Asian Journal of Chemistry Vol. 20, No. 6 (2008), 4647-4658

Interaction of Transfusion Gelatin Molecule with Cetylpyridinium Chloride

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The experiments on the binding of cetyl pyridinium chloride to transfusion gelatin have been carried out at various pH, whereby the ionic type of binding has been studied by pH-titrations. The binding of this surfactant to protein molecule has also been studied with the help of viscometric method. The binding results have been explained in terms of binding and consequent structural disorganization within the protein molecule. The quantitative precipitation experiments suggested that the maximum precipitation and the complete dissolution of the precipitate took place at a definite ratio of the detergent concentration. The maximum amount of cationic detergent combined at each pH-value are approximately, corresponding to the base combining power of protein and so it is concluded that the first polar combination between protein molecule and cationic surfactant is accomplished at the point of the maximum precipitation. The pH-titration and dialysis equilibrium results pointed the involvement of anionic protein groups in interaction with the detergent cations. The logrithm of association constants (log K) were found to be 2.42 and 3.38 whereas the binding sites (n) 42 and 35 at pH 4.00 and 7.50, respectively. The lesser number of linkage sites showed the maximum number of moles of surfactant molecules combined upto statistical binding. The binding was found to be highly cooperative at higher concentrations of the surfactant. The flow behaviour indicated some conformational changes in the protein molecule. The pH-metric data strongly supported a mechanism in which binding between cationic surfactant and protein consists of an ionic binding while dialysis equilibrium corresponds to ionic as well as other types of binding. The precipitation and redissolution of surfactant-protein complex has been attributed to electrostatic linking above isoelectric point and nonelectrostatic type below this point involving forces which usually binds surfactant ions into micelles. It may be concluded that cationic surfactant-protein combination involved ionic hydrophobic and hydrogen bonding in forming complexes depending upon the pH and concentration of the cationic surfactant.

Key Words: Transfusion gelatin, Cetyl pyridinium chloride.

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INTRODUCTION

The interest in surfactants with biopolymers and synthetic polymers has been the subject of many investigators[1-8]. It has been reported that the interaction depends upon some factors, among which the ratio of surfactant to protein seems to be of great importance in determining its mechanism[9]. Inspite of the considerable biochemical interest of the cationic synthetic surfactants only a few studies have been made in this direction[10]. Timasheff and Nord[11] have reported the binding of dodecylamine hydrochloride (DAH) with ovalbumin while Tamaki and Tamamushi[12] have compared the surfactant ion binding of gelatin with its acid and base binding capacity. Yang and Foster[13] have studied the binding of cetylpridinium bromide and cetyltrimethyl ammonium bromide by serum albumin and ovalbumin. Birdi[14] has examined the binding of cetyltrimethyl ammonium bromide to many proteins and revealed the perturbation of their tyrosyl groups. Tanaka *et al.*[15] have reported the interaction between cationic surfactants and bovine serum albumin molecule. The binding of cationic surfactants to many globular proteins was reported by Nozakil *et al.*[16], while Jones *et al.*[17] have established that anionic surfactants bind more strongly than the cationic surfactant to bovine serum albumin (BSA).

A literature survey shows that little work has been done on the binding of cationic surfactants with transfusion gelatin[18-20] although it is a well characterized fibriller protein[21,22]. In this paper, the experiments on the binding of cetylpyridinium chloride (CPC) to transfusion gelation (TG) are discussed at various pH, whereby the ionic type of binding has been studied by continuous and discontinuous pH titrations. The binding of this detergent to the protein molecule has also been investigated with the help of viscometric method. The binding results have been interpreted in terms of binding and consequent structural disorganization within the protein molecule.

EXPERIMENTAL

Transfusion gelatin (TG) supplied by National Chemical Laboratory, Poona, (India) (m.w. 75000) was used as such throughout these studies. Stock solution (1.0×10^{-2} M) of cetylpyridium chloride (CPC) was prepared in double-distilled water. Buffer-solution used were prepared from reagent grade chemicals. Solution of hydrochloric acid and carbonate free KOH (AR) were prepared for the pH-measurements. Potassium chloride solution was prepared for the maintenance of the ionic strengths.

