The Structure of the Casein Micelle of Milk and Its Changes During Processing

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Annu. Rev. Food Sci. Technol. 2012. 3:449–67

The *Annual Review of Food Science and Technology* is online at food.annualreviews.org

This article's doi: 10.1146/annurev-food-022811-101214

1941-1413/12/0410-0449$20.00

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Keywords

milk proteins, milk processing, casein micelle structure, casein micelle functionality, interactions of milk proteins

Abstract

The majority of the protein in cow's milk is contained in the particles known as casein micelles. This review describes the main structural features of these particles and the different models that have been used to define the interior structures. The reactions of the micelles during processing operations are described in terms of the structural models.

INTRODUCTION

MPC: milk protein concentrate

Casein is the major protein component of bovine milk, comprising approximately 80% of the total milk protein. There are four individual types of casein molecules, the α_{s1}-, α_{s2}-, β-, and κ-caseins in approximate relative amounts of 4:1:3.5:1.5, respectively. The properties of the proteins have been previously summarized (Swaisgood 2003, Farrell et al. 2004). In milk, the caseins, together with the essential ingredient of calcium phosphate (de Kruif & Holt 2003), form aggregates of several thousand individual protein molecules with average diameters of 150 to 200 nm (de Kruif 1998), known as casein micelles (Fox & Brodkorb 2008).[1] The micelles are highly hydrated, with approximately 3.5 kg of water per kg of protein (Jeurnink & de Kruif 1993.). Thus, although the caseins make up approximately 2.5% of the total weight of milk, the micelles occupy approximately 10% of the volume. The micelles can be moderately heated or cooled without significant aggregation or disruption of their basic structures. On the other hand, they are easily destabilized either by treatment with proteolytic enzymes or by acidification to give the coagula that are the bases of cheeses and yogurt-type products.

The structure of the micelles has been debated for many years, and several reviews have appeared in the past 10 years, giving somewhat divergent points of view (de Kruif & Holt 2003, Horne 2006, Farrell et al. 2006, Fox & Brodkorb 2008, Horne 2008, Dalgleish 2011). We need to explain how large numbers of casein molecules assemble in a controlled manner to form the particles but yet do not aggregate infinitely to give a precipitate. Because most, if not all, of the κ-casein is found on the surfaces of the micelles, it is believed to limit the growth of the particles by binding to the surface of growing aggregates formed by the three other caseins and the calcium phosphate (Horne 2006). Most of the functional properties of the micelles depend on the properties of the surface, rather than those of the interior, and to some extent the micelles can be regarded as hard spheres with a protective coating (de Kruif 1999). On the other hand, the interior of the micelle becomes important in such postcoagulation rearrangements as cheese curd formation. Also, it is possible that in the future micelles may find a use as carriers of nutritionally significant molecules, and for this an understanding of the highly hydrated interior may be important.

This review aims to describe current understanding of the structure of the casein micelle and how changes in this structure determine the behavior of the micelle during processing. However, before introducing the topic, we should point out that in many of the studies of micelle structure described below, it is not the native micelles from milk that are studied, but rather those that are reconstituted from skim milk powder, milk protein concentrates (MPCs), or phosphocaseinate. Although these particles have been subjected to processing and drying, it is generally assumed that they have similar structure and properties to the native micelles. That the particles behave similarly to native micelles there is no doubt, but whether the micelles in all of these starting materials are identical to the native particles has not been fully demonstrated.

SIZES AND COMPOSITIONS OF CASEIN MICELLES

The composition of native micelles depends on their size. Several studies have shown that smaller micelles are relatively rich in κ-casein and relatively depleted in β-casein, while the contents of α_s-caseins appear to be independent of size (Dalgleish et al. 1989, Marchin et al. 2007). These results imply that κ-casein is predominantly if not completely on the surface of the micelle, β-casein is mostly present in the interior, and the α_s-caseins are found throughout the structure.

[1]Properly speaking, the particles should always be referred to as casein micelles, to distinguish them from the soap-type micelles known from colloid chemistry. However, for the sake of conciseness, they are referred to as micelles throughout this article.

In any milk, there is a range of micelle diameters, from <100 nm to >300 nm (Dalgleish & Horne 1985). The sizes of the native micelles are determined by the amount of surface that can be stabilized by the available κ-casein; thus, the greater the proportion of κ-casein present in the total protein, the smaller are the micelles (Delacroix-Buchet et al. 1993). The average sizes depend on the composition of the milk, but, in general, the average hydrodynamic diameters are from 150 to 200 nm. Analysis of the size distribution using field-flow fractionation techniques has suggested a minimum diameter of approximately 80 nm (de Kruif 1998), although some particles may be as low as 50 nm in milk reconstituted from skim milk powder (Udabage et al. 2003). Electron microscopy generally yields smaller values for the micellar diameter, probably because sample preparation and dehydration steps can cause significant shrinkage of the micelles (Martin et al. 2006). Recently, it has been claimed that a population of so-called mini-micelles with diameters in the 20 to 40 nm range coexists with the larger particles observed by light scattering and separation methods (Müller-Buschbaum et al. 2007, Metwalli et al. 2009). However, these particles have not been separated and analyzed.

THE STRUCTURE OF THE CASEIN MICELLE

The Internal Structure of the Casein Micelle

Since the late 1960s, there has been debate about the internal structure of the micelle (Fox & Brodkorb 2008). From relatively simple coat-core models of the α_s- and β-caseins surrounded by a layer of κ-casein (Waugh et al. 1970), structural models have developed to provide two major contenders for this structure, namely the submicellar and the nanocluster models. Both of these have been reviewed in detail, giving divergent points of view (Farrell et al. 2006, Horne 2006).

