The Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. XI. The Determination of Protein Using a 1,10-Phenanthroline-Hydrogen Peroxide-Osmium(VIII) System

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Osmium(VIII) was found by the authors to show extremely sensitive catalytic activity for the 1,10-phenanthroline-hydrogen peroxide chemiluminescence system. By optimizing the measurement conditions, 3.2×10^{-11}—1.3×10^{-8} mol dm^{-3} osmium(VIII) could be determined by chemiluminescence flow-injection analysis with a detection limit of 0.4 pg. The method for the determination of protein was established; it was based on the lowering of the catalytic activity of osmium(VIII) attributable to the complex formation between osmium(VIII) and protein. Similar calibration curves were obtained for bovine serum albumin, human serum albumin, and human serum γ-globulin under the optimum conditions for the determination of protein. According to the method, 5.0×10^{-6}—1.0×10^{-3} g dm^{-3} protein could be determined with a detection limit of about 250 pg and with a coefficient of variation of 2.9% (n=10) for a 1.0×10^{-4} g dm^{-3} protein solution.

Various sensitive methods for the determination of protein have been reported previously by the present authors;[1-4] in those methods, use is made of chemiluminescence(CL) flow-injection analysis for a small amount of a metal ion or a metal complex. The method is based on the lowering of the catalytic activity of a metal ion by the complex formation between a metal ion and protein. Though protein was determined by the present authors by the use of the luminol-hydrogen peroxide(H_2O_2)-osmium(VIII) oxide (Os(VIII)) CL system, neither the stability of the CL intensity nor the detection limit of protein was satisfactory.[1]

As a method for the determination of protein using the 1,10-phenanthroline(phen)-H_2O_2-ruthenium(III) system was reported in one of the previous papers,[4] the catalytic activities of iridium(III), osmium(VIII), palladium(II), platinum(IV), and rhodium(III) for the phen-H_2O_2 CL system have been investigated in the present study. Os(VIII) has been found to be very sensitive. By optimizing the measurement conditions, a micro amount of Os(VIII) could be determined by CL flow-injection analysis. The present method for the determination of Os(VIII) is 5—60 times as sensitive as the methods using a lucigenin or luminol CL system.[5-7] Here, this sensitive method for the determination of Os(VIII) was combined with the complex formation between Os(VIII) and protein to established a sensitive method for the determination of protein using CL flow-injection analysis. According to the present method, a micro amount of protein can be rapidly determined with a stable CL intensity.

The present method is 4×10^2—1×10^6 times as sensitive as the non-labeling colorimetric or fluorometric method[8] for the determination of protein and is the most sensitive method among those reported by the present authors[1-4] for the determination of protein using CL flow-injection analysis.

Experimental

All the reagents were of a commercially available special grade. Ion-exchanged water was distilled before use. A 1.2×10^{-4} mol dm^{-3} phen solution contained 5.0×10^{-3} mol dm^{-3} ethylhexadecyldimethylammonium bromide(EHDAB), 1.0×10^{-1} mol dm^{-3} sodium hydroxide(NaOH), and 1.0×10^{-9} mol dm^{-3} tetraethylenepentamine(TEPA). A 5.0 wt% H_2O_2 solution was prepared by diluting 30 wt% H_2O_2 with water. A 1.3×10^{-3} mol dm^{-3} Os(VIII) catalyst solution was prepared as a stock solution by diluting a 4% osmium(VIII) oxide aqueous solution (2 ml) (Nakarai Chemical, Ltd.) with water; it was then further diluted to the desired concentration with a buffer solution (pH 10.2) consisting of 1.0×10^{-1} mol dm^{-3} boric acid and 1.0×10^{-1} mol dm^{-3} potassium hydroxide. Bovine serum albumin(BSA) and human serum albumin (HSA) from Nakarai Chemical, Ltd., human serum γ-globulin(HγG) from the Sigma Chemical Company, and control serum(Q-PAC-Chemistry Control Serum I) from Hyland were prepared by diluting them with the above-mentioned buffer solution.

