# Mechanism for the Ethanol-Dependent Heat-Induced Dissociation of Casein Micelles

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An explanation as to how casein micelles dissociate when heated in the presence of ethanol is presented. Dissociation of casein micelles in milk−ethanol mixtures was studied using $^1$H NMR, and the effects of addition of $CaCl_2$, NaCl, or EDTA or alteration of milk pH on this dissociation were studied. It is proposed that at low temperatures, ethanol reduces the solvent quality of milk serum, but above a critical temperature (∼30 °C in a 35% ethanol solution), ethanol enhances solvent quality and dissociates the casein micelles. Ethanol reduced protein hydrophobicity and increased the p$K_a$ value of phosphoserine, effects that are likely to be significant in the dissociating effect of ethanol at elevated temperatures.

**Keywords:** *Milk; casein micelles; ethanol; dissociation*

## INTRODUCTION

As shown by Zadow (*1*) and O'Connell et al. (*2*) it appears that casein micelles dissociate when heated in the presence of ethanol [a 1:1 (v/v) mixture of milk and 65% (w/w) aqueous ethanol]. This phenomenon was observed by measuring the transmission and *L** value of milk−ethanol mixtures. Ethanol also dissociated *β*-casein micelles (*2*). It was concluded that the dissociating effect of ethanol at elevated temperatures is related to a change in solvent quality as the temperature increases.

Using additional spectroscopic (i.e., light scattering and nuclear magnetic resonance) and wet chemical techniques, the study of ethanol-dependent temperature-induced dissociation of casein micelles was extended, and a mechanism for the aforementioned effect is presented in this paper.

## MATERIALS AND METHODS

**Materials.** Reconstituted low-heat skim milk powder (10% nonfat solids) was prepared as described in ref *2*. All chemicals used were of reagent grade.

**Casein Micelle Dispersions.** Skim milk was centrifuged at 100000*g* for 1 h at 20 °C, and the pellet was dispersed, using a Potter tube, in synthetic milk ultrafiltrate, which was made up in $D_2O$ rather than $H_2O$ (*3*). This dispersion was recentrifuged under the same conditions and dispersed in synthetic milk ultrafiltrate containing $D_2O$ instead of $H_2O$.

**Measurement of Transmission.** Transmission−temperature profiles for 1:1 mixtures of milk [with and without added $CaCl_2$ (0−2 mM), EDTA (0−8 mM), or NaCl (0−50 mM)] and various alcohols (methanol, ethanol, or 2-propanol) were determined as outlined by O'Connell et al. (*2*).

**$^1$H Nuclear Magnetic Resonance ($^1$H NMR).** $^1$H NMR analysis of casein micelle dispersions in synthetic milk ultrafiltrate was carried out using a 600 MHz Bruker Avance 600 spectrometer with a quadrature detection. Large flip angles, typically 70−90°, and an interpulse delay time of 2.5 s were used. The chemical shifts quoted are relative to 3-trimethyl-2,2,3,3-tetradeuteropropionate, and for comparison of spectra as a function of temperature, the temperature in the probe was determined using a tetramethylammonium chloride solution in $D_2O$.

**Determination of p$K_a$ Value of Phosphoserine.** The p$K_a$ values of phospho-DL-serine in a 1:1 (v/v) mixture of 0.1 M phosphoserine and water or 65% (w/w) ethanol were determined by acid−base titration curves with 1.0 M NaOH. Aliquots of 1.0 M NaOH (50 or 100 μL/20 mL of test solution) were added incrementally every 2.5 min, and the pH was monitored continuously.

**Surface Hydrophobicity.** The surface hydrophobicity of 1:1 mixtures of milk and 65% ethanol at temperatures in the range 5−75 °C were determined, using anilinonaphthalenesulfonic acid, essentially as described in ref *4*.

## RESULTS AND DISCUSSION

**Proposed Explanation for the Alcohol-Dependent Temperature-Induced Dissociation of Casein Micelles.** As reported by Zadow (*1*), and subsequently supported by the results of O'Connell et al. (*2*), it appears that on heating in the presence of ethanol casein micelles dissociate. It was proposed that this effect is similar, or related, to the effect of 2,2,2-trifluoroethanol (TFE) on the stability of casein micelles, as reported by Horne and Davidson (*5*). Herskovits and Mescanti (*6*) and Griffin et al. (*7*), using circular dichroism and optical rotary dispersion, respectively, showed that alcohols induce α-helical features in $\alpha_s$- and *κ*-casein. Considering this, Horne and Davidson (*5*) proposed that the ability or tendency for caseins to associate into macromolecular colloidal aggregates is eliminated as a consequence of alcohol-induced structural transformations. However, we propose that an alcohol-dependent temperature-induced increase in casein solubility causes dissociation of casein micelles and that under such conditions the caseins undergo conformational transformations, that is, an increase in α-helical structural features.

