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Effect of calcium on the hydration of casein

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Effect of calcium on the hydration of casein. I. Water vapour sorption and fine structure of calcium caseinates compared with sodium caseinates in the pH range 4·6–8·0

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Summary. Hydration of Ca and Na caseinates which were prepared from whole casein at different pH levels (range 4·7–8·0) was determined by means of water vapour sorption measurements in the water activity (a_w) range 0·58–0·95. Water uptake of Ca caseinates was systematically lower than that of Na caseinates prepared at equal pH, the differences increasing with increasing pH and a_w. Plots of the water content *v.* the amount of added hydroxide at constant a_w revealed a linear relationship between water uptake and cation content, suggesting that the increase in water uptake is mainly determined by the amount and type of cations associated with the caseins. In the a_w range tested, Ca^{2+} adsorbed about 2–7 and Na^+ 3–18 mol water/mol of cation. This implies that replacement of one mol of casein-bound Na^+ by Ca^{2+} is accompanied by a displacement of 1–11 mol water at a_w 0·58–0·95. The loss of water is a consequence of conformational changes induced by the chelating and cross-linking effects of Ca^{2+}, which also lead to micelle formation.

The interaction between Ca and caseins plays a key role in the formation and structure of casein micelles. It also influences the behaviour of the caseins during the manufacture of milk products. The most striking effects of the binding of the divalent ion to whole casein, or its principal components, α_{s1}- and β-casein, are the aggregation and the decrease in solvation. Isolated κ-casein, which comprises about 14 % of whole bovine casein, differs somewhat in its behaviour. It remains soluble even at high concentrations of Ca^{2+}. In the presence of the divalent ion, κ-casein forms micelles with either α_{s1}- or β-casein, stabilizing the latter against precipitation. Ca-induced aggregation and precipitation of caseins have been investigated in great detail using a variety of methods. However, little information is available on Ca-induced changes in hydration. A decrease in the amount of solvation water has been observed when Ca^{2+} was added to purified caseins (Waugh *et al.* 1971; Slattery & Waugh, 1973) or casein micelle suspensions in dilute solutions (Green & Marshall, 1979). Also, a negative correlation has been found between the Ca content of casein micelles and their solvation (Sood *et al.* 1979*a*, *b*).

Ca-binding by caseins is pH-dependent (Carr, 1953; Zittle *et al.* 1958; Dickson & Perkins, 1971). A pH increase is generally accompanied by a greater Ca-binding capacity. Also, a pH shift is observed upon Ca-binding by caseins, due to the displacement of protons (Kiermeier, 1952; Kiermeier & Galanos, 1957; Waugh *et al.* 1971). The solvent systems used to study the influence of pH on Ca-binding and hydration usually contained both Ca^{2+} and monovalent cations, mainly Na^+.

Therefore, the changes in hydration observed were to some extent caused by the transformation of Na into Ca caseinate. A study of the water-binding properties of the pure caseinates is an essential preliminary to a better understanding of the way in which Ca^{2+} interacts with casein and influences its hydration. Furthermore, it seems worthwhile to make this type of study not only in dilute solutions but also in semi-dry systems with reduced water activity (a_w). Various milk products in which the interaction of Ca with casein is important have low a_w levels compared with those of most model systems used in the past.

The present paper is concerned with hydration of pure Ca and Na caseinates in the a_w range 0·58–0·95. Water vapour sorption was measured as a function of pH and water activity, using isopiestic techniques. In addition, the fine structure of the caseinates was studied by transmission electron microscopy.

MATERIALS AND METHODS

Materials

Whole casein was prepared from milk of Simmental cows by acid precipitation. With vigorous stirring diluted skim-milk was slowly brought to pH 4·6 using 5% acetic acid. The precipitate was washed 3 times with deionized water and redissolved in water at pH 7·0 with the aid of 2 M-NaOH. After removing undissolved particles by filtration the precipitation procedure was repeated twice and the final preparation lyophilized. Electrophoretic examination on polyacrylamide gel (Groves & Kiddy, 1968) revealed a mean composition of 47% a_s-, 38% β-, 15% κ-, and 1·4% γ-casein. The acid casein contained 75 μmol Ca, 13 μmol Mg, 500 μmol Na, 41 μmol K and 25 μmol P/100 g dry protein. The cations were analysed by atomic absorption spectrophotometry after mineralization in hot 65% nitric acid. Phosphorus content was measured using a standard photometric method (Schweizerisches Lebensmittelbuch, 1969).

To obtain Ca and Na caseinates at various pH levels, 5 g portions of acid casein were dispersed in 50 ml water and 0·01 M-$Ca(OH)_2$ or 0·1 M-NaOH were added. The suspensions were allowed to equilibrate over 3 d and then lyophilized. The pH range tested (pH 4·7 to about 8·0) corresponded to a cation content of 0·07–45 mmol Ca or 0·50–84 mmol Na/100 g dry casein.

Methods

Water sorption measurements. Water vapour sorption isotherms at 25·0 ± 0·1 °C were determined using an isopiestic method. The apparatus and technique of this method are described in detail by Gál (1975) and Gál & Hunziker (1977). The following saturated salt solutions were used to obtain equilibrium water contents in the water activity range 0·58–0·95: NaBr ($a_w = 0{\cdot}576 \pm 0{\cdot}004$), KI ($0{\cdot}689 \pm 0{\cdot}002$), NaCl ($0{\cdot}753 \pm 0{\cdot}001$), KCl ($0{\cdot}843 \pm 0{\cdot}003$), $CaCl_2$ (0·902), and KNO_3 ($0{\cdot}936 \pm 0{\cdot}006$) (Stokes, 1949; Greenspan, 1977). The highest humidity level ($0{\cdot}950 \pm 0{\cdot}001$) was produced with a sulphuric acid solution (Rüegg, 1980). In preliminary measurements, the dry samples were exposed directly to each humidity (integral sorption). This procedure was found not to be suitable because of extremely long equilibration times and irregular weight gain curves. Better results were achieved by increasing the humidity stepwise from the lowest to the highest level at 7-d intervals (differential sorption). The values of equilibrium water content are based on the dry weight obtained after heating for 6 h at 60 °C in a vacuum oven and subsequent cooling over P_2O_5.

Mathematical analysis of sorption data. The sorption equation according to the model of Guggenheim, Anderson and de Boer, (G.A.B. equation), proposed for food materials by van den Berg (1981), has been used to fit an isotherm to the experimental points. This 3-parameter equation can be rearranged into a second degree polynomial and is then mathematically identical to the isotherm equation derived from the model of Hailwood & Horrobin (1946):

$$\frac{a_w}{W} = \alpha \,.\, a_w^2 + \beta \,.\, a_w + \gamma$$

(W: water uptake on dry basis; α, β, γ: coefficients related to number of primary sorption sites, heat of sorption and factor correcting properties of water in multilayers). Regression analyses were made using BMDP computer programs (BMDP Statistical Software 1981, University of California Press, Berkeley, CA, USA) and special plots were drawn with the aid of DISSPLA subroutines (DISSPLA Version 8·2, Integrated Software Systems Corp., San Diego, CA, USA.)

Electron microscopy. The caseinates were fixed with glutaraldehyde and acrolein and embedded in agar for better handling during the dehydration and epoxy resin embedding procedure (Blanc *et al.* 1980). The very fine agar fibrils are visible in the electron microscope but can clearly be distinguished from casein aggregates (Fig. 1). The procedure of Goldsmith (1967) was used to estimate the distribution of casein particles from the distribution of their cross-sections observed in the electron microscope. (A slice thickness of 30 nm was assumed).