Methods and techniques: Varying amounts of hydrochloric acid (0.0861 M) and potassium hydroxide (0.0605 M) were taken and 1 mL of isoionic (6 %) protein were added to each. The total volume was made up to 10 mL by adding water and KCl to make the ionic strength 0.15 M. Similar set was also arranged having 1.0 mL of CPC of 0.01 molar concentration.

Water and CPC alone were also mixed with varying amounts of hydrochloric acid and potassium hydroxide. The pH values of the various mixtures were recorded with the help of a systronic pH-meter using a wide range glass electrode. The instrument was standardized by means of standard buffers in the acidic and basic ranges, respectively. Different samples of protein and CPC were adjusted to the same initial pH and titrated and the pH values of the mixtures were recorded. Purified nitrogen gas was bubbled through the reaction mixtures in alkaline range to ensure completely inert atmosphere.

For equilibrium dialysis measurements cellophane tubings were filled with 5.0 mL of 0.66×10^{-4} molar TG and then immersed in 5.0 mL of a solution of CPC (1.0 to 70.0×10^{-4} mol/L) contained in different boiling test tubes. Two similar sets, one at pH 4.0 and the other at pH 7.50 were prepared and then subjected to constant shaking at 25 ºC for 72 h, a time just sufficient to attain the equilibrium. The dialysis tubings were then removed and the external solution were analyzed by volumetric precipitation method using sodium dodecyl sulphate (SDS) as a precipitant and rhodamine-6G as an indicator. Controls were also run to determine the CPC binding to the material of the tubing and these were found to be negligibly small. The accuracy of detergent estimation was found to be ± 2 per cent in these measurements.

The viscosity measurements were made with the help of an Ostwald viscometer of flow time of water 55 s at a temperature of 25 ºC in a water thermostat. Protein and surfactant stock solution were centrifuged at 16000 rpm of 1 h to remove particulate matter. The density of the solvent and solutions were determined with the help of a pyknometer. The viscosity values were determined by the following relation:

$$\eta_{rel} = \frac{\eta}{\eta_o} = \frac{t.p.}{t_0 p_0}$$

where η_{rel} is the relative viscosity, t and p are the flow time and density of the solution, while t_0 and p_0 are the flow time and density, respectively for water. The following sets of solutions were prepared for viscosity measurements. (i) A fixed amount of TG (5 mg/mL) was taken in different tubes and varying amounts of 0.0861 M HCl and 0.0605 M KOH were added keeping the total volume 10 mL (ii). A fixed amount of TG (5 mg/mL) and CPC (0.001 mol) was taken as in (i) and different amounts of HCl/KOH added. (iii) to a fixed amounts of TG (5 mg/mL) were added varying amounts of CPC (0.0 to 0.02 mol) and the total volume was made 10 mL. The viscosity of this set was recorded at pH values 7.0, 8.0, 9.0 and 10.0, respectively and (iv), varying amounts of TG (2 mg to 16 mg/mL) were taken with the same amounts of CPC and the pH was adjusted to 2.60, 3.49 and 5.40,

respectively. The viscosity results are plotted in the form of reduced viscosity protein concentration curves.

RESULTS AND DISCUSSION

Complex formation: Different amounts of CPC were added to a (5 mg/mL) TG solution at various values of pH of the solution, which had been controlled by adding HCl or KOH to the solution. It has been found that TG was precipitated at pH 7.0 to 10.0 in certain range of CPC concentration. When precipitate appeared, the mixtures were centrifuged and the concentration of CPC in the supernatant solution was determined, the amounts of the CPC combined with the precipitant was obtained by taking the difference of concentration which existed before and after the mixing Fig. 1 shows the amount of the combined CPC per gm of TG in relation to the ratio of CPC to the TG. It is evident from the figure that the precipitation zone is greatly influenced by the pH of the mixed solution and the precipitation zone is displaced to the higher ratio of CPC to TG as pH become greater. It may be stated that the maximum precipitation and the complete dissolution of the precipitate takes place at the definite ratio of the CPC concentration. In fact, the maximum values of CPC combined at each pH are approximately, corresponding to the base combining power of TG and therefore, it is concluded that the first polar combination between TG molecules and cationic surfactant (CPC) is accomplished at the point of the maximum precipitation.