The submicellar hypothesis was formulated first. It derived from the observation that the proteins in sodium caseinate (the protein extract from micelles when the calcium phosphate has been removed by acidification) form small aggregates via noncovalent interactions when they are dispersed in aqueous media (Waugh et al. 1970). It was therefore a straightforward hypothesis to postulate that the micelles were formed from these aggregates (submicelles) linked together by small domains of calcium phosphate (Schmidt 1982). The knowledge that κ-casein is mainly to be found on the surfaces of the micelles implies that at least two types of submicelle (κ-casein rich and κ-casein poor) must exist. Although it has not been clearly demonstrated that such different particles exist either in sodium caseinate or in micelles, it is known that κ-casein does tend to self-aggregate via the formation of intermolecular disulphide bonds (Groves et al. 1992); such aggregates are present in caseinate (Rose et al. 1969) and could possibly represent the κ-casein rich type of submicelle. Electron microscopy also appeared to suggest that the micellar interior was of a granular appearance consistent with the presence of submicelles (Schmidt 1982), although later studies have tended to disagree with this assessment (McMahon & McManus 1998, McMahon & Oomen 2008).

The alternative, nanocluster, model is derived from the observation that the phosphopeptides from β-casein are capable of stabilizing calcium phosphate in solutions at concentrations in which it would precipitate (Little & Holt 2004). Study of this phenomenon showed that the phosphopeptides bound to and stabilized small domains of calcium phosphate, termed nanoclusters. The nanoclusters of calcium phosphate have radii of 2.3 nm and are surrounded by approximately 50 phosphopeptide chains (Holt et al. 1998). Similar nanoclusters could be formed in the mammary gland by an interaction between the more phosphorylated caseins and calcium phosphate, via the phosphate centers of the α_s- and β-caseins (Holt et al. 1998, Holt 2004), although they will be stabilized by fewer chains of intact protein (Ono et al. 1998, Horne 2006). Crosslinking of the

calcium phosphate nanoclusters by the more highly phosphorylated caseins (α_{s1} and α_{s2}) would allow growth into the micellar particles (de Kruif & Holt 2003). However, the 18 nm distances between adjacent nanoclusters described by these authors are so large that bridging by individual proteins may be unlikely (Horne 2006). The alternative is that aggregation of the calcium phosphate/protein nanoclusters can occur via noncovalent interactions between themselves and with other caseins to lead to the formation of micelles (Horne 1998, 2006; Dalgleish 2011). Given that κ-casein cannot participate in the formation of nanoclusters because it lacks phosphate centers, it will be available to associate with the aggregating proteins via noncovalent interactions to form the surface layer, thereby acting as a monofunctional chain terminating agent as described in Horne's dual-binding model (Horne 1998, 2008) and stabilizing the assemblies of nanoclusters. Taken from this point of view, the micelles would have a substructure, rather than a distribution of calcium phosphate within a rather uniform protein matrix, but it would not be the same as that envisaged in the original submicellar hypothesis (Dalgleish 2011). In effect, this embodiment of the nanocluster model can be taken to invert the submicellar model; instead of casein particles linked by calcium phosphate, there are calcium phosphate/casein particles linked by noncovalent bonds. What is important about the nanocluster model is that it provides a distinct mechanism for the formation of the casein micelles via the interaction between the serine phosphate groups of the caseins and calcium phosphate (Holt 2004).

SANS: small angle neutron scattering

SAXS: small angle X-ray scattering

SEM: scanning electron microscopy

TEM: transmission electron microscopy

It is difficult to distinguish between the models on the basis of chemistry. In principle, it should be possible to dissociate the micelles and measure the composition and size of the resulting particles to reinforce one of the models. However, there is no way of dissociating the micelles with the certainty of maintaining the integrity of the putative subunits. Treatment of the micelles by acidification or by EDTA dissolves the calcium phosphate (Dalgleish & Law 1989, Griffin et al. 1988), so that the nanoclusters can no longer exist. On the other hand, use of urea as a dissociating agent would keep the nanoclusters intact [and evidence has been found for this (Aoki et al. 1986)], but it will also dissociate the submicelles, if they exist, to leave simply the calcium phosphate with whatever proteins from the submicelles are attached to it. Therefore, simple dissociation experiments cannot provide proof one way or the other. On the basis of controlled dissociation of micelles in calcium phosphate–depleted sera and in solutions of κ-casein with and without urea, it has been argued that the observed patterns of dissociation are inconsistent with a submicellar model (Holt 1998).

The main details of micellar substructure come from physical measurements from two sources, electron microscopy and small angle scattering of neutrons (SANS) or X-rays (SAXS). Electron microscopy, both transmission (TEM) and scanning (SEM), has been used for many years to try to elucidate micellar structures. In particular, the earlier work by TEM on gold replicas of freeze-fractured micelles showed a granular appearance that was taken to indicate the presence of submicelles (Schmidt 1982, Karlsson et al. 2005, Karlsson et al. 2007). However, as pointed out above, the nanocluster model also provides inhomogeneities within the micelle. A recent study of the TEM of micelles has severely criticized much of the earlier results from microscopy, suggesting that many observed structures could be artifacts arising from the fixation and staining procedures that are essential in TEM (McMahon & Oomen 2008). This study gave evidence that the structure was more of an extended web rather than one consisting of subunits and suggested that water channels could exist throughout the micelle. It provided some support for the nanocluster model, where the nanoclusters surround regions of low density associated with hydration. Similar results have been proposed by Harte (2011), where an open structure of the micelle with water channels is proposed. Other studies, using cryo-TEM, where staining is not used, again give images that do not appear to be consistent with the submicellar model, but rather show a semiregular dispersion of calcium phosphate clusters within a reticulated protein matrix (Marchin et al. 2007,

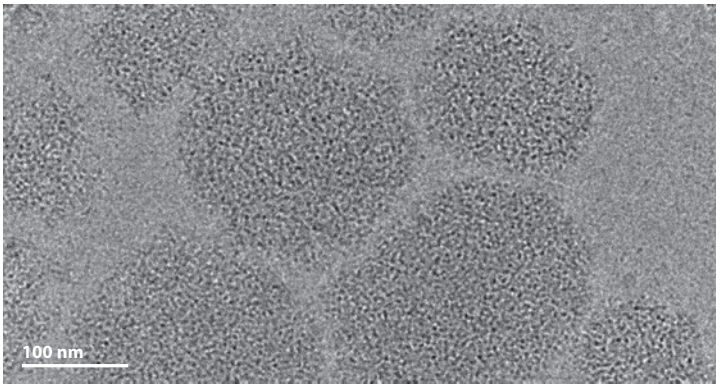

Figure 1

Cryo-transmission electron microscopy image of casein micelles, showing the relatively even distribution of calcium phosphate clusters (*dark points*) within a distributed matrix of protein. Samples were prepared by vitrification in liquid ethane, and the measurement was made at a temperature below 97 K.

