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Binding of Cations to Caseins. Site Binding, Donnan Binding, and System Characteristics[*]

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ABSTRACT: Ion–protein interactions in the formation of core polymers and cores of casein micelles are determined by examining α_s-casein, β-casein, and their unit weight ratio mixture at final pH 6.6 and 37°. Increasing protein association is correlated with monomer net charge, protein solvation, and site binding of calcium and hydrogen ions. To obtain these data, isoionic proteins are used and proton releases are obtained (a) as monovalent cation concentration, I, is brought to 0.04, 0.08, and 0.16 M, and (b) at each I, as calcium concentration is brought progressively to 0.08 M. Protein solvations are measured for precipitates, and calculated from apparent monomer net charge for protein in solution. Apparent molar calcium binding, $\bar{\nu}_{\mathrm{Ca,A}}$, is measured after equilibrium dialysis. Since net fixed charge occurs on short, solvent-accessible acidic peptides, Donnan binding of calcium ion is examined. A model consistent with the theory of Overbeek is used to obtain monomer net charge, and Donnan binding corrections, $\bar{\nu}_{\mathrm{Ca,D}}$. Site-bound calcium is obtained by difference. Results and conclusions are: $\bar{\nu}_{\mathrm{Ca,D}}$ are small at low $\bar{\nu}_{\mathrm{Ca,A}}$ ($\sim$15%) and decrease as ionic strength and/or $\bar{\nu}_{\mathrm{Ca,S}}$ increase. For α_s-casein, β-casein, and the mixture; and independent of I, (a) precipitation is initiated at the same $\bar{\nu}_{\mathrm{Ca,S}}$ and $\bar{Z}$ (respectively, 9.3, 5.4, and 10.4, and −9, −6, and −1.5), and (b) at 0.08 M calcium, proton release and $\bar{\nu}_{\mathrm{Ca,S}}$ are essentially constant (6.6, 4.8, and 6.3, and 20, 11.3, and 16.3). At these $\bar{\nu}_{\mathrm{Ca,S}}$ there is apparently a reversal of $\bar{Z}$ in precipitate (to approximately +10.4, +4.4, and +8.9). Since solubility and solvation do not reverse significantly, core polymer cross-linking by calcium is proposed as a source of interaction energy. The mixture is not intermediate in precipitate characteristics of $\bar{\nu}_{\mathrm{Ca,S}}$, $\bar{Z}$, and solvation. In the mixture, comingling of monomers in core polymers produces interactions which convert potential interpolymer cross-linking sites into intrapolymer cross-linking sites.

Casein micelles, according to the model of Waugh and Noble (1965), consist of cores of insoluble α_s- and β-caseinates stabilized by a coat layer containing κ-casein. Cores are variable in size, and this leads to a micelle size distribution. The coat-core model suggests that the properties of the core can be determined through investigation of the precipitation characteristics of α_s- and β-caseins. Waugh *et al.* (1970), using individual proteins or a unit weight ratio mixture, have shown that an increasing degree of monomer association is produced both by increasing ionic strength due to mono-

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valent ions and by the introduction of strongly binding divalent cations, such as calcium, which have effects much larger than predicted by their ionic strength contributions. Before or at precipitation, monomers associate radially to form polymers of limited size (core polymers). These interact at their surfaces to form precipitate. The most important sequences of interactions between proteins and ions are those which occur as a result of divalent cation binding, since stable micelles will form only when such ions are present.

Little information on ion binding is available. The interactions of mixtures of caseins, with monovalent cations (Carr and Topol, 1950; Carr and Engelstad, 1958) and with divalent cations (Chanutin *et al.*, 1942; Zittle *et al.*, 1958) have been studied, but studies of components are limited to the data of Ho and Waugh (1965a) on the binding of sodium and potassium to α_s-casein. For divalent cations, Ho and Waugh (1965b) report that the organic phosphate groups on α_s-casein appear to be the strongest binding sites for calcium and Dickson and Perkins (1969) report that the relative order of binding constants to α_s-, β-, and κ-caseins is $Ca^{2+} > Ba^{2+} > Sr^{2+}$, and that the relative order of binding capacities is $\alpha_s > \beta > \kappa$.

For the following studies α_s-casein, β-casein, and a unit weight ratio mixture were used. Present purposes are to present, for 37° and pH 6.6 (the normal conditions of milk), correlated sequences of average monomer net charge, proton release, and site binding of calcium at constant monovalent cation concentrations, I, of 0.04, 0.08, and 0.16 M. To obtain site binding of calcium a model independent examination is made of apparent binding to determine the extent to which this result from the Donnan distribution. That this is necessary is indicated by two observations. First, according to Osterberg (1964) and Peterson *et al.* (1958), the monomers of α_s-casein and β-casein contain short acidic peptides which carry essentially all organic phosphate groups and in addition several carboxylate groups. Consequently, peptides carry essentially all net monomer charge. Local regions of high charge density are therefore expected. Second, according to Overbeek (1956), under such conditions the fraction of fixed charge compensated by the accumulation of counterions increases with local fixed charge density.

In a later publication correlated sequences of site-bound calcium and hydrogen ions will be examined using intrinsic binding constants and models having specific geometry for the calculation of electrostatic effects.

