

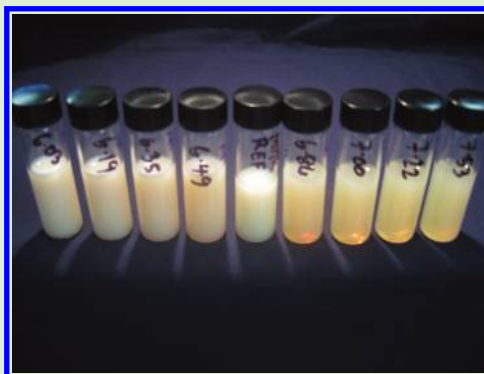
# Interaction of Lactoferrin and Lysozyme with Casein Micelles

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**ABSTRACT:** On addition of lactoferrin (LF) to skim milk, the turbidity decreases. The basic protein binds to the caseins in the casein micelles, which is then followed by a (partial) disintegration of the casein micelles. The amount of LF initially binding to casein micelles follows a Langmuir adsorption isotherm. The kinetics of the binding of LF could be described by first-order kinetics and similarly the disintegration kinetics. The disintegration was, however, about 10 times slower than the initial adsorption, which allowed investigating both phenomena. Kinetic data were also obtained from turbidity measurements, and all data could be described with one equation. The disintegration of the casein micelles was further characterized by an activation energy of 52 kJ/mol. The initial increase in hydrodynamic size of the casein micelles could be accounted for by assuming that it would go as the cube root of the mass using the adsorption and disintegration kinetics as determined from gel electrophoresis. The results show that LF binds to casein micelles and that subsequently the casein micelles partly disintegrate. All micelles behave in a similar manner as average particle size decreases. Lysozyme also bound to the casein micelles, and this binding followed a Langmuir adsorption isotherm. However, lysozyme did not cause the disintegration of the casein micelles.



## INTRODUCTION

The interaction of proteins is highly relevant for living organisms as the interaction provides the signaling pathways in living cells (see for example the special issue of "Nature Structural & Molecular Biology"<sup>1</sup>). For example, DNA is a strong anionic polyelectrolyte and interacts strongly with proteins.<sup>2,3</sup> In addition, the interactions between anionic and cationic polymers is a topic of recent interest.<sup>4</sup> Bungenberg de Jong and Kruyt<sup>5</sup> and Bungenberg de Jong<sup>6,7</sup> were among the first to investigate the interaction between oppositely charged proteins and polysaccharides. The observed phase separation was called complex coacervation. The word complex was used to distinguish it from the usual phase separation on mixing polymers, which was called coacervation, since each polymer "heaped" together with its own sort. In complex coacervation the different polymers coacervate to form a new phase. For further information on complex coacervation, see recent reviews by de Kruif et al.,<sup>8</sup> Weinbreck et al.,<sup>9</sup> and Cooper et al.<sup>10</sup> and references therein.

Bungenberg de Jong and Kruyt<sup>5</sup> and Bungenberg de Jong<sup>6,7</sup> extensively investigated the gum Arabic–gelatin system. However, it was Voorn<sup>11</sup> and Overbeek and Voorn<sup>12</sup> who developed the first theoretical descriptions for complex coacervation. In their thermodynamic model, the coacervation was mainly entropy driven through the "liberation" of the counterions surrounding isolated charges. The fact that salt influences coacervation strongly and temperature usually very little also suggested a predominantly entropy-driven process.

The interactions between oppositely charged proteins and polymers are of high relevance for the food industry. Many

drinks are stabilized through the addition of polysaccharides, such as pectin in yogurt drinks<sup>13</sup> or carrageenans in milk/chocolate drinks.<sup>14</sup> These polymers bind to oppositely charged proteins and thus form a network. For further information on this subject, the extensive review by Tolstoguzov<sup>15</sup> provides details on the interaction of polysaccharides and proteins and their phase behavior.