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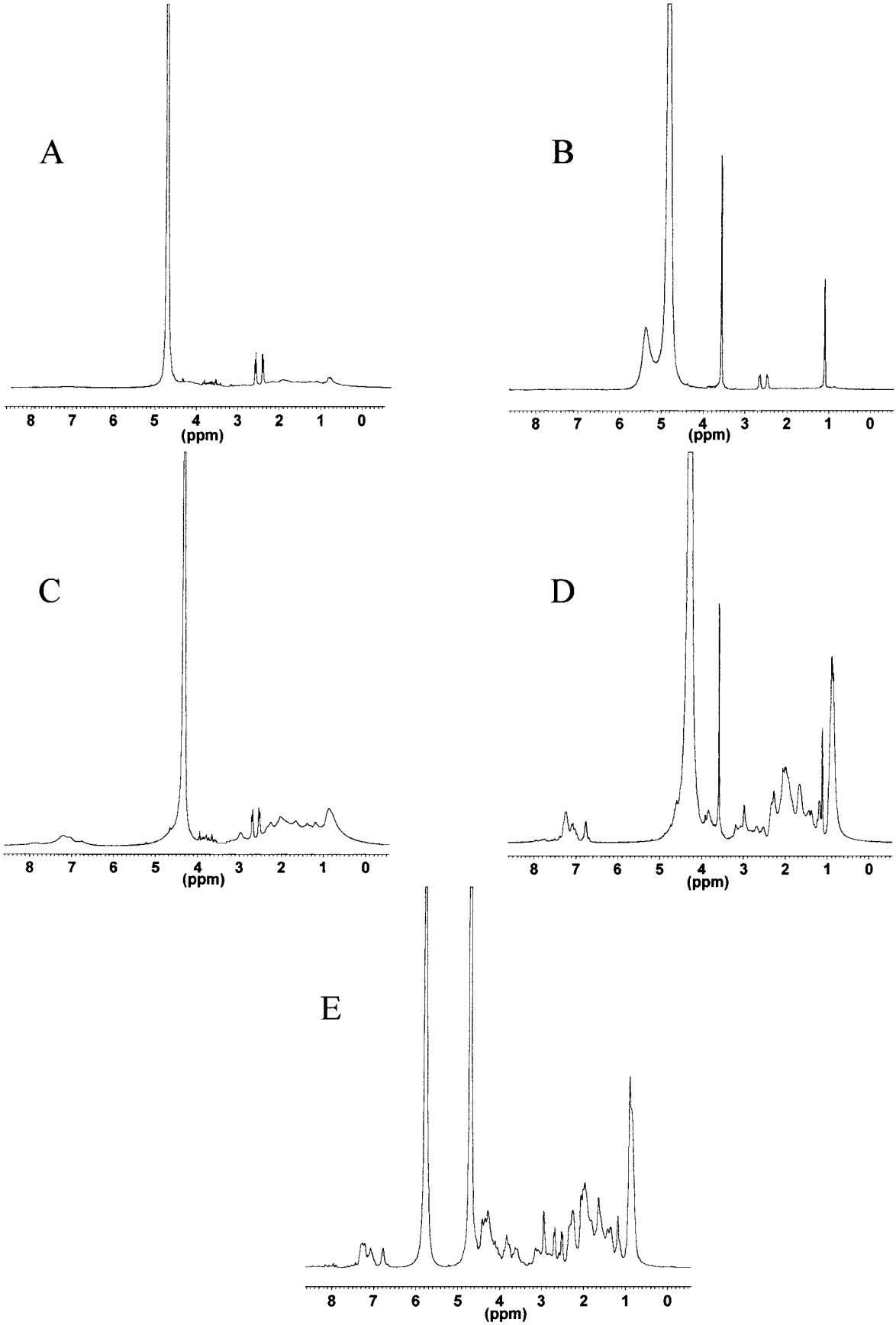


**Figure 1.** [1]H NMR spectra of a 1:1 mixture of milk and water (A, C) or 65% ethanol (B, D) at 20 (A, B) or 70 (C, D) °C or of milk containing 6 M urea at 20 °C (E).