The experiment was carried out using the same apparatus as in a previous paper.[9] A phen-H_2O_2 solution and the above-mentioned buffer solution as a carrier were mixed at the point X of the apparatus. The optimum distance from the point X to a photometric flow cell and the optimum flow rates were decided by experimentation. A definite volume of Os(VIII) catalyst solution was added to a sample solution containing protein, and the resulting solution was heated for a definite period for the complexation reaction of Os(VIII) with protein. After the solution has then cooled to room temperature, an aliquot of the solution (50 mm^3) was injected into the line of a buffer solution as a carrier and the CL intensity-time profile was measured at the flow cell.

Results and Discussion

Cl Flow-Injection Analysis for a Micro Amount of Os(VIII). As the method for the determination of protein using a phen-H_2O_2-ruthenium(III) system was reported in the previous paper, the catalytic activities of iridium(III), osmium(VIII), palladium(II), pla-

tinum(IV), and rhodium(III) for the phen-H_2O_2 CL system were investigated in the present study; Os(VIII) was found to be very sensitive. Then, the effects of the flow rates, the tube length, and the reagent concentration on the CL intensity of a Os(VIII) catalyst solution were examined in the following way, and the conditions for the determination of a micro amount of Os(VIII) were established.

The CL intensity of a 6.3×10^{-9} mol dm^{-3} Os(VIII) catalyst solution was measured by the use of 10-, 100-, 200-, and 300-cm portions of tubing, which corresponded to the distance between the point X and a photometric flow cell. The maximum CL intensity was observed by the use of a 200-cm tube. Both the flow rate of a phen solution and that of a H_2O_2 solution were chosen at the same time as follows: 0.5, 0.8, 1.0, 1.3, 1.5, and 1.7 ml min^{-1}, and the CL intensity of a 6.3×10^{-9} mol dm^{-3} Os(VIII) catalyst solution (volume injected, 50 mm^3) was measured. The CL intensity increased with the increase in the flow rate, but was accompanied by an increasing noise. The flow rate of a carrier buffer solution was always set at 2.3 ml min^{-1}, the maximum capacity of the pump, so as to deal with as many samples as possible within a definite time. On the basis of the experimental results, the optimum conditions were established as follows: tube length, 200 cm; flow rates of a phen, a H_2O_2, and a buffer solution, 1.3, 1.3, and 2.3 ml min^{-1} respectively.

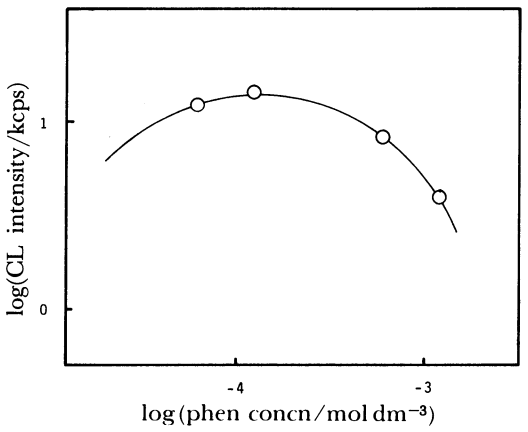

Fig. 1. Effect of phen concn on CL intensity. Conditions: 5.0×10^{-3} mol dm^{-3} EHDAB, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-9} mol dm^{-3} TEPA, 7.5 wt% H_2O_2, and 1.3×10^{-8} mol dm^{-3} Os(VIII).