The interaction of globular proteins with polysaccharides has been extensively investigated and the picture that emerges is that the globular proteins bind to the polysaccharides and then form complex coacervate particles. These particles may initially be soluble but on increased interaction the system tends to phase separate, forming a new liquid phase or a precipitate. Interaction is strongest at pH between the respective  $pK_a$  and  $pK_b$ , or at optimum charge stoichiometry. Adding salt destroys the complex.

A system that has been investigated quite extensively is that of spherical polyelectrolyte brushes (PE) and proteins.<sup>16,17</sup> One may say that such a system mimics the casein micelles (CM) in milk as the CM is stabilized by a brush of  $\kappa$ -casein that may be considered as a PE brush.<sup>18</sup> Witteman and Ballauff<sup>16</sup> and Ballauff and Borisov<sup>17</sup> showed that a grafted PE binds much stronger to the protein (lysozyme (LZ)) than a linear PE. The main driving force is, again, the release of the counterions. In a somewhat more remote system of cross-linked poly(NIPAM-co-acrylic acid) microgels, Johansson et al.<sup>19</sup> found similar

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results for the binding of LZ to the gels. Actually, the cross-linked microgel shrunk at high LZ concentrations as the osmotic swelling of the gel through the presence of the counterions disappeared. Malmsten et al.<sup>20</sup> reviewed the binding of drugs to microgels. Again, CM can be considered as a nanogel, especially if they are internally cross-linked by transglutaminase.<sup>21,22</sup>

The interaction of positively charged LZ (a bacteriostat) with negatively charged nonglobular proteins such as casein was investigated by Thapon and Brule,<sup>23</sup> and they found a strong binding at pH above 5 and no binding at low pH.  $\kappa$ -Casein only bound a small amount of LZ compared with  $\alpha_{s1}$ -casein and  $\beta$ -casein. Salt addition diminished the binding. De Roos et al.<sup>24</sup> investigated the binding of LZ to different casein systems including casein-stabilized emulsions and found basically similar results to those of Thapon and Brule<sup>23</sup> with binding  $\alpha_{s1} > \beta > \kappa$ -casein. They used the equation  $y = ax/(b + x)$  to fit the data which is equal to a Langmuir adsorption isotherm if  $b = 1/a$ ; however, they did not use that condition. Considering their data, a Langmuir isotherm would have given a similar fit but of course with one parameter less.

In an extremely interesting paper, Yamniak et al.<sup>25</sup> investigated the binding of lactoferrin (LF) to osteopontin (bone sialoprotein I (BSP-1 or BNSP), a largely unstructured phosphorylated and sialated protein present in cheese whey) using isothermal titration calorimetry. Osteopontin resembles the caseins present as CM in mammalian milk, which are responsible for calcium transport to the neonate. Osteopontin has a role in bone formation. It was shown that osteopontin has three different binding sites which are occupied sequentially and that the driving force was mainly entropical. LF binds particularly strongly (at neutral pH) to osteopontin.<sup>25</sup>

Our interest for the LF–casein system derives from the fact that human milk contains almost 10-fold higher levels of LF than bovine milk. On fortifying (skim) bovine milk with LF we observed that the milk became translucent with time (Figure