Knudsen & Skibsted 2010). In these measurements, as exemplified by **Figure 1**, there is minimal sample preparation.

Studies using SEM also depend on the methods of sample preparation and whether or not the samples are metal coated. The most detailed micrographs of uncoated specimens using field-emission SEM do not show submicelles; rather, they show an irregular structure with apparently tubular features and with deep clefts that seem to be leading to the interiors of the micelles (Dalgleish et al. 2004 and **Figure 2**). Coating of these structures with gold for standard TEM does give a more submicellar appearance (McMahon & McManus 1998), but this may be an artifact arising from the gold coating. Thus, more recent methodologies in electron microscopy result in images that do not appear to support a classical submicellar structure.

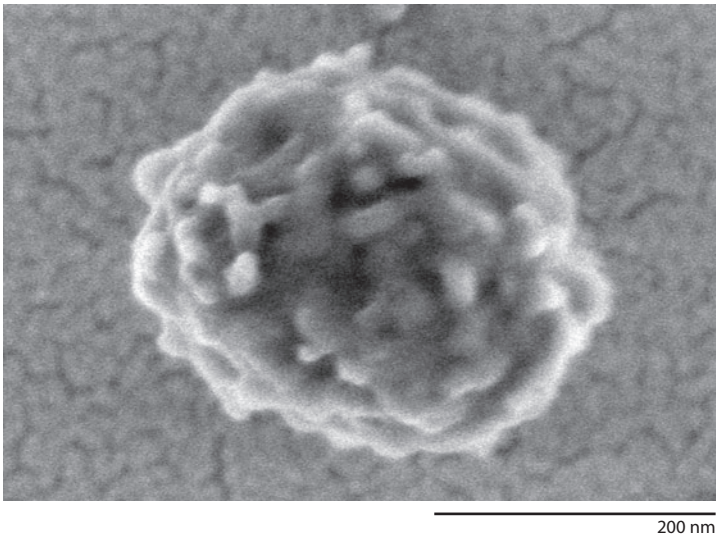

Figure 2

Field-emission scanning electron microscopy image of a casein micelle, chemisorbed to a carbon film and fixed but not stained or metal coated.

The earliest studies using SANS were interpreted as showing that the micelles had some internal structure, which was thought to be indicative of the presence of submicelles (Stothart & Cebula 1982). However, detailed analysis of the SANS results required the submicelles to have different sizes and varying densities (Hansen et al. 1996), which may cast some doubt on the interpretation. More recent studies of SANS have been mainly interpreted in terms of the calcium phosphate nanoclusters surrounded by caseins. It is possible to change the contrast between solvent and micellar proteins by changing the ratio of D_2O to H_2O in the solvent, thus minimizing the scattering of the protein. With contrast matching, a shoulder in the scattering profile at a scattering vector of $q \sim 0.35$ nm^{-1} becomes more pronounced, suggesting that it arises from the nonhomogeneous distribution of calcium phosphate in the micelle (Holt et al. 2003). Similarly, SAXS and SANS studies have shown that the shoulder is removed by acidification of the micelles (Marchin et al. 2007), which partly dissolves the calcium phosphate but retains the integrity of the protein part of the micellar structure, and concentration by diafiltration, which removes some of the calcium phosphate (Alexander et al. 2011). On the basis of SANS and other measurements, the micelle consists of a protein matrix without long-range order in which calcium phosphate clusters are randomly distributed. According to Holt et al. (2003), the average micelle is 108 nm in radius, has a particle mass of 7.2×10^8 Da, and contains 830 domains of calcium phosphate.

Recent SAXS studies have generally been interpreted on the basis of a nanocluster model (Marchin et al. 2007), although detailed interpretations of the scattering profiles differ substantially. Shukla et al. (2009) proposed an uneven distribution of ellipsoidal calcium phosphate nanoclusters within the micelles. On the other hand, using a variant of SANS on thin films of micelles, Metwalli et al. (2009) postulated the existence of mini-micelles, which are present in micellar suspensions prepared from powders derived from the ultrafiltration and diafiltration of milk. A most recent interpretation of the SAXS profile has been given by Bouchoux et al. (2010), on the basis of studies of the scattering of micelles that are progressively concentrated and dehydrated by osmotic stress, where it is claimed that the micelle contains hard regions of protein/calcium phosphate within a highly hydrated sponge-like structure. This last interpretation is interesting because it explicitly takes into account the large hydration of the micelle and how the loss of this water during osmotic stressing affects the micellar structure. The proposed structure is similar to that suggested by Dalgleish (2011) in a recent review and shown in **Figure 3**.

This question of the location of the large amount of water in the micellar interior has not generally been considered in most models. It is important because it is known that the micelle is to some extent porous given that β-casein can be removed from the micelle by cooling (Creamer et al. 1977) and large molecules can penetrate the micelle (Colsenet et al. 2005, Le Feunteun & Mariette 2007). The dual binding model (Horne 2008) invokes hydrophobic interactions as being important in linking the caseins or nanoclusters together, but such interactions would appear to preclude the presence of water in the micelle interior. The suggestion has been made that water channels within the micelle can be stabilized by β-casein interacting with the hydrophobic portions of the nanoclusters, to give a structure such as is shown in **Figure 3** (Dalgleish 2011). Such a model shows a relatively even distribution of the nanoclusters but also contains pores stabilized by β-casein; this accounts for the hydration of the particles, the presence of β-casein in the micelle interior, and the observation of more rigid regions formed from linked nanoclusters.

