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Solvent-Mediated Disruption of Bovine Casein Micelles at Alkaline pH

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The disruption of casein micelles at alkaline pH was investigated using turbidity measurements. The rate and extent of disruption of casein micelles at alkaline pH (8.0−11.0) increased with pH. Furthermore, the extent of alkaline disruption increased with increasing temperature (5−40 °C). Preheating milk for 10 min at 90 °C did not influence the extent of alkaline disruption of casein micelles, suggesting that whey proteins do not influence the alkaline disruption process. Levels of ionic calcium and serum calcium and phosphate decreased in a logarithmic fashion with increasing pH, indicating precipitation of calcium phosphate onto the casein micelles. A mechanism for alkaline disruption of casein micelles is proposed, in which increasing the milk pH improves the solvent quality for the caseins, thereby leading to the disruption of casein micelles into their constituent nanoclusters; increases in the net-negative charge on the caseins on increasing pH may contribute to micellar dissociation.

KEYWORDS: Milk; casein micelle; pH; disruption; solvent quality

INTRODUCTION

Milk is the fluid secreted by the females of all mammalian species, primarily to meet the complete nutritional requirements for the neonate, i.e., energy, essential amino acids and fatty acids, vitamins, minerals, and water. As such, milk has been a fascinating area of scientific research for centuries for the nutritionist and biologist. However, from an industrial and technological point of view, the ease with which milk, in particular bovine milk, can be converted into a wide variety of attractive products has been its most important feature. The constituent of milk of most significance in this respect is the casein micelle.

Casein micelles are highly hydrated association colloids consisting of 94% protein and 6% inorganic constituents on a dry matter basis (*1*). The protein fraction of the casein micelles consists of the caseins, a class of four phosphoproteins (α_{s1}-, α_{s2}-, β-, and κ-casein), which represent ~80% of total proteins in bovine milk (*1*). The inorganic constituents in the casein micelles is referred to as micellar calcium phosphate (MCP) and consists primarily of calcium and phosphate but also of low levels of magnesium and citrate and trace levels of other minerals (*1*). MCP is primarily present in the form of nanoclusters, which consist of a nanometer-sized core of amorphous calcium phosphate surrounded by a stabilizing shell of α_{s1}-, α_{s2}-, and β-caseins, which inhibit the core from further growth; these caseins contain at least one center of phosphorylation, i.e., at least three phosphorylated residues in close proximity, and can hence bind to the amorphous core through calcium phosphate ions pairs (*2*, *3*). The nanoclusters may be cross-linked, since the α_s-caseins contain more than one center of phosphorylation and can hence participate in multiple nanoclusters. Furthermore, nanoclusters may associate due to attractive hydrophobic and Van der Waals interactions and hydrogen bonding of the hydrophobic regions of the surrounding casein shell (*3*). Cross-linking and association of the nanoclusters lead to the formation of particles of colloidal dimensions. This growth process is inhibited when the surface of the aggregates becomes hydrophilic, which occurs because of the association of κ- and, to a lesser extent, β-casein at the surface (*3*). The surface layer of κ- and β-casein provides the colloidal stability of the casein micelle, primarily in the form of steric repulsion, because it acts as a salted brush, i.e., a polyelectrolyte brush, in a medium of high ionic strength (*4*, *5*). Aggregation of casein micelles may be induced by the removal (i.e., enzymatically, as in cheese making) or collapse (i.e., acid-induced, as in yogurt manufacture) of this salted brush (*6*).

The intramicellar stability refers to the ability of the casein micelle to maintain its structural integrity under the influence of adverse environmental conditions. From the above, it is apparent that reductions in intramicellar stability can be achieved either by dissociation of the calcium phosphate ion pairs, through which the surrounding shell of caseins is attached to the core of the nanoclusters, or by disruption of the cohesive interactions between hydrophobic areas of the caseins of the surrounding

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shell. Dissociation of calcium−phosphate ion pairs is commonly achieved by the addition of a calcium-chelating agent, e.g., trisodium citrate or ethylenediaminetetraacetic acid, to milk (*3*, *7*, *8*) as well as treatment of milk at high hydrostatic pressure (*9*). Attractive interactions between the caseins are reduced on addition of urea (*3*, *10*, *11*, *12*) or sodium dodecyl sulfate (*13*, *14*) or by heating milk in the presence of ethanol (*15*, *16*).