Materials and Methods

Materials and methods previously described include chemicals, laboratory distilled water, and treatment of dialysis tubing (Waugh *et al.*, 1962). Fresh milk obtained from individual Guernsey cows typed as α_s-casein B and β-casein A was skimmed, and pure α_s- and β-caseins obtained as previously described (Waugh *et al.*, 1970).

Deionization of Protein. Ho and Waugh (1965a) deionized by introducing mixed-bed ion-exchange resins. However, considerable protein is lost by precipitation onto resin beads. To avoid this protein solutions are first brought to pH 2.5 by the addition of 1.0 N HCl, so that protons displace even strongly bound cations. At this pH, α_s-casein is in solution, but β-casein is mostly precipitated. Into each solution at 2° is introduced a dialysis sac containing a Teflon-covered stirring bar and a mixture of carefully washed resins in hydroxyl and hydrogen form (Dowex 50W-X-4 cation-exchange resin from Baker, and Rexyn-201 anion-exchange resin from Fisher). The sac of ion exchanger is rotated to stir the system. Two or three sacs of freshly mixed resins, over a 48-hr period, bring the pH to approximately 4.0 where both α_s- and β-caseins exist as fine particles. The deionized proteins are titrated to pH 6.6 with 1.0 N NaOH, frozen, and stored at −15°. These will be referred to as "protein just at pH 6.6."

Protein Concentration. Absorbances were used. A correction was made for scattering by subtracting 1.7 times the apparent absorbance at λ 320 nm from the apparent absorbance at λ 280 nm. Absorptivities ($E^{1\%}_{1\,\mathrm{cm},\lambda 280}$) of 10.0 for α_s-casein and 4.7 for β-casein were used. Concentrations are generally reported in mg per ml.

Ionic Strength. Protein solutions contained various amounts of sodium chloride, calcium chloride, and sodium hydroxide, and 0.001 M sodium cyanide. The symbol I will refer to sodium concentration, and calcium chloride concentrations will be reported separately.

Determination of Proton Release. To maintain pH, it was necessary to determine the proton release on adding sodium or calcium chlorides. To do so, 10 ml of protein solution at 7.5 mg/ml and just at pH 6.6 were placed in a jacketed beaker and stirred at 37°. The pH was measured using a Radiometer PHM 4 with a GK 2021 B combination glass electrode. Nitrogen, saturated with water vapor, was flowed over the surface of the solution to minimize absorption of CO_2. First, sufficient 2.0 M NaCl was added to give the appropriate I. Proton release was determined by the amount of 0.10 N NaOH required to return the solution to pH 6.6. Then, 0.568 M $CaCl_2$ was added at each I in the following sequence of increments, which total 1.0 mmole: four at 2.5×10^{-3}, one at 0.01, two at 0.02, four at 0.01, one at 0.1, and four at 0.2 mmole. Each incremental addition was followed by titration to pH 6.6. Reagents were added by syringe microburets, Model No. SB 2 (Micro-Metric Instrument Co.), equipped with 1.0-ml tuberculin syringes. By gravimetric calibration 0.04 ml can be delivered with an accuracy of ±1%.

Dialysis Equilibration and Solvation. The majority of binding experiments were performed using dialysis equilibration. The dialysis tubing, $^{18}/_{32}$ (Union Carbide), does not bind calcium under the conditions used. At equilibrium, all solutions contained I values equal to 0.04, 0.08, or 0.16. Each sac contained 75 mg of protein in 5 ml of solution, and also 0.002 M NaCN. The diffusate, also 5 ml, contained the proper amounts of $CaCl_2$ and NaOH to give an equilibrium pH of 6.6 at each level of calcium. Equilibration was carried out in 50-ml polycarbonate centrifuge tubes sealed with polypropylene caps lined with Parafilm. Solutions were gently agitated for 24 hr at 5°, and finally for 16 hr at 37°.

At calcium levels where precipitate did not form, the Ca^{2+} concentration was measured on both protein and diffusate solutions and the pH of both solutions was checked. Since colloid osmotic pressure causes dilution of protein solutions, final volumes were calculated from protein concentrations after equilibration.

When the calcium level produced precipitate inside the dialysis sac, the diffusate was saved for calcium determination. The contents of the sac were transferred to a polyallomer tube and placed in the bucket of a SW39 rotor maintained at 37°. The precipitate was packed by centrifuging at 67,000g for 35 min (measured solvation is essentially independent of field). The supernatant was decanted. The end of the centrifuge tube was sliced off and excess moisture blotted from the protein–polyallomer junction. The pellet plus tube end was weighed, the pellet was dissolved in 10 ml of purified 9.0 M urea solution, and the tube end was recovered and weighed. The pro-

TABLE I: Proton Release of the Caseins.[a]

Protein	I^b	δH_I^+	$\bar{Z}_I$	ξ	Total δ
α_s-Casein	6×10^{-4}	22.0	−22		
	0.04	2.8	−24.8	0.21	6.58
	0.08	0.65	−25.4	0.18	6.69
	0.16	0.85	−26.3	0.13	6.64
β-Casein	3.7×10^{-4}	13.0	−13.0		
	0.04	1.25	−14.2	0.37	4.95
	0.08	0.67	−15.9	0.30	4.92
	0.16	0.48	−16.3	0.26	4.90
α_s- + β-Caseins	5×10^{-4}	17.2	−17.2		
	0.04	1.95	−19.2	0.30	6.3
	0.08	0.75	−19.9	0.25	6.3
	0.16	0.60	−20.5	0.21	6.3

[a] The reference materials are the isoionic proteins. Sodium concentrations are given in column 2 and the increments in proton release between sodium concentrations in column 3. The monomer net charge for each sodium concentration is given in column 4. Column 5 records ξ, the average proton release per apparent bound calcium, and column 6 the total proton release at 0.08 M calcium. [b] These are sodium concentrations. The first for each protein does not accurately represent the ionic strength.