[1]H NMR was used to determine the effect of temperature on the molecular mobility, which is an index of solvent quality, of caseins, both in the absence and in the presence of ethanol. The [1] H NMR spectra of a 1:1 mixture of milk with water or 65% ethanol were similar at 20 °C but differed markedly at 70 °C (Figure 1). The

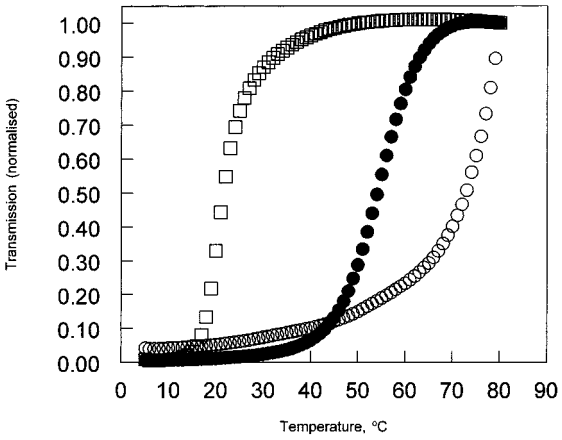


**Figure 2.** Effect of temperature on the transmission ($\lambda = 600$ nm) of a 1:1 (v/v) mixture of milk and 65% (w/w) methanol (○), ethanol (●) or 2-propanol (□).

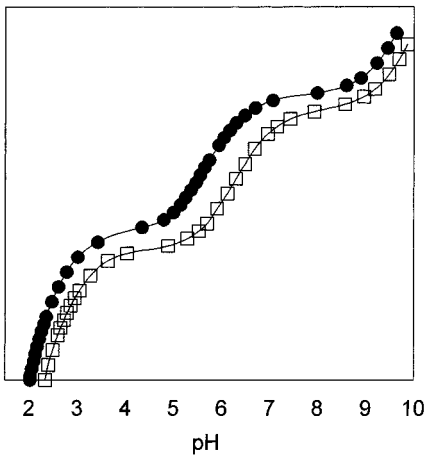


**Figure 3.** Acid−base titration of a 1:1 (v/v) mixture of 0.1 M phospho-DL-serine and water (●) or 65% (w/w) ethanol (□).

methyl (leucine, isoleucine, valine, and threonine) peaks at ∼1 ppm and aromatic (tyrosine, tryptophan, and phenylalanine) peaks at ∼7 ppm of the milk−ethanol mixture at 70 °C were markedly larger than in the same system at 20 °C or in the milk−water mixture at 70 °C (Figure 1). This suggests that protein mobility, and solvent quality, is greater in the milk−alcohol mixture at 70 °C and that the micelles in the milk−ethanol mixture have dissociated at the elevated temperature. This conclusion is supported by comparing the spectra of the milk−ethanol mixture at 70 °C with that of milk containing 6 M urea, which is known to enhance protein solubility (and dissociate casein micelles) by inhibiting hydrophobic bonding. As shown, the apparent increase in protein mobility was similar (Figure 1).

From the data presented, it appears that native casein micelles undergo an alcohol-dependent temperature-induced dissociation, the mechanism of which appears to be related to an increase in solubility when heated in the presence of ethanol. However, this observation is contrary to the established destabilizing effect of ethanol on the stability of the milk protein system (*8*−*12*). Alcohols reduce the dielectric constant of the system, resulting in a commensurate reduction in solvent quality, among other ill-defined reactions. A decrease in the dielectric constant reduces micellar stability by collapsing the protruding C-terminal region of $\kappa$-casein located on the micelle surface, reducing the net negative charge of the protein (i.e., causes an increase in the p$K_a$ of aspartate and glutamate residues with little effect of the basic amino acid residues lysine, arginine, and histidine) and precipitating calcium phosphate (*12*−*15*). Alcohols may cause coagulation of casein micelles directly (*10*, *12*) or, alternatively, increase the susceptibility of casein micelles to thermal (*9*) or enzymatic coagulation (O'Connell and Fox, unpublished data).

Horne and Parker (*8*) and Fox and Mohammed (*9*), who studied the effect of different solvents on the alcohol and heat stability of milk, respectively, showed that the effect of these solvents was related to their ability to reduce the dielectric constant; that is, the destabilizing effect of propanol is greater than that of ethanol, which in turn is greater than that of methanol. In the current study, a relationship between the ability of a given alcohol to reduce the dielectric constant of milk and to dissociate the micelles was also observed (Figure 2). The apparent dissociation temperature of casein micelles in a 1:1 mixture of milk and 65% 2-propanol was ∼30 °C (Figure 2). This is consistent with the effect of TFE, which dissociates micelles at ambient temperature (*5*) and has a greater ability to reduce the dielectric constant of aqueous systems than 2-propanol. Geerts et al. (*16*) reported that reducing the dielectric constant decreases the calcium ion activity of milk, which may be significant. However, as is the case with the effect of the dielectric constant on the alcohol stability (*10*), it remains unclear whether the relationship between dielectric constant and the dissociation of casein micelles at elevated temperatures is significant or merely casual.