The Surface of the Casein Micelle

It is now well established that most, if not all, of the κ-casein is present on the surfaces of the particles (Dalgleish et al. 1989). This κ-casein causes the micelle to be stable against aggregation, because part of the molecule, the macropeptide (residues 106–169 of the protein), appears to be

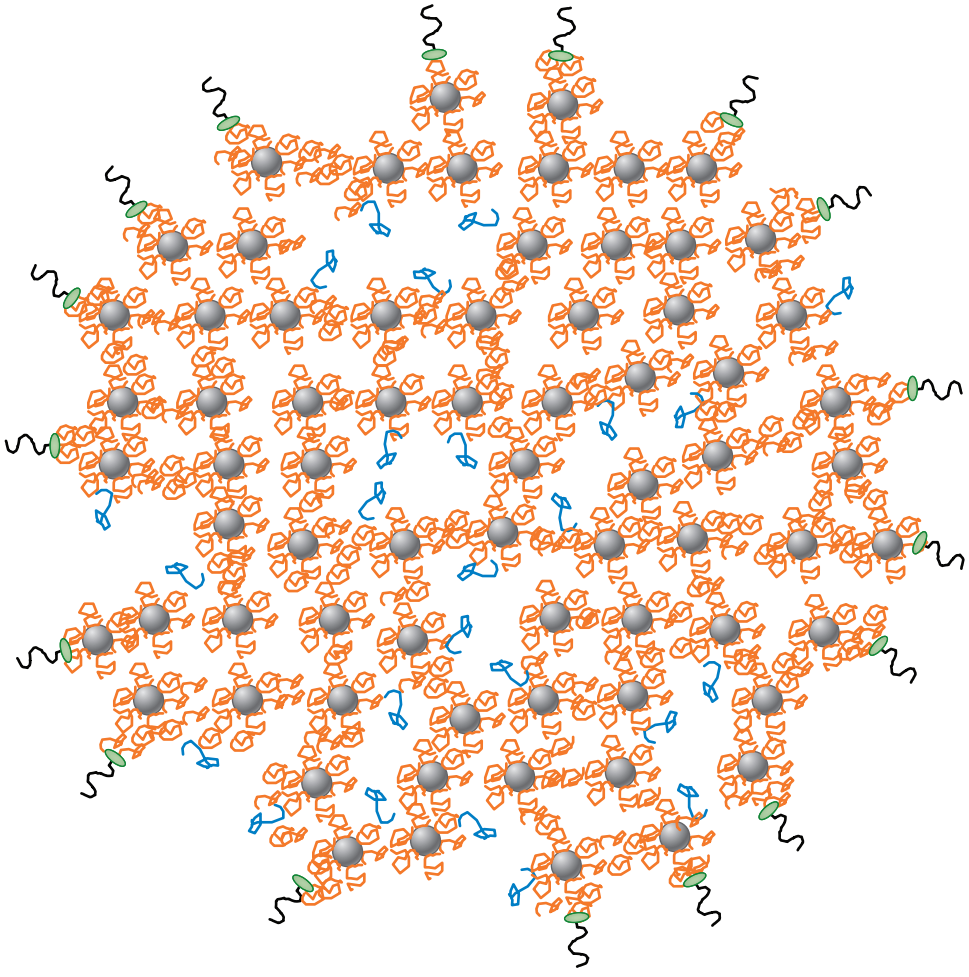

Figure 3

Schematic section through a micelle, showing the regions of water within the structure. The α_s- and β-caseins (*orange*) are attached to and link the calcium phosphate nanoclusters (*grey spheres*). Some β-casein (*blue*) hydrophobically binds to other caseins and can be removed by cooling. The para-κ-casein (*green*) and the caseinomacropeptide chains (*black*) are on the outermost parts of the surface. Not drawn to scale, and the sizes of the water channels are exaggerated for clarity.

extended from the micellar surface to create a layer, estimated to be 5–10 nm thick, around the particles (Horne 1986, de Kruif & Zhulina 1996). This hairy layer provides steric stabilization to the micelles so that they cannot approach each other closely (**Figure 4*a***). It is not so dense, however, that it prevents individual protein molecules from passing through [β-casein can leave the micelle and re-enter it during cooling and rewarming (Creamer et al. 1977); whey proteins can penetrate the layer and form disulphide bonds with the inner part of the κ-casein (Anema & Li 2003, Donato et al. 2007a); chymosin can approach and cut the κ-casein; trypsin can attack the β-casein (Diaz et al. 1996)].

The presence of the hairy layer has been inferred from the observations that the hydrodynamic diameter of micelles decreases during renneting (Walstra et al. 1981). Because the only action