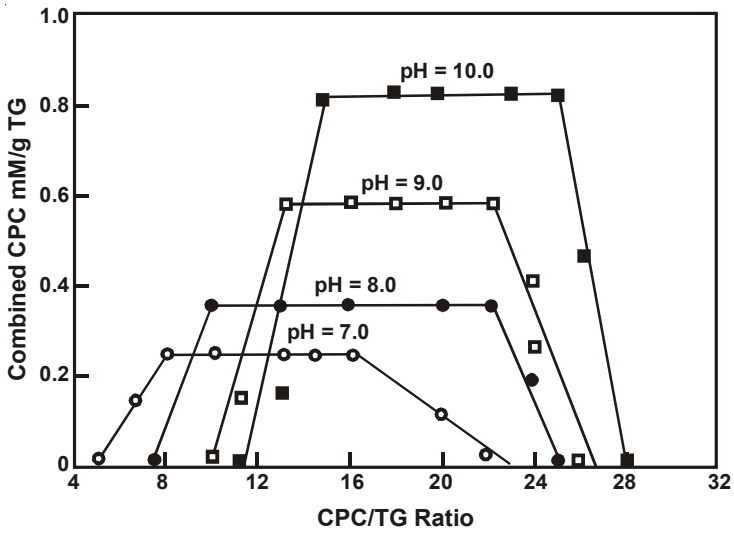

Fig. 1. Amount of CPC combined per g gelatin in relation to CPC/TG ratio

Effect of CPC on the hydrogen-ion equilibrium of TG: From the pH-measurements the value of hydrogen ions (r) dissolved per mole of TG was calculated both in the presence and absence of CPC by means of Tanford method[23] and the results are shown in Fig. 2. The hydrogen ion equilibria curves were used to determine the number of hydrogen ions displaced by the surfactant cations. The extra hydrogen ions displaced by the surfactant ions is equal to V_M the number of surfactant ion bound to per mole of TG, based on Gurd and Murray[24] concept of one to one binding. The curve of r against pH (Fig. 2) shows that surfactant binding progressively increase with increase in pH of the mixed solutions. This is in agreement[22] with the deprotonation of TG side chain groups with rising pH. Since the ions bound are cations, there is possibility of interaction with anionic groups of the protein molecule. A lesser value of r at low pH could be explained by the fact that although all the 84 carboxyl groups were fully ionised at pH 5.50, but the cationic protein sites (amino, imidazole and guanidino) would produce repulsive electrostatic influence on the tenside cations. The regular increase of binding with rising pH may be due to deprotonation of imidazole (pk = 7.50) and amino groups (pk = 9.30 to 10.50) thereby reducing the net positive charge and consequent increase in the net negative charge on the TG molecule.

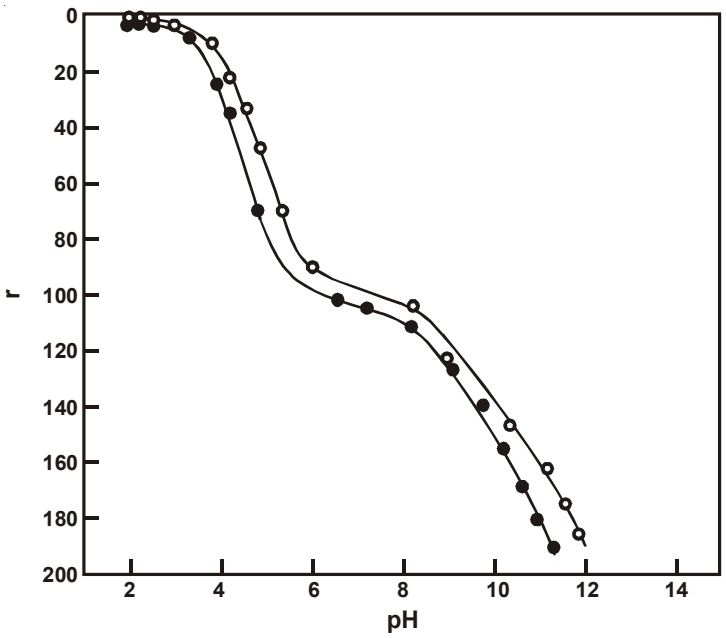

Fig. 2. Hydrogen ions dissociated per mol of TG (r) in the absence (o—o) and presence of CPC (●—●) at different pH values μ = 0.15 M, Temp. = 25 ºC

