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Study of the spectrophotometric analysis of protein solution with *p*-iodochlorophosphonazo as adsorbate by the microphase adsorption–spectral correction technique

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A microphase adsorption–spectral correction (MPASC) technique was established, which consists of Langmuir adsorption and the spectral correction technique. The hypothesis of a microelectric field formed in macromolecules is proposed, the existence of which results in the adsorption and aggregation of dye molecules in macromolecules. This paper describes the spectrophotometric analysis of protein solution with *p*-iodochlorophosphonazo (*p*-ICPA) dye. The results showed that the adsorption ratios of *p*-ICPA to proteins (bovine serum albumin, ovalbumin, myoglobin and γ-globulin) at pH 2.56 were 54, 25, 14 and 90, respectively, their adsorption constants 2.86×10^5, 1.42×10^6, 2.38×10^5 and 1.09×10^5 and their absorptivities 8.00×10^5, 4.02×10^5, 1.77×10^5 and 1.15×10^6 l mol^{-1} cm^{-1} at 620 nm. The analysis of practical samples showed that the recovery of protein was between 92.3 and 108% and the relative standard deviation was 4.7%.

Introduction

Nowadays, many chemists and biologists are interested in research on supramolecular chemistry. The study of supramolecular interactions of organic dyes with proteins is useful for understanding their structures and functions. The quantitative analysis of proteins is very important in clinical tests and laboratory practice. There are many methods for determining the content of proteins in samples, but the most commonly used methods are the biuret,[1] Bradford,[2] Lowry,[3] and Bromcresol Green[4] method. Recently, based on the above interactions, the resonance light scattering (RLS) technique was proposed[5,6] and applied successfully in the investigation of biological macromolecular complex with organic dyes.[7,8] In recent reports, the interactions of the chromophores, porphines and porphyrins with macromolecules have been increasingly studied.[1–3,9–13] However, the interaction between biological macromolecules and organic dyes has not been elucidated satisfactorily and earlier observations have not been explained clearly and reasonably, *e.g.*, the Pesavento equation[14] and Scatchard model.[15]

Some chromophores and dyes, *e.g.*, Bromophenol Blue and Neutral Red, as the adsorption indicators are usually used in the determination of halogen anions by precipitation titrimetry. In the laboratory, the same color change was observed when we used proteins in place of halogen anions. Consequently, the reaction between biological macromolecules and dye molecules has the same interaction mechanism as the precipitation adsorption reaction. Commonly, the biological macromolecule contains a complex spatial structure. The winding, folds, coils and other arrangements lead to many holes, gullies and helix grooves. Many secondary bonds are close to each other, such as van der Waals bonds, salt bonds, hydrogen bonds and other polar bonds. The charge density becomes very high around the holes, grooves and gullies. The like charges aggregate together to form many microelectric fields, as shown in Fig. 1. They will selectively attract charged organic compounds until kinetic equilibrium is reached, (Fig. 2). The existence of a microelectric field is the basis of the adsorption of dye molecules in macromolecules. In addition, we found that the equilibrium equation of the biochemical reaction[16] is same as the Langmuir adsorption equation.[17] The microelectric field is so narrow that organic molecules (with charge) were adsorbed only in a monolayer. Therefore, the interaction between macromolecules and organic dyes is regarded as monolayer adsorption of the microelectric field.

Because the absorption spectrum of macromolecule–dye products often approaches that of the dye in most biochemical reactions, the spectral correction technique[18,19] should be used

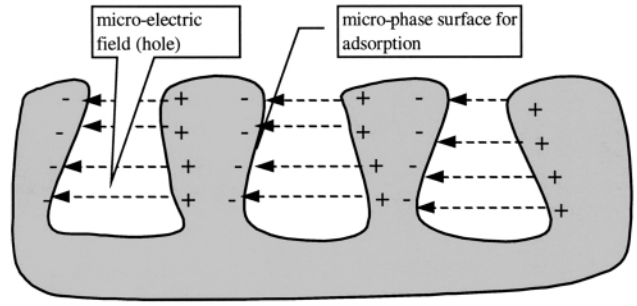

Fig. 1 The microelectric field hypothesis proposed for macromolecules.