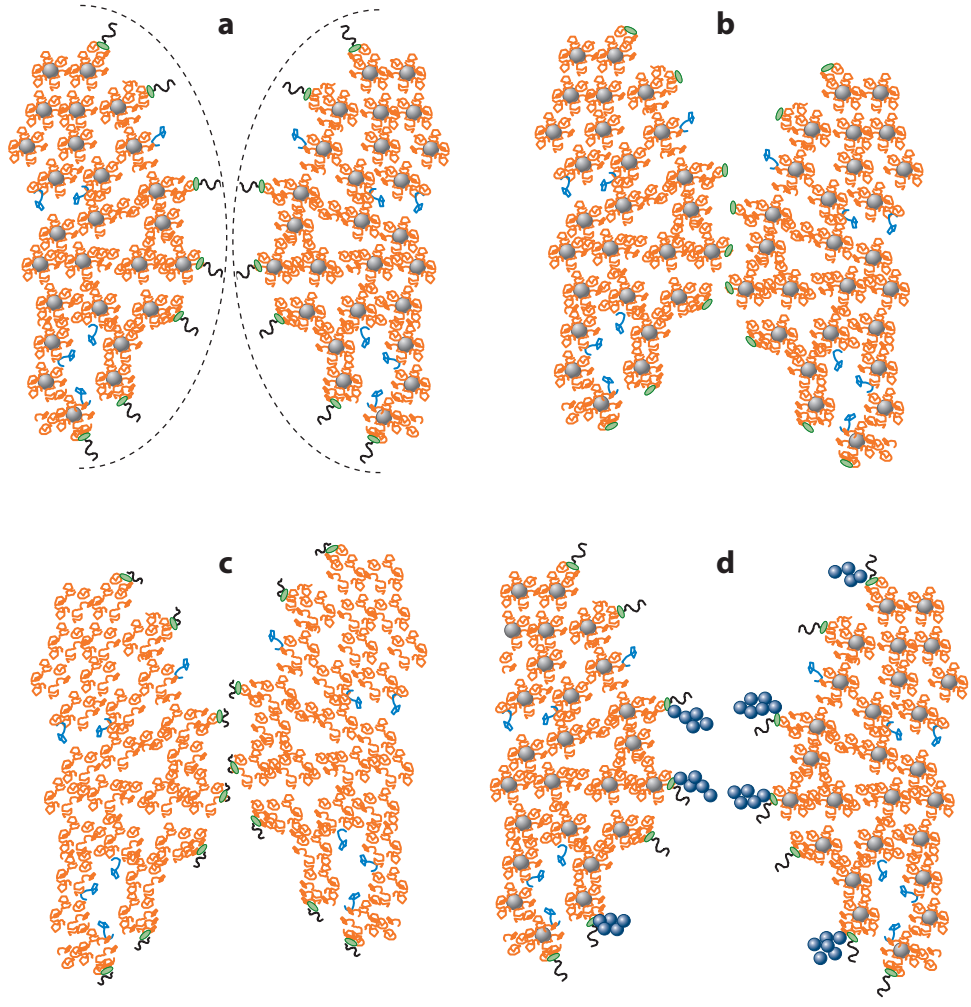

Figure 4

Diagrams of interacting micelles (only the interacting parts are shown, not the whole particles). (*a*) Native micelles are sterically stabilized by macropeptide hairs. The zone of action of the steric effect is indicated by dashed lines. (*b*) Renneted micelles where the hairs have been removed by chymosin, allowing close approach of the micellar surfaces. (*c*) Acidified micelles where the calcium phosphate has been dissolved and the hairs have been collapsed. (*d*) Micelles from heated milk with attached whey protein/κ-casein complexes. These complexes prevent coagulation even if the κ-casein is removed and provide points of attachment between micelles during acid gelation. Para-κ-casein is green, the caseinomacropeptide chains are black, α_s- and β-caseins are orange, and calcium phosphate nanoclusters are represented by grey spheres. In panel d, whey proteins are represented by dark blue spheres. Some β-casein (*blue*) hydrophobically binds to other caseins and can be removed by cooling. Not drawn to scale.

of the chymosin in the short term is to cut the κ-casein at the bond 105–106 and liberate the C-terminal macropeptide (Hyslop 2003), it can be inferred that the decrease in the observed diameter during renneting results from the loss of the stabilizing hairy layer rather than from a shrinkage of the micelle (de Kruif & Zhulina 1996). It has been shown that if the hairy layer is collapsed by ethanol (a poor solvent for protein), the decrease in diameter during renneting is no

longer apparent (Horne 1984). Given that the existence of the hairy layer is universally accepted, there remain some questions to be answered as to how the κ-casein is distributed on the available surface. It is known that κ-casein can form disulphide-linked oligomeric aggregates (Groves et al. 1998), and it is not known whether the κ-casein is present on the micellar surface in this form, so that the micellar surface might be covered by bunches of hairs, rather than single ones. It has also been suggested, on the basis of the known surface area covered by individual caseins on the interfaces of emulsion droplets, that the amount of available κ-casein is insufficient to cover all of the micellar surface and that other caseins must be present in the surface layer (Dalgleish 1998). This calculation assumed that the micelles are rather smooth spheres whose available surface is dependent on the cube of the hydrodynamic radius. However, if the surface of the micelle is deeply indented as shown by SEM (Dalgleish et al. 2004) (**Figure 2**), the outermost regions of the micelle take up much less area than that of the equivalent sphere; if the κ-casein is located only in these regions, then problems of coverage are diminished.

The question becomes, indeed, where is the surface of the micelle? For a deeply indented structure such as is shown in **Figures 2** and **3**, the hydrodynamic diameter depends mainly on the most protruding parts of the structure (**Figure 4*a***). Two micelles approaching one another see only these highest points of the approaching particles. So, for the approach of large particles, only the outermost part of the surface is important. On the other hand, for the approach of small molecules (relative to the size of the micelle), the micellar surface includes not only the highest areas but the indented areas as well. Therefore, the concept of the micellar surface depends on the specific interactions that are being considered.

The details of the relevance of the surface layer to processing properties are considered in later sections of this review. However, it is worth considering at this point an interaction involving the surface κ-casein, namely the interaction between κ-casein and the polysaccharide κ-carrageenan. This interaction is important because it creates a weak gel (Spagnuolo et al. 2005) and prevents the phase separation in, for example, ice-cream mixes (Vega et al. 2005). The interaction is apparently anomalous, because it occurs between two negatively charged molecules, κ-casein and κ-carrageenan. It has been postulated that there is a specific interaction between the negative κ-carrageenan and the positively charged region of κ-casein in the vicinity of the site cleaved by chymosin (Snoeren et al. 1975). If this is the case, the association of κ-carrageenan with the micelles offers another example of the accessibility to large molecules of certain parts of the κ-casein that might be imagined to be shielded by the hairy layer.

STRUCTURE AND FUNCTION DURING PROCESSING OF CASEIN MICELLES

The behavior of micelles during processing depends almost entirely on the structure and properties of the micellar surface. That is, different processing operations cause different changes to the hairy surface structure. These can affect the micellar stability in different ways.

