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# Determination of Proteins by Using the Color Reaction with Pyrocatechol Violet–Molybdenum(VI) Complex[1)]

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A method for the determination of proteins (as albumin) with pyrocatechol violet(PV)–molybdenum(VI) [Mo(VI)] complex in the presence of polyvinyl alcohol was established. This method is based on the formation of the PV–Mo(VI)–protein complexes, which have absorption maxima at around 680 nm in weakly acidic media. The method could be used to determine up to *ca.* 300 $\mu$g/10 ml of albumin at 680 nm, and is superior to the Coomassie brilliant blue method and the pyrogallol red(PR)–Mo(VI) method in terms of sensitivity and reproducibility. Recovery of albumin added to human urine was good.

**Keywords**——protein determination; albumin; color reaction; polyvinyl alcohol; pyrocatechol violet–molybdenum(VI) complex

In the previous report,[2)] the color reaction between pyrogallol red(PR)–molybdenum(VI)[Mo(VI)] complex and protein was investigated, and a spectrophotometric method for the assay of proteins by using the PR–Mo(VI) complex was developed. On the other hand, it was found that the color contrast between pyrocatechol violet(PV)–Mo(VI)–protein solution (a green color) and PV–Mo(VI) solution (a brownish-yellow color) was much greater than that obtained with the PR–Mo(VI) complex.[2)] In addition, the method utilizing PV–Mo(VI) complex offered better sensitivity and reproducibility.

In this paper, suitable conditions for the spectrophotometric determination of proteins (as albumin) based on the color reaction between PV–Mo(VI) complex and protein were examined.

## Experimental

**Reagents and Materials**——Albumin (human serum), cytochrome C (horse heart), α-chymotrypsinogen A (bovine pancreas) and insulin (bovine pancreas) from Sigma Chemical Co., and γ-globulin and protease from Tokyo Kasei Kogyo Co., Ltd. were used. Each standard solution (500 $\mu$g/ml) of protein was prepared with distilled water. A solution ($5.0 \times 10^{-4}$ M, M = mol dm$^{-3}$) of Mo(VI) was prepared by dissolving sodium molybdate in water. A $1.0 \times 10^{-3}$ M methanol solution of PV (Dojindo Lab.) was prepared without purification. A 2.0% aqueous solution of polyvinyl alcohol (PVA, $n = 500$, Kishida Chemical Co., Ltd.) was also prepared. A buffer solution, pH 3.0, was made by titrating 0.2 M sodium acetate with 0.2 M hydrochloric acid.

**Apparatus**——A Shimadzu UV-240 recording spectrophotometer with 1.0-cm silica cells was used to take absorption spectra and for absorbance measurements. A Hitachi-Horiba F-7 AD glass electrode pH meter was used for pH measurements.

**Standard Procedure for the Determination of Albumin**——The following components were mixed in a 10-ml calibrated flask: 0.4 ml of $5.0 \times 10^{-4}$ M Mo(VI) solution, 1.0 ml of 2.0% PVA solution, 3.0 ml of the buffer solution (pH 3.0), 0.5 ml of $1.0 \times 10^{-3}$ M PV solution, and a solution containing up to *ca.* 300 $\mu$g of albumin. The mixture was diluted to 10 ml with water, and kept at room temperature for 20 min. The absorbance was measured at 680 nm against the reagent blank without protein.

## Results and Discussion

The absorption spectra of PV, PV–albumin, PV–Mo(VI) and PV–Mo(VI)–albumin

solutions in the presence of PVA at pH 3.0 are shown in Fig. 1.

On addition of a protein such as albumin to PV–Mo(VI) solution, the color of PV–Mo(VI) solution changed from brownish-yellow to green. The net absorbance at around 680 nm was proportional to the concentration of protein and was constant in the pH range of 2.5 to 3.8.

The PV–Mo(VI)–protein complex precipitated immediately in the absence of a surfactant. PVA ($n=500$) was found to be the most effective dispersion agent in terms of sensitivity and stability of the chromophore among various nonionic surfactants tested: PVA, polyvinyl pyrrolidone, Tween 20, Triton X-100, gum arabic, *etc.* With surfactants other than PVA, the PV–Mo(VI)–protein complex solution was unstable and liable to precipitate immediately. The maximum and constant absorbance was obtained by adding more than 0.5 ml of 2.0% PVA solution in a final volume of 10 ml.