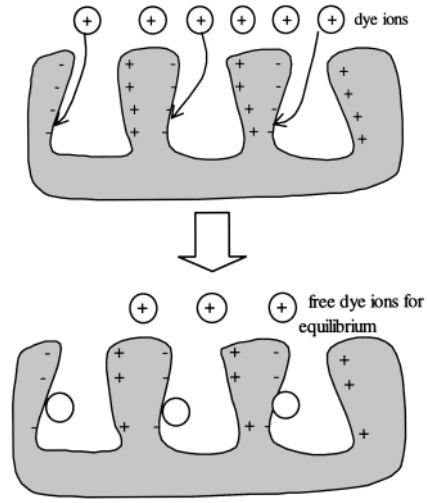

Fig. 2 The adsorption process of small dye molecules on a microphase surface.

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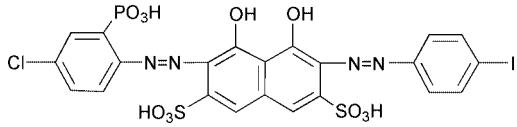
instead of ordinary methods. It was applied earlier in the investigation of metal complexes and the determination of trace amounts of components.[20,21] It will give accurate determinations of both the fractional absorbance of the free dye and the real absorbance of the product. The combination of the Langmuir adsorption and the spectral correction technique will provide a very useful experimental strategy for the study of dye aggregation on macromolecular surfaces. The new method is named the microphase adsorption–spectral correction (MPASC) technique.

In this work, we studied the reaction between proteins (bovine serum albumin, ovalbumin, myoglobin and γ-globulin) and the organic compound *p*-iodochlorophosphonazo (*p*-ICPA), the structure of which is given below.



This reagent was earlier applied in the determination of Bi,[22] Pd[23] and rare earth elements.[24] The relationship between the adsorption ratio of *p*-ICPA to proteins (bovine serum albumin, ovalbumin, myoglobin and γ-globulin) and the concentration of the free *p*-ICPA in solution accorded with the Langmuir adsorption. The results showed that at pH 2.56 the maximum adsorption ratios of bovine serum albumin, ovalbumin, myoglobin and γ-globulin to *p*-ICPA are 54, 25, 14 and 90, respectively, and their adsorption constants are $K_{\text{BSA–}p\text{-ICPA}} = 2.86 \times 10^5$, $K_{\text{OVA–}p\text{-ICPA}} = 1.42 \times 10^6$, $K_{\text{Mb–}p\text{-ICPA}} = 2.38 \times 10^5$ and $K_{\gamma\text{-G–}p\text{-ICPA}} = 1.09 \times 10^5$. The determination of the protein content in practical samples by MPASC gave satisfactory results.

Theory of MPASC technique

Spectral correction method

We observe the following reaction process between a macromolecule (M) and dye (L):

$$\text{M}(C_\text{M}) + \text{L}\ (C_{\text{L}0}) \longrightarrow \text{ML}_N\ C_\text{M}) + \text{L}\ (C_\text{L}).$$

The species before the arrow represent the composition of the solution at the beginning and the mixture after the arrow refers to the composition of the equilibrium solution. *N* is the binding ratio of L to M. The *C* terms in parentheses represent the molarity of M, L and product ML_N. It is impossible for the real absorbance (A_c) of product ML_N to be measured directly with a spectrophotometer because of the serious influence of the free L in the equilibrium solution. However, it may be calculated by the following equation, developed earlier:[18]

$$A_\text{c} = \frac{\Delta A - \beta \Delta A'}{1 - \alpha\beta} \tag{1}$$

where ΔA and $\Delta A'$ indicate the absorbances of the M–L solution, measured respectively at the peak absorption wavelength λ_2 and the valley absorption wavelength λ_1. α and β are the correction coefficients and they are calculated with the following equation by measuring directly ML_N and L solutions:

$$\alpha = \frac{\varepsilon_{\text{ML}_N}{}^{\lambda_1}}{\varepsilon_{\text{ML}_N}{}^{\lambda_2}} \tag{2}$$

and

$$\beta = \frac{\varepsilon_\text{L}{}^{\lambda_2}}{\varepsilon_\text{L}{}^{\lambda_1}} \tag{3}$$