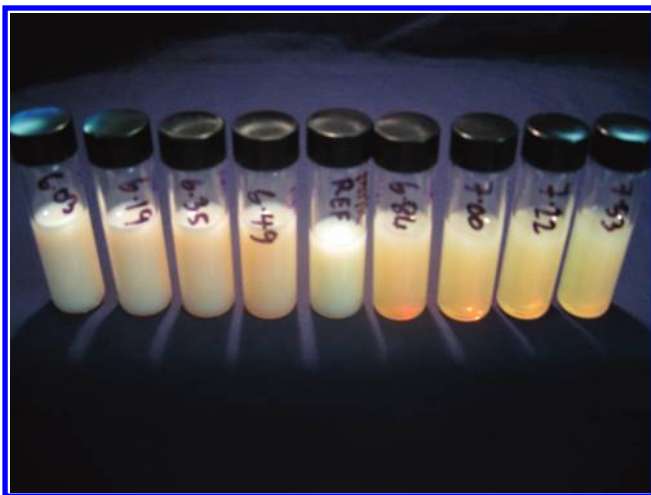


**Figure 1.** Skim milk samples with added LF (4% w/w) at various pH, from left to right pH = 6.03 to 7.53. The reference sample in the middle is skim milk with no added LF. The samples were held for 72 h after LF addition. Clearly the samples become more transparent, and the effect was more pronounced at higher pH.

1), indicating that, surprisingly, the CM were disintegrating. The effect was more pronounced at higher LF addition levels and at higher pH. At first, we thought this might be due to

calcium sequestration by the LF, which is known to disintegrate the CM, or that the LF induced proteolysis. Early experiments excluded both these hypotheses. For that reason we decided to investigate the phenomenon more thoroughly. What we found is that LF binds strongly to caseins and (for some reason) leads to a disintegration of the CM. LZ also binds to the CM but does not seem to cause a disintegration of the micelles.

This paper reports on our findings, and if appropriate we compared our experimental results to simple physical chemical principles to see whether they are self-consistent and whether the parameters from one experiment can be used to explain results from another experiment.

## EXPERIMENTAL SECTION

**Experimental Solutions.** Skim milk was prepared from skim milk powder (low heat, from Fonterra Cooperative Group, New Zealand) and water to 20% total solids (TS) on a w/w basis. LF (>90% purity; Fonterra Cooperative Group, New Zealand) was added to water to give an 8% solution (w/w). Chicken egg white LZ (>90% purity, Sigma-Aldrich, St. Louis, MO) was added to water to give a 4% solution (w/w). Skim milk, LF solution, and water were combined to give skim milk solutions (10% total solids, w/w) with 0–4% (w/w) LF. Skim milk, LZ solution, and water were mixed to give skim milk solutions (10% total solids, w/w) with 0–1% (w/w) LZ. Where required, the pH of the milk solutions was adjusted using 1 M HCl or NaOH.

**Centrifugation and Electrophoresis.** For binding/adsorption studies, the CM were separated from the milk serum by centrifugation (~27000g, 25 °C, 1 h in a bench centrifuge), and the level of protein in the original milk samples and supernatants was determined by conventional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and microfluidic chip SDS-PAGE, as has been described previously.<sup>26</sup>

**Dynamic Light Scattering and Turbidity/Transmission Measurements.** Dynamic light scattering (DLS) was performed using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, Worcestershire, U.K.) using the techniques described previously.<sup>27</sup> Turbidity/transmission measurements were performed using a Jasco V580 spectrophotometer (Japan Spectroscopic Co., Hachioji City, Japan) using the techniques described previously.<sup>28</sup>

## RESULTS AND DISCUSSION

**Adsorption Measurements.** After adding LF to skim milk it was observed that the milk samples became progressively more transparent with time (Figure 1). The decrease in turbidity was not only time dependent but also pH dependent (Figure 1) and temperature dependent. Further experiments showed that LF initially bound to the CM, and then the CM disintegrated with prolonged holding. First we measured the pH dependence of this behavior.

LF (2% w/w, ~0.25 mM) was added to skim milk at different pH, and the samples were held for 6 h before measuring the adsorption of LF to the micelles. The adsorption of LF to the CM as a function of pH from 6 to 7.5 is shown in Figure 2A. LF was found to adsorb to the CM. Low levels were adsorbed at the low pH, and the adsorption increased with increasing pH because the net charge of the casein micelles increases due to increased dissociation of carboxylic groups. The adsorption levels off near pH 7.5 at about 70% binding and would probably decline at higher pH. We assume that the binding originates from the interaction between negatively charged carboxylic and/or phosphate groups of the caseins with the oppositely charged amino groups of the LF. As the natural pH of the milk solutions (pH ~6.7) is almost exactly halfway between the isoelectric point of LF (pH 8.9) and the casein