The above-described methods for disruption of casein micelles have been studied quite extensively and are quite well-understood. However, there is another method to reduce the intramicellar stability of the casein micelles: Increasing the milk pH to >9 leads to an extensive reduction in the turbidity of skimmed bovine milk, indicating considerable disruption of casein micelles (*17*, *18*). Although the process of alkaline disruption is utilized in various applications, e.g., milk protein isolation (*19*), the mechanism underlying the disruption of casein micelles by this means is, at present, poorly understood. By utilizing the great progress in our understanding of casein micelle structure over the past decade, we aim to clarify the mechanism for alkaline dissociation of casein micelles by a series of systematic studies presented in this article.

MATERIALS AND METHODS

Sample Preparation. Skimmed bovine milk was prepared by reconstituting low-heat skimmed milk powder (Irish Dairy Board, Dublin, Ireland) in demineralized water at a level of 6, 9, 12, 15, or 18% (w/w); sodium azide (0.05%, w/w) was added to prevent microbial growth. Subsamples of milk were preheated by heating the milk to 90 °C and holding it at this temperature for 10 min, followed by rapid cooling in ice water. The MCP content of casein micelles was altered by adjusting the milk pH to 5.5, 6.0, 6.6, 8.0, or 9.0, followed by exhaustive dialysis against 2 × 20 volumes of bulk milk for 48 h.

Determination of the Extent of Alkaline Disruption of Casein Micelles. Milk samples, prepared as described above, were equilibrated at 5, 20, or 40 °C and adjusted to pH 6.6, 8.0, 9.0, 10.0, or 11.0 by the addition of 2 M NaOH. Immediately after the desired pH was reached, samples were transferred into a quartz cuvette (path length, 1 mm) and the turbidity of the samples was determined at 600 nm over time at the desired temperature. Turbidity values were normalized using values for the untreated sample ($\tau_{untreated}$) and milk serum (τ_{serum}), according to

$$\tau_{normalized} = \frac{(\tau_{sample} - \tau_{serum})}{(\tau_{untreated} - \tau_{serum})} \quad (1)$$

Determination of the Influence of Alkaline pH on the Mineral Balance of Milk. Milk, equilibrated at 20 °C, was adjusted to pH 6.6−11.0 as described above. The level of ionic calcium in milk was determined over time using a calcium-selective electrode (Radiometer, Copenhagen, Denmark) and calculated from a standard curve of 1−10 mmol L^{-1} $CaCl_2$ in 0.1 M KCl. To determine the effect of pH on the concentration of nonmicellar calcium and inorganic phosphate, the serum phase of milk was isolated, 30 min after pH adjustment, by filtration through a 10 kDa molecular mass cutoff membrane under centrifugal force using Vivaspin 6 centrifugal concentrators (Vivascience AG, Hannover, Germany). The concentration of calcium in milk and its serum (i.e., nonmicellar calcium) was determined by atomic absorption spectroscopy (*20*). The concentration of inorganic phosphorus in milk and milk serum (i.e., nonmicellar inorganic phosphorus) was determined spectrophotometrically (*21*).

RESULTS

Casein Micelle Stability at High pH. Increasing the milk pH reduced the turbidity of milk (**Figure 1**); because casein micelles are the main light-scattering particles in skimmed milk, these decreases in turbidity suggest disruption of casein micelles.