Here $\varepsilon_{\text{ML}_N}{}^{\lambda_1}$, $\varepsilon_{\text{ML}_N}{}^{\lambda_2}$, $\varepsilon_{\text{L}_N}{}^{\lambda_1}$ and $\varepsilon_{\text{L}_N}{}^{\lambda_2}$ are the molar absorptivity of ML_N and L at wavelengths λ_1 and λ_2, respectively. In general, both α and β are often constants. However, a dimerization or polymerization reaction[25,26] will possibly occur in some dye solutions, hence the β value will change with the concentration of L ($C_{\text{L}0}$).

The molar ratio (γ) of the effective L to M is expressed as follows:[19]

$$\gamma = \eta \times \frac{C_{\text{L}0}}{C_\text{M}} \tag{4}$$

where

$$\eta = \frac{\alpha\Delta A - \Delta A'}{(1 - \alpha\beta)A'_0} \tag{5}$$

where η indicates the effective fraction of and A'_0 the absorbance of the reagent blank, directly measured at wavelength λ_1. Within increase in L concentration, γ will approach a maximum, called the binding ratio N.

In addition, the absorptivity (real $\varepsilon_\text{r}{}^{\lambda_2}$ not apparent $\varepsilon_\text{a}{}^{\lambda_2}$) of the product ML_N at λ_2 may be directly calculated by the following equation:

$$\varepsilon_\text{r}{}^{\lambda_2} = \frac{NA_\text{c}}{\delta\gamma C_\text{M}} \tag{6}$$

where δ is the cell thickness (cm) and the others have the same meanings as in the equations above.

Micro phase adsorption

Langmuir proposed[17] the molecular monolayer adsorption theory, which was applied extensively to adsorption on solid surfaces. He postulated that adsorbate molecules being adsorbed by a solid surface cannot form a layer more than a single molecule thick. He visualized the adsorption process as consisting of two opposing actions, condensation of molecules from the solute phase on to the surface and evaporation of molecules from the surface back into the body of the solution. The two rates, condensation and desorption, will eventually become equal, and when this happens an adsorption equilibrium will be established. In solution, the mutual random intertwining of long amino acid chains of protein molecules will form microelectric fields (Fig. 1) because of the presence of various polar bonds. It is easy for organic dyes or compounds with charges to be adsorbed on the microphase surfaces (Fig. 2). The biochemical reaction between a biological macromolecule (M) and a small molecule ligand (L) is just the dynamic adsorption equilibrium. In fact, all of the intercalative binding, groove binding and long-range assembly of L on M are just the different ways of arrangement of L molecules in microelectric fields. Such an adsorption equilibrium occurs: L (solution phase, C_L) $\rightleftharpoons$ ML_N (micelle phase, C_M) in L–M solution. The Langmuir adsorption equation may be used as follows:

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KNC_\text{L}} \tag{7}$$

where *K* is the equilibrium constant and the others have the same meanings as in the equations above. We calculated the *N* and *K* values by plotting $1/\gamma$ against $1/C_\text{L}$. In addition, we can also calculate *K* by replicate analyses of a certain solution where γ/N is between 0 and 1. We find that the Pesavento hypothesis and Scatchard model are same as the Langmuir adsorption [eqn. (7)]. Consequently, in fact, their principle is just microelectric field adsorption of small dye molecules in macromolecules.