The fixed pH titrations of TG against added CPC also supported the electrostatic nature of surfactant-protein combination. It is evident from the data that when cationic surfactant is added to a solution to TG below its isoelectric point (IEP), say at pH 2.0, no charge in pH occured indicating absence of electrostatic binding owing to the existence of positive charge on the protein as well as on the surfactant cations. Furthermore, the dissociation of CPC in highly acidic solution would be negligible due to a large concentration of hydrogen ions in the reaction mixtures. As the pH of the mixed solution is raised the pH of the reaction mixture decrease, which is an evidence of the progressive deportonation of protein. The pH decrease is greater up to pH 8.90 while lesser above this pH presumably owing to hydrolysis of the surfactant and consequently an indication of the decreased ionic surfactant binding to the negatively chraged groups of TG molecule. At extremely higher pH (pH 11.50) it seems that about 95% of the surfactant was hydrolyzed because the reaction mixtures turned as yellowish liquids, side by the protein also underwent degradation.

The shift in pH of TG was taken as the index of surfactant ion binding. In Fig. 3 the ΔpH values are plotted against added cation surfactant. The negative ΔpH values go to indicate the release of protons from the reactive site of the protein molecule *i.e.* H^+ ions are displaced from protonated groups by the surfactant cations. The ΔpH values start rising from pH 2.50 and attains a maximum value at pH 8.90 and it then starts decreasing and attain a minimum value at pH 11.50 and upto pH 9.00 the rising negative ΔpH values may be atttributed to the deprotonation of the TG and ionisation of CPC, but at higher pH-values either there is hydrolysis of CPC or degradation of TG molecules, or both the factors may operate in deciding the decrease of negative ΔpH values.

The fixed pH titration could be used for the computation of number of surfactant ions bound to per mole of TG (m.w. 75000) by means of the following relation of Scatchard *et al.*[25].

$$\Delta pH = -\frac{2W}{2.3}\Delta Z = \frac{2W}{2.3}\Delta V \quad \text{or} \quad \Delta V = \frac{2.3}{2W}\cdot\Delta pH$$

where ΔV represents the ligand bound per mole of TG *i.e.* V_M and W is the electrostatic interaction factor[22]. In all the cases, saturation in ΔpH values is attained at higher CPC concentrations. It means that only the ionic binding could be detected from pH-measurements and not the other types as described in surfactant-protein binding studies.

Fig. 4 shows the data on the binding of CPC by TG at pH 4.0 and 7.50 at a temperature of 25 ºC. The isotherms show that the mode of binding changes with increasing free equilibrium concentration of surfactant. In the lower concentration range (region A) the curve is a straight line at both

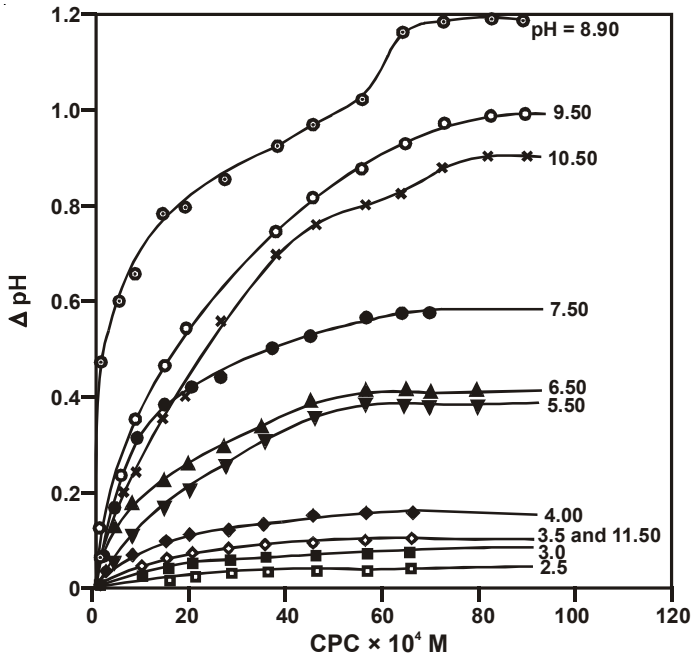

Fig. 3. pH (Protein minus surfactant-CPC mixture) plotted against varying concentration of CPC × 10^4 M at fixed TG (6.0 g/L) conc. at varying pH values (2.5 to 11.50) at μ = 0.15 M, Temp. = 25 ºC