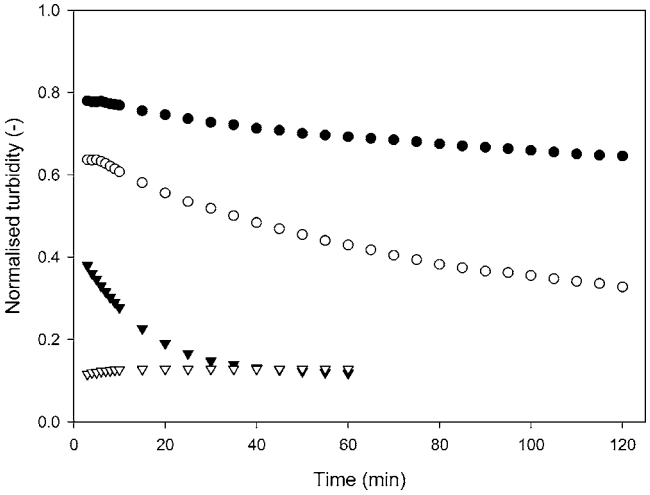

Figure 1. Normalized turbidity of skim milk (9%, w/v, milk solids) at pH 8.0 (●), 9.0 (○), 10.0 (▼), or 11.0 (▽) during incubation at 20 °C for up to 120 min. Time $t = 0$ min indicates the time point at which the desired pH was reached. Values are means of data from three experiments on individual milk samples; the coefficient of variation was <5% for each data point.

The extent of the reduction in turbidity, and hence micellar disruption, increased with pH with the turbidity at pH 11.0 being close to that of milk serum (**Figure 1**). The rate of alkaline-induced disruption of casein micelles also increased with pH; at pH 8.0, turbidity decreased progressively with time, whereas at pH 11.0, maximum disruption of casein micelles was observed at the first measurement point, i.e., 3 min after reaching the desired pH, with no further change on prolonged storage (**Figure 1**). The turbidity of milk maintained at pH 6.6 did not change during storage at 20 °C.

The extent of alkaline disruption was influenced considerably by temperature; at both pH 9.0 (**Figure 2A**) and pH 10.0 (**Figure 2B**), both the rate and the extent of alkaline disruption of casein micelles increased considerably with temperature (5−40 °C). At pH 9.0, only small differences in alkaline disruption of casein micelles were observed between samples at 5 and 20 °C, but samples at 40 °C showed considerably faster and more extensive alkaline disruption of casein micelles (**Figure 2A**). At pH 10.0, turbidity values after >45 min were comparable for samples at 20 and 40 °C, while those at 5 °C remained considerably higher. These data indicate a synergistic destabilizing effect of increasing pH and temperature on casein micelles.

Preheating milk at 90 °C for 10 min prior to increasing the pH to 9.0 or 10.0 had little effect on the alkaline disruption of casein micelles (**Figure 3**). At both pH values, differences between samples were very small, suggesting that heat-induced association of whey proteins with the casein micelle surface does not affect the mechanism by which alkaline pH diminishes micellar integrity.

Increasing the concentration of milk solids progressively reduced the extent of alkaline disruption of casein micelles (**Figure 4A**,**B**). At both pH 9.0 and pH 10.0, the rate and extent of alkaline disruption of casein micelles were highest in unconcentrated milk and decreased progressively with an increasing concentration of milk solids (**Figure 4**). These results suggest an antagonistic effect between pH and casein micelle concentration on alkaline destabilization of casein micelles.

Mineral Balance of Milk at High pH. The milk used in this study (9% total solids) contained ∼28.6 mmol L^{-1} calcium, of which 10.7 mmol L^{-1} was found in the milk serum and 1.7 mmol L^{-1} in ionic form at pH 6.6, and 18.9 mmol L^{-1} inorganic