RESULTS AND DISCUSSION

When $Ca(OH)_2$ was added to the suspension of whole casein a colloidal dispersion of spherical particles was formed above a pH of about 5·8. The Ca caseinate particles had a volume/surface average diam. of $d_{vs} = 380$ nm. (The observed number-average diam. was 200 nm.) The distribution width, expressed as coefficient of variation of d_{vs}, was 54 %. Fig. 1 shows electron micrographs typical for the undissolved Ca caseinate in the pH region before and after formation of a colloidal dispersion. The material in the pH range 4·6 to ~ 5·7 appears mostly homogeneous. The spherical particles showed a substructure, but not as pronounced as natural and artificial micelles which also contain phosphate and citrate (Schmidt *et al.* 1974; Knoop *et al.* 1979).

During the course of titration with NaOH the casein gradually dissolved. Compared with the individual casein components (Bingham, 1971) the solubilization of whole casein occurred at higher pH values. The solubility increased from about 0·05 mg/ml at pH 5·7 to 2·0 mg/ml around pH 6·0.

At the same pH levels, the Na caseinates always absorbed a higher amount of water than in Ca caseinates. Fig. 2 shows 2 water sorption isotherms typical for the caseinates in the a_w range 0·55–0·95. The difference between the water uptake of Na and Ca caseinate increased with increasing pH and a_w. This can be clearly seen in Fig. 3, where the measured sets of isotherms are represented in 3-dimensional plots. The surface formed by the isotherms of Ca caseinates if flat over the whole range tested compared with that for Na caseinates.

The curves parallel to the pH axis in Fig. 3 represent isopsychric lines. They are not smooth because they were drawn by connecting points, representing equal a_w-levels, on the regression lines of the isotherms. Two isopsychric functions, one for a medium and the other for a high water activity, are shown in detail in Fig. 4. In

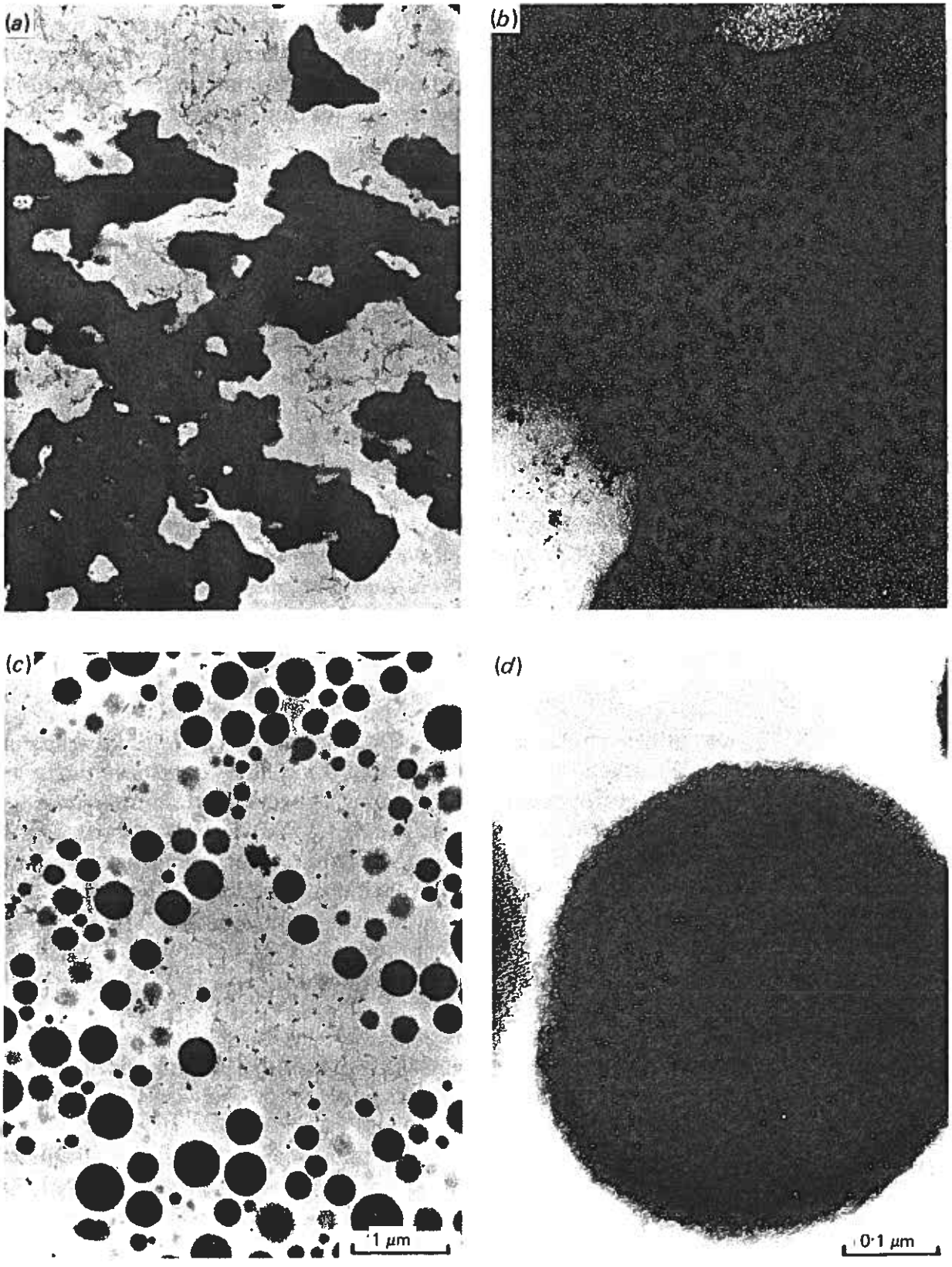

Fig. 1. Electron micrographs of Ca caseinates. (*a*) and (*b*) Fine structure of insoluble Ca caseinate at pH 5·1 (0·033 mmol Ca/g casein), typical for the samples in the pH range of 4·6 to 5·8 (0·002 to ~ 0·18 mmol Ca/g casein). (*c*) and (*d*) Micelles of Ca caseinate at pH 6·1 (0·24 mmol Ca/g casein), typical for the dispersions formed in the approximate pH range 5·8–8·0 (0·18–0·45 mmol Ca/g casein).

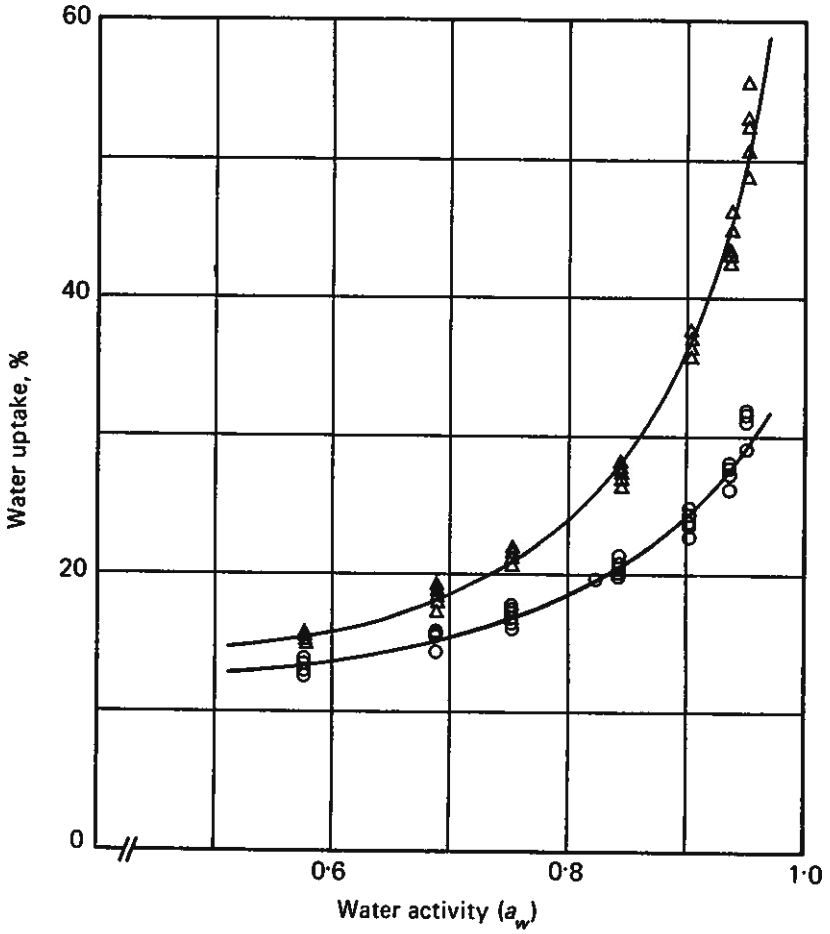

Fig. 2. Water sorption isotherm of Ca and Na caseinate at 25 °C and pH 7·5. The curves correspond to the G.A.B.-isotherm obtained by regression analysis. △, Na caseinate; ○, Ca caseinate.

the pH range 4·6 to about 5·5, the water uptake by the 2 caseinates was not significantly different. At higher pH levels, however, the isopsychric curves begin to separate. The separation coincides with the formation of Ca caseinate micelles.