Experimental

Apparatus and reagents

Absorption spectra were recorded with a UV/VIS 265 spectrophotometer (Shimadzu, Kyoto, Japan). A DDS-11A conductivity meter, (Tianjin Second Analytical Instrument Works) was used to measure conductivity together with a DJS-1 conductivity immersion electrode (electrode constant 0.98) (Shanghai Tienkuang Device Works) in the production of non-ionic water of < 0.3 μΩ cm^{-1}. The pH of the solution was measured with a pHS-2C acidity meter (Leici Instrument, Shanghai, China) and Model 620D pH Pen (Shanghai Ren's Electric). The temperature was adjusted and remained constant in a Model 116R electrically heated thermostatic bath (Changjiang Test Instrument of Tongjiang, China).

All reagents were of analytical-reagent grade and used without further purification. Stock standard solutions of proteins were prepared by dissolving commercial bovine serum albumin (BSA) (Beitai Biochemical, Chinese Academy of Sciences, Beijing, China), human γ-globulin (γ-G) (Serva, Heidelberg, Germany), horse myoglobin (Mb) (Shanghai Chemical Reagents Center) and ovalbumin (OVA, Shanghai Chemical Reagents Center) in non-ionic water. The γ-G solution was 1.00 mg ml^{-1} and the others 0.500 mg ml^{-1}.

A *p*-ICPA organic dye solution, 0.500 mM, was prepared by dissolving 0.1922 g of *p*-ICPA (purified and provided by Changke Reagent Institute, Shanghai, China) in 500 ml of non-ionic water. Britton–Robinson buffer solutions (between pH 2.21 and 8.69) were used to control the acidity of the reaction solution. NaCl solution (2 M) was used to adjust the ionic strength of the aqueous solutions. Na_2EDTA solution (5%) was prepared to mask the foreign metallic ions possibly co-existing in practical samples. The surfactant solutions, Triton X-100, sodium dodecylbenzene sulfonate (SDBS) and cetyltrimethylammonium bromide (CTMAB) (all 1%), were prepared for testing their effect on the sensitivity.

General procedures

In a 25 ml calibrated flask were placed an appropriate working solution of proteins, 2.5 ml of Britton–Robinson buffer solution and appropriate *p*-ICPA solution. The mixture was then diluted with non-ionic water to 25 ml and mixed thoroughly. All the absorption measurements were obtained against a blank treated in the same way but without proteins.

In the determination of proteins in samples, 1 ml of the masking reagent, Na_2EDTA solution (5%), was added to complex metal ions, *e.g.* Pb(II), Cd(II), Hg(II) and others.

Results and discussion

Absorption spectra

The adsorption between *p*-ICPA and proteins (BSA as representative) was carried out. The absorption spectra of the *p*-ICPA and BSA–*p*-ICPA solution at pH 2.56, are shown in Fig. 3. From curves 1 and 2, we find that the absorption peak of *p*-ICPA is located at 550 nm and that of the BSA–*p*-ICPA product at 600 nm. The spectral red shift is only 50 nm at pH 2.56. Therefore, the free *p*-ICPA in its BSA solution will affect the measurement of the real absorbance of the product. From curve 3, the two wavelengths 620–nm (peak) and 540–nm (valley) should be selected as the measurement wavelengths because the relative absorbances both reach a maximum. From curves 1 and 2, the correction coefficients were calculated to be $\beta = 0.370$ and $\alpha = 1.27$. Because of the large β, the spectral correction method should be used instead of ordinary spectrophotometry for the correct analysis of the interaction of dye molecules with protein macromolecules.

Effect of pH

By varying the pH of the solution, the absorbances of the BSA–*p*-ICPA solution were measured and the binding ratio of *p*-ICPA to BSA is shown in Fig. 4. We observe that a lower pH will give a higher ratio. It is possible that protonation of *p*-ICPA occurs in acidic solution: $L + H^+ = LH^+$. The LH^+ cation is attracted easily into microelectric fields in protein molecules.