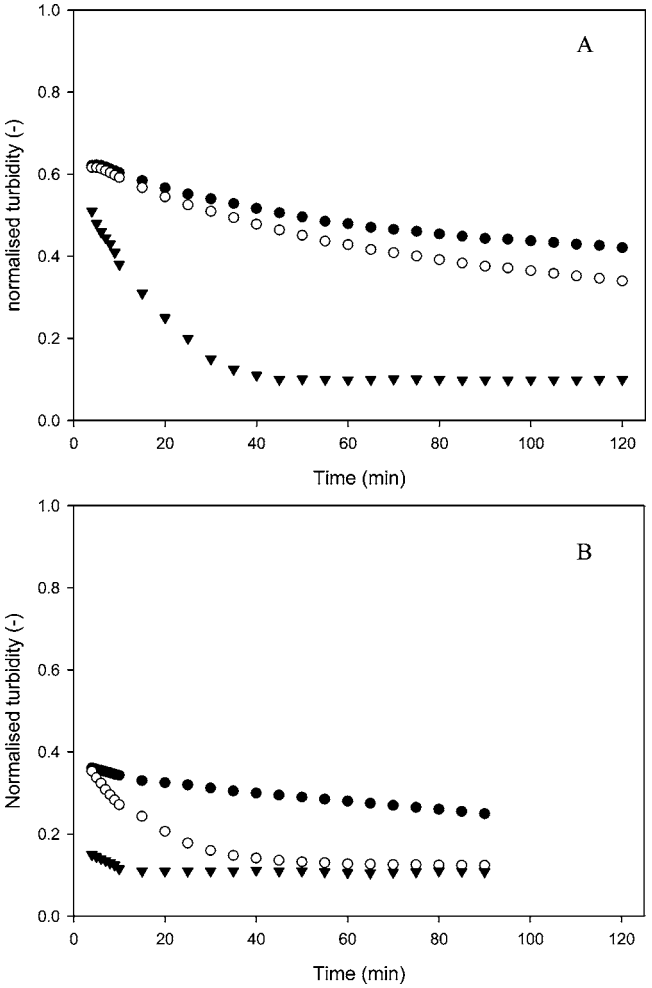

Figure 2. Normalized turbidity of skim milk (9%, w/v, milk solids) at (**A**) pH 9.0 or (**B**) pH 10.0 during incubation at 5 (●), 20 (○), or 40 (▼) °C. Time $t = 0$ min indicates the time point at which the desired pH was reached. Values are means of data from three experiments on individual milk samples; the coefficient of variation was <5% for each data point.

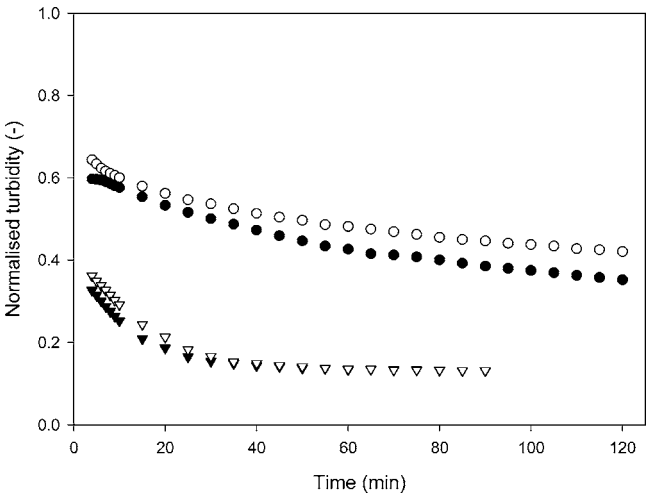

Figure 3. Normalized turbidity of unheated (●,▼) or preheated (10 min, 90 °C; ○,▽) skim milk (9%, w/v, milk solids) at pH 9.0 (●,○) or 10.0 (▼,▽) during incubation at 20 °C for up to 120 min. Time $t = 0$ min indicates the time point at which the desired pH was reached. Values are means of data from three experiments on individual milk samples; the coefficient of variation was <5% for each data point.

phosphorus, of which 9.3 mmol L^{-1} was found in the milk serum at pH 6.6. Increasing milk pH to values in the range 6.6−11.0 progressively reduced concentrations of serum calcium, serum inorganic phosphorus, and ionic calcium in logarithmic fashion (**Figure 5**). The pH-induced changes in concentrations of ionic and nonmicellar calcium were closely related and at pH 11.0 were <2% of that in milk at pH 6.6 (**Figure 5**). The pH-induced reductions in the level of nonmicellar inorganic phosphorus were less extensive than those in ionic or nonmicellar calcium, with ∼25% of original nonsedimentable inorganic phosphorus remaining at pH 11.0 (**Figure 5**).