In Figs 2 and 3 the comparison of water absorption by caseinates is made in relation to pH. It must be considered that in the case of Ca and Na caseinate an equal degree of protonization represents a difference in cation content of a factor of about 2. Therefore, it is meaningful to discuss the water sorption capacity of the caseinates also as a function of the amount of cation associated with the proteins. The data in Fig. 4(*a*) have been replotted in Fig. 4(*b*) as a function of the concentration of Ca and Na in units of mmol/g dry and cation-free casein. The experimental points now apparently follow a straight line. This phenomenon has already been observed for Na caseinates and casein hydrochlorides in a limited a_w and pH range (Signer & Gál, 1961; Rüegg & Blanc, 1976). Linear least squares regression lines are drawn in Fig. 4(*b*). Although close inspection of the residuals of the regression analysis indicated a sigmoid shaped isopsychric curve, the deviation from linearity was not statistically significant. The slope and intercept values for the regression lines are given in Table 1. The intercept corresponds to the water content of whole casein at pH 4·7, before the addition of hydroxide. The slope values represent the contribution of the protein-bound cations. The apparently linear relationship suggests that the increase in water uptake is mainly determined by the amount and type of cations bound by whole casein. The amount of water associated with the peptide chain seems to play a minor role. The slope values thus are an estimate of the hydration of the casein-bound cations in units of g water/100 mmol of Ca^{2+} or Na^{+}.

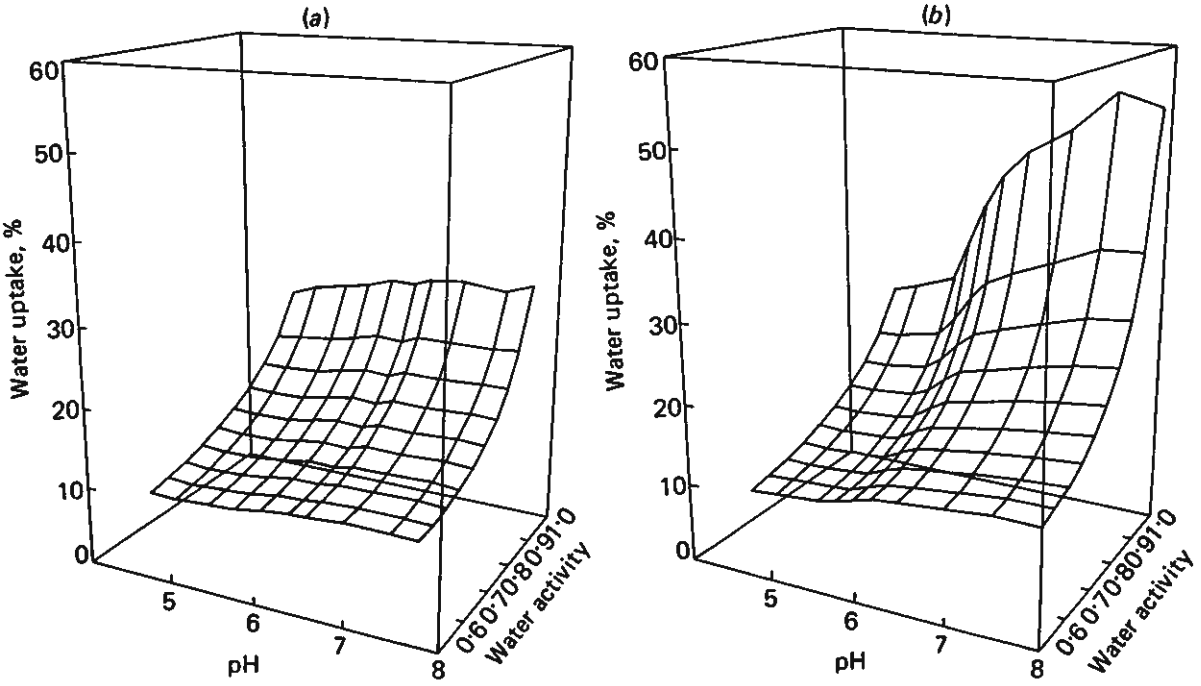

Fig. 3. Water uptake of (*a*) Ca and (*b*) Na caseinates in the high a_w-range as a function of pH at 25 °C. The 2 sets of sorption isotherms (water content *v.* a_w functions) were obtained by regression analysis of the experimental points and represent G.A.B.-functions (details are given in the experimental section). Water content *v.* pH curves in the surface are isopsychric lines.

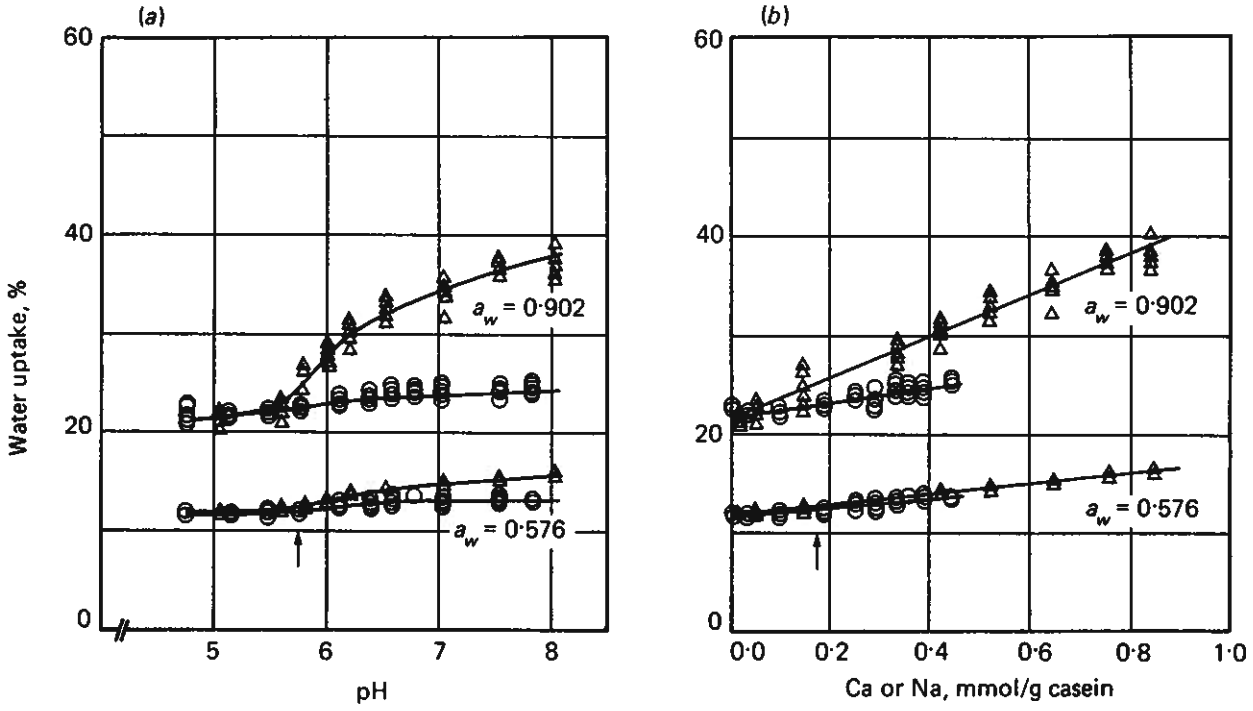

Fig. 4. Isopsychric curves for Ca and Na caseinates at 25 °C. (*a*) Water uptake at a_w = 0·576 and 0·902 as a function of pH. (*b*) Same data plotted as a function of the amount of $Ca(OH)_2$ and NaOH added to isoelectric whole casein. Linear least squares regression lines are shown for the data in Fig. 4(*b*). Arrows point to the region of formation of Ca caseinate micelles. (Ordinate: water content on dry and metal-free basis). △, Na caseinate; ○, Ca caseinate.