To elucidate the role of specific interactive forces on the alcohol-dependent temperature-induced dissociation of casein micelles the effect of $CaCl_2$ (0−2 mM) or EDTA (0−8 mM) on the dissociation temperature of casein micelles was determined, but neither had an effect (results not shown). This is surprising as calcium markedly affects the colloidal stability of casein micelles toward heat, alcohol, and rennet (*12*, *17*, *18*). The inability of calcium (or EDTA) to affect the alcohol-dependent temperature-induced dissociation of casein micelles may be explained by the fact that alcohol causes a shift in the p$K_a$ value of the phosphoserine residues to more alkaline values (Figure 3), thereby reducing their relative contribution to associative interactions. The possible significance of electrostatic interactions was investigated by measuring the effect of pH (pH 6.55−7.30) or NaCl (0−50 mM) on the apparent dissociation temperature (Figures 4 and 5). A reduction of pH (Fgiure 4) or the addition of NaCl (Figure 5) markedly increased the dissociation temperature (as denoted by the inflection point in the temperature−transmission profile), which suggests that electrostatic repulsive forces play a major role in the alcohol-dependent temperature-induced dissociation of casein micelles. As shown in the insets of Figures 4 and 5 there is a linear relationship between dissociation temperature and pH or NaCl concentration. Perhaps the initial decrease in protein charge on the addition of ethanol, that is, as a consequence of the shift of the p$K_a$ values of glutamate and aspartate, is counteracted by the fact that the basic amino acids become less protonated as the temperature increases (*19*).

From the data presented, it is clear that casein micelles dissociate when heated in the presence of alcohols. Considering the effect of pH and NaCl on the alcohol-dependent temperature-induced dissociation, it appears that electrostatic repulsive forces play a major

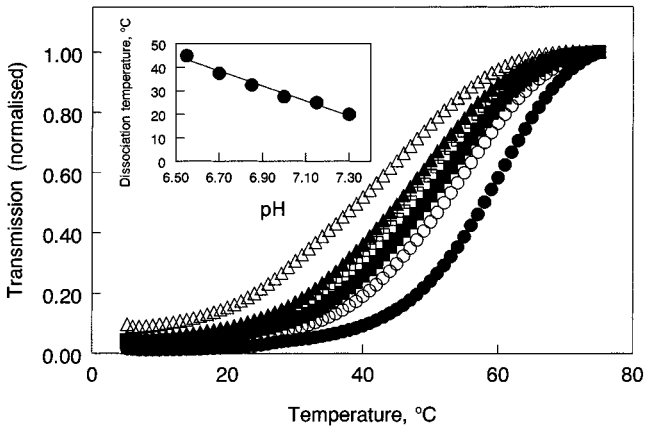


**Figure 4.** Effect of temperature on the transmission ($\lambda = 600$ nm) of a 1:1 (v/v) mixture of 65% (w/w) ethanol and milk at pH 6.55 (●), 6.70 (○), 6.85 (■), 7.00 (□), 7.15 (▲) or 7.30 (△).

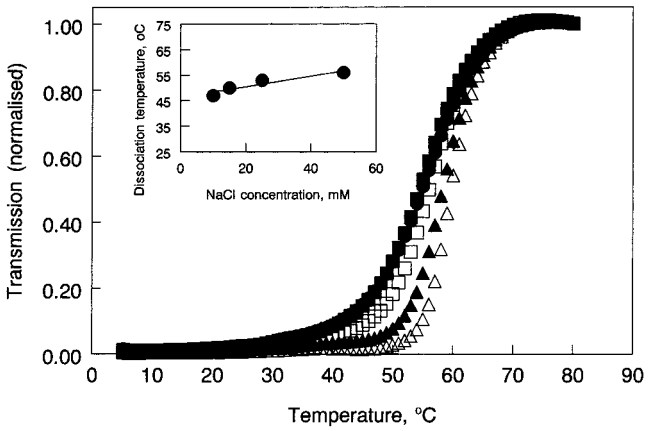


**Figure 5.** Effect of temperature on the transmission ($\lambda = 600$ nm) of a 1:1 (v/v) mixture of 65% (w/w) ethanol and milk supplemented with 0 (●), 5 (○), 10 (■), 15 (□), 25 (▲) or 50 (△) mmol $L^{-1}$ NaCl.