DISCUSSION

The results presented in this article show a strong influence of increasing milk pH on the mineral balance of milk and the stability of casein micelles. Increasing the temperature and pH showed a synergistic effect (**Figure 2**), whereas increasing the milk solids concentration and pH showed an antagonistic effect (**Figure 4**). Alkaline pH-induced changes in the mineral balance can be explained as a result of the influence of pH on the solubility of calcium phosphate, as outlined in more detail in the next section; furthermore, it is proposed that alkaline disruption of casein micelles is primarily a result of an increased solvent quality of milk serum at high pH, leading to disruption of protein−protein interactions, as will be discussed further in Alkaline Disruption of Casein Micelles: A Proposed Mechanism.

Alkaline pH-Induced Changes in the Mineral Balance of Milk. The data in **Figure 5** show decreasing concentrations of nonmicellar (or serum) calcium and phosphate and ionic calcium with increasing pH. This is probably the result of supersaturation of milk serum with respect to calcium phosphate, since the solubility product of calcium phosphate decreases with increasing pH (*22*). A logarithmic decrease in the concentration of ionic calcium with increasing pH was previously observed (*23*). The theoretical basis for this relationship can, as suggested by Holt (*24*), be demonstrated by combining the Henderson−Hasselbach equation for the second ionization of phosphate:

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{\{\mathrm{HPO_4}^{2-}\}}{\{\mathrm{H_2PO_4}^{-}\}}\right) \quad (2)$$

with the solubility product of MCP (*25*):

$$K_S = \{\mathrm{Ca}^{2+}\}\{\mathrm{PO_4}^{3-}\}^{0.2}\{\mathrm{HPO_4}^{2-}\}^{0.7} \quad (3)$$

From eq 3 it follows that:

$$\{\mathrm{Ca}^{2+}\} \propto \frac{1}{\{\mathrm{HPO_4}^{2-}\}^{0.7}} \quad (4)$$

which, for low values of $\{Ca^{2+}\}$ and $\{HPO_4^{2-}\}$ (as found in milk serum, e.g., <5 mmol L^{-1}), may be approximated by:

$$\{\mathrm{Ca}^{2+}\} \propto \frac{1}{\{\mathrm{HPO_4}^{2-}\}} \quad (5)$$

Combining eqs 2 and 5 leads to the empirical relationship:

$$(\mathrm{pH} - \mathrm{p}K_a) \propto -\log\{\mathrm{Ca}^{2+}\} \quad (6)$$

which predicts that calcium activity decreases in a logarithmic fashion with increasing pH, as is indeed observed in **Figure 5** and predicted by Holt (*24*). Because the level of serum calcium and consequently also the level of serum phosphate are closely