Effect of ionic strength and surfactant

The influence of the ionic strength of the solution on the binding ratio is shown in Fig. 5. The ratio of *p*-ICPA to BSA decreases with increase in ionic strength between 0 and 0.2 M. More Na^+

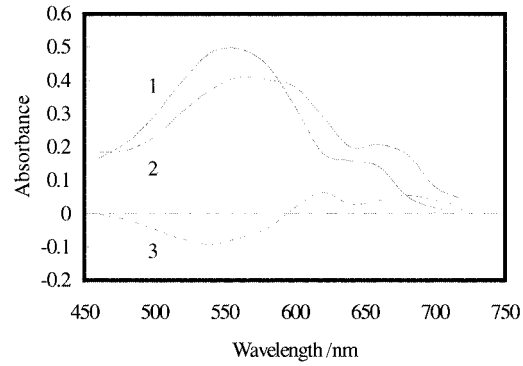


Fig. 3 Absorption spectra of *p*-ICPA and its BSA (0.50 mg) solutions at pH 2.56: 1, 0.500 μmol of *p*-ICPA; 2, 0.250 μmol of *p*-ICPA with 2 μmol of BSA; 3, 0.500 μmol of *p*-ICPA with 0.250 mg of BSA. Both 1 and 2 are against water and 3 against the blank.

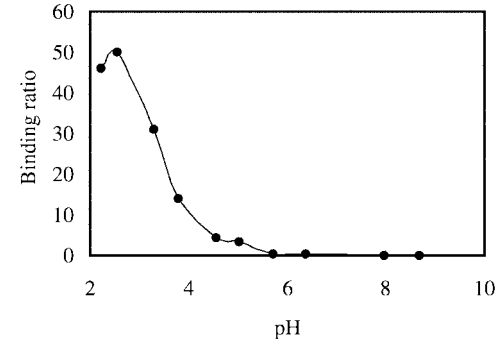


Fig. 4 Effect of pH on the binding ratio of *p*-ICPA to BSA in the reaction solution: 0.500 μmol of *p*-ICPA with 0.250 mg of BSA.

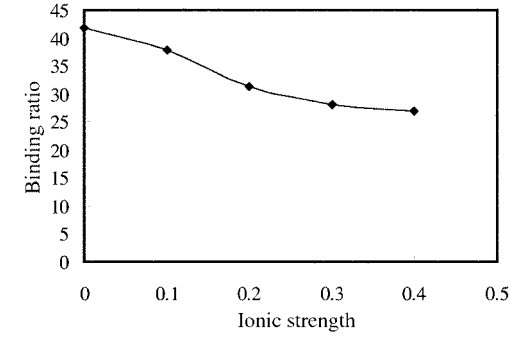


Fig. 5 Effect of ionic strength of solution on the binding ratio of *p*-ICPA to BSA at pH 2.56: 0.500 μmol of *p*-ICPA with 0.250 mg of BSA.

ions are attracted into microelectric fields to take up the microphase surface, hence a decrease in the adsorption ratio occurs. The effect of various surfactants on the binding ratio of *p*-ICPA to protein showed that the ionic surfactants CTMAB and SDBS can stop the adsorption of *p*-ICPA, because they can be adsorbed strongly instead of *p*-ICPA molecules to take up the limited surface available. The non-ionic surfactant Triton X-100 does not affect the binding ratio because its molecules carry no charge.

Effect of *p*-ICPA concentration

By varying the addition of *p*-ICPA solution, the absorbances of the various protein solutions were measured. The calculated γ and its curves are shown in Fig. 6(a). Fig. 6(b) gives the absorbance ratio, $A_{540\ nm}/A_{620\ nm}$, for *p*-ICPA solutions of various concentration measured at 540 and 620 nm. The ratios remain constant so the *p*-ICPA dye will not form dimers or polymers in the addition range 0–3.0 ml of 0.500 mmol l^{-1} *p*-ICPA. From Fig. 6(a), the binding ratio remains almost constant when the addition of *p*-ICPA solution is from 1.0 ml up to 3.0 ml. The final binding ratios are 55 (*p*-ICPA to BSA), 25(*p*-ICPA to OVA), 14 (*p*-ICPA to Mb) and 77 (*p*-ICPA to γ-G). Fig. 7 shows the relationship between the reciprocal of the binding ratio (γ), $1/\gamma$ and that of free *p*-ICPA concentration (C_L)