The effect of the amount of Mo(VI) on the development of the color was examined, the amounts of PV and albumin being kept constant. As shown in Fig. 2, the maximum and almost constant absorbance was obtained when the molar ratio of Mo(VI) to PV was approximately 1 : 2. Furthermore, the color reaction between PV–Mo(VI) and albumin was found to be optimal when the molar ratio of Mo(VI) to PV was 1 : 2, as determined by the molar ratio and continuous variation methods. On the other hand, the less the amount of Mo(VI), the clearer was the color contrast between the sample and blank. Accordingly, all further work was carried out with $2.0\times10^{-5}$ M Mo(VI) and $5.0\times10^{-5}$ M PV solutions in a final volume of 10 ml.

Constant absorbance at 680 nm was obtained when the reaction was done at room temperature for 15—60 min. However, the reaction mixture containing proteins gradually formed precipitates on further standing.

The calibration curve for the determination of albumin was prepared by the standard procedure described in Experimental. The present method was found to be applicable to the determination of albumin in quantities of up to *ca.* 300 $\mu$g. Coefficients of variation ($n=5$) for 50 and 200 $\mu$g of albumin were 0.37 and 0.94%, respectively. As shown in Fig. 3, the

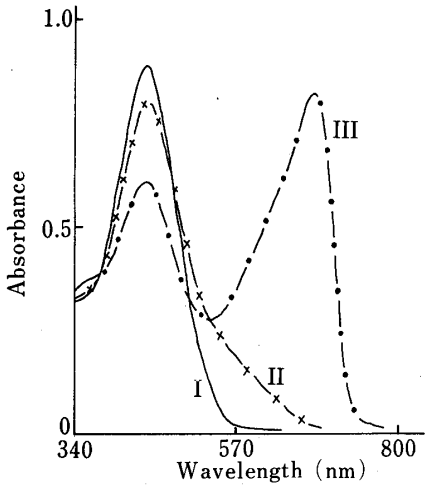


Fig. 1. Absorption Spectra of PV, PV–Albumin, PV–Mo(VI) and PV–Mo(VI)–Albumin Solutions in the Presence of PVA at pH 3.0

Albumin, 300 $\mu$g/10 ml; Mo(VI), $2.0\times10^{-5}$ M; PV, $5.0\times10^{-5}$ M; PVA, 1.0 ml of 2.0% PVA solution/10 ml; reference, water.
Curve I, PV or PV–albumin; curve II. PV–Mo(VI); curve III, PV–Mo(VI)–albumin.

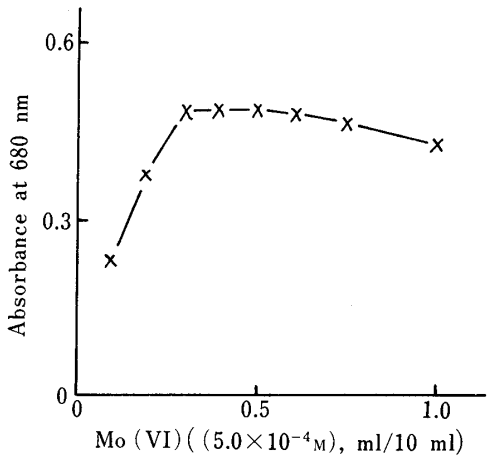


Fig. 2. Effect of Mo(VI) Concentration

Albumin, 200 $\mu$g/ml; PV, $5.0\times10^{-5}$ M; PVA, 1.0 ml of 2.0% PVA solution/10 ml; pH, 3.0; reference, the reagent blank without albumin.