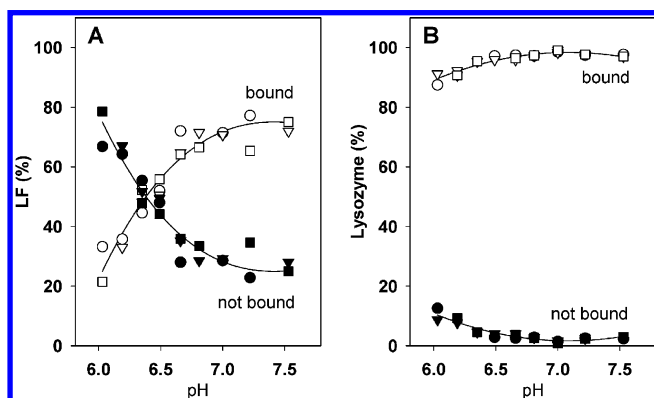


**Figure 2.** pH dependence of the adsorption of LF (A) and LZ (B) to casein micelles in skim milk. The level of LF added to the milk was 2% (~0.25 mM) and LZ was 0.5% (~0.36 mM), and the milk samples were held 6 h before measuring adsorption levels. Duplicate measurements are shown.

micelles (pH 4.6), maximum binding would be expected (and was observed) at this pH. Further deprotonation (at higher pH) of the LF and decreased dissociation of carboxylic and/or phosphate groups of caseins (at lower pH) will reduce binding between LF and the caseins.

We decided to investigate whether LZ, another basic protein, also adsorbed to the CM. LZ (0.5% w/w, ~0.36 mM) was added to the milk samples and found to adsorb strongly to the micelles with almost all bound regardless of pH (Figure 2B). There was only a small pH dependence with slightly lower levels (~90%) bound at low pH milk samples and almost 100% bound at pH above ~6.5.

We measured the adsorption of LF to the CM at the natural pH (~6.7) by mixing skim milk with different levels of LF. The milk samples were held for 6 h at 20 °C as this corresponded to the maximum increase in particle size, and the CM had not started disintegrating. After 6 h we centrifuged the CM down and measured the total LF content and the LF content of the supernatant. The difference was attributed to the pellet and represented the LF bound to the CM (Figure 3). The gel

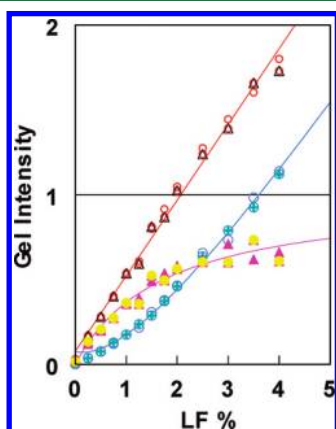


**Figure 3.** Binding of lactoferrin to casein micelles in skim milk. Two independent experiments. Curves are based on a Langmuir type adsorption. (△, red ○) Total protein added. Solid symbols: (▲, ●) amount of protein adsorbed; (⊕, blue ○) protein amount in supernatant.

intensity data for the total LF at increasing addition levels was linear and in accordance with the amount added. Several

repeated measurements validated the procedure. Some pellet samples were also analyzed and confirmed that LF had associated with the CM fraction.

We assumed a simple Langmuir adsorption mechanism with independent adsorption sites called S on the CM and that LF bind to these sites. All sites are equal, and empty sites ( $S^*$ ) obey a mass action law equilibrium with LF. Thus

$$S^* + LF \xrightleftharpoons{K} SLF, \quad K = \frac{[SLF]}{[S^*][LF]},$$

$$\theta = \frac{\alpha[LF]}{1 + \alpha[LF]} \quad (1)$$

Here  $\theta$  is the fraction of occupied sites which goes to 1 for high concentrations of LF.  $\alpha$  is constant and a measure for the binding affinity. The adsorption data were fitted to a Langmuir isotherm and then normalized so that  $\theta = 1$  for high lactoferrin concentrations (Figure 3). The parameter  $\alpha$  was found to equal 0.578 if the LF concentration is in % w/w.