Table 1. *Isopsychric lines for Ca and Na caseinates. Regression coefficients of the apparent linear relationship between water uptake (W) at 25 °C and molar concentration of cations (c): $W = \alpha + \beta . c$**

	Ca caseinate					Na caseinate				
a_w	α	s_α	β	s_β	R^2	α	s_α	β	s_β	R^2
0·576	11·59	0·10	4·60	0·34	0·705	11·81	0·04	5·11	0·09	0·979
0·689	13·46	0·10	4·87	0·36	0·714	13·47	0·10	6·95	0·22	0·943
0·753	15·09	0·13	5·12	0·43	0·639	15·21	0·07	8·57	0·15	0·980
0·843	18·22	0·11	6·44	0·36	0·801	18·26	0·11	12·90	0·24	0·979
0·902	21·58	0·14	7·45	0·48	0·751	21·68	0·23	20·43	0·50	0·964
0·936	23·99	0·19	10·17	0·67	0·745	24·46	0·37	26·27	0·84	0·939
0·950	26·65	0·28	12·52	1·00	0·685	28·14	0·48	32·40	1·03	0·944

* Water uptake (W) in g/100 g dry and cation-free protein; cation concentration in mmol/g protein.
s_α, s_β, standard deviations.
R^2, coefficient of determination.

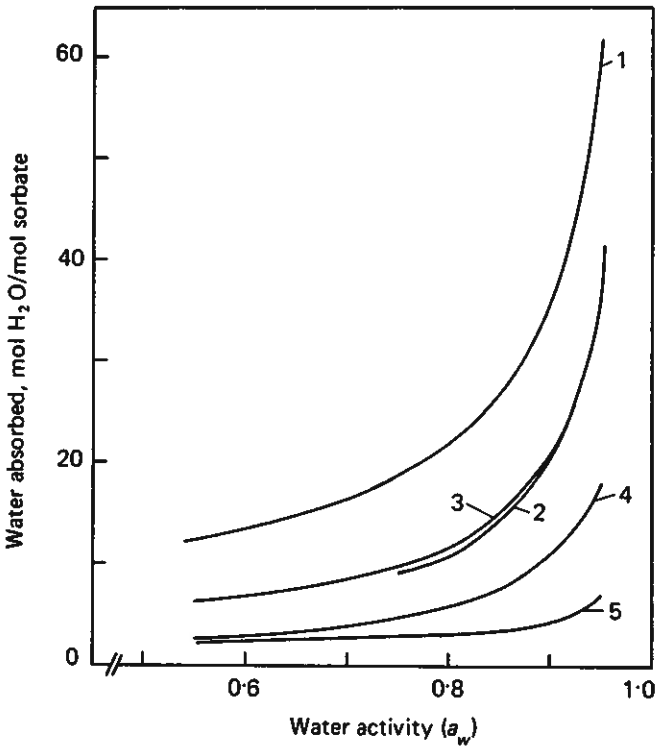

Fig. 5. Hydration of Ca^{2+} and Na^+ in caseinates in the a_w-range 0·58–0·95, compared with the hydration of the corresponding chlorides and NaOH. The sorption isotherms for the individual cations in the caseinates were estimated from the slopes of the isopsychric curves (see Fig. 4(*b*) and Table 1). The isotherms for the chlorides and for NaOH were calculated from data of Robinson & Stokes (1959). 1, $CaCl_2$; 2, NaCl; 3, NaOH; 4, Na^+ (caseinate); 5, Ca^{2+} (caseinate).

It is interesting to note that the hydration of casein-bound Na^+ is systematically higher than that of Ca^{2+}. This can clearly be seen in Fig. 5, which shows the water sorption isotherms of the casein-bound cations. (The slope values in Table 1 have been recalculated on a molar basis and plotted against the water activity). For comparison, water uptake of NaCl, NaOH and $CaCl_2$ has been calculated from other data (Robinson & Stokes, 1959) and the corresponding curves included in Fig. 5. In protein-free aqueous salt solutions, primary hydration numbers for Ca^{2+} are systematically greater than for Na^+. From entropy and ion-mobility data, the number of tightly bound water molecules in a first shell has been estimated as 7–12 for Ca^{2+} and 2–4 for Na^+ (see e.g. review by Kortüm, 1962). Ions of high charge density usually increase

the order of the water structure and have higher primary hydration numbers. The different situation in caseinate systems can be explained by the chelating and cross-linking effect of Ca^{2+}. For steric reasons, water is displaced from the primary and secondary shell of hydration of Ca^{2+} after binding to deprotonized groups at protein side chains. The chelating and cross-linking effects eventually lead to micelle formation. Hydrophobic interactions are also important in the polymerization process as electrostatic repulsion is reduced by Ca^{2+}-binding (Slattery, 1979).

Fig. 5 also shows that the difference between the hydration of Ca^{2+} and Na^{+} in caseinates increases from about 1 to 11 mol water/mol cation in the a_w range 0·70–0·95.

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Der Einfluss von Calcium auf die Hydratation des Caseins

I. Wasserdampf-Sorption und Feinstruktur von Calciumcaseinat im Vergleich zu Natriumcaseinat im pH-Bereich von 4.6 bis 8.0

Zusammenfassung

Ca- und Na-Caseinate wurden aus isoelektrischem Casein durch Titration mit $Ca(OH)_2$ und NaOH bei verschiedenen pH-Werten hergestellt und deren Wasserbindungsvermögen mit Hilfe von Sorptionsmessungen im a_w-Bereich von 0.58 - 0.95 gemessen. Ca-Caseinate nahmen im Vergleich zu Na-Caseinaten, die bei gleichen pH-Stufen präpariert wurden, durchwegs weniger Wasser auf. Der Unterschied nahm mit steigenden pH- und a_w-Werten zu. Es wurde eine lineare Abhängigkeit der Wasseraufnahme von der zugesetzten Kationenmenge festgestellt. Dies deutet an, dass die Erhöhung der Wasseraufnahme weitgehend durch die Art und Menge der zugesetzten Kationen bestimmt wird und dass strukturelle Aenderungen der Proteine eine untergeordnete Rolle spielen. Im untersuchten a_w-Bereich adsorbierten die gebundenen Ca^{2+}-Ionen 2-7 und die Na^+-Ionen 3-18 Mole Wasser pro Mol Kation. Dies bedeutet, dass im a_w-Bereich 0.58–0.95 der Austausch eines Na^+-durch ein Ca^{2+}-Ion am Caseinat mit der Verdrängung von 1-11 Mol Wasser verbunden ist. Die Verdrängung des Wassers kann mit der chelierenden und vernetzenden Wirkung des zweiwertigen Kations erklärt werden, die auch zur Mizellenbildung führt.