Effects of Heat and of High Pressure Treatments

The heat stability of milk is affected by pH, usually with a maximum at the native pH of milk (pH 6.7) and a minimum at a pH of approximately 6.9 (Singh & Creamer 1992). However, the instability of milk to heat is not simply caused by the micelles, but by the denaturation of the whey proteins and their interaction with the micelles, as well as changes in calcium equilibrium (Singh & Creamer 1992). At temperatures above 100°C, solubilization of colloidal calcium phosphate and calcium precipitation occur, as a function of pH (O' Connell & Fox 2001). However, these

changes do not appear to cause the disruption of the overall basic structure of the micelles or colloidal destabilization, although there is extensive heat-induced dissociation of κ-casein from micelles heated in the absence of whey proteins at pH > 6.5 (Anema & Klosternmeyer 1997). This dissociation of κ-casein, as well as some α_s-casein from the micelles, has been attributed to the increase in electrostatic repulsion on the surface of the micelles with increasing pH (Singh & Fox 1987). However, the interior of the micelles must also be modified during heating of milk at pH values greater than 6.9 because significant amounts of α_s- and β-caseins are liberated into the serum (Donato & Dalgleish 2006).

During heating in the presence of whey proteins, the surface of the micelles is modified, because of the association of whey proteins with κ-casein. At temperatures above approximately 70°C, whey proteins denature and start to aggregate. In the presence of micelles, the aggregating whey proteins form complexes with micellar or serum κ-casein and α_{s2}-casein to give particles with sizes ranging from 30 to 100 nm (Anema & Li 2003, Donato & Dalgleish 2006, Donato et al. 2007b). These complexes are distributed between the micelles and the serum, and the fraction attached to the micellar surfaces decreases with increasing pH of heating: At the natural pH of milk, approximately 30% of the denatured whey protein is associated with the micelles, and the rest is present in solution as soluble complexes (Kethiredipalli et al. 2010). At a lower pH (for example, pH 6.3) there is an increasing binding of the whey protein complexes to the micelles (Anema & Li 2003, Donato & Dalgleish 2006). It has been suggested that the heat-induced aggregates coating the micelles become less homogeneous as the pH decreases, with larger proportions of whey proteins binding to one κ-casein site (Vasbinder & de Kruif 2003, Vasbinder et al. 2003a). These changes in the structures on the micellar surface (**Figure 4*d***) have important consequences in the processing behavior of milk (see below).

Although heating at close to the normal pH of milk appears to have little effect on the internal structure of the micelles, ultrahigh pressure processing at >250 MPa results in their dissociation. In addition to solubilization of the colloidal calcium phosphate, there is a decrease in the size of the micelles and depletion of κ- and β-casein (Gaucheron et al. 1997). These rearrangements, however, still do not cause full colloidal destabilization and precipitation, suggesting that proteins other than κ-casein can stabilize the particles. There appears to be a pressure above which the changes in the micelles are not reversible: At moderate pressures (<200 MPa), the micelles reform to something similar to their original size distribution once the pressure is released; however, if this pressure is exceeded, the micelles do not reform but exist as much smaller (~30 nm) caseinate particles of unknown structure (Gebhart et al. 2006). However, results from different laboratories do not totally agree on the sizes of the final particles (Anema et al. 2005), perhaps because the original starting materials were different. It is not known what mechanism stabilizes these particles.

In the presence of whey proteins and at pressures greater than 100 MPa, the surface of the micelle is also modified by the association of denatured whey protein (Needs et al. 2000). Unlike the effect of heating, however, such association of the whey proteins with micelles does not hinder the rennet-induced aggregation of the caseins, as high pressure–treated milk shows a reduced coagulation time, unlike heated milk (Lopez-Fandino et al. 1996).

Compared to static high pressure, there are few changes in the micelles subjected to dynamic high pressure. Homogenization at 180 MPa causes a reduction in size of less than 10 nm for both heated and unheated milk, and there is a slight reduction in the rennet-induced clotting time (Sandra & Dalgleish 2007). However, in the presence of oil, semi-intact micelles as well as micellar fragments adsorb on the surface of the fat globules (Dalgleish et al. 1996). This results from high-energy collisions between micelles and fat surfaces, which may cause physical disruption and subsequent spreading of the micelles across the oil surface. In that way, it is possible to use micelles as emulsifiers, although they are much less effective than the equivalent amount of sodium caseinate

because of the highly aggregated state of the protein in the micelles. Using a microfluidizer, it has been found possible to create casein micellar nanoparticles with a lipid core (Dalgleish et al. 1996).

Conversely, micelles can be stabilized against dissociation by forming covalent interprotein bonds in the inner core using transglutaminase. This enzyme forms bonds between glutamine and lysine, and when added to unheated milk, crosslinks the caseins within the micelles with very little intermicellar aggregation. This enzymatic treatment has been shown to limit the extent of dissociation of the micelles by urea, acid, and high pressure, and prevents the hydrolysis of κ-casein by rennet (O'Sullivan et al. 2002, Smiddy et al. 2006).

Rennet-Induced Instability of Casein Micelles

Given that the micelles are stabilized by steric repulsion generated by the polyelectrolyte layer of κ-casein, any process that removes the C-terminal region of κ-casein causes a decrease in the colloidal stability of the micelles and causes a sol-gel transition (**Figure 4*b***).

Chymosin specifically breaks κ-casein molecules at a specific position and releases the C-terminal region of the κ-casein [caseinomacropeptide (CMP)]. During the primary stage of the enzymatic reaction, there is an increase in the diffusion coefficient of the micelles because of the gradual removal of their polyelectrolyte hairy layer (de Kruif 1992). The milk viscosity decreases until more than 85% of the κ-casein is hydrolyzed (Tunier & de Kruif 2002). At this point, the steric stabilization generated by the few remaining κ-casein hairs is insufficient to keep the micelles apart, and they begin to aggregate and eventually to gel (Dalgleish 1979, Sandra et al. 2006, Kethiredipalli et al. 2010). Thus, gelation occurs only after a nearly full release of CMP in solution. A number of mechanisms have been invoked to explain this phenomenon, ranging from detailed physicochemical analyses of the steric stabilization mechanism (de Kruif 1999, Tunier & de Kruif 2002) to more geometrically based descriptions of the formation of hot spots via which aggregation can occur (Dalgleish & Holt 1988). As the pH is decreased, the charges on the κ-casein layer are decreased, and this causes its partial collapse so that it is less effective in providing steric stabilization (de Kruif 1999). Thus, when renneting is carried out at a lower pH, the gelation occurs at a lower degree of κ-casein breakdown (van Hooydonk et al. 1986, Ferrer et al. 2008). Similarly, the secondary stage of aggregation commences earlier in the presence of ethanol because of a reduced dielectric constant of the solution, a collapse of the κ-casein layer, and a decrease in steric repulsion (O'Connell et al. 2006).

