Interactions of Bovine α_s-Casein with Small Ions[1]

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Deionized α_s-casein, isoionic pH 5.16 at 20°, is a stable colloid which binds potassium (or sodium) ions, releasing a much smaller number of hydrogen ions in the process. Application of electrostatic theory to the KCl concentration–pH data suggests two classes of binding sites which number, in g.-atoms per mole of 27,300 g., $n_1 = 2$ and $n_2 = 3$ with binding constants of $K_1° = 323$ and $K_2° = 7.1$, respectively. No precipitation takes place up to ionic strength (I) = 0.04, but thereafter the logarithm of the colloid solubility is proportional to the added ionic strength. Direct measurement of binding at isoionic and neutral pH values by means of an ion-exchange electrode reveals a total of five prominent binding sites per mole for K^+ or Na^+. Hydrogen ion binding was studied by titration of α_s-casein at two ionic strengths (I = 0.05 and 0.4) at 20° and between pH 1.9 and 12. Within these limits the titration is completely reversible. The titration results are in agreement with the amino acid composition. The protein exists as a colloid and/or as a precipitate over the pH range 5.3 to 3.5 at I = 0.05 and as a precipitate over pH 6.4 to 1.9 at I = 0.4. The customary protein titration equation and Scatchard equation giving the electrostatic factor for impenetrable spheres have been modified and applied to a portion of the titration curve near the isoionic point. At I = 0.05 an apparent electrostatic factor w' has a maximum at approximately +4 charges per monomer and decreases to one-tenth of this value at approximately +16 and 0 charges, respectively. A correspondingly low value of apparent electrostatic factor is observed at I = 0.4 over the range from +15 to −15 charges per monomer. Variation in the apparent electrostatic factor with experimental conditions is attributed to some appropriate change in a combination of the degree of aggregation and a geometric factor associated with monomer conformation.

Introduction

Micelle formation in the casein system may involve several proteins, notably α_s-, β-, and κ-caseins, and a divalent cation.[2] Of these the minimum requirements are α_s-casein, κ-casein, and a divalent cation.[3–5] The set of interactions is complex: our eventual objective is an understanding of the mechanism of formation, structure, and sources of stability of the micelle.

The major caseins are the α_s-casein group. According to Waugh, *et al.*,[6] this group makes up 45% of the total casein and consists of closely related proteins which have common properties in their phosphorus content (1%), C- and N-terminal groups, and molecular weight (27,300).[6,7] The interactions of α_s-casein with κ-casein, β-casein, and divalent cations are known to be sensitive to hydrogen ions and to monovalent salts such as KCl and NaCl. As a step toward understanding these we describe here some of the interactions of α_s-casein with monovalent cations.

Carr reported in 1955 that both casein and vitellin bind sodium ions,[8] the former to the extent of 4.8 sodium ions per 27,300 g. of casein at pH 7.4, 0.01 *N* sodium chloride, and 1% casein. Our results on total binding are in agreement with the values obtained at pH 7.5 by Carr and co-workers.[9] However, in the later paper Carr and Engelstadt find that binding decreases as the pH decreases, reaching zero at pH 5.7. We do not observe this decrease, possibly because we have used α_s-casein rather than whole casein. At the same time, Carr and Topol reported that whole casein does not bind significant chloride ion at pH 5 and 0.012 *M* chloride ion.

Carr found that most proteins, such as blood proteins, serum albumin, and hemoglobin, do not bind potassium or sodium ions at pH near 7.4.[10] Since both casein and vitellin are phosphoproteins, he concluded that phosphate groups are responsible for interactions with the alkali metal ions.[8] We report here studies of the binding of potassium, sodium, and hydrogen ions to α_s-casein and a correlation of the data so obtained by the electrostatic theories of Scatchard and co-workers.[11,12]

Experimental

Materials. *α_s-Casein.* α_s-Casein refers to $\alpha_{s1,2}$-caseins, a mixture of two closely related proteins as fractionated and purified according to the procedure described by Waugh, *et al.*[6] Lyophilized samples (1–4%) were dissolved in 0.01 *M* potassium citrate and dialyzed at 4° initially against 0.01 *M* potassium citrate for about 8–12 hr., then against 0.01 *M* KCl for another 8–12 hr. to remove the citrate, and finally against "boiled" distilled water for 20–30 hr., with at least three changes of distilled water and until the dialysate was chloride free. The ratio of protein to dialysate volume is about 100 in each case. The final pH was 6 to 8. The concentration of α_s-casein is determined from optical density. The extinction coefficient at 280 mμ in a 1-cm. cell is 10 for a 1% solution.[6]

Chemicals. All were analytical reagent grade and were used without further purification. Potassium hydroxide stock solution was made carbonate free by

(1) This material was presented in part at the 148th National Meeting of the American Chemical Society, Chicago, Ill., Sept. 3, 1964.
(2) Normally calcium.
(3) D. F. Waugh and P. H. von Hippel, *J. Am. Chem. Soc.*, **78**, 4576 (1956).
(4) D. F. Waugh, *Discussions Faraday Soc.*, **25**, 186 (1958).
(5) D. F. Waugh, *J. Phys. Chem.*, **65**, 1793 (1961).
(6) D. F. Waugh, M. L. Ludwig, J. M. Gillespie, B. Melton, M. Foley, and E. S. Kleiner, *J. Am. Chem. Soc.*, **84**, 4929 (1962).
(7) P. Dreizen, R. W. Noble, and D. F. Waugh, *ibid.*, **84**, 4938 (1962).
(8) C. W. Carr in "Electrochemistry in Biology and Medicine," T. Shedlovsky, Ed., John Wiley and Sons, Inc., New York, N. Y., 1955, Chapter 14.
(9) C. W. Carr and L. Topol, *J. Phys. Colloid Chem.*, **54**, 176 (1950); C. W. Carr and W. P. Engelstadt, *Arch. Biochem. Biophys.*, **77**, 158 (1958).
(10) C. W. Carr, *ibid.*, **66**, 476 (1956).
(11) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
(12) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

adding minimal quantities of BaO followed by filtration. It was standardized against potassium acid phthalate. Hydrochloric acid was prepared from Acculute vials (Anachemia, Ltd., Montreal) and standardized against the potassium hydroxide. Sodium hydroxide was prepared from fresh carbonate-free Acculute vials.

Distilled Water. The general supply of laboratory distilled water was passed through a Barnstead mixed-bed resin cartridge and then through an HA Millipore filter. The pH of the distilled water was between 6.0 and 7.0.