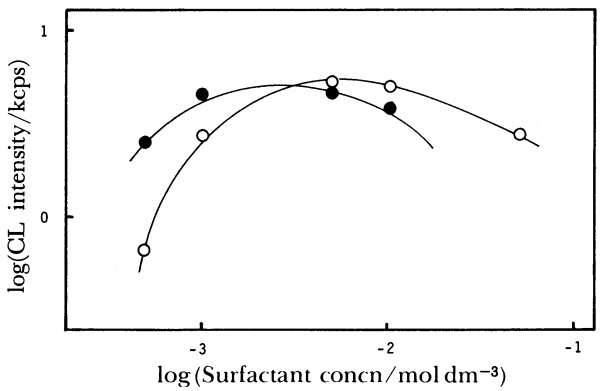

Fig. 2. Effect of surfactants on CL intensity. ○: EHDAB and ●: TOAC. Conditions: 1.2×10^{-4} mol dm^{-3} phen, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-9} mol dm^{-3} TEPA, 5.0 wt% H_2O_2, and 6.3×10^{-9} mol dm^{-3} Os(VIII).

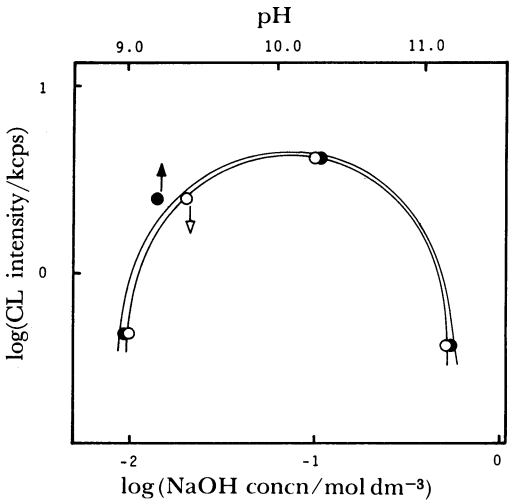

Fig. 3. Effect of NaOH concn or pH[1] on CL intensity. ○: CL intensity against NaOH concn and ●: CL intensity against pH. Conditions: 1.2×10^{-4} mol dm^{-3} phen, 5.0×10^{-3} mol dm^{-3} EHDAB, 1.0×10^{-3} mol dm^{-3} TEPA, 5.0 wt% H_2O_2, and 6.3×10^{-9} mol dm^{-3} Os(VIII). 1) The pH value refers to the value of a solution just before CL intensity is measured.

The effect of the phen, surfactant, and NaOH concentration in the phen solution on the peak CL intensity are shown in Figs. 1—3 under the optimum flow rates, as has been described above. Trimethyloctadecylammonium chloride(TOAC) and EHDAB were examined as cationic surfactants, for they had been known to show a high, enhanced action for a phen-H_2O_2-copper(II) system.[10] As can be seen from Figs. 1—3, 1.2×10^{-4} mol dm^{-3} phen, 5.0×10^{-3} mol dm^{-3} EHDAB, and 1.0×10^{-1} mol dm^{-3} NaOH were chosen as the optimum concentrations. A phen solution also contained 1.0×10^{-9} mol dm^{-3} TEPA as a masking reagent for a small amount of the metal ion. The effect of the H_2O_2 concentration on the CL intensity is shown in Fig. 4. The CL intensity increased with the increase in the concentration of H_2O_2, but was accompanied by an increasing noise. From these results, 5.0 w% H_2O_2 was chosen as the optimum concentration.

The relationship between the CL intensity and the Os(VIII) catalyst concentration was obtained under the above-mentioned conditions and is shown in Fig. 5. According to the present method, a micro amount of Os(VIII) could be determined over a range of concentration of 3.2×10^{-11}—1.3×10^{-8} mol dm^{-3}, with the detection limit of 0.4 pg at the rate of about 40 samples per hour. The coefficient of variation was 2.2% for 10 analyses of a 6.3×10^{-9} mol dm^{-3} Os(VIII) catalyst solu-