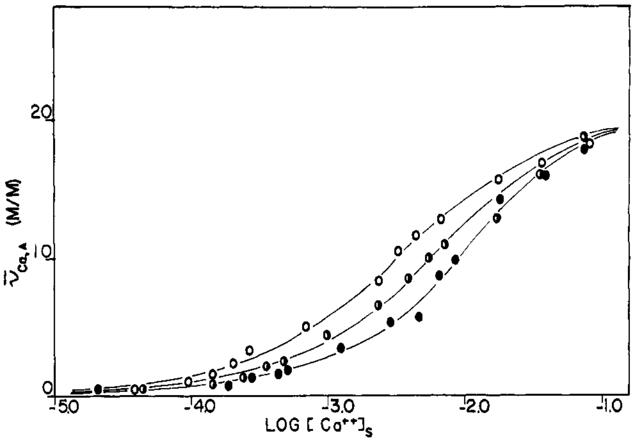

FIGURE 1: Apparent binding of calcium, $\bar{\nu}_{Ca,A}$, in moles of calcium per mole of protein for α_s-casein *vs.* the logarithm of the solution calcium concentration, $[Ca^{2+}]_s$. Total sodium concentrations are: 0.04 M (○), 0.08 M (◑), and 0.16 M (●).

tein–urea solution was diluted to 100 ml with distilled water. Protein was determined by absorbance and calcium content as described below. These data permit calculation of precipitate (pellet) solvation, *G*, in grams of water per gram of protein, and the total apparent binding of calcium, $\bar{\nu}_{Ca,A}$, as moles of calcium per mole of protein. For the latter, the molecular weights used were 27,300 for α_s-casein (Waugh *et al.*, 1962), 24,100 for β-casein (Sullivan *et al.*, 1955), and 25,600 for the unit weight ratio mixture.

Precipitate solvation determinations were also made with the reaction mixture established directly in a polyallomer tube held in a centrifuge bucket maintained at 37°. Calcium chloride was added directly rather than by dialysis and the tubes were incubated at 37° for 30 min before centrifugation and measurement of pellet solvation. Equilibrium calcium concentrations were obtained from measurements on supernatants.

Calcium Determinations. Measurements were made on both protein and diffusate using a dual-channel, double-beam IL-153 atomic absorption spectrophotometer (Instrumentation Laboratories). One channel was used to determine calcium and the other to monitor a strontium internal standard, which was in a "lanthanum diluent" containing 0.072 M $LaCl_3$, 1.14×10^{-4} M $SrCl_2$, and 0.1 M HCl. A 0.5-ml aliquot of the solution to be analyzed was diluted to 10.0 ml with lanthanum diluent. Protein is precipitated by lanthanum, and bound calcium released into solution. Samples were compared with a series of known calcium concentrations, ranging from 5×10^{-4} to 6.25×10^{-6} M, containing equivalent lanthanum diluent. At the lowest calcium concentration the standard deviation of the single measurement was just under 4%, at the highest concentration it was less than 0.4%. A number of standard solutions were prepared containing fixed amounts of Ca^{2+} but varying amounts of protein. A protein effect was not detected.

To measure calcium content of precipitates, calibration standards were prepared by distilled water dilution to 100 ml of the proper amount of standard calcium solution and 10 ml of 9.0 M urea. A concentration series was obtained by diluting these 20-fold with lanthanum diluent, as for the unknown samples.

Results

Proton Release. When sodium chloride is added to protein just at pH 6.6, protons are released and alkali must be added to maintain the pH. There is an additional release when calcium chloride is added. Table I summarizes the results. Column 1 gives the protein examined and column 2 the concentration of sodium. The first row for each protein gives the sodium hydroxide concentration required to bring the isoionic protein into solution at pH 6.6. Little chloride ion is present and the ionic strength is uncertain. The following three rows record for each protein the three added concentrations of sodium chloride, 0.04, 0.08, and 0.16 M. Column 3 gives increments in proton release δH_I^+/mole of protein. The first row gives δH^+ between isoionic protein and protein just at pH 6.6, and the remaining rows the increments due to changes in *I* between values of column 2. Column 4 gives $\bar{Z}_I$, the charge per monomer, exclusive of site-bound sodium. Column 5 gives ξ, the average increment in δH^+ per molecule per apparent bound calcium, and column 6 gives the total proton release between protein just at pH 6.6 and 0.08 M calcium chloride. Total δH^+ per molecule is essentially constant at 6.6 protons for α_s-casein, 4.9 protons for β-casein, and 6.3 protons for the α_s–β-casein mixture.

