Determination of Basic-Proteins Based on Color Reaction with the 3,4,5,6-Tetrachloro-2-carboxyphenylfluorone-Manganese(II) Complex¹

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We have been studying the determination of proteins using dye-metal ion-complex systems in micellar media.²⁻⁸ In this series we have suggested that the sensitivity and selectivity of the proposed methods are dependent on the dye, metal ion and/or surfactant, and that the reactivity of the protein to the dye-metal ion-complex seems to be correlated with the iso-electric point (pI) of each protein. In a continuation of this investigation we have found that 3,4,5,6-tetrachloro-2-carboxyphenylfluorone(TCl·CPF)-manganese(II)[Mn(II)] complex exhibits a considerable red shift in the presence of a basic-protein in weakly basic aqueous-methanol, and that the color reaction is fairly selective and sensitive. Here, we report on a new, simple and selective spectrophotometric method for the assay of basic-proteins with the TCl·CPF-Mn(II) complex.

Experimental

All of the proteins and polyamino acids used were purchased from Sigma Chemical Co., and were prepared by dissolving each material in water or dimethyl sulfoxide. TCl·CPF (5.0×10^{-4} M) and Mn(II) (1.0×10^{-3} M) solutions were prepared in methanol and deionized water, respectively, as described in previous papers.^{9,10} A 1.0% solution of poly(*N*-vinylpyrrolidone) (PVP, K-90) was prepared in methanol. A solution (1.0×10^{-1} M) of sodium tetraborate (borax, pH 9.4) was prepared as a buffer solution. The absorption spectra and absorbance were measured using a Shimadzu Model UV-160 spectrophotometer with 10-mm quartz cells.

Results and Discussion

In order to establish a selective determination of basic-proteins, it is necessary to select a metal ion which reacts with a dye in basic media. Mn(II) reacts with xanthene dyes in basic media and a xanthene dye-Mn(II) complex produces a color complex with basic organic com-

pounds¹¹⁻¹³, such as streptomycin and chlorhexidine. We, therefore, selected Mn(II) as a metal ion and histone (Type II-AS, from Calf Thymus) as a basic-protein for all further work. The dyes used were *o*-hydroxyhydroquinonephthalein (QP), TCl·CPF (the tetrachloro derivative of QP), *o*-sulfophenylfluorone (SPF)^{6,7}, phenylfluorone (PF), *m*-fluorophenylfluorone (FPF), salicylfluorone (SF), *o*-nitrophenylfluorone (NPF), Pyrogallol Red (PR)², Pyrocatechol Violet³, Methylthymol Blue, tetrachlorogallein (TCl·Gall)^{4,5}, 4-(2-pyridylazo)resorcinol, 1-(2-pyridylazo)-2-naphthol and so on. The addition of histone to a xanthene dye-Mn(II) mixture produced a bathochromic effect on the absorption band of a xanthene dye-Mn(II) complex solution. Dyes could be arranged in the following order with respect to sensitivity: TCl·CPF > QP, SPF > PF, SF, FPF, NPF, PR, TCl·Gall >> the others. Therefore, TCl·CPF was used for sensitive determinations. Various nonionic surfactants were examined regarding their ability to stabilize the colored complex and to increase the sensitivity: PVP (K-90, K-30, K-15), polyvinyl alcohol, Triton X-100, Brij 35, Tween 20 and gum arabic. PVP (K-90) was the best. The absorbance values gradually increased with increasing amount of methanol concentration, showing a maximum with 60% methanol.

The recommended procedure for the assay of basic-proteins is as follows. The following components were mixed in a 10-ml volumetric flask: 1.0 ml of 1.0% PVP solution, 1.5 ml of 1.0×10^{-1} M borax solution (optimal pH range between 9.2 and 9.8), 0.5 ml of 1.0×10^{-3} Mn(II) solution, 2.0 ml of 5.0×10^{-4} M TCl·CPF solution, 3.0 ml of methanol and a solution containing up to about 200 μ g of basic-protein. The mixture was diluted to 10 ml with water, transferred into a test tube, mixed well and kept at 40°C for 15 min. After the solution had been in water for 5 min the absorbance of the resultant solution was measured at 590 nm against a reagent blank.

The addition of basic-proteins to the TCl·CPF-Mn(II) complex solution exhibited an extreme increase in the absorbance at about 590 nm. On the other hand, the addition of non-basic proteins, such as human serum

Fig. 1 Absorption spectra of TCl·CPF-Mn(II)-protamine, TCl·CPF-Mn(II)-histone, TCl·CPF-Mn(II)-HSA and TCl·CPF-Mn(II) solutions. Protamine, histone and HSA taken: 200 μ g. Curves: A, TCl·CPF-Mn(II)-protamine; B, TCl·CPF-Mn(II)-histone; C, TCl·CPF-Mn(II)-HSA; D, TCl·CPF-Mn(II); reference, water for A–D.

albumin (HSA), slightly increased the absorbance at about 580 nm. Figure 1 shows the absorption spectra of the samples and blank. For solutions containing 100 μ g/10 ml histone, the relative standard deviation was 1.0% ($n=5$). The color reactions of various proteins and polyamino acids with the TCl·CPF-Mn(II) complex were examined. The sensitivity of proteins and polyamino acids seems to be correlated with the pI^{14} of these substances (Fig. 2). Basic-amino acids (arginine, lysine and histidine) and basic-low molecular peptides, such as bradykinin, angiotensin III and substance P, did not produce any colored complex, even in large amounts. Among the foreign ions and substances tested, large amounts of iron(III), zinc(II) and copper(II) ions caused a slight increase in the absorbance at 590 nm. Large amounts of citrate, oxalate and phosphate ions, and deoxyribonucleic acid caused a minor decrease in the absorbance. Other ions and substances (such as sodium, chloride, sulfate, fluoride and cyanide ions, and glucose, amino acids, caffeine and adenine) interfered very little with the determination of histone.

In conclusion, the application of the present method to biological sample should be feasible, in spite of the fact that further investigations are necessary.

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Fig. 2 Relationship between pI and absorbance of the complex in various of proteins and polyamino acids. Proteins and polyamino acids (100 μ g, uncorrected): a, protamine (salmine); b, protamine (clupeine); c, histone (Type III); d, histone (Type II); e, lysozyme; f, cytochrome *c*; g, ribonuclease A; h, avidin; i, α -chymotrypsinogen; j, hemoglobin; k, γ -globulin; l, transferrin; m, catalase; n, HSA; o, superoxide dismutase; p, poly-L-arginine; q, poly-L-lysine; r, poly-L-histidine; s, poly-DL-tryptophan; t, poly-D-tyrosine; Absorbance measurement: a–f, p and q, at 590 nm; g–o and r, at 580 nm.

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