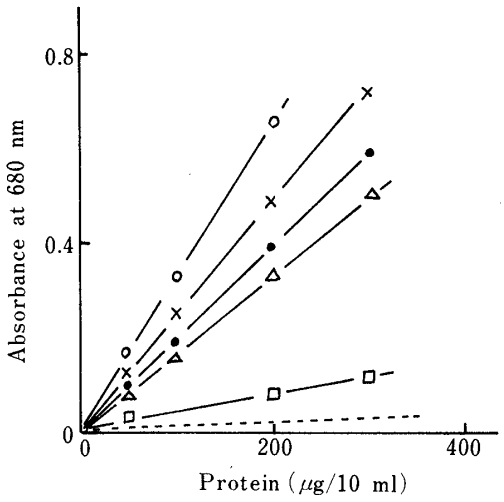


Fig. 3. Calibration Curves for Various Proteins Obtained by the Standard Procedure

Mo(VI), $2.0 \times 10^{-5}$ M; PV, $5.0 \times 10^{-5}$ M; PVA, 1.0 ml of 2.0% PVA solution/10 ml; pH, 3.0; reference, the reagent blank without albumin.
—○—, cytochrome c; —×—, albumin; —●—, γ-globulin; —△—, insulin; —□—, α-chymotrypsinogen A; ------, protease.

TABLE I. Effects of Various Substances on the Determination of Albumin

| Substance | Added (μg/10 ml) | Albumin found (μg/10 ml) |
|---|---|---|
| — | — | 200.0 |
| Fe(III) | $2.8 \times 10$ | 176.2 |
| Cu(II) | $5.9 \times 10$ | 200.0 |
| Sodium chloride | $5.8 \times 10^5$ | 200.0 |
| Phosphoric acid | $9.8 \times 10^4$ | 200.0 |
| Oxalic acid | $4.5 \times 10$ | 176.7 |
| Citric acid | $4.8 \times 10$ | 170.0 |
| Glycine | $1.5 \times 10^4$ | 200.0 |
| Pyruvic acid | $4.4 \times 10^3$ | 180.0 |
| Creatinine | $3.5 \times 10^4$ | 200.0 |
| Hippuric acid | $3.6 \times 10^4$ | 200.0 |
| Urea | $1.2 \times 10^5$ | 200.0 |
| D-Glucose | $4.5 \times 10^4$ | 200.0 |
| Sodium chondroitin sulfate | $5.0 \times 10^4$ | 128.8 |
| Ampicillin | $3.5 \times 10^4$ | 200.0 |
| Caffeine | $3.9 \times 10^4$ | 200.0 |
| Salicylic acid | $1.4 \times 10^4$ | 200.0 |
| Chlorpheniramine maleate | $3.9 \times 10^4$ | 227.9 |

Albumin, 200 μg/10 ml; Mo(VI), $2.0 \times 10^{-5}$ M; PV, $5.0 \times 10^{-5}$ M; PVA, 1.0 ml of 2.0% PVA solution/10 ml; pH, 3.0; reference, the reagent blank without albumin.

calibration curves for other proteins also passed through the origin, though the sensitivities were different depending on the individual proteins.

Under the standard conditions, various substances were examined for interference. Among the substances tested, iron(III), citric acid, oxalic acid, pyruvic acid, and sodium chondroitin sulfate caused a decrease in absorbance at 680 nm. The interference by iron(III) could be overcome by adding L-ascorbic acid or ethylenediaminetetraacetic acid (EDTA) solution. The presence of other substances such as amino acids, D-glucose, creatinine, urea, hippuric acid, phosphoric acid and sodium chloride in large amounts did not interfere. Among various drugs tested, large amounts of chlorpheniramine caused an increase in absorbance. The interference by other drugs was minor. The results are summarized in Table I. The recovery of albumin added to human urine was examined, and was 99.4% (the average

of 5 determinations for 50 $\mu$g of albumin).

Though further investigation is necessary, detection of protein on a spot plate or filter paper was more efficient than with the previous method[2] by using PR–Mo(VI) complex.

In conclusion, the present method has the disadvantage of the difference in sensitivity to various proteins, but the sensitivity of the method is equal to that of the Coomassie brilliant blue method,[3] and it about 1.2 times more than the PR–Mo(VI) method.[2] In addition, the proposed method has higher accuracy, with C.V. = 0.37% (for 200 $\mu$g of albumin), and the presence of surfactants or drugs scarcely affects the determination of protein.

Application of this method to biological samples should be feasible, as with the PR–Mo(VI) method.

**References and Notes**

1) This paper is Part XLI of a series entitled "Application of Xanthene Derivatives for Analytical Chemistry," Part XL, *Bunseki Kagaku*, **33**, E195 (1984).
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