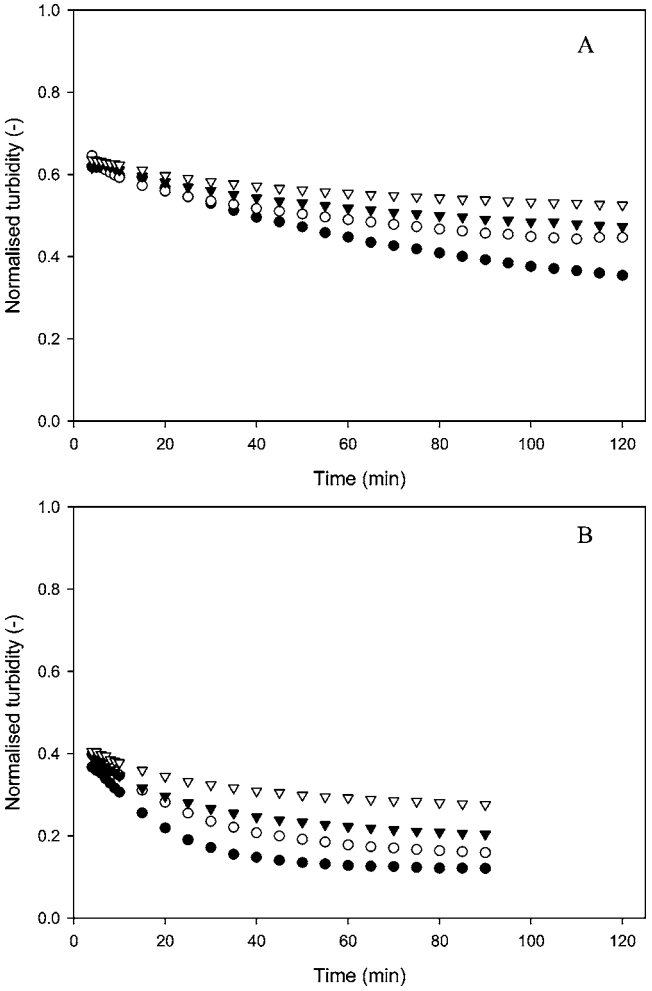

Figure 4. Normalized turbidity of skim milk containing 9 (●), 12 (○), 15 (▼), or 18 (▽) % (w/w) milk solids at (**A**) pH 9.0 or (**B**) pH 10.0 during incubation at 20 °C. Time $t = 0$ min indicates the time point at which the desired pH was reached. Values are means of data from three experiments on individual milk samples; the coefficient of variation was <5% for each data point.

related to the level of ionic calcium, their logarithmic decrease in concentration with increasing pH (**Figure 5**) follows logically from eq 6.

At present, the nature of the alkaline-precipitated calcium phosphate in milk is not known and several options exist; precipitated calcium phosphate may become part of MCP nanoclusters, which can thus either grow in size or number. Ionic calcium may bind to phosphoseryl residues not involved in nanocluster formation; furthermore, evidence for calcium binding to carboxylate groups of glutamic or aspartic acid residues exists (*26*). Elucidation of alkaline precipitation of calcium phosphate in milk, using techniques such as small-angle neutron or X-ray scattering, may be of interest for future research.

Alkaline Disruption of Casein Micelles: A Proposed Mechanism. From the data presented in this article, it is apparent that a plausible mechanism for alkaline disruption of casein micelles must account for the increased extent of disruption with increasing pH (**Figure 1**), increasing temperature (**Figure 2**), and reducing concentration of milk solids (**Figure 4**). As outlined previously, disruption of casein micelles can be achieved by diminishing the stability provided by either one, or a combination of, two types of interactions: (i) ion pairing

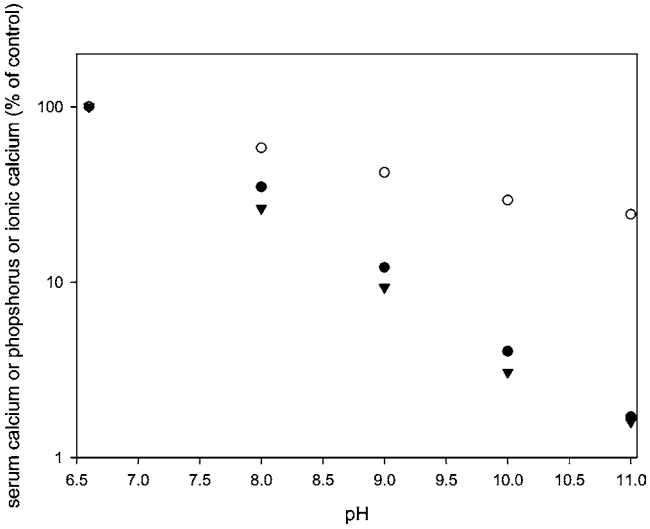

Figure 5. Influence of pH on the concentration of serum calcium (●), serum phosphorus (○), or ionic calcium (▼) in skim milk (9%, w/w, milk solids). Values are means of data from three experiments on individual milk samples; the coefficient of variation was <2% for each data point.

of phosphoseryl residues of the caseins with MCP and (ii) cohesive interactions between the hydrophobic domains of the caseins (*3*).