Calcium Binding at pH 6.6. Figure 1 refers to α_s-casein and shows, for the three values of *I*, the relation between apparent calcium binding, $\bar{\nu}_{Ca,A}$, and the logarithm of the solution calcium concentration, $[Ca^{2+}]_s$. Curves for β-casein and the α_s–β-casein mixture are similar to these, and full data are given in Tables II, III, and IV. It is apparent from Figure 1 that at constant $[Ca^{2+}]_s$, increasing the concentration of sodium ion results in lowered binding, with the effect diminishing as $[Ca^{2+}]_s$ increases.

Solvation of Ca–Caseinate Precipitates. In Figure 2, *G*, in grams of water per grams of protein, is plotted against site-bound calcium, $\bar{\nu}_{Ca,S}$. The latter were obtained from $\bar{\nu}_{Ca,A}$ by a

TABLE II: System Characteristics of α_s-Casein at Three Sodium Concentrations, I.

Log $[Ca^{2+}]_s^{-1}$	$G - U$	$\bar{\nu}_{Ca,A}$	$\bar{\nu}_{Ca,D}$ (%)	$\bar{\nu}_{Ca,S}$	$\bar{Z}$
I = 0.04					
4.627	12.17	0.25	16	0.21	−24.4
4.023	11.59	0.90	16	0.76	−23.5
3.857	10.95	1.61	12	1.41	−22.3
3.672	10.24	2.40	12	2.12	−21.1
3.598	9.65	3.06	10	2.75	−19.9
3.158	7.91	4.99	13	4.31	−17.2
2.734	4.93	8.30	13	7.23	−12.0
2.485[a]	3.14	10.29	11	9.35	−8.6
2.307	1.78	11.53	10	10.32	−6.5
2.180	1.48	12.73	4	12.25	−2.8
1.788	1.14	15.50	−2	15.88	+3.8
1.442	1.04	16.70	−5	17.52	+6.7
1.098	1.08	18.20	−10	20.08	+11.2
I = 0.08					
4.345	8.95	0.22	14	0.19	−25.1
3.846	8.52	0.88	10	0.79	−24.2
3.601	8.25	1.31	11	1.16	−23.5
3.450	7.77	2.03	10	1.83	−22.3
3.320	7.53	2.40	10	2.15	−21.6
3.039	6.36	4.19	7	3.79	−18.7
2.635	4.96	6.35	13	5.57	−15.5
2.420[a]	3.65	8.37	11	7.44	−12.1
2.268	3.03	9.88	9	9.01	−9.2
2.151	1.95	11.05	7	10.20	−7.0
1.771	1.22	12.96	4	12.45	−2.9
1.436	1.07	16.10	−4	16.74	+4.8
1.115	1.05	18.73	−9	20.38	+11.0
I = 0.16					
4.646	6.60	0.22	5	−0.21	−26.2
3.738	6.40	0.65	8	0.60	−25.4
3.570	6.17	1.13	10	1.01	−24.4
3.375	5.95	1.59	6	1.49	−23.5
3.248	5.77	1.98	6	1.85	−22.9
2.909	5.09	3.40	7	3.15	−20.4
2.546	4.20	5.26	9	4.79	−17.5
2.341	4.07	5.54	13	4.81	−17.4
2.195[a]	3.77	8.76	6	8.20	−11.3
2.060	2.87	9.87	6	9.25	−9.2
1.743	1.55	14.23	0	14.23	0
1.420	1.18	15.98	−4	16.47	+4.4
1.113	0.98	17.86	−7	19.10	+9.1

[a] First precipitate.

TABLE III: System Characteristics of β-Casein at Three Sodium Concentrations, I.

Log $[Ca^{2+}]_s^{-1}$	$G - U$	$\bar{\nu}_{Ca,A}$	$\bar{\nu}_{Ca,D}$ (%)	$\bar{\nu}_{Ca,S}$	$\bar{Z}$
I = 0.04					
3.960	6.79	0.42	26	0.31	−13.8
3.811	6.25	1.08	14	0.93	−12.8
3.577	5.89	1.52	15	1.29	−12.2
3.449	5.47	2.03	14	1.74	−11.5
3.321	5.13	2.45	15	2.09	−11.0
2.967	4.10	3.72	17	3.09	−9.5
2.578	2.95	5.12	21	4.03	−8.1
2.347	2.57	5.59	29	4.00	−8.3
2.224[a]	2.30	6.52	17	5.39	−5.9
2.106	1.80	6.97	17	5.80	−5.2
1.750	1.48	7.77	12	6.82	−3.5
1.425	1.29	8.92	−1	9.04	+0.5
1.106	1.29	10.62	−13	11.97	+5.6
I = 0.08					
4.144	5.24	0.14	21	0.11	−14.7
3.682	4.98	0.57	14	0.49	−14.1
3.513	4.73	0.99	11	0.88	−13.5
3.285	4.64	1.13	16	0.95	−13.4
3.192	4.41	1.51	14	1.30	−12.8
2.861	3.96	2.25	18	1.84	−12.0
2.775	3.58	2.88	15	2.46	−10.9
2.332	2.55	4.57	19	3.71	−8.9
2.202	2.29	5.01	20	3.99	−8.5
2.080[a]	2.28	5.75	16	4.81	−7.0
1.743	1.56	7.46	9	6.78	−3.6
1.455	1.34	8.65	1	8.54	−0.6
1.140	1.38	10.38	−10	11.34	+4.6
I = 0.16					
4.277	3.86	0.10	10	0.09	−15.1
3.484	3.84	0.14	30	0.09	−15.1
3.422	3.66	0.56	12	0.50	−14.5
3.281	3.55	0.80	10	0.72	−14.1
3.138	3.51	0.88	12	0.77	−14.0
2.822	3.15	1.70	12	1.50	−12.8
2.496	3.00	2.04	20	1.64	−12.6
2.299	2.49	3.17	17	2.64	−10.9
2.151	2.19	3.86	17	3.21	−10.0
2.066	1.59	5.21	14	4.65	−7.4
1.724	1.73	6.55	10	5.92	−5.2
1.417	1.43	8.00	4	7.67	−2.0
1.064	1.35	10.00	−6	10.62	+3.3

