. Then the present method was applied to the estimation of the A/G ratio.

Various A/G ratio of mixtures of HSA and human serum γ-globulin were subjected to the fractionation

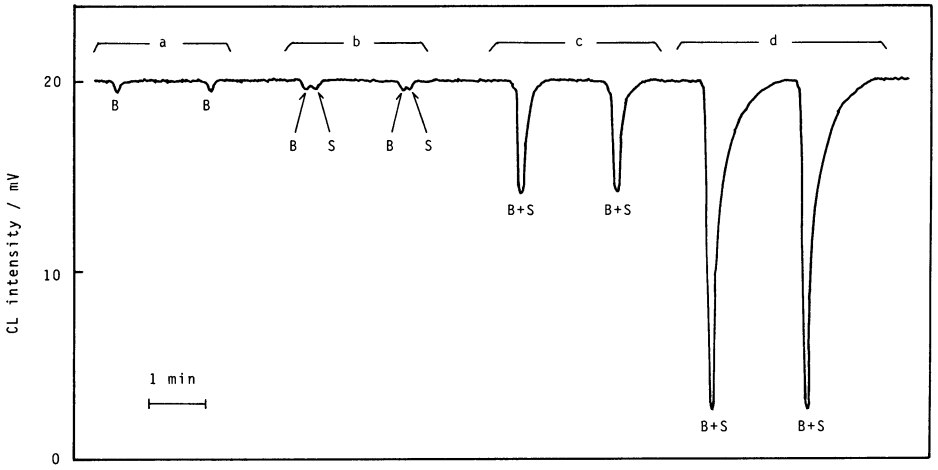

Fig. 7. Flow chart in duplicate measurements.
a: Blank value, b: 1.0×10^{-4} g dm^{-3}-, c: 5.0×10^{-3} g dm^{-3}-, d: 5.0×10^{-2} g dm^{-3}-BSA, B: blank (shock peak by the change of the flow path), and S: sample peak.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol, 5.0×10^{-4} mol dm^{-3} H_2O_2, 2.0×10^{-6} mol dm^{-3} Cu(II), and 1.5 cm min^{-1} chart speed.

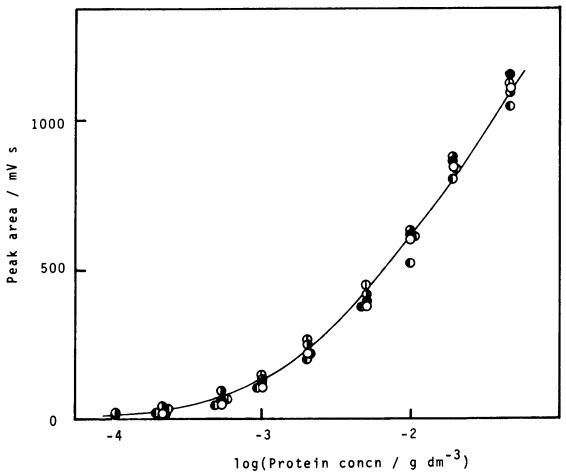

Fig. 8. Response pattern of flow-injection method for various proteins.
○: HSA, ●: human serum γ-globulin, ◑: BSA, ◐: bovine serum α-globulin, and ⊕: bovine serum γ-globulin.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol, 5.0×10^{-4} mol dm^{-3} H_2O_2, and 2.0×10^{-6} mol dm^{-3} Cu(II).