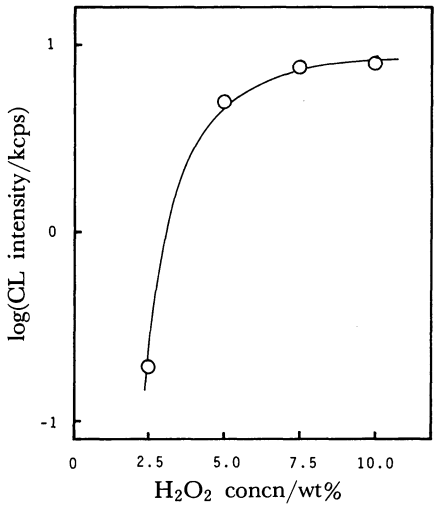

Fig. 4. Effect of H_2O_2 concn on CL intensity. Conditions: 1.2×10^{-4} mol dm^{-3} phen, 5.0×10^{-3} mol dm^{-3} EHDAB, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-9} mol dm^{-3} TEPA, and 6.3×10^{-9} mol dm^{-3} Os(VIII).

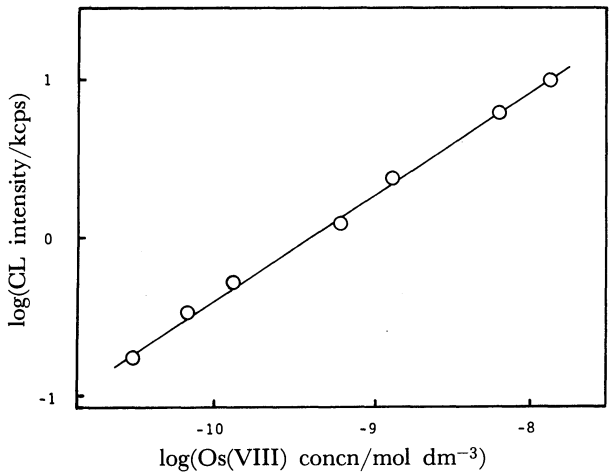

Fig. 5. Relationship between CL intensity and Os(VIII) concn. Conditions: 1.2×10^{-4} mol dm^{-3} phen, 5.0×10^{-3} mol dm^{-3} EHDAB, 1.0×10^{-1} mol dm^{-3} NaOH, 1.0×10^{-9} mol dm^{-3} TEPA, and 5.0 wt% H_2O_2.

Table 1. Detection Limit of Elements of the Platinum Group

Sample	Detection limit/mol dm^{-3}
OsO_4(VIII)	3.2×10^{-11}
$RuCl_3$(III) $3H_2O$	1.0×10^{-7}[4]
$PdCl_2$(II)	1.1×10^{-6}
$IrCl_3$(III) $1.5H_2O$	5.0×10^{-6}
$RhCl_3$(III) $3H_2O$	1.0×10^{-5}
H_2PtCl_6(IV)	1.0×10^{-4}

tion. The present method for the determination of Os(VIII) was 5—60 times as sensitive as the methods using the lucigenin or luminol CL system. By the present CL flow-injection analysis, iridium(III), palladium(II), platinum(IV), and rhodium(II) were determined with the detection limits of 5.0×10^{-6}, 1.1×10^{-6}, 1.0×10^{-4}, and 1.0×10^{-5} mol dm^{-3} respectively (Table 1).

Determination of Protein. Though protein was formerly determined by the authors by the use of the luminol-H_2O_2-Os(VIII) system, both the stability of the CL intensity and the detection limit of protein were not satisfactory. Here, a sensitive method for the determination was carried out by the use of a CL flow-injection technique using a phen-H_2O_2-Os(VIII) system.

Since protein was determined after the reaction between Os(VIII) and protein, the reaction time, the reaction temperature, and the Os(VIII) concentration required for the determination of protein were investigated in advance. Figure 6 shows the relationship between the relative CL intensity and the reaction time at 20, 60, and 95 °C. The relative CL intensity is represented as the value against the CL intensity of a BSA-free sample solution at the reaction time of 0, for