Methods. Isoionic α_s-Casein. By a modification of the method of Dintzis,[13] deionized α_s-casein was obtained by combining, with stirring, aliquots of dialyzed protein solution and mixed-bed ion-exchange resin (manufactured by Bio-Rad Laboratories, Richmond, Calif., AG 501 × 8, 20–50 mesh). The time required for deionization varied from minutes to hours, depending upon the ratio of the amount of protein to that of the resin.

During deionization the protein, originally in solution, aggregates to form a colloid. On cessation of stirring the ion-exchange beads settle easily under gravity. The isoionic pH of α_s-casein is the pH of the colloidal suspension. We have used this method to measure the isoionic point of insulin (Lilly Iletin insulin, U-80) and have obtained 5.66 at 20°. This is in good agreement with the value of 5.60 reported by Tanford and Epstein.[14] The isoionic point of crystalline bovine serum albumin (Armour Co. lot No. R 370295) was found to be pH 5.13 at 20°, in good agreement with the value of pH 5.15 reported by Scatchard, *et al.*[15]

Titration of α_s-Casein. All pH measurements were made with a Radiometer pH meter Model 4, using a combination electrode of type GK 2021 B. The pH meter was standardized with 0.05 *M* potassium acid phthalate buffer and standard phosphate buffer (0.025 *M* KH_2PO_4 in 0.025 *M* Na_2HPO_4).[16] The pH meter has an accuracy of 0.002 pH unit. All measurements were made at 20° (±0.1°). A solution was prepared by mixing appropriate amounts of dialyzed solutions of α_s casein (pH 6 to 8), HCl and/or KOH, and KCl. The total final ionic strength was kept constant. We have neglected the contribution of the macroions (protein) in calculating the ionic strength. The protein concentration varied from 0.5–1%. Prepurified nitrogen of Airco was passed over the surface of the solution during the whole course of titration to minimize the interference of carbon dioxide at higher pH.

Reversibility of titration was tested according to the method suggested by Tanford.[17,18] A series of protein solutions are prepared containing a sufficient amount of acid or base to bring each solution to pH 2 or 12. The solutions are equilibrated for 10 min. at 20°. Following this, base or acid is added so that the final pH values cover a suitable range. If the resulting pH values fall on the titration curve obtained without pre-treatment with acid or base, the titration represents true equilibrium with H^+ ions.

Under our experimental conditions, it is necessary to make an electrode correction to obtain the free H^+ or OH^- ions for measured pH less than 4 or greater than 10. We have used the method of Tanford.[17,18] It is assumed that the activity coefficient of the free hydrogen or hydroxyl ion is not affected by the presence of protein.[19] In essence, the molality of H^+ or OH^- at a given pH is obtained directly from a plot of measured pH *vs.* known molality of HCl or KOH for the ionic strengths used. There is a negligible difference between molar and molal concentrations under our experimental conditions.

Ion-Exchange Electrode Measurements of Binding of K^+ and Na^+ Ions to α_s-Casein. Our ion-exchange electrode is a slight modification of the one used by Scatchard, *et al.*,[20] and we are indebted to Professor Scatchard for the use of some of his equipment. The advantages and the disadvantages of this technique in the binding study with proteins have been discussed extensively by Scatchard and co-workers[15,20–22] and by Carr.[8] A cation-exchange membrane (Permutit Company, Ltd., England, Lot No. "Permaplex" C-20) was converted either to the K^+ or Na^+ form before use. The membrane separates two compartments of a lucite cell. Each compartment makes connection through a capillary with Beckman standard calomel electrode (No. 39970). To minimize the effects of small temperature changes, the calomel electrodes are immersed in 4.5 *m* KCl. Our ion-exchange cell was kept in a thermally insulated Faraday cage (±3°). Most potentials were measured with a Leeds and Northrup Type K-1 potentiometer and General Electric Type G-9 galvanometer. A few were measured with pH meter 4 of Radiometer Co.

The ion-exchange cell was calibrated by using KCl or NaCl over the range from 0.01 to 0.1 *m*. Measured e.m.f. was within 2% of those calculated by eq. 1.

$$\Delta E = \frac{RT}{F} \ln \frac{m\gamma_{\pm}\ (\text{in half cell a})}{m\gamma_{\pm}\ (\text{in half cell b})} \qquad (1)$$

Here ΔE is the electromotive across the cation-exchange membrane, m is the molality of salt, R is the gas constant, T is the absolute temperature, and F is the Faraday constant. The mean ionic activity coefficient, $\gamma_{\pm}$, for KCl as a function of concentration is given by Lietzke and Stoughton[23] and that for NaCl by Scatchard, *et al.*[24]

The cation exchange membrane in K^+ or Na^+ form behaves as though it is a reversible potassium or sodium electrode, respectively. The e.m.f. across the calomel electrodes and eq. 1 permits calculation of ion bound per α_s-casein molecule, $\bar{\nu}$, using eq. 2, where m_2 is the mo-

$$\bar{\nu} = (m_3 - m^+)/m_2 \qquad (2)$$

(13) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.
(14) C. Tanford and J. Epstein, *J. Am. Chem. Soc.*, **76**, 2163 (1954).
(15) G. Scatchard, J. C. Coleman, and A. L. Shen, *ibid.*, **79**, 12 (1957).
(16) R. G. Bates, "Electrometric pH Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954, p. 74.
(17) C. Tanford, *J. Am. Chem. Soc.*, **72**, 441 (1950).
(18) C. Tanford in "Electrochemistry in Biology and Medicine," T. Shedlovsky, Ed., John Wiley and Sons, Inc., New York, N. Y., 1955, Chapter 13.
(19) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 454.
(20) G. Scatchard, Y. V. Wu, and A. L. Shen, *J. Am. Chem. Soc.*, **81**, 6104 (1959).
(21) G. Scatchard in "Electrochemistry in Biology and Medicine," T. Shedlovsky, Ed., John Wiley and Sons, Inc., New York, N. Y., 1955, Chapter 5.
(22) G. Scatchard, I. H. Steinberg, and S. H. Armstrong, *J. Am. Chem. Soc.*, **72**, 535, 540 (1950).
(23) M. H. Lietzke and R. N. Stoughton, *J. Phys. Chem.*, **66**, 508 (1962).
(24) G. Scatchard, A. C. Batchelder, and A. Brown, *J. Am. Chem. Soc.*, **68**, 2610 (1946).