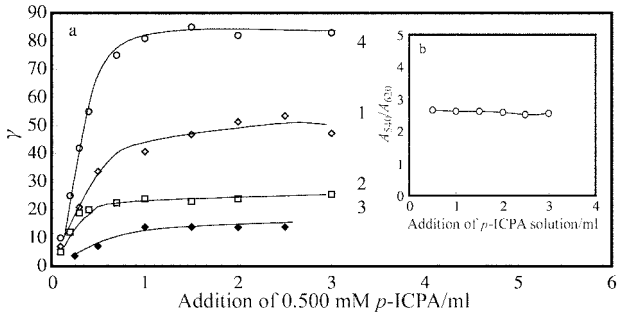


Fig. 6 Effect of the addition of *p*-ICPA solution on the binding ratio γ at pH 2.56: 1, BSA; 2, OVA; 3, Mb; and 4, γ-G.

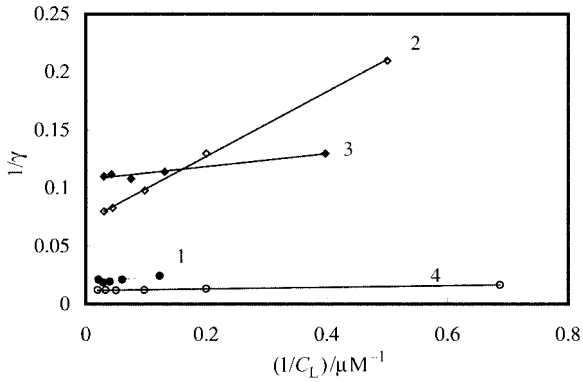


Fig. 7 Relationship between of $1/C_L$ and $1/\gamma$. 1, −BSA; 2, OVA; 3, Mb; and 4, γ-G.

in solution, $1/C_L$. We observe that they are all linear. Therefore, the interaction between *p*-ICPA and each protein accords with the Langmuir adsorption. The linear regression equations are as follows:

$$\frac{1}{\gamma} = 0.0185 + \frac{6.23 \times 10^{-8}}{C_{p\text{-ICPA}}}\ (\text{BSA} - p\text{-ICPA}),$$

$$\frac{1}{\gamma} = 0.040 + \frac{4.22 \times 10^{-8}}{C_{p\text{-ICPA}}}\ (\text{OVA} - p\text{-ICPA}),$$

$$\frac{1}{\gamma} = 0.071 + \frac{2.75 \times 10^{-7}}{C_{p\text{-ICPA}}}\ (\text{Mb} - p\text{-ICPA}),$$

and

$$\frac{1}{\gamma} = 0.011 + \frac{1.06 \times 10^{-8}}{C_{p\text{-ICPA}}}\ (\gamma\text{-G} - p\text{-ICPA}),$$

From these equations, the maximum binding ratios of bovine serum albumin, ovalbumin, myoglobin and γ-globulin to *p*-ICPA are calculated to be 54, 25, 14 and 90, respectively, all of which approach to those observed in Fig. 6. Simultaneously, from the equations, the equilibrium constants (*K*) of the protein–*p*-ICPA products are 2.55×10^5, 1.07×10^6, 2.60×10^5 and 1.05×10^6, respectively. In addition, we may also calculate *K* and ε from replicate determinations of protein–*p*-ICPA interactive solution. The preparation of the solution and the results are given in Table 1. We find that the *K* values are near to the above results obtained from the curve of $1/\gamma$ *vs.* $1/C_L$. The binding constants of proteins decrease in the order OVA > γ-G > BSA > Mb. From each ε value in Table 1, we observe that the higher the absorptivity of the product becomes, the greater the binding ratio *N* becomes; their order is γ-G > BSA > OVA > Mb. This accords with the common reaction law. In the determination of the binding ratio and equilibrium constant, the spectral correction technique has special advantages in operation and principle over classical methods such as molar ratio,[27] continuous variation[28] and equilibrium movement.[29]

We calculated the adsorption (*R*) *p*-ICPA per milligram of protein by $R = N/W$, where *W* indicates the molecular weight of the protein. The results are as follows: 0.782×10^{-3} (BSA), 0.568×10^{-3} (OVA), 0.813×10^{-3} (Mb) and 0.600×10^{-3} (γ-G). The relative standard deviation (RSD) is 17.8%. We think that 1.0 mg of protein can bind an almost constant number of *p*-ICPA molecules even if various proteins have different structures. It is possible that one of the microelectric fields consisted of constant amino acid residues due to folding.