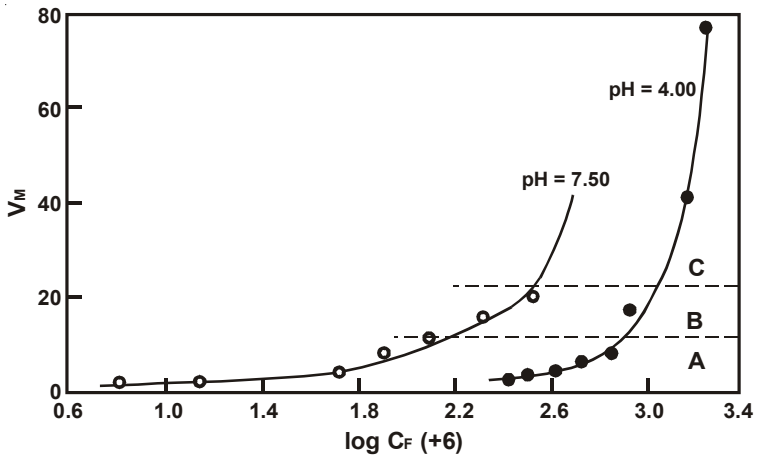

Fig. 4. V_M Plotted against log of free equilibrium conc. of TG-CPC system

pH-values which indicates the distribution of surfactant molecules over all the anionic protein *i.e.* in a more or less statistical manner. This has been tested by fitting the experimental data according to the reciprocal plotting method which are linear and anomalous over the entire range of CPC concentration (Fig. 5). The slope of the linear portion corresponds to the association constant (K); while the intercept on the ordinate indicates the reciprocal of the binding sites (n). The association constants (log K) are found to be 2.42 and 3.38 for CPC, while the binding sites being 42 and 35 at pH 4.0 and 7.50, respectively. The lesser number of sites show the maximum number of V_M upto which statistical binding takes place. The binding appears to be highly cooperative at higher concentration of the surfactant. Beyond region A (*i.e.* in region B) it may be assumed that after a certain number of sites have been occupied, the surfactant disrupts the tightly folded protein structure and enters into combination with the less accessible sites. In the region C, the value of V_M may even exceed the total anionic sites present in TG. It is therefore probable that the progressive rise of binding is due to the expansion of the protein molecule[13].

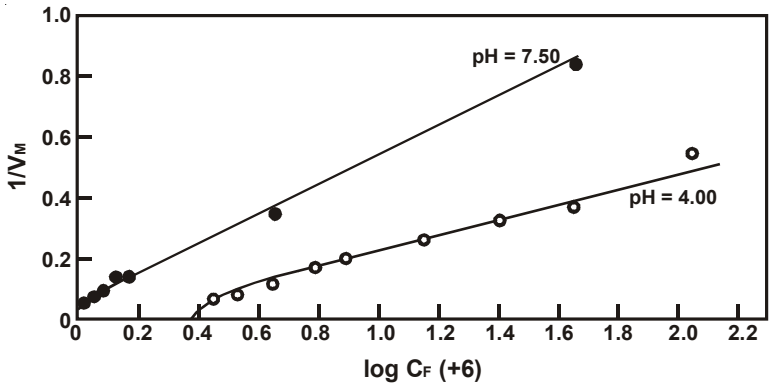

Fig. 5. Plots of $1/V_M$ *vs.* 1/CF for TG-CPC system at pH 4.0 and 7.50, Temp. = 25 ºC (μ = 0.15 M)

Flow properties of CPC-TG system: The relative viscosity of protein solutions in the absence and the presence of CPC are shown in Fig. 6 as a function of pH. The curves possess a minimum at the isoelectric point (pH 5.20) and a maximum on either side of this pH. The macromolecule is probably in the contracted from due to the attractive forces between balanced charges, whereas on both sides of isoelectric point, the molecule has a net overall charge which may cause of the molecule to expand by repulsive forces. The viscosity of CPC-TG mixture also shows a minimum at the isoelectric point, by the viscosity values are much higher than TG alone.