[a] First precipitate.

process to be described below. Figure 2 refers only to precipitates, where G is measured directly. Evidently, for the three protein systems, as $\bar{\nu}_{Ca,S}$ increases, G decreases to essentially plateau levels of 1.6–1.7 for both α_s-casein and the α_s-β-casein mixture, and to 1.9 for β-casein. There is an indication that, if $\bar{\nu}_{Ca,S}$ were to be increased sufficiently, G would increase. The curve for α_s-casein indicates that G is dependent essentially on $\bar{\nu}_{Ca,S}$, but that it increases slightly as I is increased at constant $\bar{\nu}_{Ca,S}$. The points for α_s-casein show some scatter. An examination of the data suggests that this is greater than expected from errors in experimental measurements. Scatter is attributed to the varying conditions which prevail locally due to imperfections in mixing; for example, precipitate will form in regions where the local calcium concentration is different from the final concentration. The pellet composition will be an average of precipitate in all regions, and unless there is a rapid rearrangement, local regions will differ from true equilibrium values. Experiments were carried out on α_s-casein in which pellets were first formed at high and low solvation levels, and these were then placed in ionic environments ex-

TABLE IV: System Characteristics of an Equal Weight Mixture of α_s- and β-Caseins at Three Sodium Concentrations, I.

Log $[Ca^{2+}]_s^{-1}$	$G - U$	$\bar{\nu}_{Ca,A}$	$\bar{\nu}_{Ca,D}$ (%)	$\nu_{Ca,S}$	$\bar{Z}$
I = 0.04					
3.997	9.26	0.25	52	0.12	−18.8
3.965	8.59	1.04	13	0.91	−17.5
3.818	8.10	1.58	11	1.41	−16.6
3.608	7.55	2.26	11	2.00	−15.6
3.470	7.13	2.75	12	2.42	−15.0
3.058	5.63	4.53	14	3.88	−12.6
2.671	3.27	7.30	11	6.42	−8.3
2.453	1.81	9.02	9	8.25	−5.2
2.284[a]	1.84	10.60	2	10.45	−1.3
2.157	1.72	10.92	1	10.84	−0.6
1.774	1.19	11.80	−2	12.03	+1.5
1.449	1.54	13.90	−7	14.88	+6.3
1.116	1.50	15.00	−14	17.06	+10.5
I = 0.08					
3.921	6.87	0.26	30	0.20	−19.4
3.621	6.47	0.91	10	0.80	−18.4
3.487	6.17	1.39	11	1.24	−17.6
3.368	5.84	1.92	9	1.74	−16.7
3.295	5.47	2.50	8	2.30	−15.7
2.920	4.65	3.82	10	3.42	−13.8
2.823	4.12	4.66	10	4.22	−12.4
2.370	2.58	7.13	10	6.37	−8.7
2.088[a]	2.10	10.50	2	10.35	−1.7
1.757	1.44	10.90	2	10.74	−1.0
1.434	1.30	13.20	−5	13.87	+4.6
1.091	1.27	14.50	−10	15.97	+8.2
I = 0.16					
4.084	5.08	0.22	10	0.20	−20.0
3.580	4.94	0.52	10	0.47	−19.5
3.406	4.82	0.78	11	0.70	−19.0
3.272	4.67	1.12	9	1.02	−18.5
3.138	4.48	1.53	7	1.40	−17.9
2.838	4.05	2.48	10	2.24	−16.4
2.521	3.25	4.20	10	3.80	−13.6
2.333	2.56	5.73	9	5.24	−11.1
2.185	2.38	6.12	10	5.50	−10.6
2.084	1.88	7.21	9	6.59	−8.6
1.753	1.66	10.70	1	10.55	−1.6
1.435	1.33	12.00	−2	12.19	+1.4
1.148	1.28	14.60	−8	15.76	+8.0

[a] First precipitate.

pected to decrease or increase solvation, respectively. Changes in G in the expected directions were observed, but these changes were slow and incomplete.

It is noteworthy that α_s-casein centrifuged from three-fourths saturated ammonium sulfate has G = 1.6, but that centrifuged isoionic α_s-casein has G = 0.79.