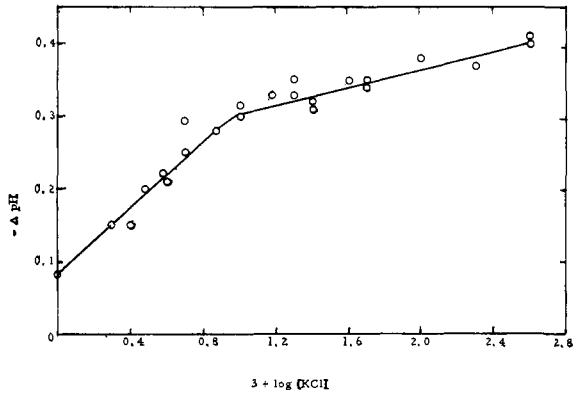

Figure 1. Effects of potassium chloride on the isoionic α_s-casein. Plot of $-\Delta$pH *vs.* the logarithm of total concentration of KCl at 20°.

lality of the protein, m_3 is the initial molality of KCl or NaCl (half cell a), and m^+ is the molality of unbound KCl or NaCl in the protein solution (half cell b); m^+ is calculated by eq. 1.

It is understood that the validity of eq. 1 depends on certain assumptions: first, that the only effect of the protein is on the concentration of K^+ or Na^+ and not on the ionic species activity coefficient, γ_+; second, γ_+ is the same as the mean coefficient, $\gamma_\pm$; and third, the ionic strength of the solution is equal to m_3. These assumptions have been discussed by Scatchard and coworkers.[15,20-22]

Amino Acid Composition. Amino acid composition of α_s-casein was analyzed with a standard Technicon amino acid analyzer.[25] α_1-Casein was hydrolyzed in 6 *N* HCl at 105° for 24 hr.

Results

1. Relationship between KCl Concentration and pH of Isoionic α_s-Casein. The deionized α_s-casein colloid has a pH of 5.16 ± 0.09 at 20°.[26] This value, the isoionic pH, is the average of eight independent determinations of several different preparations over a period of one year. The protein concentration varied from approximately 0.25–1%. The colloidal α_s-casein suspension is quite stable at room temperature for a period of several hours and at 4° for a period of several days, as judged by opacity and the absence of precipitate.

The pH of an isoionic protein decreases upon the addition of potassium chloride. In addition, the isoionic α_s-casein precipitates at low concentrations of KCl. We will discuss the effects of KCl on colloidal stability later. Figure 1 shows the effects of KCl on the pH of isoionic α_s-casein. We have plotted ΔpH *vs.* the logarithm of the total concentration of potassium chloride at 20°. There is a decrease of about 0.4 pH unit when the isoionic suspension is brought to 0.4 *M* KCl. These results indicate that potassium ions are bound to α_s-casein and, that when they bind, the dissociation constants of acidic groups increase.

2. Binding of Potassium and Sodium Ions to α_s-Casein. Direct binding studies were carried out between isoionic pH values and pH 7.3. The concentration of deionized protein varied from 0.7 to 2%. In all cases when the pH of the isoionic protein was adjusted upward with sodium or potassium hydroxide before adding neutral salt, the colloidal α_s-casein went into solution and no difficulties were encountered. Addition of neutral salt to the isoionic colloid produced the expected decrease to a new pH and gross protein precipitate which was removed by mild centrifugation before ion activities were measured. Even so, an additional problem encountered in such isoionic systems was the precipitation of protein in the junction capillary (containing 4.5 *m* KCl) when the protein colloid contained less than 0.04 *m* KCl.

For concentrations of KCl or NaCl greater than 0.08 *m*, the measured e.m.f. of a 2% α_s-casein solution is less than 1 mv., which is not much greater than the liquid junction (or membrane) potential correction. A second limitation relates to measurements at higher pH. In order to bring the isoionic α_s-casein to neutral pH, the introduction of about 0.017 *m* KOH or NaOH is necessary for 2% α_s-casein. This puts a lower limit on the concentration of K^+ or Na^+ ions which can be used in binding studies.

The accuracy of the e.m.f. measurements is estimated to be about 0.2 mv. For low protein concentrations or high salt concentration, this may correspond to an error of 50% in $\bar{\nu}$. For most of our results it corresponds to about 20%.

Table I gives the experimental results. Since reliable e.m.f. measurements could not be made at high salt concentrations, we cannot be sure that the maximum binding of K^+ ions is five per molecule (even though the data in Table I indicate that the binding of K^+ ions appears to be independent of the concentration of KCl from 0.0221 to 0.0600 *m*). The difference in the binding of K^+ and Na^+ on α_s-casein is within the experimental accuracy (Table I).

Table I. Binding of K^+ and Na^+ to α_s-Casein

Salt	$m_3 \times 10^2$	$m_2 \times 10^4$	pH	$\bar{\nu}$ (exptl.)	$\bar{\nu}$ (calcd.)[a]
KCl	1.96	8.35	Isoionic (4.84)[b]	2.5	1.4
KCl	6.98	7.93	Isoionic (4.81)[b]	2.8	2.0
KCl	8.00	2.96	Isoionic (4.80)[b]	4.7	2.0
KCl	1.69	2.92	7.01	1.7	5.0
KCl	2.21	7.85	7.19	4.8	5.0
KCl	5.00	2.89	5.71	5.2	4.4
KCl	5.00	2.82	6.76	4.6	5.0
KCl	6.00	7.54	6.98	5.0	5.0
NaCl	1.79	8.13	7.23	4.0	
NaCl	6.00	7.77	6.97	4.0	

[a] See text for the method of calculation. [b] The isoionic pH at a given KCl concentration is estimated from Figure 1.

Calculated values of $\bar{\nu}$ shown in the last column of Table I will be discussed below.

3. Effects of Potassium Chloride on the Colloid Properties of Isoionic α_s-Casein. Deionized isoionic α_s-casein is a milky colloid which is stable at room temperature and at 4° in the absence of added salts. Isoionic α_s-casein will start to precipitate at concentrations of KCl near 0.04 *m*. Figure 2 is a summary of experimental results obtained at 20°. Precipitates were removed by centrifuging at about 400 *g* for 1 min. The

(25) We wish to thank Dr. Paul Melnychyn of the Research Laboratories of the Carnation Company for the analysis of the amino acid composition of α_s-casein.