Effect of Calcium on the Hydration of Casein. II. Binding of Calcium Chloride to Whole Casein and Calcium Caseinate

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The effect of $CaCl_2$ on water vapour sorption by whole casein and Ca caseinate was investigated in the water activity range a_w = 0.58–0.95. The $CaCl_2$–Ca caseinate mixtures adsorbed more water than isoelectric casein. The water uptake of the mixtures was lower than the sum of the equilibrium water contents of the pure components, because of salt binding, which also decreased the pH. The maximum salt binding capacity could be estimated from plots of water uptake versus $CaCl_2$ content (isopsychric curves). An equation was derived which allowed estimation of the binding capacity from plots of the isopsychric points using nonlinear regression procedures. It was found that binding of $CaCl_2$ by casein increased with decreasing a_w, from very low values near a_w = 1.0 to values greater than 30% (g salt/100 g salt-free casein) at a_w = 0.58. Ca caseinate had a lower binding capacity for $CaCl_2$ than did whole casein.

In a previous article, the effect of replacing sodium by calcium ions in caseinates at various pH levels upon protein hydration was discussed (1). It was shown that binding of Ca^{2+} is accompanied by a significant displacement of water, which also leads to micelle formation. The loss of water was determined quantitatively from water sorption isotherms in the water activity range a_w = 0.58–0.95. The present study investigates the effect of $CaCl_2$ addition to whole casein and Ca caseinate on protein hydration, as well as the influence of a_w on the calcium binding capacity of the caseins.

Many reports are available in the literature concerning the binding of Ca^{2+} and other electrolytes by whole casein and its pure fractions. A summary of some literature data on the calcium binding of caseins is shown in **Table 1**. When comparing the values compiled in this Table it is necessary to take into account that binding of Ca to caseins depends on metal ion concentration, pH value and ionic strength, as well as on temperature (2). The relative order of binding capacities was found as α_s > whole casein > β > κ (3, 4). Literature values of the binding constants for the complexes of Ca^{2+} with whole casein for pH levels between about 5.9 and 7.7 range from pK = 2.2–2.8 (5–7). For α_s-casein, apparent pK values in the range 0.8–16 have been reported (2,3). These studies were carried out in dilute solutions containing mostly Ca^{2+} but with other cations from the buffers used for pH control. It was considered useful to study the calcium binding of casein in the absence of other types of cations and also in systems with reduced water activity. Many milk products in which the interaction and diffusion of Ca is important have low a_w levels compared with those of most model systems used in the past (8). Therefore the results obtained for those dilute systems are not transferable to milk products at reduced a_w.

Information concerning electrolyte–protein interactions at reduced a_w can be obtained from water sorption measurements. For example, the phase diagram of the casein–NaCl–water system was determined by Gal and Hunziker (9,10) from water sorption isotherms. The present paper reports the results of a similar study of the casein–$CaCl_2$–water system in the a_w range 0.58–0.95. Metal-ion-free whole casein, isolated near its isoelectric point, was investigated as well as Ca caseinate prepared at a pH near that of milk, in order to obtain additional information concerning the effect of pH and presence of cations

Table 1 Literature data on maximum calcium binding capacity of caseins in dilute solutions

	Ca^{2+} bound (mmol/100 g casein)				
pH	Whole casein	α_s	β	κ	Reference
5.6/6.2/7.3/9.1	0.1/1.1/9.9/13	—	—	—	Car (7)
5.0/5.7/6.5/7.4	0/15/28/35	—	—	—	Zittle *et al.* (20)
7.4	—	36	20	11	Dickson and Perkins (3)
6.6	—	73	41	—	Waugh *et al.* (13)
7.0	—	43	—	—	Holt *et al.* (21)
6.9	16	25	14	6	Imade *et al.* (4)
7.1	—	40	29	11	Yoshikawa *et al.* (22)
7.4	11*	—	—	—	Rajput and Ganguli (23)
4.6	0.2		—	—	Sadhukhan and Chattoraj (24)

* Buffalo casein; —, not determined

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on salt binding. The article also includes a new mathematical approach to determine the salt binding capacity of proteins from isopsychric curves.

Materials and Methods

Preparation of materials

The preparation and composition of whole casein from the milk of Simmental cows has been described previously (1). Calcium caseinate was prepared by adding 0.01 M $Ca(OH)_2$ to acid casein dispersed in water until a pH value of 6.7 was attained (approximately 34 mmol/100 g casein). The suspension was allowed to equilibrate overnight. The small pH shift occurring during equilibration was corrected with $Ca(OH)_2$ before freeze drying.

Mixtures of casein and $CaCl_2$ were obtained by resuspending 4-g portions of acid casein or Ca caseinate in 100 ml of water and adding 2.8–34.8 g of $CaCl_2$ per 100 g of casein (25–314 mmol/100 g casein). The mixtures were stirred and allowed to stand for six days at 5°C. The pH shift was measured and the mixtures freeze-dried.

Methods

Water vapour sorption isotherms at 25°C were determined in the a_w range 0.58–0.95 using a gravimetric isopiestic method (10). Saturated salt and sulphuric acid solutions were used to obtain the desired water vapour pressures, and the a_w-dependent water uptake of the mixtures was determined by increasing the humidity stepwise from the lowest to the highest level at seven-day intervals (1). Salt-free acid casein, Ca caseinate and $CaCl_2$ were measured under the same conditions in order to obtain reference values for the unreacted components. For mixtures containing less than 10% $CaCl_2$, seven or eight determinations were performed at each a_w level. At higher concentrations, about 12 measurements were made because of the increasing experimental error. The dry weight of the samples was determined after the adsorption measurements by predrying for 3 hours at 105°C in a vacuum oven and subsequent heating for 6 hours at 105°C.

For graphical representation of the isotherms the coefficients of the so-called G.A.B. equation (Guggenheim–Anderson–De Boer model (11)) were calculated. Regression analyses were done using BMDP computer programs (BMDP Statistical Software, 1981, University of California Press, Berkeley, CA, USA), and DISSPLA routines were used to draw special plots (DISSPLA Version 8.2, Integrated Software Systems Corp., San Diego, CA, USA).

Results and Discussion

When $CaCl_2$ was added to the suspensions of acid casein or Ca caseinate the pH decreased due to the displacement of protons upon Ca binding (12, 13). The observed pH shifts, which are shown graphically in **Fig. 1**, were smaller than those reported by Kiermeier and Galanos (12) for acid and renneted casein, although these authors used shorter equilibration times. The pH shift in the Ca caseinate suspensions was 2.5–3 times greater compared to that for isoelectric casein. **Figure 1** shows a steep decrease in the pH curves up to about 10% $CaCl_2$, followed by a levelling off at about −0.22 and −0.58 pH units at 40% $CaCl_2$. **Figure 2** shows three water sorption isotherms typical of the mixtures in the a_w range 0.58–0.95. The experimental error increased with increasing a_w and $CaCl_2$ content. For example, the relative standard deviations of the measurements shown in **Fig. 2** increased in the a_w range tested from 1–4%, 3–6% and 8–11% for 0, 12.5 and 34.8 g $CaCl_2$ per 100 g casein, respectively. The dependence of the experimental error on a_w is inherent to sorption measurements at high humidities. Small inhomogeneities in the distribution of the unbound $CaCl_2$ within the mixtures are probably the main reason for the increase of the standard deviation at higher $CaCl_2$ contents.