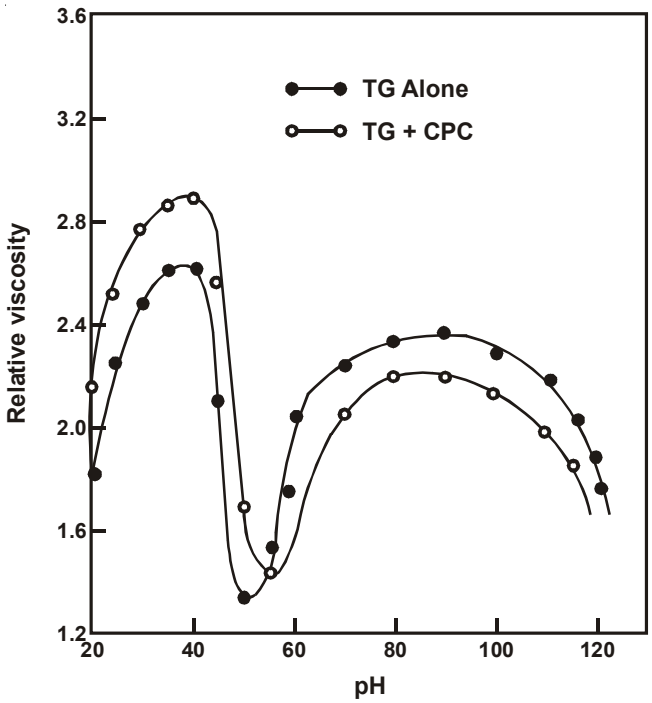

Fig. 6. Relative viscosity of TG (5 g/L) in the presence and absence of CPC (0.001 M)

This may be due to the interaction of surfactant cations with neutral TG molecule to give it a net charge and consequently cause the molecule to expand itself. The shift of isoelectric point can be explained in terms of the reaction of CPC cation with the negatively charged groups of the protein. These results are in agreement with those obtained from pH and equilbirum dialysis measurements.

In Fig. 7 the changes in viscosity are plotted against added surfactant concentration to fixed TG concentration at various pH-values above the isoelectric point of the protein. The viscosity values decrease on addition of increasing amounts of surfactant until precipitation takes place. The precipitate dissolves in excess of surfactant and the mixture revealed nearly Netwtonian behaviour while it was non-Newtonian before the precipitation. The mechanism of precipitation and its redissolution can be explained as follows. The more or less expanded protein molecule at the alkaline side isoelectric point may be changed into the folded hydrophobic state by the continuous combination of the surfactant cations until precipitation occured. On addition of more surfactant the second adsorption layer of surfactant ions is formed by van der Wall's attraction forces between carbon chain, which makes the molecule hydrophilic, hence the precipitate dissolves