Destabilization of casein micelles through disruption of ion pairs is observed when the solubility of calcium phosphate is increased, e.g., on addition of a sufficiently high concentration of a calcium-chelating agent (*3*, *7*, *8*) or at high pressure (*9*), and can readily lead to the extent of micellar disruption observed in the current study (**Figures 1**−**4**). However, disruption of calcium phosphate ion pairs is unlikely to be the cause of alkaline disruption of casein micelles since the solubility of calcium phosphate decreases with increasing pH (**Figure 5**; *22*) so the calcium phosphate ion pairs in the nanocluster should be stabilized, rather than disrupted, at high pH.

Because alkaline disruption of casein micelles is unlikely to occur as a result of ion pairs, it seems plausible to assume that this process occurs through disruption of the cohesive interactions between hydrophobic areas of the caseins. In the literature, such interactions are often attributed to hydrophobic bonding. However, it is important to realize that the hydrophobic effect, from which hydrophobic bonding arises, seems to exist by virtue of the extensive hydrogen bonding in water and is as such influenced significantly by all solutes which affect water structure (*27*, *28*). Hence, it is more appropriate to describe the cohesive interaction between micellar caseins as a solvent-mediated association, where interactions are strengthened or weakened as a result of increasing or decreasing solvent quality, as well as attractive and repulsive interactions between the proteins.

For pH > pI, the ionization of acidic side groups of the proteins increases, whereas that of the basic side groups decreases with increasing pH; as a result, the net-negative charge, and hence intermolecular repulsion, increases, which may cause disruption of casein micelles. Hydrophilic areas of the caseins are not involved in cohesive protein−protein interactions (*29*, *30*), so it is important to focus only on the hydrophobic areas of the caseins. The occurrence of amino acids with ionisable side groups in caseins is given in **Table 1**, from which κ-casein was omitted because it contributes little to intramicellar stability (*3*). Although cysteine and phosphoserine also contain ionizable side groups, both were omitted from **Table 1**; the former because it exists in the form disulfide bonds

Table 1. Overview of Amino Acids with Acidic and Basic Side Groups in Caseins

amino acid	type of side group	pK of side group[a]	no. of residues (values between brackets indicate the no. of residues located in the hydrophobic areas)		
			α_{s1}-casein[b]	α_{s2}-casein[c]	β-casein[d]
glutamic acid	carboxylic acid	4.5	25 (10)	24 (0)	19 (2)
aspartic acid	carboxylic acid	4.0	7 (4)	4 (0)	4 (1)
tyrosine	phenol	9.6	10 (10)	12 (3)	4 (3)
lysine	ϵ-amino	10.5	14 (7)	24 (1)	11 (2)
histidine	imidazole	6.4	5 (2)	3 (0)	5 (2)
arginine	guanidine	>12.5	6 (4)	6 (1)	4 (2)

[a] Values from ref *37*. [b] Values are for α_{s1}-CN B-8P (*31*); hydrophobic areas contain residues 1–44 (*37*), 90–110, and 140–190 (*31*). [c] Values are for α_{s2}-CN A-11P; hydrophobic area contains residues 90–120 (*31*). [d] Values β-casein A^2-5P; hydrophobic areas contain residues 55–90 and 130–209 (*31*).

