Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. II. Determination of Albumin as a Model Protein by Means of the Flow-injection Analysis Using a Cobalt(III) Complex Compound as a Catalyst

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Synopsis. A new procedure for the determination of 8×10^{-9}—3×10^{-7} mol dm^{-3} bovine serum albumin as a model protein has been established on the basis of the fact that the catalytic activity of *cis*-tetraamminediaquacobalt(III) sulfate for the chemiluminescent reaction between luminol and hydrogen peroxide decreases in the presence of bovine serum albumin.

In the previous paper,[1] several proteins were determined by use of the fact that the catalytic activity of copper(II) for the chemiluminescent reaction between 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and hydrogen peroxide (H_2O_2) decreased in the presence of those proteins. In the present work, *cis*-tetraamminediaquacobalt(III) sulfate which is known to give a longer luminescence time and more stable luminescence intensity than those in copper(II)[2,3] was used in place of copper(II) in the previous method. It was experimentally observed that the catalytic activity of the cobalt(III) complex compound for the chemiluminescent reaction between luminol and H_2O_2 decreased in the presence of bovine serum albumin (BSA), human serum albumin, bovine serum α-globulin, and bovine serum γ-globulin. BSA was chosen as a model protein. Though the present method is similar to the previous method in its detection limit and concentration range suitable for the determination, the standard curve of BSA in the present method is linear, and different from the non-linear one in the previous method. This means that the present method is superior to the previous method.

Experimental

All reagents were of commercially available special grade. Ion exchange water was distilled for use. A luminol solution, a H_2O_2 solution, and a buffer solution were prepared as in the previous paper. A 1.0×10^{-3} mol dm^{-3} cobalt(III) catalyst solution was prepared as a stock solution by dissolving *cis*-tetraamminediaquacobalt(III) sulfate[4] in water and it was diluted to 1.0×10^{-5} mol dm^{-3} with water, followed by the dilution with a buffer solution for use. BSA (Sigma Chemical Co., mol wt 66000) was used as a model protein.

The experiment was carried out as in the previous paper using the same apparatus. A sample solution containing BSA was dissolved and diluted with a buffer solution, and to it a cobalt(III) catalyst solution was added to give a definite concentration of cobalt(III) solution. After the solution was held at 30 °C for 50 min, an aliquot of the solution was subjected to the flow-injection analysis and the chemiluminescence (CL) intensity in the luminol-H_2O_2-cobalt(III) catalyst system was measured.

Results and Discussion

A calibration curve was obtained for the solution which contained only cobalt(III) catalyst (Fig. 1), and it is quite different from the non-linear calibration curve obtained for the copper(II) catalyst solution.

The relationship between reaction time and the CL intensity at 3.0×10^{-8}, 7.6×10^{-8}, and 3.0×10^{-7} mol dm^{-3} BSA was obtained (Fig. 2). Figure 2 shows that the reaction between cobalt(III) catalyst and BSA goes to completion in 50 min at 30 °C.

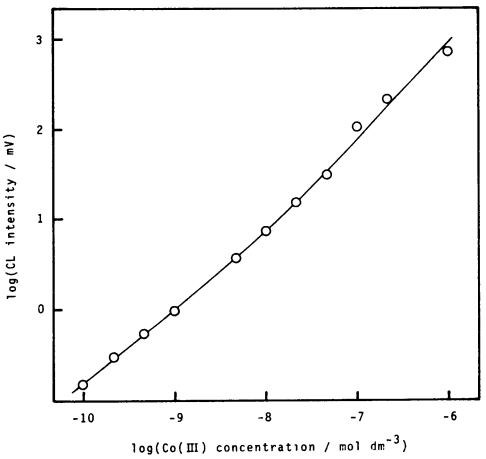

Fig. 1. Relationship between cobalt(III)[a] concentration and CL intensity. Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2. a) $[Co(NH_3)_4(H_2O)_2]_2(SO_4)_3$

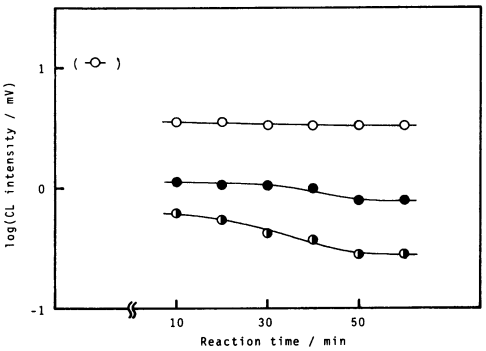

Fig. 2. Relationship between reaction time and CL intensity.
○: 3.0×10^{-8} mol dm^{-3}-, ●: 7.6×10^{-8} mol dm^{-3}-, ◑: 3.0×10^{-7} mol dm^{-3}-BSA, and (○): BSA free.
Conditions: 1.5×10^{-8} mol dm^{-3} Co(III), 1.0×10^{-3} mol dm^{-3} luminol, and 2.5×10^{-3} mol dm^{-3} H_2O_2.