Discussion

Monomers of α_s- and β-caseins (Waugh *et al.*, 1970) are considered to consist of compact elongated bodies to which are attached solvent accessible acidic peptides. As environmental ionic strength, calcium concentration, or temperature are increased, the degree of monomer association of α_s- and/or β-caseins increases to form near-spherical polymers of limited size. These, termed core polymers, have radii near 10 nm (three monomer body length) and contain approximately 30 monomers. Solvations are in the range G = 2.2–4.4. In core polymer formation, attractive energy is assigned to hydrophobic and hydrogen-bond formation between bodies, and the monomers are arranged radially with their acidic peptides placed near core polymer surfaces to minimize electrostatic free energy.

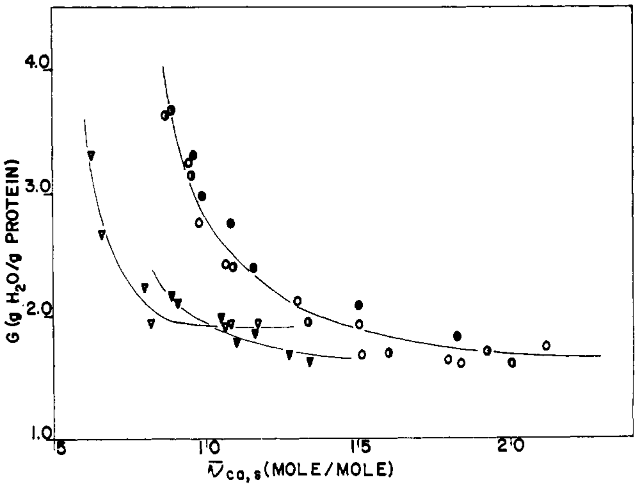

FIGURE 2: Precipitate solvation, G, *vs.* site-bound calcium, $\bar{\nu}_{Ca,S}$. Sodium concentrations for α_s-casein (circles) are: 0.048 M (○), 0.085 M (◑), and 0.154 M (●). Sodium concentration for β-casein (▽) and a unit weight ratio mixture (▼) are 0.048 M.

The precipitates at pH 4 are used here as the starting point. By titration, between pH 4 and 5.5 (limits for the true isoionic points) the increase in negative net charge per monomer is less than 1 for α_s-casein and less than 3 for β-casein. Using this information and hydrogen ion release between pH 4.0 and 6.6, the monomer net charge on protein just at pH 6.6 is −22 ± 0.5 for α_s-casein and −13 ± 1 for β-casein.

The acidic peptide of α_s-casein contains 7 of the 9 organic phosphate groups, 11 carboxylate groups, and 2 amino groups (Osterberg, 1964). The acidic peptide of β-casein contains all 5 organic phosphate groups, 5 carboxylate groups, and 2 arginine groups, both of which are terminal in the peptide (R. F. Peterson, unpublished data; Peterson *et al.*, 1958). Simple calculations, using any reasonable hydrogen ion binding constants for phosphate and carboxylate groups show that (exclusive of possible sodium binding) the acidic peptides must carry essentially all monomer net charge (the body is −2 to −3). The peptides, then, give rise to regions of high local fixed charge density, and within these regions will occur the principle effects both of ion binding and of Donnan distribution.

It is evident that the concentration of charge on the acidic peptides eliminates models, such as that used by Scatchard and coworkers (see Scatchard and Yap, 1964) in which fixed charge is distributed over a surface of constant geometry. It is expected that electrostatic effects are important in establishing peptide geometry, and other system characteristics which affect the distribution of fixed charge.

According to Overbeek (1956), when fixed charge is located so that the charge density on some portion of a polyelectrolyte is high, the classical Donnan (1911) theory does not apply. In particular, the mobilities of the counterions decrease as local charge density is increased, and an increasing fraction of fixed charge is compensated for by the accumulation of counter ions, and less by the expulsion of co-ions. Thus, it is possible that, in spite of the appreciable ionic strengths used in this study, a significant fraction of the apparent bound calcium $\bar{\nu}_{\text{Ca,A}}$, is due to Donnan binding, $\bar{\nu}_{\text{Ca,D}}$. Present interest in the extent of Donnan binding results also from calculations, to be given elsewhere, which show that the fixed charge distributions in specific monomer and system models are sensitive to ionic strength and the levels of ion site binding. From the following it is evident that the most important ions for site binding are hydrogen and calcium. As a result of ion concentrations the Donnan distribution will contribute significantly to the apparent binding of calcium but negligibly to the apparent binding of protons.

No evidence has been found which suggests that sodium site binds to carboxylate groups. With respect to phosphate groups, Smith and Alberty (1956) determined sodium binding to orthophosphate by means of pH measurements. They obtain an apparent binding constant of $k_{\text{Na,P}} \sim 4$. Strauss and Ross (1959), using conductance and electrophoresis methods, which are considered to be more accurate when the binding is small, obtain an intrinsic binding constant, $k^{\circ}_{\text{Na,P}}$, of approximately unity. Although local electrostatic effects will give k_{NaP} larger than $k^{\circ}_{\text{Na,P}}$, accepting an apparent constant between 1 and 4 suggests that site binding of sodium is low (about 1 per α_s-casein at $I = 0.04$).[1] In calculating the Donnan corrections which follow it is assumed that sodium binding is negligible; otherwise the calculations become model dependent. However, if sodium binding is obtained from model-dependent calculations, to be described elsewhere, the calculated Donnan corrections, $\bar{\nu}_{\text{Ca,D}}$, are decreased only slightly.