A similar experiment was made for the LZ addition to milk (Figure 4). The binding of LZ was much stronger than for LF.

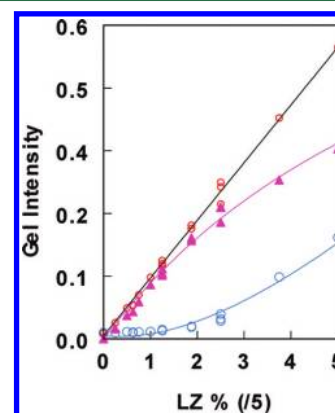


**Figure 4.** Adsorption of LZ to casein micelles in skim milk. Note that LZ concentrations values were multiplied by 5 for comparison with LF (Figure 3). (red ○) Total protein added. Solid triangles (▲) amount of protein adsorbed. (blue ○) Amount of protein in supernatant. Concentrations were measured twice.

The binding affinity,  $\alpha$ , was 0.1196 for LZ, which confirms the stronger binding. This seems reasonable as the isoelectric point for LZ (~10.9) is much higher than that for LF (~8.9).

**Transmission Measurements.** We decided to measure the transmission of the skim milk samples (at 850 nm) at the natural pH of 6.7 with four different addition levels of LF (0.5%, 1%, 2%, and 4% w/w) and at various holding temperatures (from 4 to 50 °C) (Figure 5 for 30 °C samples). It was observed that before disintegration took place the transmission decreased (not visible on the scale of Figure 5). For samples at 20 °C, DLS also showed that the scattering intensity and hydrodynamic radius of the CM initially increased before decreasing after a few hours. SDS-PAGE indicated that there was no proteolysis of protein in the milk samples. Thus, it seemed that LF bound to the CM and then initiated the disintegration process. Subsequent measurements of the LF and casein content of the supernatants with time confirmed this.

After the initial binding of the LF to the CM, the transmission of the samples clearly increases after several hours. Figure 5 illustrates the measured transmission as a

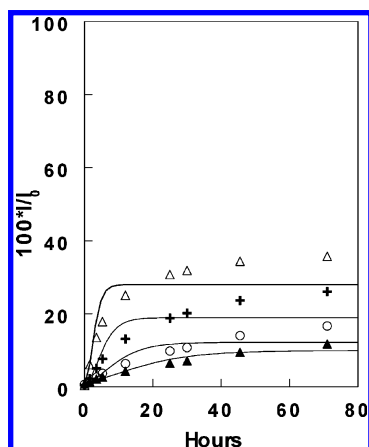


**Figure 5.** Transmission (%) of skim milk. Lactoferrin samples with ( $\Delta$ ) 4%, (+) 2%, (O), 1%, and ( $\blacktriangle$ ) 0.5% w/w LF added. The lines are from a model calculation with fixed parameters (see text).

function of time at 30 °C for the four different LF additions. In order to model the disintegration we assumed the simplest possible model consistent with our observations. We suppose that the LF binds to the CM forming CM-LF which then disintegrate according to the following scheme:



The rate constant  $K_1$  is much larger than  $K_2$ . The adsorption kinetics of LF disappearing from the supernatant can be described as

$$\frac{d[\text{LF}]}{dt} = -k'_1[\text{LF}][\text{CM}], \quad [\text{LF}] = [\text{LF}_0]e^{-k_1t} \quad (2)$$

This assumes that the adsorption kinetics is a quasi-first-order process because the number of adsorption sites on CM is far in excess.