(26) By this method we have also determined the isoionic points of β-casein, pH 5.35 at 20°, and of κ-casein, pH 5.37 at 20°.

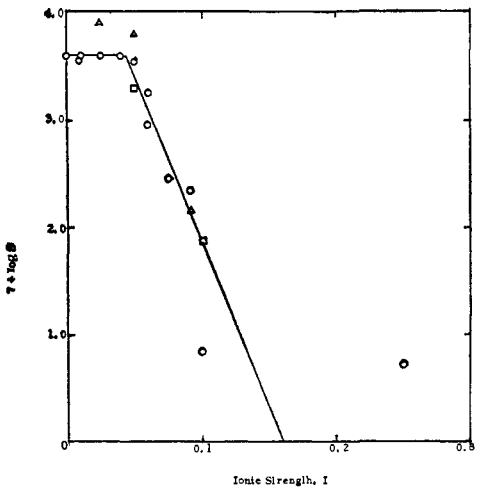

Figure 2. Effects of potassium chloride on the isoionic α_s-casein. Plot of the logarithm of the solubility of the colloidal α_s-casein as a function of the ionic strength at 20°. Open squares, □, for 0.5% α_s-casein; open circles, ○, for 1% α_s-casein; and open triangles, Δ, for 2% α_s-casein.

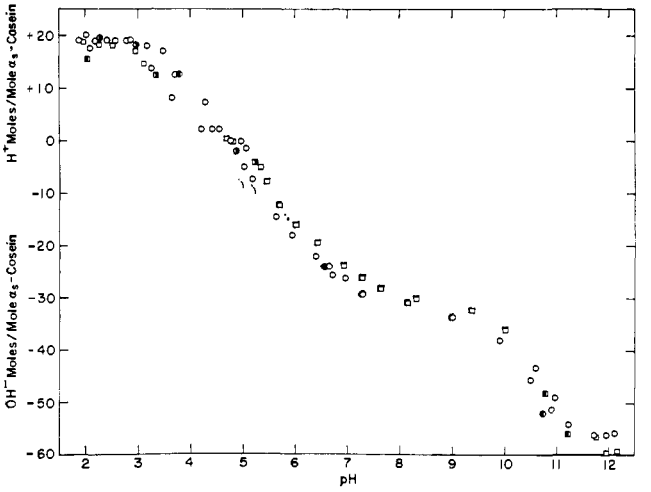

Figure 3. Titration curves of α_s-casein at 20°. Ionic strength, I = 0.4; ○ forward titration point; ◑ reverse titration point from the acid end; and ◐ reverse titration point from the alkaline end. Ionic strength, I = 0.05: □, forward titration point; ◨, reverse titration point from the acid end; and ◧ reverse titration point from the alkaline end. The concentration of α_s-casein varied from 0.5 to 1%.

protein concentration varied from 0.5 to 2%. The most interesting part of these results is that more than 90% of the isoionic colloid can be precipitated at 0.075 *m* and that almost complete precipitation can be achieved at 0.1 *m* KCl. This is in contrast to the salting out behavior of most proteins which require much higher concentrations of salt.[27,28]

Cohn and Edsall[28] describe solubility behavior for a number of proteins by means of eq. 3.

$$\log S = \log S_i - K_s I \qquad (3)$$

where I is the ionic strength, S_i is the hypothetical ideal solubility at zero ionic strength, and K_s is the salting out constant. According to this simple relation, the salting out constant is independent of the electric charge on the protein molecule and of temperature. It depends only on size, shape, and the extent of hydration.

Figure 2 is a plot of the logarithm of the supernatant protein as a function of the ionic strength. According to Figure 2, the solubility of colloidal isoionic α_s-casein is unaffected by KCl up to 0.04 *m*. This part may correspond to a so-called salting-in region. Salting in is considered to be due to the long-range forces between small ions (KCl) and the macroions (protein).[28]

Above 0.04 *m* KCl, the solubility of isoionic α_s-casein appears to be independent of the protein concentration within the experimental error. The salting out constant, K_s, is approximately 31. This value is about 15 to 30 times larger than most of the salting-out constants for proteins.[29] The two last points of Figure 2 are unreliable due to low protein solubility.

4. Acid–Base Titrations of α_s-Caseins. Figure 3 shows two titration curves for α_s-casein at I = 0.4 and 0.05, respectively. Each point on the titration curve represents a separate experiment. The ionic strength in each experiment was kept constant by adjusting the amounts of acid, base, and KCl added to the system. All solutions acid to the isoionic point (down to about pH 2) were found to be unchanged in pH over a period of several hours. On the alkaline side of the isoionic point, there is a weakly buffered region (pH 7–10.5) in which pH drift, amounting at times to several hundredths of a pH unit, was unavoidable. About pH 10.5, this drift disappeared. The experimental points at pH ~12 are somewhat scattered. Over the pH range from 2 to 11.5, the probable error is estimated to be about 0.02 pH unit. We estimate that the over-all accuracy of our titration curves is about 3 groups. Within the pH range of 2–12 the hydrogen ion titration of α_s-casein is found to be reversible within the experimental error (see Methods). The titration results were also found to be independent of protein concentration from about 0.5 to 1%.

In the case of the titration of α_s-casein at 0.4 ionic strength, it was noted that the solution became turbid and that from pH about 6.4 to 5.6 a gelatinous precipitate formed. As the pH of the protein solution decreases from pH 5.6 to 1.9 a clear supernate with coarse-grained precipitate was characteristic. These precipitates remained undissolved even at pH values as low as pH 1.9. A very different behavior was observed during titration at 0.05 ionic strength. The protein solution remained clear until pH about 5.3, at which time it became milky with the formation of some coarse-grained precipitate. More precipitate formed as the pH was lowered over the pH range from 5.3 to 3.5. The solution became clear and precipitate redissolved when the pH was less than pH 3.5.

Hipp, Groves, and McMeekin[30] reported the acid–base titration of an α-casein preparation. We have not made a direct comparison between our results and theirs because the α-casein used by them was subsequently shown to contain several components by McMeekin, Hipp, and Groves[31] and Hipp, Groves, and McMeekin.[32]

(27) A. A. Green, *J. Biol. Chem.*, **93**, 495, 517 (1931).
(28) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, pp. 586–622.
(29) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press Inc., New York, N. Y., 1958, p. 274.
(30) N. J. Hipp, M. L. Groves, and T. L. McMeekin, *J. Am. Chem. Soc.*, 74, 4822 (1952).
(31) T. L. McMeekin, N. J. Hipp, and M. L. Groves, *Arch. Biochem. Biophys.*, **83**, 35 (1959).