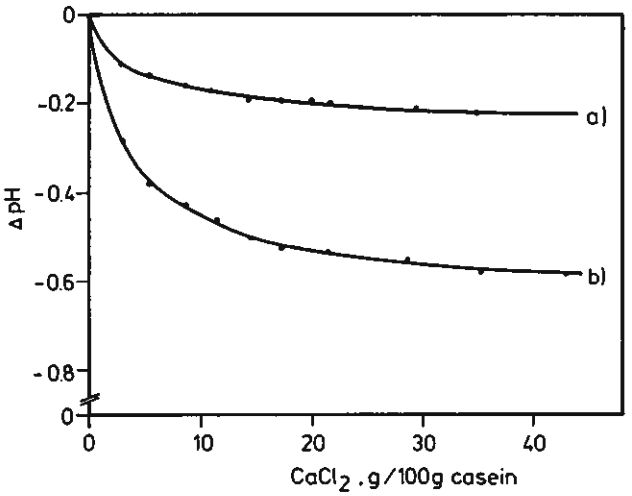

Fig. 1 Proton release after addition of calcium chloride to suspensions of (a) whole casein and (b) Ca caseinate at 20°C. pH values and protein concentrations before salt addition were (a) 4.79, 7.3 g/100 ml H_2O and (b) 6.77, 6.7 g/100 ml H_2O

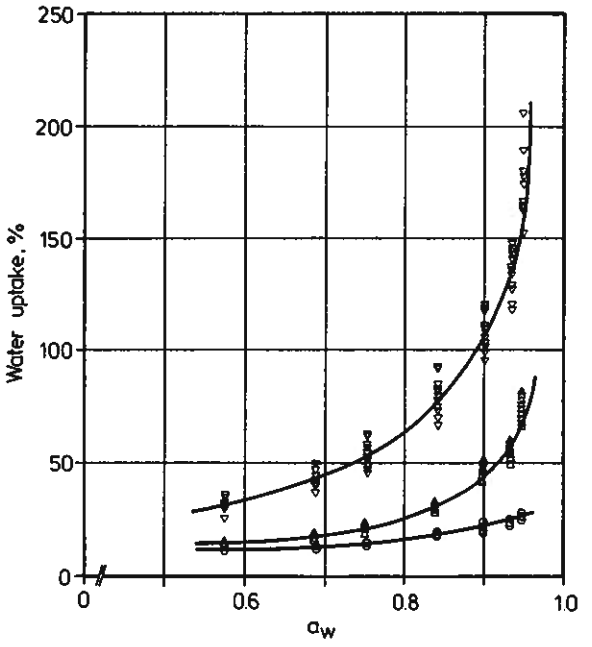

Fig. 2 Water sorption isotherms for whole casein and mixtures of casein and $CaCl_2$ at 25°C. Vertical scale: g water/100 g dry mixture. ○, Pure whole casein, isolated at pH = 4.6; △, 2.5% $CaCl_2$; ▽, 34.8% $CaCl_2$ (g salt/100 g dry and salt-free casein). The total numbers of measurements were (○) 60, (△) 101 and (▽) 92

The complete sets of sorption data, a total of 817 measurements for acid whole casein and 874 points for Ca caseinate, are represented graphically in **Fig. 3**. The smoothed isotherms (water uptake *versus* a_w curves) were drawn using the G.A.B. equation. The lines parallel to the $CaCl_2$ axis connect points at equal a_w and are usually termed isopsychric curves. The unsmoothed isopsychric lines in **Fig. 3** are drawn at a_w = 0.50–0.90 in intervals of 0.05 and at a_w = 0.96. In **Fig. 3** the surface for Ca caseinate lies above that for isoelectric whole casein, indicating that the former preparations systematically adsorbed more water under identical conditions.

Isopsychric curves have been used previously by various authors to investigate the interaction of salt with proteins (9, 14–

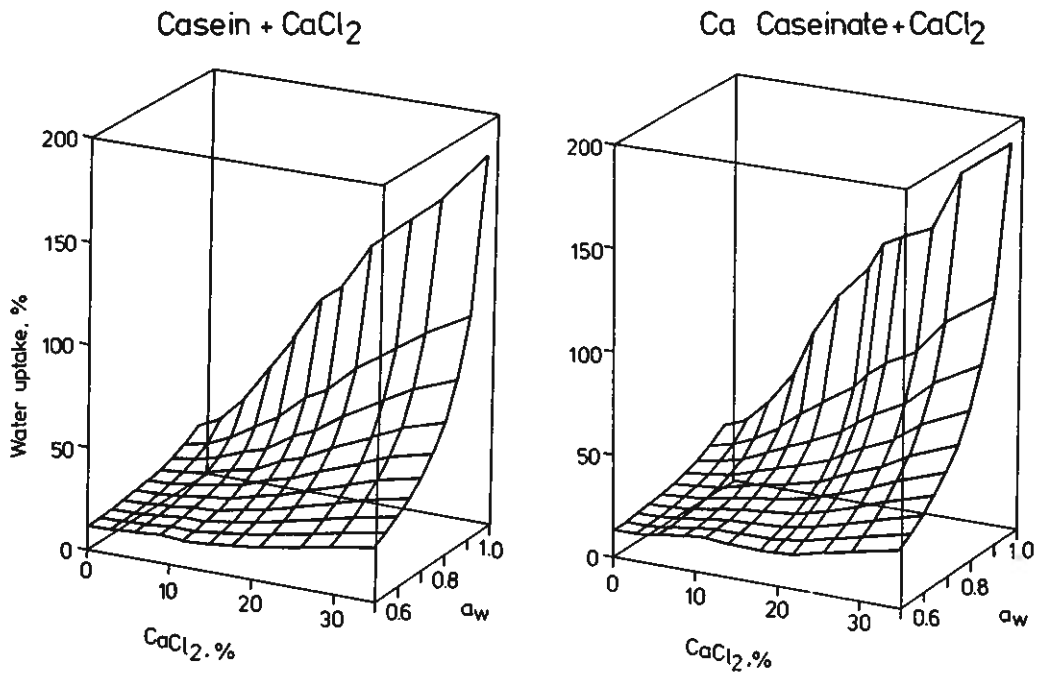

Fig. 3 Water uptake of mixtures of casein and Ca caseinate with $CaCl_2$ in the high a_w range at 25°C. The smoothed water content *versus* a_w curves were drawn using the G.A.B. equation. The unsmoothed lines parallel to the salt-content axis are isopsychric curves (water uptake as a function of salt content at constant a_w). The complete sets of data include 817 (casein) and 874 (Ca caseinate) measurements

18). The most detailed studies were carried out by Gal (9) on the casein–NaCl–water system, where a nearly complete phase diagram could be established. The information contained in isopsychric lines is most evident if the water uptake is expressed in mass of absorbed water per mass of salt-free and dry protein. If the salt content on the horizontal axis is given in mass of salt per dry mass of protein, the slope of the isopsychric lines corresponds to the water content of the salt. In the case of noninteracting components, the isopsychric lines should therefore be linear functions with slope values determined only by the a_w values and the type of solute. Deviations from straight lines indicate changes in the hydration properties associated with salt binding processes. **Figure 4** shows isopsychric plots for a_w = 0.68 and 0.90 typical of the two casein preparations. The chain-dotted lines represent the summed water uptakes of the pure components at the same water activity. It is evident that the interaction of the electrolyte with the proteins decreases the water binding capacity of the system. The difference between the hypothetical water uptake of the individual components and that of the mixture increases with increasing electrolyte content and decreasing a_w. At high $CaCl_2$ contents the experimental points may be represented by a straight line parallel to that for the pure components, suggesting the presence of unbound solute. The region where the initial curve develops into the straight isopsychric line reflects saturation of the protein with $CaCl_2$. Close inspection of the course of the isopsychric lines at the three lower a_w levels tested reveals a possible step in the initial part of the curve, at around 10–15% $CaCl_2$. However, the number of data on the horizontal axis is not sufficient for a quantitative analysis of this feature. The small plateau could be