Donnan corrections, by which site bound calcium, $\bar{\nu}_{\text{Ca,S}}$, is obtained from apparent bound calcium are calculated as follows. Overbeek (1956) approaches the Donnan distribution by considering electric potentials near polyelectrolyte ions as a function of local surface charge density. Essentially the same result can be obtained by using the classical Donnan theory and limiting the volume available for unequal distribution of counter- and co-ions to a fraction of the total solvent volume. A similar limitation in volume has been used by Strauss and Ander (1958) to interpret the Donnan distribution in polyphosphates.

In the equations which follow, a subscript "d" refers to properties in the Donnan region, V_d, where counter- and co-ion concentrations are unequal, and a subscript "s" refers to the bulk of the solution volume, V_s, where they are equal. The subscript "t" refers to total amounts. W is the total weight in grams of protein, in the total solution volume V_t. U is solvent, grams of water per gram of protein, which is tightly bound and unavailable for occupancy by mobile ions; $\bar{Z}$ is the average net charge per monomer of molecular weight M. $\bar{Z}_I$ is the charge on the protein at sodium concentration I, ξ is the average proton release per apparent bound calcium as given in Table I, $\bar{v}$ is the protein partial specific volume, and ρ is the density of water (taken as unity).

Mass balance requires that

$$\text{Ca}_t = [\text{Ca}^{2+}]_s V_s + [\text{Ca}^{2+}]_d V_d + \bar{\nu}_{\text{Ca,S}}(W/M) \tag{1}$$

$$\text{Na}_t = [\text{Na}^+]_s V_s + [\text{Na}^+]_d V_d \tag{2}$$

$$\text{Cl}_t = [\text{Cl}^-]_s V_s + [\text{Cl}^-]_d V_d \tag{3}$$

$$\bar{\nu}_{\text{Ca,S}} = \bar{\nu}_{\text{CaA}} - MV_d \frac{[\text{Ca}^{2+}]_d - [\text{Ca}^{2+}]_s}{W} \tag{4}$$

$$V_t = V_s + V_d + \left(\bar{v} + \frac{U}{\rho}\right)W \tag{5}$$

Charge balance requires that

$$[\text{Cl}^-]_s = [\text{Na}^{2+}]_s + 2[\text{Ca}^{2+}]_s \tag{6}$$

$$2[\text{Ca}^{2+}]_d + [\text{Na}^+]_d = \frac{\bar{Z}W}{MV_d} + [\text{Cl}^-]_d \tag{7}$$

$$\bar{Z} = \bar{Z}_I + \xi\bar{\nu}_{\text{Ca,A}} - 2\,\bar{\nu}_{\text{Ca,S}} \tag{8}$$

The requirement that, at equilibrium, activities of salts must be equal in all accessible regions leads to

$$a_{\text{Ca,d}}a^2_{\text{Cl,d}} = a_{\text{Ca,s}}a^2_{\text{Cl,s}} \tag{9}$$

$$a_{\text{Na,d}}a_{\text{Cl,d}} = a_{\text{Na,s}}a_{\text{Cl,s}} \tag{10}$$

In applying these equations $\bar{v}$ is taken to be 0.74 cm^3/g (McKenzie and Wake, 1959). According to eq 9, $\bar{v}$ is not critical since it affects only V_s, which is large. U is taken to be 0.5 g of water/g of protein. This is chosen to be slightly larger than $U \sim 0.3$ obtained from the gain of water vapor by dry proteins (Steinhardt and Beychok, 1964). Activity coefficients were calculated by the Debye–Huckel formula using values of the diameters of the hydrated ions as reported by Kielland (1937). The equations were then solved for $\bar{\nu}_{\text{Ca,S}}$ with the aid of an IBM 360 computer for assigned values of V_d,[2] knowing total quantities, equilibrium concentrations, $\bar{Z}_I$, ξ, and $\bar{\nu}_{\text{Ca,A}}$. An iterative process was used to solve 1–10 simultaneously.

For precipitates, values of V_d were obtained from measured precipitate solvations, *i.e.*, $V_d = (G - U)W/\rho$. For protein in solution V_d were estimated from apparent net charge, defined as $\bar{Z}_A = \bar{Z}_I - (2 - \xi)\bar{\nu}_{\text{Ca,A}}$, as follows. Values of the ratio $(G - U)/\bar{Z}_A$ were obtained for α_s-casein for the minimum calcium concentrations at which precipitates first form.

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[1] This is not in accord with Ho and Waugh (1965a), who found two strong and three weak binding sites, at apparent constants of 323 and 7.1. In addition, these authors found binding to be essentially constant over the range in I under discussion. In their experiments, apparent sodium or potassium binding to α_s-casein was calculated from the potential across a cell which included salt bridges and salt solutions, with and without added protein, separated by a cation impermeable membrane. It is proposed that at least the weak binding and apparent insensitivity to sodium concentration can be accounted for by a decrease in cation mobility, according to the theory of Overbeek (1956, see text). The potential of the cell used by Ho and Waugh is expected to be dependent on reduction of counterion activity in the protein compartment, which can take place by a decrease of ion mobility as well as by site binding. A large fraction of the total apparent binding can result from the former source.