5. Amino Acid Composition of α_s-Casein.[25] Table II gives a summary of the amino acid composition of α_s-casein. The results are tabulated in two ways: by grams of anhydro amino acid per 100 g. of α_s-casein and by the number of moles of amino acid residue for

Table II. Amino Acid Composition of α_s-Casein

Constituents	g/100 g α_1-casein		Amino acid residues/molecule α_1-casein	
	This analysis	Hipp, *et al.*[a]	This analysis	Hipp, *et al.*[a]
Total N	14.7[b]	14.1		
Total P	1.0[b]	0.85		
Glycine	2.16	1.80	10.33	8.61
Alanine	2.72	2.54	10.44	9.75
Serine	3.05	4.81	9.56	15.08
Threonine	1.83	2.55	4.94	6.89
Proline	4.93	6.58	13.86	18.50
Valine	4.37	4.53	12.03	12.47
Isoleucine	5.16	4.45	12.46	10.74
Leucine	9.00	7.46	21.72	18.01
Phenylalanine	5.40	4.51	10.01	8.36
Tyrosine	7.43	6.40	12.43	10.71
Tryptophan	1.57	1.94	2.30	2.84
Cystine/2	0	0.17	0	0.39
Methionine	2.89	2.14	6.01	4.45
Aspartic acid	5.95	6.56	14.11	15.56
Glutamic acid	21.10	18.34	44.62	38.78
Amide N (ammonia)	(0.80)[c]	(1.4)[c]	12.85	22.48
Arginine	24.97	3.35	8.69	5.85
Histidine	2.95	2.39	5.87	4.76
Lysine	8.45	7.50	17.99	15.97
Phosphate	3.48[d]	2.96[d]	10.00	8.51
Total: g. of amino acid residues per 100 g. of protein	97.41	90.98		
% of total N recovered	99.05	102.5		

[a] The data were taken from Hipp, Basch, and Gordon[33] and had been converted according to the amino acid residue. The data reported here refer to the α_1-casein according to the authors. The molecular weight of α_1-casein is taken as 27,300. [b] Data taken from ref. 6. [c] These figures were not included in the calculation of the total grams of amino acid residues per 100 g. of protein. [d] We have made the assumption that all the phosphorus is present in dibasic form, such as O-phosphoserine.

27,300 g. of α_s-casein. The total recovery in terms of grams of anhydro amino acids per 100 g. of α_s-casein is about 97% and the same recovery in terms of total protein nitrogen is about 99%. The agreement is good. We have not made corrections for the loss of serine and threonine due to acid hydrolysis. The amount of ammonia obtained was taken to be equivalent to the sum of the glutamine and asparagine present in the α_s-casein. The analysis was carried out in duplicate. The results reported in Table II are the average values. The agreement for the duplicate analysis is about 5%.

Hipp, *et al.*[33] reported the amino acid composition of a preparation they named α_1-casein. Their results are tabulated in Table II, columns 3 and 5. The agreement between amino acid compositions is within 2 or 3 groups except for serine, proline, glutamic acid, and NH_3 which differ markedly and beyond any reasonable experimental error. Puzzling is the fact that the analysis by Hipp, *et al.*[33] has a recovery of 91% in terms of grams of anhydro amino acids per 100 g. of protein but a recovery of 103% in terms of total protein nitrogen.

(32) N. J. Hipp, M. L. Groves, and T. L. McMeekin, *Arch. Biochem. Biophys.*, **93**, 245 (1961).

(33) N. J. Hipp, J. J. Basch, and W. G. Gordon, *ibid.*, **94**, 35 (1961). Values given in this manuscript in Table II have been corrected to the anhydro form by G. R. Tristram and R. H. Smith (*Advan. Protein Chem.*, 18, 227 (1963)).

Discussion

The interactions of the hydrogen ions with macroions have generally been analyzed by using eq. 4, where α is

$$\mathrm{pH} - \log \frac{\alpha}{1-\alpha} = \mathrm{p}K_i - 0.868w\bar{Z} \qquad (4)$$

the fraction ionized of groups of pK_i, K_i is the intrinsic dissociation constant of the group at a given ionic strength, $\bar{Z}$ is the average net charge (taking into account the binding of anions and cations) per macroion at any pH, and w is a parameter, depending, at constant temperature and ionic strength, mainly on the geometry (such as size and shape) of the macroion; w is commonly known as the electrostatic factor.

If the macroion is a compact impenetrable sphere with uniform charge distribution and the microion is univalent, Scatchard[11] calculated from the Debye theory the electrostatic factor to be

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa A}\right) \qquad (5)$$

where ϵ is the protonic charge, D is the dielectric constant of the solvent, κ is the usual Debye–Hückel function (proportional to the square root of the ionic strength), b is the radius of the hydrated macroion, A is the distance of the closest approach of macro and micro ions (here $A = (b + 2.5)$ Å.), k is the Boltzmann constant, and T is the absolute temperature.

Modifications of eq. 4 and 5 have been applied to the interaction of K^+ and H^+ ions with α_s-casein. These modifications are necessary since in most experiments casein is present as a precipitate, a colloid, or small polymers. An examination of Figure 3 suggests that all of the ionizable groups in α_s-casein, in whatever form the protein occurs, are accessible and completely reversible to hydrogen ions. The data of Figure 1 suggest that this also applies to potassium and chloride ions. The presence of association products, the availability of groups, and the fact that the structure of the system changes with conditions suggest that eq. 4 be modified to include an *apparent* electrostatic factor, w', as follows

$$\mathrm{pH} - \log \frac{\alpha}{1-\alpha} = \mathrm{p}K_i - 0.868w'\bar{Z}_p \qquad (6)$$

where $\bar{Z}_p$ is the average net charge per monomer and $w' = pw$ where p is the degree of aggregation and w is the electrostatic factor per aggregate.

As an approximation to the variation of electrostatic factor with ionic strength an equation analogous to eq. 5 was used. It is

$$w' = \frac{\epsilon^2}{2DkT}\frac{p}{q}\left(1 - \frac{\kappa q}{1+\kappa(q+2.5)}\right) \qquad (7)$$

where q is related to the geometry of the aggregate.