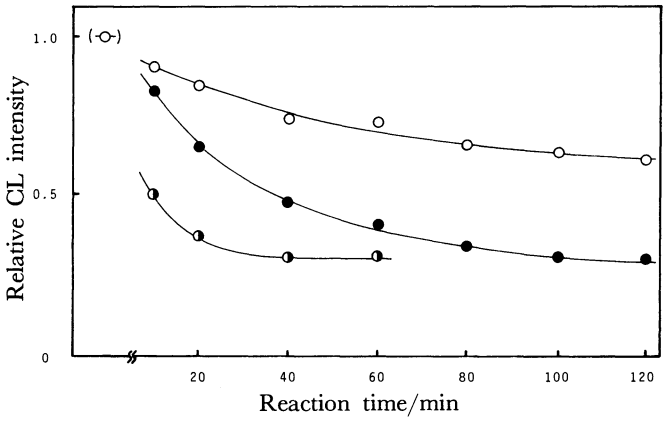

Fig. 6. Relationship between CL intensity and reaction time. ○: 20, ●: 60, ◑: 95 °C, and (—○—): BSA free. Conditions: 5.0×10^{-4} g dm^{-3} BSA and 6.3×10^{-9} mol dm^{-3} Os(VIII).

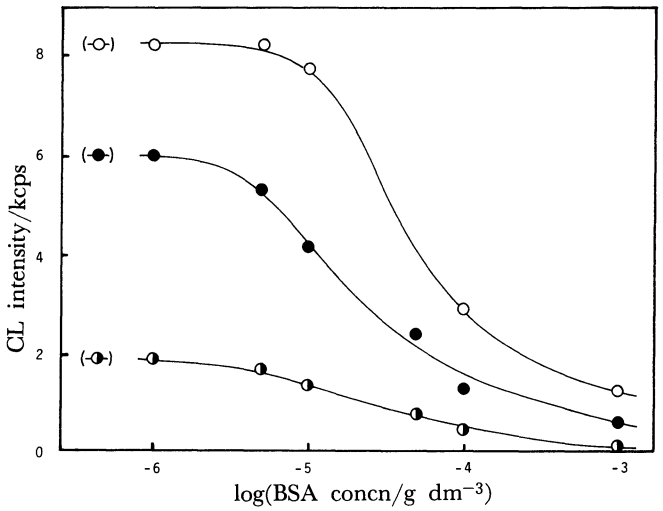

Fig. 7. Effect of Os(VIII) concn on the calibration curve of BSA. ○: 1.3×10^{-8} mol dm^{-3}-, ●: 6.3×10^{-9} mol dm^{-3}-, ◑: 3.2×10^{-9} mol dm^{-3} Os(VIII), and (—○—): BSA free.

Table 2. Lowering of CL Intensity in the Presence of 1.0×10^{-4} g dm^{-3} BSA

System	Lowering of CL intensity	Relative lowering CL intensity[a)]
phen-H_2O_2-Cu(II)	0.05	1
phen-H_2O_2-Ru(III)	0.14	2.8
phen-H_2O_2-Os(VIII)	0.75	15

a) Based on the value of the lowering of the CL intensity for the phen-H_2O_2-Cu(II) system.

the CL intensity of the BSA-free sample solution did not change upon heat treatment up to 60 min at 95 °C or up to 120 min at 20 and 60 °C. On the basis of the results pertaining to the decrease in the CL intensity and the reaction time, the CL intensity of a sample solution containing protein was examined after it had been treated for 45 min at 95 °C in the following experiments. Figure 7 shows the calibration curve of BSA obtained by the use of 3.2×10^{-9}, 6.3×10^{-9}, and 1.3×10^{-8} mol dm^{-3} portions of the Os(VIII) catalyst. The detection limit of the protein concentration and the decrease in the CL intensity for a definite amount of protein suggested that a 6.3×10^{-9} mol dm^{-3} Os(VIII) catalyst solution should be used in the present study.

The calibration curves for BSA, HSA, and HγG were obtained under the conditions specified above (Fig. 8). They could be used for the determination of protein in the concentration range of 5.0×10^{-6}—1.0×10^{-3} g dm^{-3} with a detection limit of about 250 pg. The coefficient of variation was 2.9% for 10 analyses of a 1.0×10^{-4} g dm^{-3} protein solution by the present method. The lowering of the CL activity of an Os(VIII) catalyst solution in the presence of protein seemed to be due to the formation of an Os(VIII)-polypeptide linkage, as in the previous paper.