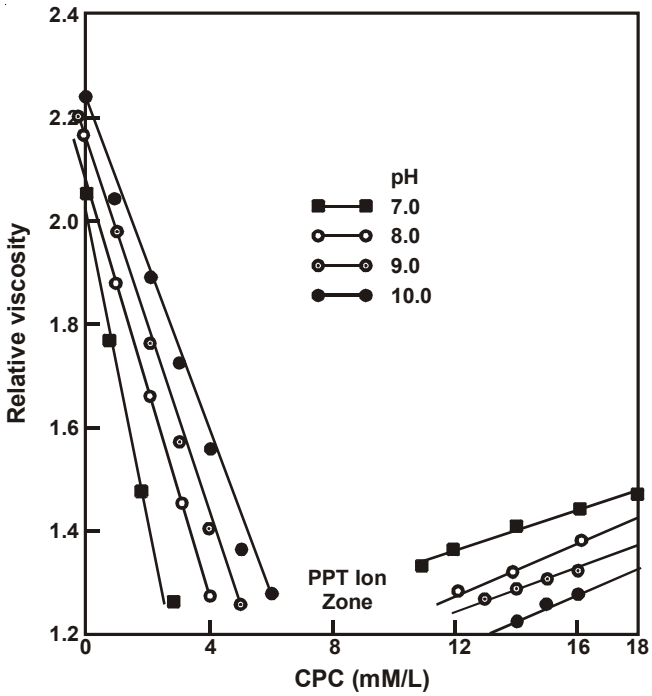

Fig. 7. Relative viscosity of TG (5 g/L) in the presence and absence of CPC

again. The mixture so formed behaves similarly to the solution at the isoelectric point without the surfactant. It may be observed that the amount of surfactant needed to produce complete precipitation increase as the pH of the system rises as we move away from the isoelectric point. This shows that the protein-surfactant ratio increases as the total negative charge on protein increase[22].

The intrinsic viscosity of protein in the presence of varying amounts of CPC was determined by plotting the reduced viscosity against protein concentration (mg/mL) and then extraplotting to zero protein concentration. These values of intrinsic viscosity are given in Table-1. It is seen that the reduced viscosity increases with increasing concentration of the surfactant. This may be correlated with the conformational changes produced in the protein molecule by the cationic surfactant. The high values of intrinsic viscosity below isoelectric point of protein could not be due to electroviscous effect as both protein and surfactant carried positive charges. However, this behaviour could be explained on the basis of protein swelling, presumably owing to coulombic repulsion between the large number of positively charged groups. The swelling of the protein in the acid solution due to repulsion of similarly charged ions may cause opening up of the protein

TABLE-1
REDUCE VISCOSITY AND INTRINSIC VISCOSITY OF TG-CPC MIXTURE AT DIFFERENT pH VALUES

CPC 10 %	pH 2.60	pH 3.49	pH 5.40	pH 8.00
0.25	10.80	5.80	12.40	10.40
0.50	13.40	6.60	14.00	11.60
1.00	15.60	8.40	16.00	12.80
1.50	17.50	9.40	17.60	14.00
2.00	20.20	12.00	19.00	15.00
2.50	22.20	13.40	21.80	16.00
[η]	10.64	5.40	12.00	10.30

and the surfactant cations can enter into the swollen sphere. The relatively higher viscosity at pH 5.40 could be explained by the fact that near isoelectric point, the protein exists as a compact molecule and additional surfactant caused marked unfolding due to the cooperative nature of surfactant binding. Probably the surfactant molecule splits off the salt linkages between anionic and cationic groups attached there. The increasing values of intrinsic groups and becomes attached there. The increasing values of intrinsic viscosity may also be due to solubilization and unfolding of TG in the presence of larger amounts of the cationic surfactant. This behaviour depends upon several factors *e.g.*, chain length, nature of distribution of polar groups, the carboxylic groups are distributed all over the polar regions while the amino and guanidino groups are concentrated on the ends of the chains[26], flexibility of chains, tightening of packing and on the number of cross links. On the other hand a large number of aliphatic hydroxyl groups from hydroxy proline and hydroxy lysine amino acid residues may also play a siginificant role in increasing the viscosity through hydrogen bond formation. This extensive increase in viscosity may also be due to the corresponding elongation of the molecule and a consequent increase in the dissymmetry of the macromolecular units.

Mechanism of interaction: The pH-metric results strongly suggested a mechanism in which the interaction between cationic surfactant and the TG molecule consists of ionic binding of the positively charged pyridinium groups of the surfactant to the negatively charged sites on the protein molecule. Any conformational change, which is brought about as a consequence of the resulting change in the balance of the polar group and non-polar interaction, can not be suggested from pH-measurements. However, the equilibrium dialysis results suggested that besides ionic binding other binding forces are also involved in the surfactant-protein interaction. Two types of binding forces can explain the precipitation and dissolution of surfactant-protein complexes. One is mainly electrostatic (in lower pH range) involving forces, which normally binds surfactant ions into micelles. In the initial

stages of surfactant addition, the electrostatic type of binding dominates while in the middle ragne the non-electrostatic linkage is more probable. At higher concentration of the surfactant cooperatively of binding results complete unfolding of the protein molecule.