1. Binding of Potassium and Sodium Ions to α_s-Casein. The pH changes produced by the addition of KCl to isoionic α_s-casein correspond to the liberation of a small fraction of one hydrogen ion per molecule (see

Figure 1). Thus, to a good approximation, the values of α and pK in eq. 6 do not change appreciably. Noting that under the experimental conditions the average net charge on the deionized isoionic protein is close to zero leads to

$$\Delta pH = pH - pH_0 = -0.868w'\sum_i \bar{\nu}_i Z_i = -0.868w'\bar{\nu} \quad (8)$$

where pH_0 is the isoionic pH in the absence of salt, pH is the measured pH after the addition of salt, and $\bar{\nu}$ is the average number of ions of charge Z_i bound per molecule of protein at the given salt concentration. A decrease in pH is expected when cations are bound. A binding equation which takes electrostatic interactions into account has been developed by Scatchard and coworkers.[15,20,22] It is

$$\bar{\nu} = \sum_i \frac{n_i K_i^\circ \beta}{1 + K_i^\circ \beta} \quad (9)$$

where $\beta = a\exp(-2w'\bar{Z}_p Z_i)$. Here, a is the activity of K^+ in solution, and n_i and K_i° are the number and intrinsic binding constant for each class of binding site.

Where more than one class of binding site is present, techniques have been developed for estimating n_i and K_i° from binding data.[15,20,34] According to Scatchard and Yap[34] a plot of log $(\bar{\nu}/\beta)$ *vs.* $\bar{\nu}$ yields an intercept at $\bar{\nu} = 0$ of

$$\log \sum_i n_i K_i^\circ = \log n_1 K_1^\circ + \log \frac{\sum_i n_i K_i^\circ}{n_1 K_1^\circ}$$

and that the asymptotic slope is

$$-0.4343 \sum_i n_i K_i^{\circ 2}/(\sum_i n_i K_i^\circ)^2 = -0.4343\left[\frac{\sum_i n_i (K_i^\circ)^2}{n_1 (K_1^\circ)^2}\right] \Big/ \frac{1}{n_1}\left(\frac{\sum_i n_i K_i^\circ}{n_1 K_1^\circ}\right)^2$$

The values of n_1 and K_1° are obtained if it is assumed that $\sum_i n_i K_i^\circ / n_1 K_1^\circ$ and $[\sum_i n_i (K_i^\circ)^2]/[n_1 (K_1^\circ)^2]$ are very nearly unity and n_1 is an integer. Having n_1 and K_1°, if the calculations are now repeated using $(\bar{\nu} - \bar{\nu}_1)$, values of n_2 and K_2° may be obtained.

The data of Figure 1 on the isoionic system were first analyzed using eq. 8 and 9. The apparent electrostatic factor, w', enters into calculations of the binding parameters.

Fortunately, the second term in eq. 7 is much less important than the first term under our experimental conditions, and probably does not depend significantly on the geometry of the particle. In using eq. 7 an assignment of a ratio of p/q and q are required. The value of p/q is most important in determining w' while the assignment of the value of q is not critical for calculating w' or, in fact, in calculating ionic strength dependence. By trial and error a value of q/p = 11 Å. was selected and for convenience a value of 11 Å. was then assigned to q. This gave a set of binding values consistent with the data in Figure 1 and the first three values of Table I, and in agreement with the binding data at higher pH values (Table I). A set of binding parameters were obtained as follows: $n_1 = 2$, $K_1^\circ = 323$, $n_2 = 3$, and $K_2^\circ =$

(34) G. Scatchard and W. T. Yap, *J. Am. Chem. Soc.*, **86**, 3434 (1964).

Table III. Binding of Potassium Ions Calculated from Electrostatic Theory at 20°[a]

3 + log [KCl], moles/kg. H_2O	$\bar{\nu}^{11}$	$\bar{\nu}^{11}$ (calcd.)	$\bar{\nu}^{11}/\beta$	$\bar{\nu}^{21}$	$\bar{\nu}^{21}/\beta$
0.000	0.32	0.42	401	0.67	840
0.100	0.43	0.49	459	0.89	942
0.301	0.62	0.64	463	1.33	992
0.500	0.85	0.79	454	1.84	973
0.700	1.07	0.99	403	2.42	909
0.800	1.21	1.10	385	2.78	891
0.900	1.36	1.17	368	3.11	840
1.000	1.41	1.28	326	3.36	750
1.100	1.51	1.39	275	3.58	653
1.500	1.85	1.84	150	4.52	366
1.700	2.07	2.07	113	5.16	281
1.900	2.28	2.29	84	5.90	217
2.100	2.62	2.50	67	6.88	171
2.301	2.81	2.80	47	8.00	135
2.500	3.14	3.08	36	9.67	112
2.600	3.36	3.23	32	10.7	101

[a] The calculations are based on the results given on Figure 1. Refer to the text for the symbols in this table.

7.1. In Table III, column 1 gives the total concentration of added KCl, column 2 gives values of $\bar{\nu}$ calculated from the ΔpH values of Figure 1, and column 3 gives the value of $\bar{\nu}^{11}$ calculated by eq. 9. It should be pointed out that columns 2 and 3 are in excellent agreement. A choice of q/p = 21 Å. predicts binding under isoionic conditions considerably greater than that at pH 7 (compare column 5, Table III with column 1, Table I). In addition, satisfactory values of n_1 and K_1°, etc., could not be obtained readily by eq. 9.

In no case could the binding data be satisfied by a single set of binding sites. Table III, columns 4 and 6, give values of $\bar{\nu}/\beta$ for assumed q/p = 11 Å. ($\bar{\nu}^{11}$) and q/p = 21 Å. ($\bar{\nu}^{21}$). These are plotted against $\bar{\nu}^{11}$ and $\bar{\nu}^{21}$, respectively, in Figure 4. Scatchard[11] points out that if there is only one class of sites, a plot of $\bar{\nu}/\beta$ *vs.* $\bar{\nu}$ should be a straight line with intercepts on the $\bar{\nu}/\beta$ axis at nK and on the $\bar{\nu}$ axis at n. The curvatures of the plots shown in Figure 4 indicate that one class of site is not sufficient.

The values of $\bar{\nu}$ given in the last column of Table I were calculated by means of eq. 9 using the values of n_i and K_i° given above and the values of net charge from titration data to be considered next. Values of $\bar{\nu}$ in the last column are generally in good agreement with measured values. Large discrepancies between the calculated and measured values occur when the experimental values are subject to large errors due to low protein concentrations. It should also be pointed out that the binding of Na^+ or K^+ ions with some of the most powerful chelating agents is also quite weak. For example, the first binding constant[35] for ethylenediaminetetraacetic acid (EDTA) with Na^+ is about 46, which is seven times less than that for the binding between the first two K^+ ions and α_s-casein.