Effect of temperature and reaction time

At various temperatures, the binding ratio of *p*-ICPA to BSA remained almost constant at 42 because the product is very stable (observed from the high constant *K*). At room temperature, the reaction between BSA and *p*-ICPA is complete in 5 min. This indicates that the adsorption between macromolecules and small dye molecules is often rapid and it is different from a chemical reaction.

Table 1 Six replicate analyses of protein and nucleic acid solutions with *p*-ICPA at pH 2.56 and calculation of the binding ratio, equilibrium constant and real absorptivities of protein–*p*-ICPA complexes

Reaction	γ/N	*K*	ε/l mol^{-1} cm^{-1} at 620 nm
BSA (0.500 mg)–*p*-ICPA (1.000 μmol)	(37.4 ± 3.4)/54	$(2.86 \pm 0.41) \times 10^5$	8.00×10^5
OVA (0.500 mg)–*p*-ICPA (0.100 μmol)	(10.5 ± 1.3)/25	$(1.42 \pm 0.33) \times 10^6$	4.02×10^5
Mb (0.120 mg)–*p*-ICPA (0.500 μmol)	(11.3 ± 0.9)/14	$(2.38 \pm 0.41) \times 10^5$	1.77×10^5
γ-G (0.500 mg)–*p*-ICPA (0.200 μmol)	(54.6 ± 3.1)/90	$(1.09 \pm 0.31) \times 10^6$	1.15×10^6

Calibration graph and precision

The standard series of proteins were prepared and measured at pH 2.56 with 1.0 ml of *p*-ICPA solution added. The regression equations are given in Table 2. They are all linear in the recommended range. We find that the slopes are very close. The real absorption per milligram of protein is similar even if various proteins have different structures. In addition, we calculated the binding ratio (γ) of the product and the effective fraction (η) of *p*-ICPA in each of the solutions above. Their variation curves are shown in Fig. 8. We find that all γ peaks are located at about 0.60 mg of protein and all η curves approach coincidence. The γ peak shows almost a direct ratio to the molecular weight of the protein. This also indicates that the adsorption of *p*-ICPA molecules in protein macromolecules bears almost no relation to the sequence of amino acids or their type.

Table 2 Linear regression equations for the determination of proteins with *p*-ICPA as adsorbate at pH 2.56

Protein	Linear range/ mg per 25 ml	Regression equation at 620 nm (x = mg protein)	Correlation coefficient
BSA	0–1.0	$A_c = 0.340x - 0.003$	0.9989
OVA	0–1.0	$A_c = 0.340x - 0.001$	0.9987
Mb	0–1.0	$A_c = 0.359x + 0.002$	0.9920
γ-G	0–1.0	$A_c = 0.324x - 0.001$	0.9969

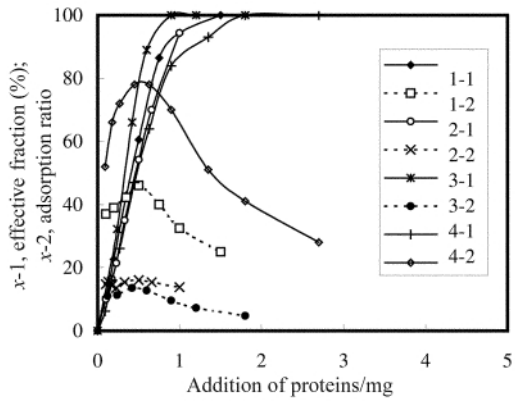


Fig. 8 Effect of addition of protein solutions on both η (x-1) and γ (x-2) where 1.00 μmol of *p*-ICPA was added: 1, BSA; 2, OVA; 3, Mb; and 4, γ-G.