The kinetics of the disintegration is written as  $p$ th order in  $[\text{LF}]$  and first order in  $[\text{CM}]$  reaction kinetics. We did not vary  $[\text{CM}]$ ; thus, we actually assume that it is first order which is highly probable because it is difficult to see how the large CM would influence the disruption of a neighboring CM. Thus

$$\frac{d[\text{CMLF}]}{dt} = -k_2[\text{LF}]^p[\text{CMLF}]$$

$$\frac{1}{[\text{CMLF}]} \frac{d[\text{CMLF}]}{dt} = -k_2[\text{LF}]^p$$

Turbidity is the integrated scattered intensity,<sup>29</sup> and in the small particle or long wavelength limit turbidity is given by

$$\tau = \frac{16\pi}{3} K^* c M, \quad K^* = \frac{2\pi n_s^2}{\lambda_0^4 N_{\text{av}}} \left( \frac{dn}{dc} \right)^2 \quad (3)$$

where  $n_s$  and  $n$  are the refractive index of the solvent and the dispersion, respectively,  $\lambda_0$  is the wavelength of the light in vacuo, and  $c$  is the concentration of the dispersed particles.  $N_{\text{av}}$  is Avogadro's number, and  $M$  is the molecular mass. We thus write

$$\tau = -\ln\left(\frac{I}{I_0}\right) = [\text{CMLF}]H$$

(where  $H$  is a constant)

$$\frac{d \ln(\tau)}{dt} = -k_2[\text{LF}]^p \quad (4)$$

Thus, the plot of  $\ln(\tau)$  vs  $t$  has a slope equal to  $-k_2[\text{LF}]^p$ . From the plot of  $\ln(\text{slope})$  vs  $\ln[\text{LF}]$  we find the exponent  $p$  equals 1, although the value was varying between 0.6 and 1.5 depending on the number of points in the fit. The initial slope; however, converged to 1, with the exception of the 4 °C and the 10 °C data. For these low temperatures the initial slope is dominated by the adsorption of LF to the CM, and so turbidity actually increases as the mass of the CM increases.

From a plot of  $\ln(\tau)$  vs time we then determined the initial slope which is  $-k_2[\text{LF}]$ . The values of  $\ln(k_2)$  vs  $1/T$  are plotted in Figure 6. The reaction rate constant is given by

$$\frac{k_2}{k_{02}} = e^{-\Delta H/RT} \quad (5)$$

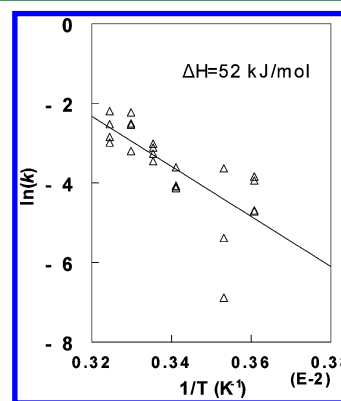


**Figure 6.** Van't Hoff plot for reaction rate constant  $k_2$  as a function of  $1/T$ . Slope leads to an activation enthalpy for the disintegration of the casein micelles of 52 kJ/mol.

We determined this from the initial slope of  $\ln(\tau)$  vs  $t$  because transmission does not go to 100% as eq 6 suggests

$$[\text{CMLF}] = [\text{CMLF}_0]e^{-k_2[\text{LF}]t} \quad (6)$$

The reason is that each CMLF is transformed into  $N$  smaller particles presumably containing LF and caseins and therefore called coacervate particles (CA). Thus, the concentration of CA will be proportional to the concentration of converted micelles. Probably a small factor,  $F$ , higher because now the LF is adhered to the caseins:

$$[\text{CA}] = F[\text{CMLF}_0](1 - e^{-k_2[\text{LF}]t}) \quad (7)$$

Since turbidity is additive, we may write

$$\tau = [\text{CMLF}]H_1 + [\text{CA}]H_2$$

in which  $H_1$  and  $H_2$  are constants depending on molecular mass and the refractive properties of the particles assuming we are in the low wavevector limit. Thus, turbidity is given by

$$\tau = H_1[\text{CMLF}_0]e^{-k_2[\text{LF}]t} + H_2F[\text{CMLF}_0](1 - e^{-k_2[\text{LF}]t})$$