The type of binding forces predicted from pH-metric and equilibrium dialysis results can also be suggested from viscometric data. The increasing values of intrinsic viscosity and Huggin constants show a large amount of electrostatic attraction at lower surfactant concentration while the phenomenon of uncurling at higher concentration of the surfactant. The view of Lundgren[27] that the secondary association of extra surfactant is in the form of a loose combination due to non-polar attraction with that which is already electrostatically bound finds support from the present investigations. It may be concluded that surfactant-protein combination involved ionic, hydrophobic and hydrogen bonding in forming the complex depending upon the pH and concentration of the cationic surfactant.

REFERENCES

1. J.P.S. Arora, D. Soam, S.P. Singh and R. Kumar, *Tenside Detergents*, **21**, 87 (1984).
2. J.P.S. Arora and S.P. Malik, *Studia Biophys.*, **100**, 181 (1984).
3. J.P.S. Arora, V.K. Singhal, S.P. Singh and R. Kumar, *Tenside Detergents*, **21**, 1 (1989).
4. J.P.S. Arora, S. Jain, C. Pal, Laxmi and M. Chand, *Bull. Soc. Chim. (France)*, 58 (1989).
5. J.P.S. Arora and K.L. Balyan, *Tenside Surfactants Detergents*, **29**, 48 (1992).
6. J.P.S. Arora, Vineeta and R.P. Singh, *Tenside Surfactants Detergent*, **29**, 418 (1992).
7. J.P.S. Arora, Vineeta and M. Chand, *Tenside Surfactants Detergents*, **30**, 136 (1993).
8. J.P.S. Arora, S.K. Arora and A.K. Duggal, *Tenside Surfactants Detergents*, **35**, 24 (1998).
9. H.N. Glassmann and D.M. Molner, *Arch. Biochem. Biophys.*, **32**, 170 (1954).
10. K. Aoki and J. Hori, *J. Am. Chem. Soc.*, **81**, 1885 (1959).
11. S.N. Timasheff and F.F. Nord, *Arch. Biochem. Biophys.*, **31**, 309 (1951).
12. K. Tamaki and B. Tamamushi, *Bull. Chem. Soc. (Japan)*, **28**, 555 (1955).
13. J.T. Yang and J.F. Foster, *J. Am Chem. Soc.*, **75**, 5560 (1953).
14. J.T. Yang and J.F. Foster, *J. Am Chem. Soc.*, **76**, 1051 (1954).
15. K.S. Birdi, *Biochem. J.*, **135**, 253 (1973).
16. M. Tanaka, S.M. Kaneshina, T. Kondo, T. Mizumo and K. Aoki, *Bull. Chem. Soc. (Japan)*, **46**, 12735 (1973).
17. Y. Nozaki, J.A. Reynolds and C. Tanford, *J. Biol. Chem.*, **294**, 4452 (1974).
18. M.N. Jones, H.S. Skinner, E. Tipping and A. Wilkinson, *Biochem. J.*, **135**, 231 (1973).
19. J.P.S. Arora, R.P. Singh, D. Soam and S.P. Singh, *Bull. Soc. Chim. (France)*, 19 (1984).
20. J.P.S. Arora, S.P. Singh, V.K. Singhal and R. Kumar, *Tenside Surfactant Detergents*, **21**, 153, 197 (1984).
21. S.L. Kalra, G. Singh and M. Ram, *Indian J. Med. Res.*, **46**, 171 (1958).
22. W.U. Malik and Salahaddin, *J. Electronal. Chem.*, **6**, 168 (1963).
23. C. Tanford and J. Epestein, *J. Am. Chem. Soc.*, **76**, 2163 (1954).
24. F.R.N. Gurd and G.R. Murray, *J. Am. Chem.*, **76**, 187 (1954).
25. G. Scatchard and J.F. Black, *J. Phys. Chem.*, **53**, 88 (1949).
26. A. Nordwing, *Z. Physiol. Chem.*, **325**, 242 (1961).
27. H.P. Lundgren, *Tex. Res. J.*, **15**, 335 (1961).

(*Received*: 1 September 2007; *Accepted*: 10 March 2008) AJC-6437