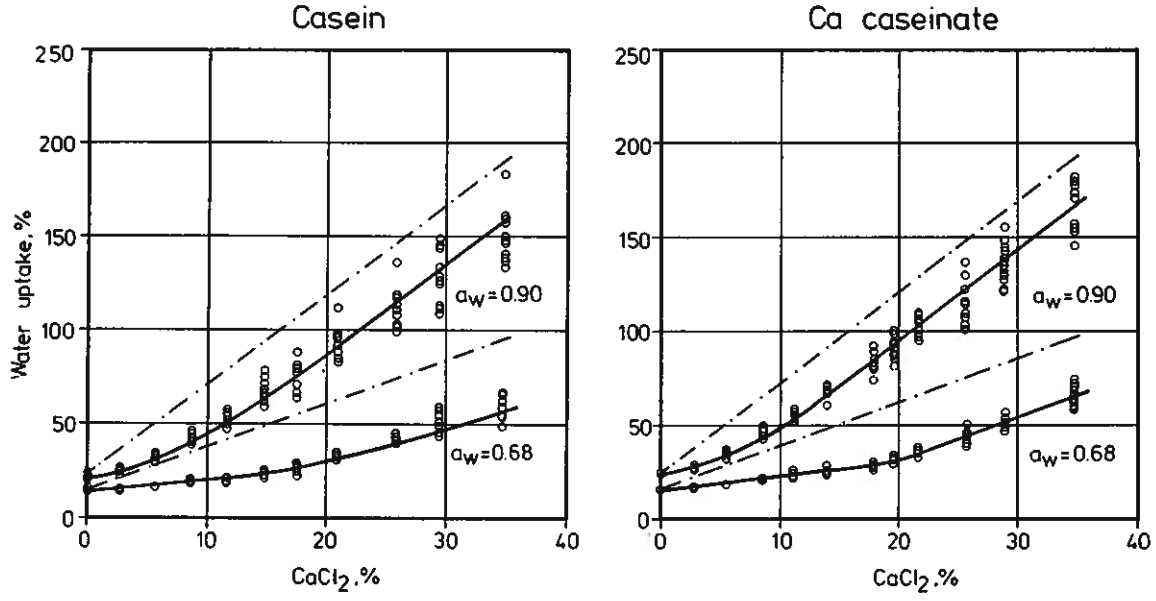

Fig. 4 Water uptake of whole casein and Ca caseinate at a_w = 0.68 and 0.90, as a function of $CaCl_2$ content (isopsychric curves at 25°C). Water content on ordinate is on a dry and salt-free basis. The chain-dotted lines correspond to the water uptakes of the pure protein and salt at the same a_w

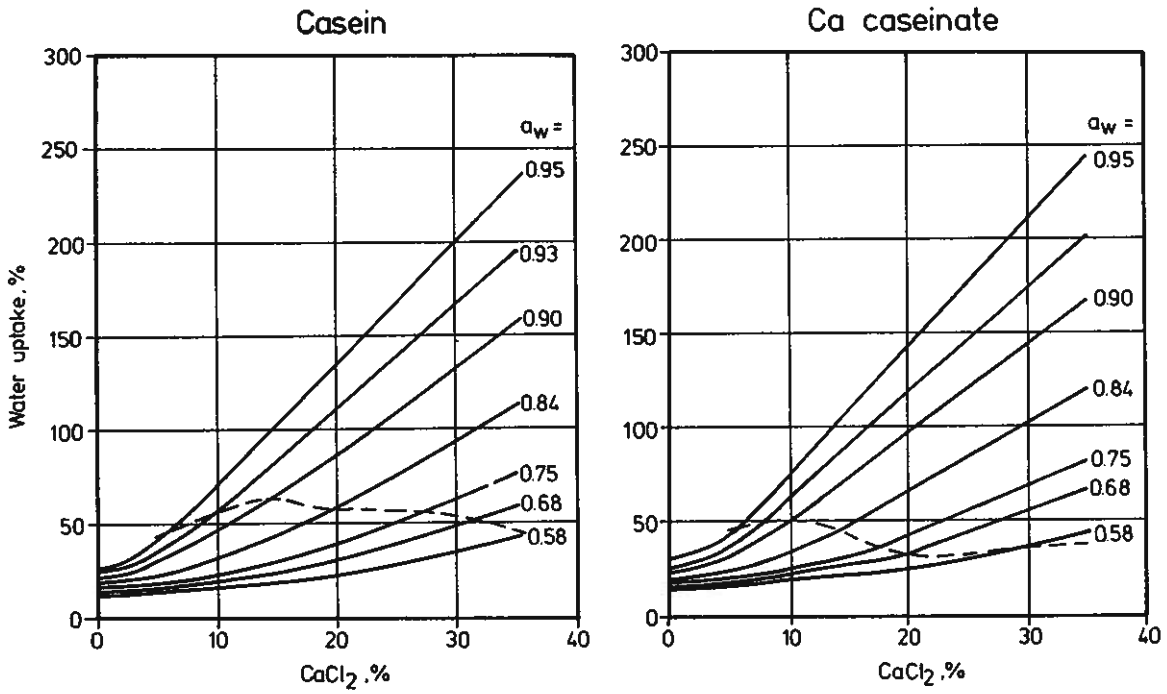

Fig. 5 Phase diagram for the casein–$CaCl_2$–water system in the a_w range 0.58–0.95. The dashed lines indicate the maximum $CaCl_2$ binding capacity of the proteins. The regions of saturation with salt were estimated from the isopsychric curves (transition of initial curve to final straight part)

explained by successive saturation of different types of binding sites or by conformational changes in the protein. The entire set of isopsychric lines is shown in **Fig. 5**. The maximum binding capacities are indicated by dashed lines. The salt content corresponding to saturation of the binding sites can be determined graphically from the isopsychric lines as suggested by Bull and Breese (14) and Gal and Hunziker (10). An attempt was made to estimate the saturation values in an objective way by mathematical procedures. The approach is based on a sorption model which considers the hydration of the pure components and the contribution made by the bound electrolyte. A five-parameter equation was obtained which describes the course of the isopsychric curves. The maximum salt binding capacity could then be estimated using nonlinear regression procedures. The development of the equation is described in detail in the Appendix. The values obtained from the coefficient of the equation are shown in **Fig. 6**. With the exception of the lowest a_w level, the maximum binding capacities estimated according to the two procedures (i.e. graphically and by curve fitting) are comparable. At the lowest a_w level a significantly higher saturation value is obtained from the fit of the experimental points to the equation. It must be considered that the isopsychric curves at low a_w exhibit a slight plateau at around 10–15% salt and that the equation does not account for stepwise saturation. Dalgleish and Parker (2), in their analysis of binding isotherms for Ca^{2+} on α_{s1}-casein in solution, considered a substitution parameter which took into account changes in the binding constant as successive cations