Table 1. The concentration of species at equilibrium

Initial Co(III) concn/mol dm^{-3}	CL intensity / mV	[Co] / mol dm^{-3} a)	[Co·BSA] / mol dm^{-3}
1.00×10^{-8}	7.0	2.1×10^{-9}	7.9×10^{-9}
1.25×10^{-8}	9.8	2.8×10^{-9}	9.7×10^{-9}
1.50×10^{-8}	14	3.7×10^{-9}	1.13×10^{-8}
1.75×10^{-8}	15	4.4×10^{-9}	1.31×10^{-8}

Initial BSA concn = 3.0×10^{-8} mol dm^{-3}. a) Estimated from Fig. 1.

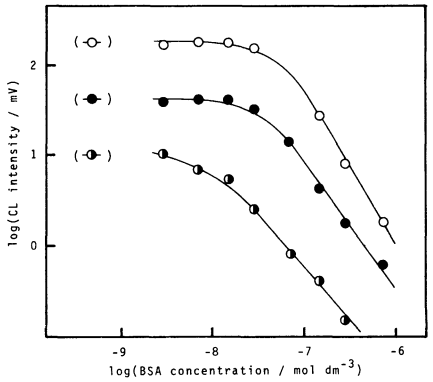

Fig. 3. Standard curves. ◑: 1.5×10^{-8} mol dm^{-3}-, ●: 5.0×10^{-8} mol dm^{-3}-, ○: 2.0×10^{-7} mol dm^{-3}-Co(III), and (○), (◑), (●): BSA free. Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

Standard curves were obtained by the reaction of BSA with 1.5×10^{-8}, 5.0×10^{-8}, and 2.0×10^{-7} mol dm^{-3} cobalt(III) catalyst solutions (Fig. 3). Lowering of the chemiluminescent activity of cobalt(III) catalyst solution in the presence of BSA seems to be due to the formation of cobalt(III)-polypeptide linkage similarly as in the copper(II) catalyst solution. As can be seen from Fig. 3, BSA in the concentration range of 8×10^{-9}—3×10^{-7} mol dm^{-3} could be determined, with a detection limit of 0.2 μg, by use of 1.5×10^{-8} mol dm^{-3} cobalt(III) catalyst solution. The present method is superior to the previous method since the standard curve in the present method is almost linear.

Lowry *et al.*[5] estimated an apparent dissociation constant between copper(II) and protein as 3×10^{-6} mol dm^{-3} on the assumption that they associated at the mole ratio 1:1 in the copper(II) concentration range of 8×10^{-6}—4×10^{-5} mol dm^{-3}. The authors also estimated an apparent association constant between cobalt(III) and BSA similarly as in the case of Lowry *et al.* The slope of the standard curve at 1.5×10^{-8} mol dm^{-3} cobalt(III) catalyst solution in Fig. 3 is approximately −1 and then the association between cobalt(III) and BSA is given by Eq. 1 as an equimolar reaction.

$$[\mathrm{Co}] + [\mathrm{BSA}] \rightleftharpoons [\mathrm{Co\cdot BSA}], \quad (1)$$

where [Co], [BSA], and [Co·BSA] are the concentrations of free cobalt(III), free BSA, and associated complex, respectively. An apparent association constant, K is defined by Eq. 2.

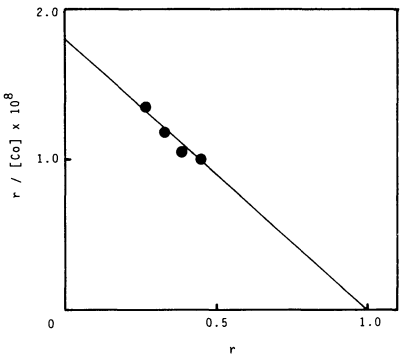

Fig. 4. Relationship between r and r/[Co]. Conditions: 1.0×10^{-3} mol dm^{-3} luminol and 2.5×10^{-3} mol dm^{-3} H_2O_2.

$$\frac{[\mathrm{Co\cdot BSA}]}{[\mathrm{Co}][\mathrm{BSA}]} = K. \quad (2)$$

From Eq. 2, Eq. 3 is derived.

$$r/[\mathrm{Co}] = K - rK, \quad (3)$$

where r means the mole ratio of bound cobalt(III) to total BSA. Figure 4 shows the plot of r/[Co] to r which was obtained by use of the data in Table 1. The straight line obtained by plotting r/[Co] against r on section paper passed through the point at r=1 in Fig. 4. This means that the derivation of Eqs. 1, 2, and 3 was reasonable. The value of K, 1.8×10^8 mol^{-1} dm^3 at 1.5×10^{-8} mol dm^{-3} cobalt(III) catalyst solution was found from the slope of the straight line in Fig. 4. The value of K thus obtained may be reasonable by considering the principle of the present method in which the concentration of a liberated cobalt(III) in the reaction between cobalt(III) and BSA is measured.

According to the present flow-injection analysis method, 8×10^{-9}—3×10^{-7} mol dm^{-3} BSA as a model protein can be determined at the rate of about 30 samples per hour after the reaction between cobalt(III) and BSA, with the detection limit of 0.2 μg BSA and with the standard curve which is almost linear in the range of 1×10^{-8}—3×10^{-7} mol dm^{-3} BSA.

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