Once decreased steric repulsion allows the micelles to approach one another closely, hydrophobic interactions cause bonding between the particles, as does the amount of ionic calcium; increased calcium also allows aggregation at progressively lower levels of CMP release (Bringe & Kinsella 1986). The rennet-induced aggregation has been described as the formation of a gel by adhesive hard spheres, as the state of the polyelectrolyte brush controls the stickiness of the particles at close contact (de Kruif 1998). However, although the inner structure of the micelle and the presence of colloidal calcium phosphate are not important in the initial stages of rennet aggregation, they become increasingly important when interparticle rearrangements occur and the micelles start to fuse. A partial loss of colloidal calcium may reduce the charge interactions, as shown by calcium depletion renneting experiments at pH 6.0, where the addition of EDTA caused the formation of a weaker, more flexible casein network (Choi et al. 2007). The firmness of the gel depends on the number of bonds created, i.e., the protein concentration, whereas the time of coagulation seems not to be affected by a protein concentration up to four times the original amount of micelles (Sandra et al. 2011).

The progressive loss of the hairy layer and the consequent instability of the micelles gives us a reasonable understanding of the renneting process in unheated milk. In milk that has been

heated, the κ-casein is broken down by chymosin as it is in unheated milk, but the micelles do not aggregate well (Vasbinder et al. 2003b, Kethiredipalli et al. 2011). At least part of this is a result of the presence of denatured whey proteins on the micellar surface, which may prevent the close approach of the potentially interacting sites on the micelles. The complexes from the serum attach to the micelles as renneting proceeds so that they do not need to be on the micelle surface before renneting starts to cause changes in the aggregation behavior (Kethiredipalli et al. 2011). In addition, sodium caseinate has been shown to inhibit the close range attachment (aggregation) of the micelles by shielding the hydrophobic or the calcium-sensitive patches on the surface of the protein particles (Gaygadzhiev et al. 2012).

Acidification of Casein Micelles

A different mechanism of instability comes into play during acid-induced coagulation of the micelles. During acidification, changes occur not only to the hairy surface of the micelle, but also to its internal structure. Acidification from pH 6.6 to 5.3 causes the progressive release of calcium phosphate, magnesium, and citrate ions from the interior of the micelles (Le Graët & Gaucheron 1999); the loss of the colloidal calcium phosphate nanoclusters is shown by SAXS (Marchin et al. 2007). All of the inorganic phosphate is solubilized when pH 5.2 is reached, and most of the remaining calcium ions are solubilized when pH 4.6 is reached (Le Graët & Gaucheron 1999). In spite of the loss of such an essential structural feature, at temperatures above 25°C there is little dissociation of the caseins at decreased pH (Dalgleish & Law 1988). This stability has been explained as a result of the decreasing negative charge on the individual caseins so that although bonds to calcium phosphate are lost, the charges on the caseins become insufficient to force them apart (Horne 2008). On the micellar surface, the decrease in charge of the CMP with diminishing pH causes the collapse of the κ-casein layer because the intra- and interchain interactions in the CMP are no longer sufficient to keep the chains extended. This decreases the steric stabilization of the micelles (de Kruif 1999). Therefore, the micelles can diffuse closer to each other, and as short range attractive forces take over, sol-gel transition occurs (**Figure 4*c***). In unheated milk, the aggregation occurs very close to the isoelectric point of the caseins, at a pH of approximately 4.8 (Vasbinder et al. 2003a, Rodriguez del Angel & Dalgleish 2005), and the network formed is weak because the interfaces between the particles in close proximity are still very hydrated: Although collapsed, the κ-casein layer still physically separates them from one another, making the bonding between them very weak (Li & Dalgleish 2006).

In heated milk, the presence of whey protein aggregates as described earlier, either in solution or on the surface of the micelles, is of great importance in acid gelation (Lucey 2002) (**Figure 4*d***). In these systems, it appears that the whey protein complexes control gel formation. When sufficient charge neutralization of the complexes on and off the casein micelles has occurred, by approximately pH 5.3, the complexes attach to the micelles and form bridges between the protein particles (Vasbinder et al. 2003a, Donato et al. 2007a). The differences between the mechanisms of aggregation in heated and unheated milk have been followed using diffusing wave spectroscopy and rheology (Alexander & Dalgleish 2005). In unheated milk, the sudden decrease in the mobility of the micelles occurs at the same pH as the rapid increase in the elastic modulus, suggesting that intramicellar interactions are the sole influence in the formation of the acid gel. On the other hand, in heated milk, the changes in the mobility of the micelles occur at a pH of approximately 5.3 and are accompanied by a moderate increase in the elastic modulus. However, this precedes the major increase in the elastic modulus at approximately pH 4.8, where the formation of a stiff network commences (Lucey et al. 1999). The gel strength is improved by a higher initial ratio of soluble to micelle-bound whey protein-κ-casein complexes, which in turn depends

on the initial pH at which the milk is heated (Vasbinder & de Kruif 2003, Vasbinder et al. 2003a; Rodriguez del Angel & Dalgleish 2005). It could be hypothesized that this reflects the ability of the whey protein aggregates to rearrange and fill the voids between micelles, resulting in a larger number of linkages in the gel network than in the case of whey proteins already attached to the surface of the protein particles before gelation.