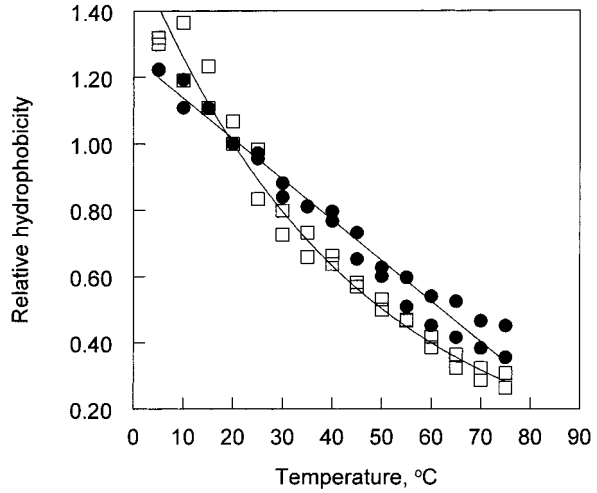


**Figure 6.** Effect of temperature on the relative surface hydrophobicity of a 1:1 (v/v) mixture of milk and water (●) or 65% (w/w) ethanol (□).

role in dissociating casein micelles. As to the factors responsible for the enhancement of the solubility of caseins, perhaps an increase in the p$K_a$ of phosphoserine residues and the concomitant reduction in calcium bridging are significant.

As shown in Figure 6, the surface hydrophobicity (determined using anilinonaphthalenesulfonic acid) of milk proteins decreased with temperature, both in the absence and in the presence of ethanol, with the hydrophobicity of the latter system being lower at elevated temperatures. This ethanol-induced change in the physicochemical properties of caseins is also likely to enhance solubility. Zadow (*1*) suggested that the alcohol-dependent temperature-induced dissociation of casein micelles can be explained in terms of the effect of ethanol on hydrophobic bonding and proposed that inhibition of hydrophobic bonding caused dissociation of casein submicelles and, consequently, of the micelles. As is the case with the effect of ethanol on the micellarization of $\beta$-casein (*2*), there appears to be a critical temperature, between 20 and 30 °C, below or above which ethanol has a destabilizing or stabilizing effect, respectively. The reduction in the surface hydrophobicity may be related to an increase in the p$K_a$ value of the phosphoseryl residues and a concomitant decrease in potential calcium binding sites. The decrease in surface hydrophobicity may also be due to protein−solute interactions, as ethanol is known to reduce the free energy of hydrophobic amino acid side chains (*20*).

**Significance of Alcohol-Dependent Temperature-Induced Dissociation in Relation to Micelle Cohesive Interactions.** Considering the fact that the solubility of calcium phosphate is reduced by ethanol (*14*) or increasing temperature (*21*), it can be concluded that the indigenous colloidal calcium phosphate present in milk remains associated with the caseins despite the dissociated state of the casein particles in the milk−ethanol system at elevated temperatures. It is therefore concluded that the presence of colloidal calcium phosphate on casein particles, although a prerequisite for the formation of casein micelles, does not guarantee that the casein particles exist in a micellar form. It is proposed that apart from a minor fraction of "authentic colloidal calcium phosphate", which interacts with the phosphoseryl residues of the caseins, calcium phosphate contributes to the cohesive interactions responsible for the micellarization process by associating with the caseins, thereby causing a reduction in protein charge. The charge shielding effect of calcium phosphate precipitation enhances the hydrophobic aggregation of casein molecules into micelles. This proposed nonspecific and indirect role of calcium phosphate in the micellarization process would appear to be consistent with the fact that its "colloidal" nature is a consequence of its low solubility rather than some elaborate mechanism. The role of calcium phosphate in modulating hydrophobic interactions would also appear to be supported by the results of Slattery (*22*), who showed that artificial casein micelles, composed of the four casein fractions and calcium, dissociated at low temperatures (at which hydrophobic attractions are minimal) but that this temperature-dependent dissociation was eliminated if phosphate was added.

**Conclusions.** From the data presented it appears that on heating in the presence of alcohols, repulsive forces between caseins increase and solvent quality is markedly enhanced, which results in swelling of the micelle and, ultimately, in dissociation. The dramatic decrease in cohesive interactions between casein molecules on heating in the presence of alcohols is most likely a consequence of a reduction in phosphoseryl-mediated cross-linking and an increase in protein hydrophilicity.

From the data presented, it would appear also that the relative contribution of calcium phosphate to the cohesive interactions responsible for the micellarization may be related to its effect on hydrophobic interactions.

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