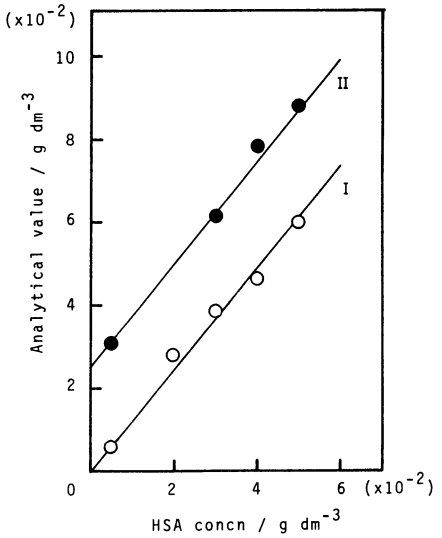

Fig. 9. Relationship between HSA concn and analytical value.
○: HSA and ●: HSA+control serum.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol, 5.0×10^{-4} mol dm^{-3} H_2O_2 and 2.0×10^{-6} mol dm^{-3} Cu(II).

using sodium sulfate as a salting-out agent, and the A/G ratio were determined on the basis of the albumin amount from the albumin fraction and of the globulin amount obtained by subtracting the albumin amount from the total amount obtained for the mixture. Neither sodium sulfite and ammonium sulfate were suitable as a salting-out agent, because the sulfite worked as a reducing agent and the ammonium salts produced a ammine complex in an alkaline solution. To 0.5 cm^3 of protein solution, the concentration of which is the same as that of protein in control serum, 0.5 cm^3 of water and 0.18 g sodium salfate were added and dissolved by shaking in a centrifugal tube. The contents was centrifuged at 10000 min^{-1} for 10 min. An aliquot volume of supernatant liquid was diluted to 1000 times of volume with the buffer solution, and the albumin amount in it and the A/G ratio were determined by the above-mentioned procedure. The relationship between theoretical and analytical values of A/G ratio is shown in Fig. 10. Though the present fractionation did not seem to give complete separation of albumin from globulin, there was a linear correlation,as shown in Fig. 10. The analytical value of the A/G ratio in a control serum was also determined similarly and is plotted in Fig. 10 against the A/G ratio of 1.71, which stood for the mean value of 1.47—2.00 estimated by different methods in eight laboratories as the A/G ratio for the same control serum. The result was almost satisfactory, but a better result would be obtained by the complete separation of albumin from globulin. The possibility of estimating the A/G ratio in human serum by the present method suggests that the combination of the present method with a continuous separation procedure for a mixed protein sample enables the system used in the present method to be a promising detector for the sensitive determination of protein.

On the basis of the results obtained, the flow-injection analysis method of protein by direct injection has been established by use of the luminol-H_2O_2

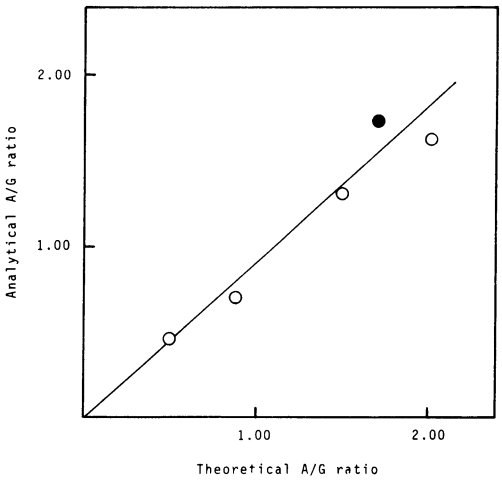

Fig. 10. Relationship between theoretical A/G ratio and analytical A/G ratio.
○: Mixture of HSA and human serum γ-globulin and ●: control serum.
Conditions: 5.0×10^{-4} mol dm^{-3} luminol, 5.0×10^{-4} mol dm^{-3} H_2O_2, and 2.0×10^{-6} mol dm^{-3} Cu(II).

luminescence system. According to the present method, the previous reaction between Cu(II) and protein as in the previous paper is unnecessary and a protein sample solution can be directly injected into the flow system, followed by the reaction between Cu(II) and protein in the flow-path and the determination of protein. The measurement can be done at the rate of about 30 samples per hour with the concentration range of 2×10^{-4}—1×10^{-1} g dm^{-3} protein and the detection limit of about 40 ng. The present method is expected to serve as a promising detector in the near future because the method is simple, rapid, and applicable to the sensitive determination of a wide range of protein concentration.

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References

1) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 1382 (1983).
2) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 289 (1984).