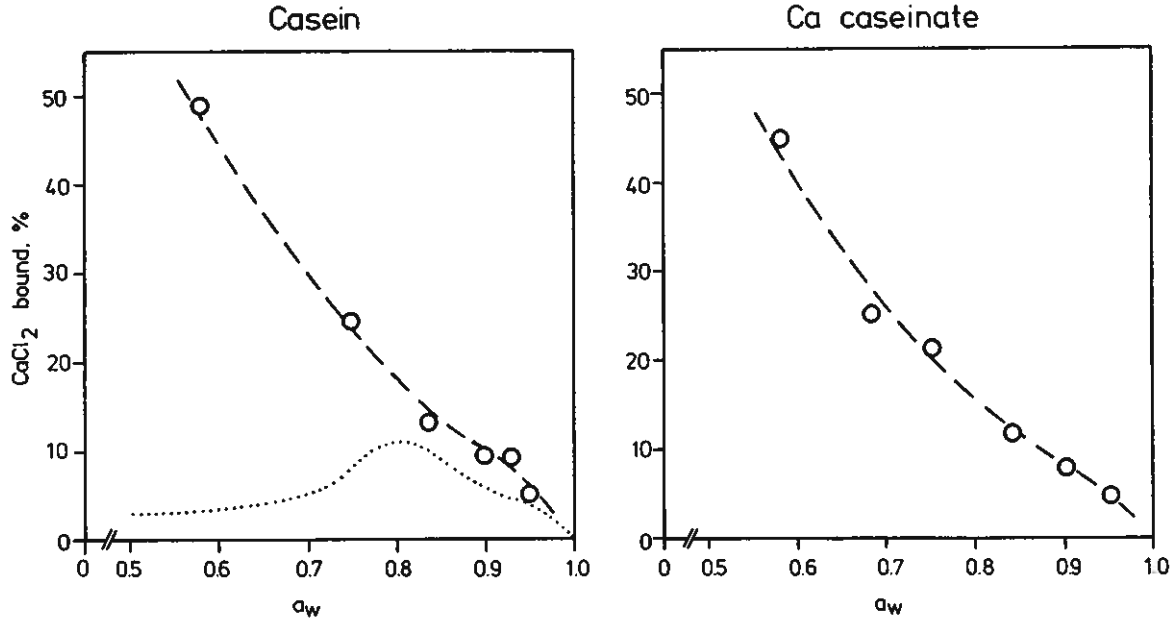

Fig. 6 Binding of $CaCl_2$ by whole casein and Ca caseinate in the high a_w range. Values were estimated by nonlinear regression from isopsychric curves. Vertical axis: g salt/100 g dry and salt-free casein. For comparison, the binding capacity of NaCl by whole casein, determined by Gal and Hunziker (9, 10), is shown as the dotted line

were bound to the protein. Isoelectric casein bound slightly more $CaCl_2$ than Ca caseinate.

For comparison, the binding curve of whole casein for sodium chloride, as determined by Gal and Hunziker (9, 10), is shown in **Fig. 6**. The binding capacity of casein is lower for NaCl than for $CaCl_2$. Furthermore, the binding curve for $CaCl_2$ does not show a maximum at around $a_w = 0.80$, a value which is in the region corresponding to a saturated NaCl solution (9). At $a_w = 0.58$ both casein and Ca caseinate bind more than 30% $CaCl_2$ (g/100 g salt-free and dry protein). The binding capacity decreases with increasing a_w and attains very low values near $a_w = 1.0$.

Summarising the results, it may be concluded that salt binding by caseins reduces hydration of the protein–electrolyte system and that the maximum salt binding capacity depends strongly on a_w. At reduced a_w the salt binding properties of isoelectric casein and of Ca caseinate are similar. Ca caseinate adsorbs more water but binds somewhat less salt. Caseins bind significantly more $CaCl_2$ than NaCl.

Acknowledgment

We are grateful to Dr S. Gal for valuable suggestions and his interest in this work.

Appendix

Derivation of an equation describing isopsychric curves for protein–salt–water systems

The water adsorbed by protein–salt mixtures (W_M) is equal to the sum of the water uptakes of the pure protein (W_P) and salt (W_S), if no interaction takes place. At constant a_w and temperature, it increases proportionally to the total salt content s:

$$W_M = W_P + W_S s \qquad \text{Eqn [1]}$$

Formation of a protein–salt complex changes the hydration. If the system is considered as a mixture of protein–salt complex, unbound salt and salt-free protein, the water sorption results from the sum of the following terms:

$$W_M = W_P f + W_S(s - s') + W_P f' + W'_S s' \qquad \text{Eqn [2]}$$

where f and f' are the mass fractions of unreacted protein and protein in the complex, and s' is the concentration of salt associated with the protein. It has been shown that the change in hydration of caseins after binding of cations or anions is determined mainly by the type and amount of ions, and that the protein *per se* does not change its sorption behaviour significantly (1, 18, 19). It may therefore be assumed that the water adsorbed by the protein part of the complex (W'_P) is about equal to that of the salt-free part (W_P). This assumption reduces Eqn [2] to:

$$W_M = W_P + W_S s - s'(W_S - W'_S) \qquad \text{Eqn [3]}$$

The third term in Eqn [3] includes the difference in hydration of the salt in solution (W_S) and in the associated form (W'_S). W'_S may be estimated from the isopsychric hydration curves for Ca caseinate and casein hydrochlorides. The slope of the isopsychric lines for these systems represents mainly the hydration of the ions. **Table 2** shows the values of W'_S used in this study.

The shape of the isopsychric lines shown in this article and reported for other protein–salt systems suggests a binding situation with a limited number of similar binding sites. The difference between the hypothetical straight line corresponding to Eqn [1] and the observed water content was therefore assumed to follow a Langmuir-type binding function. Replacing s', the amount of bound salt, in Eqn [3] by such a term gives:

$$W_M = W_P + W_S s - \frac{as}{b + s}(W_S - W'_S) \qquad \text{Eqn [4]}$$

Table 2 Hydration of Ca^{2+} and Cl^- in caseinates at various water activities, calculated from the slope of isopsychric hydration curves for Ca caseinates (1) and casein hydrochlorides (19)

	Hydration (g H_2O/mol)			
a_w	Ca^{2+}	Cl^-	$CaCl_2$	W'_S (g/100 g)
0.576	46	< 1	46	42
0.689	49	< 1	49	44
0.753	51	< 1	51	46
0.843	65	< 1	65	58
0.902	75	19	113	101
0.936	102	60	222	200
0.951	125	78	281	253

where a represents the saturation concentration and b is a constant, which in the Langmuir equation is related to the enthalpy of adsorption. W_P and W_S can be determined from the water sorption isotherm of the pure protein and salt.

List of symbols

- W_M water adsorbed by the protein–salt mixture (g/100 g dry and salt-free protein)
- W_P water adsorbed by pure protein (g/100 g dry protein)
- W'_P water uptake of protein in protein–salt complex (g/100 g dry and salt-free protein)
- W_S water adsorbed by unbound salt (g/100 g dry salt)
- W'_S water adsorbed by salt associated with protein (g/100 g dry salt)
- s total salt concentration (g/100 g dry salt-free protein)
- s' concentration of salt associated with protein (g/100 g dry protein)
- f mass fraction of unreacted protein
- f' mass fraction of protein in complex ($f + f' = 1$)
- a constant in Langmuir-type binding equation, related to total number of binding sites
- b constant in binding equation, related to binding energy

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Der Einfluss von Calcium auf die Hydratation des Caseins

II. Bindung von Calciumchlorid durch Casein und Calciumcaseinat

Zusammenfassung

Es wurde der Einfluss von $CaCl_2$-Zusätzen auf die Wasserdampfsorption von Casein und Ca-Caseinat im Wasseraktivitätsbereich von a_w = 0.58 bis 0.95 untersucht. Die $CaCl_2$-Ca-Caseinat Mischungen adsorbierten mehr Wasser als die $CaCl_2$-Casein Mischungen. Die Wasseraufnahme der Mischungen war geringer als aufgrund der Bindungsfähigkeit der isolierten, reinen Komponenten zu erwarten war. Die geringere Wasseraufnahme beruht auf der Bindung des Calciumchlorids durch Casein. Die Salzbindung erniedrigte ebenfalls den pH-Wert der Mischungen. Die maximale Salz-Bindungsfähigkeit konnte indirekt aus der Abhängigkeit der Wasseraufnahme vom $CaCl_2$-Zusatz ermittelt werden (Isopsychren). Es wurde ein mathematisches Modell entwickelt, das die Berechnung der maximalen Salz-Bindungsfähigkeit mit Hilfe nichtlinearer Regression ermöglichte. Die Bindung von $CaCl_2$ an Casein nahm mit abnehmendem a_w-Wert stark zu. Bei a_w = 0.58 betrug die Bindungsfähigkeit mehr als 30% (g $CaCl_2$/100 g salzfreies Casein). Ca-Caseinat hatte eine geringfügig kleinere Bindungsfähigkeit als Casein.