in α_{s2}-casein (*31*), while the latter is associated with calcium in the pH range studied. The Henderson−Hasselbach equation shows that for acidic side groups, the degree of ionization increases in a sigmoidal fashion, from ~1% at pH = (pK − 2) to ~99% at pH = (pK + 2), whereas a sigmoidal relationship between the pH and the degree of ionization exists for basic side groups. Taking their pK values into account (**Table 1**), it is clear that in the pH range studied here (6.6−11.0), glutamic acid, aspartic acid, and arginine remain completely dissociated and will thus not contribute to a pH-induced change in protein charge, whereas influence of histidine will also be limited >pH ~ 7.5. Any charge-induced disruption of cohesive interactions between the hydrophobic areas of the caseins would thus have to arise from reduced ionization of the ϵ-amino group of lysine and increased ionization of the phenol group of tyrosine. Increasing the milk pH from 6.6 to 9.0 induces considerably disruption of casein micelles (**Figures 1**−**4**), but the degree of dissociation of the ϵ-amino group of lysine decreases only from 100 to ~97%, whereas that of the phenol group of tyrosine increases from 0 to ~20%. These changes in charge may contribute to disruption of cohesive protein−protein interactions but are unlikely to be of sufficient magnitude to initiate micellar disruption, particularly considering that (i) the calcium, which becomes insoluble on increasing pH (**Figure 5**), may readily interact with the carboxylic acid residues in glutamic acid and aspartic acid (*32*) and hence counteract the increase in net-negative charge; (ii) milk has a high ionic strength, which will result in extensive screening of charges (*4*); and (iii) a reduced degree of ionization of basic side groups will reduce their solubility and hence enhance cohesive protein interactions.

Because, as discussed above, pH-induced changes in the caseins are insufficient to account for alkaline disruption of casein micelles and association of caseins is strongly influenced by solvent quality, pH-induced changes in the solvent quality of milk serum should be considered. On increasing milk pH to >9, a number of changes that can influence the quality of the milk serum as a solvent occur, of which perhaps the most significant is a reduction in the concentration of ionic calcium (**Figure 5**), and presumably ionic phosphate. Calcium and phosphate ions can be classified as ionic kosmotropes, i.e., small ions of high charge density (*33*). Ionic kosmotropes promote association of hydrophobic areas of proteins by binding water strongly and thus reducing the volume of water available to hydrate exposed protein surfaces (*33*). Increasing the pH reduces the concentration of ionic calcium ~50-fold (**Figure 5**) and will thus increase the solvent quality of milk serum for caseins considerably. From the data in **Figure 5**, a reduction in the concentration of ionic phosphate with increasing pH is also expected, which would further increase solvent quality.

This proposed mechanism of alkaline disruption of casein micelles as a result of increased casein solubility at alkaline pH arising from reduced concentrations of ionic calcium and phosphate can account for the observed influence of temperature (**Figure 2**) and milk solids concentration (**Figure 4**). The concentration of serum calcium, and hence presumably ionic calcium, in milk decreases with increasing temperature (*34*) so solvent quality would increase with increasing temperature. Furthermore, the strength of hydrogen bonding also decreases with increasing temperature (*35*), indicating a less dense water structure. Hence, the amount of water available for hydration of hydrophobic casein areas increases with temperature, facilitating micellar disruption. Increasing the concentration of milk solids increases the concentration of ionic calcium in milk (data not shown) and thereby the stability of casein micelles against alkaline-induced disruption.

It is interesting to note that the above-proposed mechanism of increased solvent quality as a result of reduced concentrations of ionic calcium and phosphate may also apply for ethanol-mediated temperature-induced dissociation of casein micelles, which was attributed to disruption of hydrophobic bonds (*16*). However, it should be noted that the concentration of calcium in milk serum, and hence presumably ionic calcium, decreases linearly with increasing ethanol concentration (0−25%, v/v) in milk, by ~0.22 mmol L^{-1} calcium per % ethanol (*35*). Furthermore, increasing the temperature significantly reduces concentrations of serum calcium (*33*). Hence, the combination of added ethanol and increased temperature could reduce the concentrations of ionic calcium and phosphate sufficiently to contribute significantly to micellar disruption.

Conclusions. On the basis of the observations presented in this article, a mechanism for alkaline disruption of casein micelles is proposed based as a result of decreased levels of ionic calcium and phosphate in milk at elevated pH. Such decreases in ionic calcium and phosphate increase the solvent quality of the milk serum, leading to diminished cohesive interactions between the hydrophobic regions of the caseins. An increased net-negative protein charge at higher pH may contribute to micellar disruption. Because calcium phosphate ion pairing is not disrupted at alkaline pH, it appears that alkaline pH disrupts casein micelles into individual or cross-linked nanoclusters, similar to effects observed on addition of high levels of urea or heating in the presence of ethanol.

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