2. Binding of H^+ to α_s-Casein. Table IV is a comparison of the number of the charged groups as determined by titration and by amino acid analysis. In general, the agreement between these two methods is good, *i.e.*, within two groups, with the exception of the

(35) G. Schwarzenbach and H. Ackermann, *Helv. Chim. Acta*, **30**, 1798 (1947).

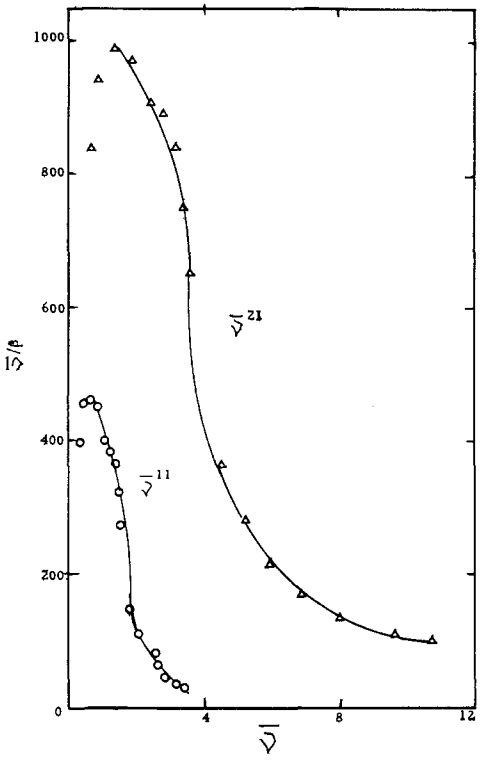

Figure 4. Binding of K^+ to isoionic α_s-casein. Calculated from the electrostatic theory at 20°.

side-chain carboxyl groups which are 40 ± 3 per molecule from titration whereas amino acid analysis gives a value of 46 ± 3. Limitations in estimation from titration are: first, uncertainty in values for the ionization constants of the phosphates (pK_1 and pK_2) and imidazoles, and, second, the possibility that some of the phosphorus atoms may be in the form of phosphodiesters or pyrophosphate.[36,37] We have made the assumption that all are present as monoesters. The main difficulty in the estimation of the number of side-chain carboxyls by amino acid analysis lies in the determination of the numbers of asparagines and glutamines from the amount of ammonia released during acid hydrolysis.

The isoionic point of α_s-casein as determined from the mixed-bed ion-exchange resins is 5.16 ± 0.09 at 20°. Combined data from titration and amino acid analysis can furnish a separate value. The total number of cationic groups from amino acid analysis is 34. If all are charged at the isoionic pH the latter should be located when 34 anionic groups are also present. If we assume that the first ionization constant of the phosphate group is about 1.8, at pH 2 there are approximately eight of the ten phosphate groups still ionized. Consequently, the net number of the cationic groups at pH 2 is approximately 26. According to Figure 3, 26 anionic groups will appear at approximately pH 5.3.

The groups most likely to be titrating over a small range near the isoionic pH are side-chain carboxyl groups. The normal pK for these groups in proteins varies from 4.3 to 4.7.[38] Tanford and co-workers[18,39]

(36) G. Perlmann, *Biochim. Biophys. Acta*, **13**, 452 (1954).
(37) J. Belec and R. Jenness, *ibid.*, **63**, 512 (1962).
(38) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press Inc., New York, N. Y., 1958, p. 534.

Table IV. Charged Groups in α_s-Casein

Group	This study: Titration	This study: Amino acid analysis	Hipp, *et al.*[h] amino acid analysis
α-Carboxyl		3[a]	
Side-chain carboxyl	40 ± 3	46 ± 3	33
Phosphate		20[b]	18
Imidazole	5[c]	6	5
α-NH_2 (α-amino)	...	1[d]	
Phenolic	28 ± 2[e]	12	11
Side-chain NH_2		18	16
Guanidyl	10 ± 2[f]	9	6
Total anionic	63 ± 3	69	
Total cationic	34 ± 2[g]	34	

[a] See ref. 6. [b] See ref. 6. It is assumed that all phosphorus is in the form of *o*-phosphoserine. There are 10 P atoms per 27,300 g. of α_s-casein. [c] The number of ionizable groups from pH 6–8.5 is about 15. Since there are 10 phosphate groups with a pK similar to that of imidazole, the number of imidazole groups is estimated as 5. [d] See N. Manson, *Arch. Biochem. Biophys.*, **95**, 336 (1961). [e] This is assumed to be equal to the number of ionizable groups between pH 8.5 and 11.5. [f] The number of guanidyl groups can be estimated from the titration according to the method suggested by Tanford [C. Tanford, "Physical Chemistry of Macromolecules," John Wiley and Sons, Inc., New York, N. Y., 1961, p. 564.] This value of 10 is equal to the total number of cationic groups (34) minus imidazole groups (5) minus the α-amino group (1) and minus the number of side-chain amino groups (18) (taken from the amino acid analysis). [g] The number of cationic groups can be estimated by summing the number of protons added from the isoionic point of α_s-casein (pH 5.16 at 20°) to pH 2 and the number of phosphate groups uncombined with proton at pH 2. [h] Refer to Table II of this manuscript and ref. 33.

have shown that intramolecular bonding between carboxyl and some other kind of group or a molecular expansion at acid pH can shift the carboxyl pK to a value as low as 4 (serum albumin). Laskowski and Scheraga[40] pointed out that carboxyl–carboxyl hydrogen bonding can give a value as high as 5. In addition, Tanford and Kirkwood have shown that both the distribution of the ionizable sites and the location of these sites with respect to the surface of a spherical impenetrable protein molecule can affect the electrostatic factor as well as the intrinsic dissociation constant for a given group in protein titrations.[41,42]

Equation 4 has been used extensively to analyze titration data.[18,43,44] The experimental titration curve is considered to be made up of subcurves (such as those due to side-chain carboxyls, imidazoles, etc.), each of which represents the titration of groups identical except for electrostatic interaction. The pK values of the groups are assumed not to overlap significantly.