In the formation of acid gels from both heated and unheated milk, the differences in the surfaces of the micelles compared with renneted micelles are very important, as the micelles do not have true bare hydrophobic patches, as in renneting, but still possess κ-casein or whey proteins at the particle-particle interface. This difference seems to be responsible for the ability of the micelles to retain their spherical shape longer during aggregation, as has been suggested by microstructural observations (Park et al. 1996).

In acid-induced gels where the pH of gelation is relatively high, there is some residual colloidal calcium phosphate at the time of gelation. Therefore, some rearrangement of the micellar structure and hence of the gel may occur as the pH decreases further. It has been claimed (Horne 2003) that the gradual solubilization of the residual calcium and calcium phosphate will result in an electrostatic imbalance within the micelles, as many sites in the core of the casein particles will not yet be neutralized (Ozcan-Yilsay et al. 2007). This enhances protein-protein repulsion within the gel network, causing a weakening of the gel until charge neutralization finally occurs and the gel again strengthens. This phenomenon is especially marked in acid gels made at high temperatures (40°C) or with very rapid acidification, where the interior of the micelle may not be in equilibrium with the serum (Horne 2003).

In many cheese-making processes, acid- and rennet-based aggregations are combined, and the stability of the micelles depends on the relative rates of pH change and release of CMP. If the rennet action is slow relative to acidification, then the gelation occurs at a low pH, although at a higher pH than that of simple acid gelation. On the other hand, rapid renneting causes gel formation at a high pH. However, if even only small amounts of CMP have been removed by rennet, the acid gel is much stronger, as judged by the elastic modulus, than it is in untreated milk (Tranchant et al. 2001, Castillo et al. 2006). If the rennet action is stopped at a low level of κ-casein breakdown before acidification starts, then the elastic modulus of the final acid gels is higher than in untreated milk (Gastaldi et al. 2003, Li & Dalgleish 2006). Although this phenomenon can qualitatively be explained by the removal of CMP diminishing the steric stabilization of the micelles and allowing them to aggregate at an earlier pH than normal, it is not easy to see why the removal of only a small amount (<20%) of the CMP creates such a very large effect on the gel properties. In these mixed rennet and acid gels, the micelles may also still contain substantial amounts of colloidal calcium phosphate that may subsequently dissolve and weaken the gel, as described above. Thus, as acidification proceeds, the gel may weaken before it becomes stronger (Lucey et al. 2000).

Casein Micelles and Concentration

Evaporation or membrane technology is employed to concentrate micelles during the manufacture of concentrated milk products or skim milk powders. During evaporation, heating may cause interactions between whey proteins and micelles, and it increases the amount of insoluble calcium and phosphate (Le Graët & Brulé 1982). All of the components are retained in the milk, so that the equilibria between the mineral ions and the proteins are altered. On the other hand, in the manufacture of MPCs or phosphocaseinate using membrane filtration, the protein is retained and free ions are transmitted through the membrane, increasing the volume fraction of the caseins, and decreasing the ratio between the soluble and colloidal minerals. The removal of the diffusible ions also alters the interactions between the micelles.

Only small changes occur to the micelles during concentration by membrane filtration, and the amount of colloidal calcium phosphate remains constant unless there is an extensive addition of water (during diafiltration) or the pH changes (Singh 2007, Ferrer et al. 2011). However, even under such harsh conditions, the size of the micelles does not change greatly (Ferrer et al. 2011). Because of their stabilizing layer of κ-casein, the micelles cannot approach closely and behave as hard spheres at normal concentrations of milk (de Kruif 1999, Alexander et al. 2002). In fact, milk can be extensively concentrated and still behave like a dispersion of hard spheres (Mezzenga et al. 2005; Bouchoux et al. 2009b, 2010; Sandra et al. 2011). That is, it can be concentrated and rediluted without any change in the micellar properties, and viscosity and light scattering show the behavior typical of hard-sphere suspensions (Ferrer et al. 2011). However, concentration to greater than a volume fraction of ~0.65 (about 178 g L^{-1} of protein) starts to cause overlap, compression of the hairy layers, and distortion of the micellar structure, and the concentrated milk forms a soft solid (Bouchoux et al. 2009a,b; Dahbi et al. 2010). Further compression leads to expulsion of the interior water, and during this stage it appears that some micellar fusion can occur (Bouchoux et al. 2009a, 2010); presumably, the hairy layers are flattened and distorted so that the interior parts of the micelles can come into contact. After this, redilution does not create micelles with normal properties.

Thus, at critical concentrations, the elastic modulus increases rapidly and the dispersions turn into gels. Similar processes may occur during ultrafiltration of milk, during which a layer of compressed micelles can be found at the surface of the ultrafiltration membrane (Pignon et al. 2004, Jimenez-Lopez et al. 2008), where they have been caught and compressed by the outward flow of serum through the membranes. Therefore, it appears that the physical compression of the hairy layer can lead to fusion of adjacent micelles. This may again suggest that the hairy layer is not sufficiently complete and that on collapse there may be gaps in the layer through which other caseins of the micelle can interact. It is therefore possible that the casein micelles in an ultrafiltration process may be irreversibly damaged. Changes in the functionality have been observed during storage of milk concentrate powders, when hydrophobic interactions and crosslinking occur between the casein proteins on the surface of the powder (Havea 2006, Mimouni et al. 2010).

CONCLUDING REMARKS

It can be seen from the preceding discussion that it is possible to consider the casein micelle as a sponge-like structure consisting of linked calcium phosphate/casein nanoclusters. Such a model agrees at least in general terms with the physical measurements that can be made by scattering techniques and electron microscopy. Likewise, the interactions of the micelles can be understood in terms of the structure of the surface layer that changes with conditions. What is less clear is the understanding of the details of the properties of the superstructures formed when the micelles are caused to aggregate. This is especially the case for the strengths of gels formed by a combination of rennet and acidification. These must depend on the intimate structures and interactions of the micellar surfaces. On the other hand, the description of the interior of the particles demonstrates how it may be feasible to introduce specific noncasein molecules into the particles' structure so that the micelles can be used as transport or delivery vehicles for desirable small molecules.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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