Table 2 shows the lowering of the CL intensity by the use of copper(II), ruthenium(II), or osmium(VIII) as a catalyst for the phen-H_2O_2 CL system in the presence of 1.0×10^{-4} g dm^{-3} BSA. The values for the lowering of the CL intensity were obtained by subtracting the relative CL intensity for a sample solution in the

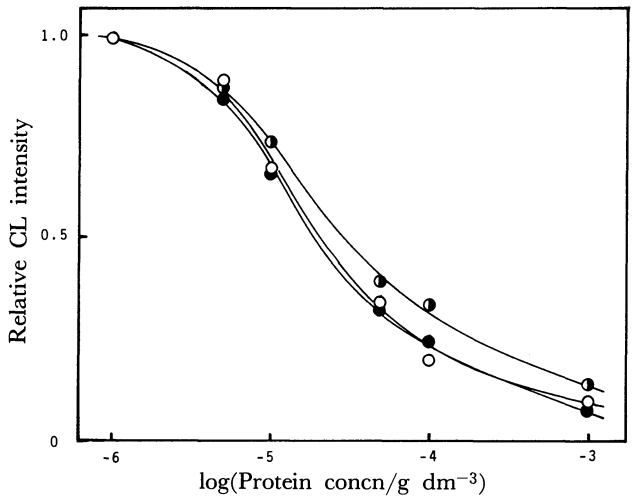

Fig. 8. Calibration curves of various proteins. ○: BSA, ●: HSA, and ◑: HγG. Conditions: 6.3×10^{-9} mol dm^{-3} Os(VIII).

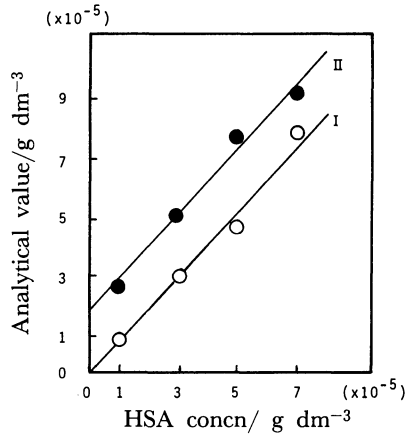

Fig. 9. Relationship between HSA concn and the observed protein concn. ○: HSA alone and ●: HSA+control serum. Conditions: 2.2×10^{-5} g dm^{-3} control serum and 6.3×10^{-9} mol dm^{-3} Os(VIII).

presence of 1.0×10^{-4} g dm^{-3} BSA from the relative CL intensity (=1.00) for a sample solution in the absence of BSA. As can be seen from these values, the present method using the Os(VIII) catalyst for a phen-H_2O_2 CL system was 5—15 times as sensitive as those using ruthenium(III) or copper(II). The present method was 4×10^2—1×10^6 times as sensitive as the non-labeling colorimetric or fluorometric method for the determination of protein and was the most sensitive method among those reported by the present authors for the determination of protein using CL flow-injection analysis.

Application to Serum Analysis. A human serum sample was measured after having been diluted because the present method was too sensitive to estimate protein in an original human serum sample and these was the possibility of interference with the coexisting constituents in human serum. Various amounts of HSA were estimated in either the presence or absence of a definite amount of control serum (diluted to a volume of 2×10^6 times with a buffer solution), and the relationship between the HSA concentration and the analytical value was obtained by the use of the calibration curve of HSA shown in Fig. 8. All the analytical values of the ordinate in Fig. 9 are represented as the amount of HSA. The difference between the straight lines, I and II, in the analytical value was approximately equal to the amount of protein in the added control serum, which was determined to be 2.2×10^{-5} g dm^{-3} by the use of the biuret method. This means that the present method was applicable to the determination of protein in a serum sample, without

any interference from coexisting constituents, when a sample solution was diluted to 2×10^6 times with a buffer solution and the resulting solution was then measured.

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