According to eq. 6 the intercept of a plot of $pH - \log \frac{\alpha}{1-\alpha}$ *vs.* $\bar{Z}_p$ at $\bar{Z}_p = 0$ will give pK_i. For molecules or particles of constant size, shape, and pK_i, a linear plot is expected with a slope of $-0.868w'$. If there is a change in any of these parameters a value of w' at any

(39) C. Tanford, S. A. Swanson, and W. S. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).
(40) M. Laskowski, Jr., and H. A. Scheraga, *ibid.*, **76**, 6305 (1954).
(41) C. Tanford and J. G. Kirkwood, *ibid.*, **79**, 5333 (1957).
(42) C. Tanford, *ibid.*, **79**, 5340 (1957).
(43) K. Linderstrøm-Lang, *Compt. rend. trav. lab., Carlsberg*, **15**, No. 7 (1924).
(44) J. Steinhardt and E. M. Zaiser, *Advan. Protein Chem.*, **10**, 151 (1955).

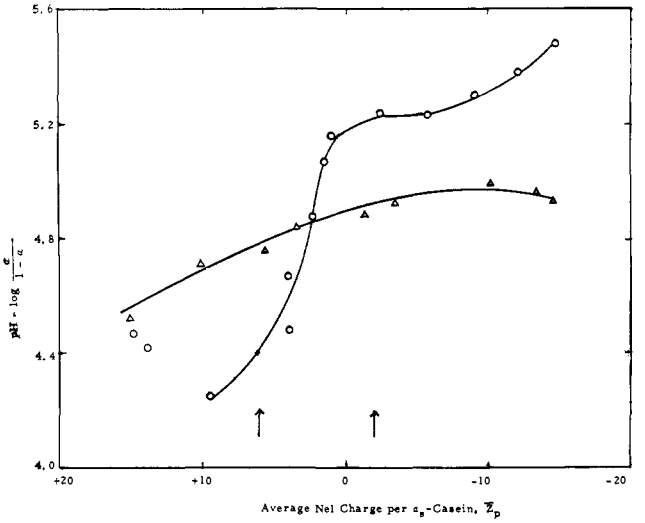

Figure 5. Plot of pH − log (α/1 − α) *vs.* the average net charge per 27,300 g. of α_s-casein for the titration of the side-chain carboxyl groups at 20°. Open circles, ○, for I = 0.05, and open triangles, Δ, for I = 0.4.

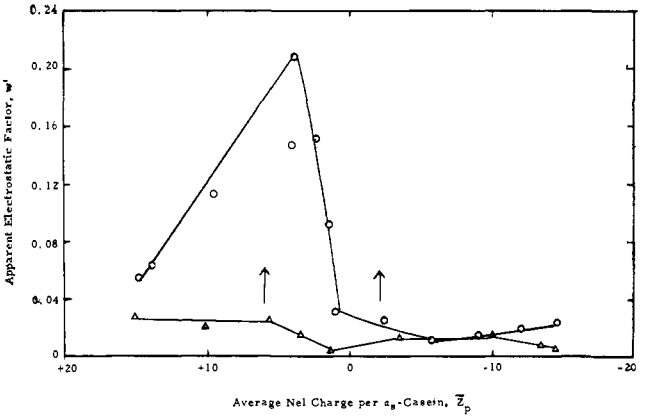

Figure 6. Apparent electrostatic factor, w', as a function of the average net charge per 27,300 g. of α_s-casein for the side-chain carboxyl groups at 20°. Open circles, ○, for I = 0.05, and open triangles, Δ, for I = 0.4.

$\bar{Z}_p$ can be calculated by substituting the value of pK_i into eq. 6 along with values of α and pH. Figure 5 shows plots of (pH − log (α/1 − α)) *vs.* $\bar{Z}_p$ at 0.05 and 0.4 ionic strength, respectively. In these, ordinate values of 5.18 and 4.88 were taken from Figure 5 for $\bar{Z}_p$ = 0 at ionic strength 0.05 and 0.4, respectively. Nonlinearity suggests that the apparent electrostatic factor (or pK_i) is strongly dependent on $\bar{Z}_p$ at the lower ionic strength. In these and the following calculations Z_p has been corrected for the binding of K^+ ions. This was accomplished by using the data of columns 4 and 5 of Table I.

Figure 6 is a plot of w' *vs.* the net charge per monomer. It is noted that w', at 0.05 ionic strength, goes through a maximum at about +4 charges and that there is more than a 10-fold variation in w' over the carboxyl region of the titration curve. The variation of w' with net charge at 0.4 ionic strength is small compared with that at the lower ionic strength. The two arrows on the abscissae of Figures 5 and 6 indicate the region over which the protein, at 0.05 ionic strength, is in the form of a colloid and/or precipitate. At ionic strength 0.4 the protein is mainly precipitate over the entire range shown.

Outside of the range of colloid or precipitate formation, α_s-casein aggregates or associates to a degree which is sensitive to pH, ionic strength, temperature, and the presence of additives such as urea. Some of these have previously been examined,[2,3] but not for the ionic strengths used here. Condition-sensitive association is indicated by the fact that the sedimentation constant of α_s-casein varies from 1.3 S. at 0.01 ionic strength to 4.6 S. at 0.1 ionic strength at pH 7 and at 20°. At 0.4 ionic strength, pH 7 and 20°, the sedimentation pattern of α_s-casein reveals polydispersity with 8.5 S. for a major peak and minor peaks of 7.6 and 10.2 S.

According to eq. 7, variations in w' with experimental conditions can be attributed to some appropriate change in a combination of the degree of aggregation (p) and geometric factor (q). An approach to resolution of this combination might be through a determination of the dependency of w' on gross particle size. Referring again to Figure 6, the maximum in w' is associated with the conditions where there is a maximum in p (or p/q). We expect this to occur when the net charge is close to zero, as indicated by the formation of colloid. Since α_s-casein at 0.4 ionic strength exists as a gross precipitate throughout the titration of the carboxyl region, we would expect less variation in p (or p/q) and for this reason less variation in w' with $\bar{Z}_p$, as is shown in Figure 6. The fact that pK_i is 4.88 at 0.4 ionic strength *vs.* 5.18 at 0.05 ionic strength suggests that increasing ionic strength decreases those special interactions which lead to an unusual pK_i at low ionic strength. Equation 6 also points out that pK_i depends on both w' and $\bar{Z}_p$. Any unusual variation in w' or $\bar{Z}_p$ can affect the value of pK_i.

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