For $A_c = 0.010$, the detection limit of proteins was calculated to be 0.03 mg. Seven replicate determinations of 0.100 mg of standard BSA were carried out. The mean result was 0.109 ± 0.005 mg of protein with RSD 3.6%.

Effect of foreign ions

By adding Na_2EDTA (1 ml of 5% solution) to the *p*-ICPA–protein reacted solution, the effect of foreign substances such as cations, anions, sugars and surfactants on the determination of proteins was tested at pH 2.56 and 0.01 M ionic strength. We observed that high concentrations of metal ions can be allowed in the tested solution. None of the following ions affected the direct determination of 0.20 mg of BSA (< 10% error): 5 mg of K^+, NH_4^+, Ca(II), SO_4^{2-}, CO_3^{2-}; 1 mg of Cl^-, F^-, PO_4^{3-}; NO_3^-, SO_3^{2-}, $C_2O_4^{2-}$, I^-, Triton X-100, OAc^-, glucose, amino acids, Mg(II), Al(III), Fe(III); 0.5 mg of acetone, ethanol, Cu(II), Mn(II), Zn(II), Ti(IV), Pb(II), Ge(IV), Ni(II), Co(II), Sn(II), Cd(II), 0.1 mg of Be(II), Hg(II), CTMAB and SDBS.

Sample analyzed

Two samples were analysed; one was Duoqi Children's Drink and the other was synthesized by mixing the protein and the following compounds or ions: 1 mg of Ca(II), ethanol, acetate, ctDNA, yRNA, glucose and PO_4^{3-}, 0.5 mg of Ca(II), Mg(II), SO_4^{2-}, NO_3^- and Cl^-, 0.1 mg of F^-, Cu(II), Mn(II) and Zn(II) and 0.02 mg of Pb(II), Hg(II) and Cd(II), where the background was drinking water. The former sample was prepared by diluting 1.00 ml of original drink to 100 ml with non-ionic water and ten 4.00 ml aliquots were taken for analysis. The analysis of samples and the recovery of standard proteins are given in Table 3. An average of 0.661 mg of protein was contained in 4.00 ml of the diluted sample. The content of protein was 1.7% in the original Duoqi Drink and is close to the 'about 2% protein' marked the container. Standard BSA was added to the Duoqi Drink sample and the recovery was 92.3% with RSD 4.7%. In addition, the recovery of standard proteins in the synthetic sample was between 96.2 and 108%.

Conclusion

This investigation of the reaction between *p*-ICPA and proteins supports the hypothesis of the formation of a microelectric field

Table 3 Determination of proteins in two samples with *p*-ICPA as reactant at pH 2.56 and in the presence of EDTA for masking other metal ions

Sample	Added	Found/mg
Duoqi Drink (containing 2% protein)	0.040 ml of sample	0.706 0.676 0.676 0.618 0.647 0.647 Av. 0.661 ± 0.031 RSD. 4.7%
	0.400 mg of BSA added to 0.040 ml of sample	0.970 1.12 1.00 Av. 1.03 ± 0.08 Recovery 92.3%
Synthetic sample with drinking water base (three replicate determinations of 5.0 ml of sample)	BSA 0.160 mg	0.166 ± 0.009 Recovery 104%
	OVA 0.600 mg	0.649 ± 0.040 Recovery 108%
	γ-G 0.500 mg	0.481 ± 0.019 Recovery 96.2%

and its monolayer adsorption on *p*-ICPA. The MPASC technique cannot give higher sensitivity than other methods such as RLS.[5] However, it may improve the precision and accuracy of trace analysis and offers the additional benefits of simplicity and versatility. We have described the basic physics behind the MPASC technique and surveyed some ongoing research on applications to different macromolecular or micellar solutions. We also understand that classical spectrophotometry can still play important role in the recognition of biological macromolecules and the determination of their property constants.

Acknowledgements

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