[2] In the absence of calcium, in accord with the observations of Overbeek (1956), as the density of the fixed charge is increased by decreasing V_d, at constant $\bar{Z}$ progressively more of the fixed charge is calculated to be compensated by increasing the concentration of counterion, and less by the expulsion of co-ion.

From Table II, for $I = 0.04$ the ratio is 0.5, for $I = 0.08$ it is 0.36 and for $I = 0.16$ it is 0.26. For protein in solution it was then assumed that $V_d = (\text{ratio})\,\bar{Z}_A(W/\rho)$. These ratios were used at corresponding I for all systems. The results for α_s-casein, β-casein, and the mixture are shown in Tables II, III, and IV. Column 1 gives log $[\mathrm{Ca}^{2+}]_s$; column 2, $G - U$; column 3, $\bar{\nu}_{\mathrm{CaA}}$; column 4, the Donnan correction $\bar{\nu}_{\mathrm{CaD}}$, in per cent of $\bar{\nu}_{\mathrm{CaA}}$; and column 5, $\bar{\nu}_{\mathrm{Ca,S}}$. Column 6 gives $\bar{Z}$. Comments on Tables II, III, and IV follow.

Except for a few values at lowest $\bar{\nu}_{\mathrm{Ca,A}}$, the Donnan corrections are approximately 15% of $\bar{\nu}_{\mathrm{Ca,A}}$. At a particular I, the correction is almost constant until precipitate formation, after which it decreases. For protein in solution this behavior is expected from the use of $(G - U)/\bar{Z}_A$ ratios, a concept consistent with the Debye–Huckel (1923) characterization of an ion atmosphere. In general, the Donnan correction decreases as ionic strength increases and $\bar{Z}$ decreases, as it should.

The importance of the selection of V_d in determining Donnan corrections has been examined. That reasonable values have been used is indicated by the following. Examination of the parameters of Tables II, III, and IV over the entire range of increasing $[\mathrm{Ca}^{2+}]_s$ shows that, with one exception, values of $\bar{\nu}_{\mathrm{Ca,S}}$ and $\bar{Z}$ increase monotonically, and $G - U$ decreases monotonically, as expected. Monotonic increases are also calculated, generally, for two additional untabulated parameters: the ionic strength and calcium concentration in the Donnan region.

However, for β-casein there are slight discontinuous decreases in the ionic strength and calcium concentration in the Donnan region as the systems at $I = 0.04$ and $I = 0.08$ go from solution to precipitate. For the α_s–β-casein mixture at $I = 0.04$, the decrease is pronounced. These decreases suggest that solution V_d near precipitation have been underestimated for these systems, and this would be consistent with the observation that the core polymer solvation of the mixture is higher than that for α_s-casein alone (Waugh *et al.*, 1970).

The possibility that the V_d have, if anything, been underestimated prompted an examination of the effects of increasing V_d. Calculations show that physically unrealistic increases in V_d would be required to decrease Donnan corrections by only 10% of their recorded values. Small increases would give monotonic sequences in all parameters. The corrections given in Table II, III, and IV therefore appear to be reasonable.

Each protein initiates precipitation, independent of I, at about the same values of $\bar{\nu}_{\mathrm{Ca,S}}$ and $\bar{Z}$. These are respectively, for α_s-casein, β-casein, and the α_s–β-casein mixture; $\bar{\nu}_{\mathrm{Ca,S}} = 9.3$, 5.4, and 10.4, and $\bar{Z} = -9$, -6, and -1.5. It is clear that the mixture is not intermediate, but has the largest $\nu_{\mathrm{Ca,s}}$ and smallest $\bar{Z}$. The same is evident for $G - U$: at precipitate initiation, α_s-casein has the largest value ($\sim$3), β-casein is intermediate ($\sim$2.1), and the α_s–β-casein mixture is least (1.9).

At high $\bar{\nu}_{\mathrm{Ca,S}}$ there is apparently a large reversal of monomer net charge for all systems. However, $G - U$ and solubility do not reverse significantly. Since ionic strength alone could not cause precipitation using β-casein or the α_s–β-casein mixture (Waugh *et al.*, 1970), it is proposed that an important source of attractive energy for precipitation is cross linking of core polymers by calcium. Other interaction bond types are probably also involved.

Interactions between α_s- and β-caseins appear to alter significantly inter- and intracore polymer interactions. For example, while both α_s- and β-caseins alone precipitate at relatively high $\bar{Z}$, the mixture requires a low $\bar{Z}$. It has been shown that α_s- and β-caseins comingle at the monomer level (Waugh *et al.*, 1970). The present results suggest that when either protein alone forms a core polymer, interpolymer cross-linking by calcium is favored compared to the mixed polymer. In the latter, acidic peptides may interact in such a way as to convert potential interpolymer cross-linking sites into intrapolymer sites.

The data given in Tables II, III, and IV are based, to as great an extent as possible, on calculations which are independent of specific peptide and body structure, and of the way in which monomers interact in developing polymers and precipitate. In a following publication specific models are examined. Binding constants, including that of sodium, are assigned, and further possible characteristics of interaction are revealed.

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