After collecting constants this may be written as

$$\tau = a_0e^{-k_2[\text{LF}]t} + a_2(1 - e^{-k_2[\text{LF}]t})$$

So in all we have three unknowns:  $a_0$ ,  $a_2$ , and  $k_{02}$ .

$$\tau = (a_0 - a_2)e^{-k_{02}e^{-6289/T}[\text{LF}]t} + a_2 \quad (8)$$

Thus, a plot of turbidity as a function of time will have an ordinate of  $a_0$  and a horizontal asymptote of  $a_2$ .

Turbidity data were fitted to eq 8. The LF concentrations were 0.5, 1, 2, and 4% w/w. The molar mass of LF is ~80 kDa; therefore the lowest LF concentration is  $5/80 = 6.25 \times 10^{-2}$  mol/m<sup>3</sup>. The subsequent concentrations are 2, 4, and 8 times greater. After fitting the data, we found an almost constant value for  $k_{02}$  of  $1.37 \times 10^{15}$  m<sup>3</sup>/(mol h). (Note that  $k_{02}$  would be the hypothetical value at infinite temperature. At room temperature  $k_2$  was  $9 \times 10^5$  m<sup>3</sup>/(mol h). After inserting  $k_{02}$  into the equation, we refitted the data with only two adjustable parameters, i.e.,  $a_0$  and  $a_2$ , the ordinate and asymptote value. The obtained average values for these parameters for each concentration of LF are given in Table 1. The ordinate values,

**Table 1. Fitted Coefficients of Eq 8, for the Evolution of Turbidity with Time, on Adding LF to Casein Micelles**

| coeff | [LF]            |                 |                 |                 |
|-------|-----------------|-----------------|-----------------|-----------------|
|       | 0.5%            | 1%              | 2%              | 4%              |
| $a_0$ | $4.48 \pm 0.30$ | $4.80 \pm 0.30$ | $5.30 \pm 0.30$ | $5.64 \pm 0.30$ |
| $a_2$ | $2.40 \pm 0.25$ | $2.17 \pm 0.25$ | $1.73 \pm 0.25$ | $1.30 \pm 0.25$ |

$a_0$ , increase linearly with LF concentration, which is expected when LF initially binds to the CM. The horizontal asymptote decreases linearly with LF concentration, which is expected because the CM are transformed into CA particles more rapidly as the LF concentration increases.

By setting the reaction rate constant  $k_{02}$  as a real constant, the fit of the data becomes somewhat poorer because we lose an adjustable parameter. The only parameters left were  $a_0$  and  $a_2$ , and they determine, in fact, only the ordinate and the asymptote. Nevertheless, considering all this, the quality of the fits was quite satisfactory as illustrated in Figure 7, in which we plot turbidity of the milk samples as a function of time at 25 °C for the different concentrations of LF. We purposely reduced the number of parameters so as to see whether the system can be understood on the simplest level, but realizing that it is not correct at all conditions.

#### Concentration of LF and Caseins in Supernatants.

After these initial more global observations and measurements, we looked in more detail into the adsorption and disintegration kinetics. We measured the concentration of caseins and LF in the supernatant as a function of time in terms of peak areas (as measured by gel electrophoresis) rather than in mol/m<sup>3</sup> (Figures 8 and 9). The concentration (peak area) of caseins initially remains constant at a very low level for the first ~10 h and then increases (Figure 8). Clearly, the LF concentration in the supernatant initially diminishes over the first ~10 h and then increases (Figure 9).

We expected that the increase of LF in the supernatant was due to LF bound to caseins. From the turbidity measurements

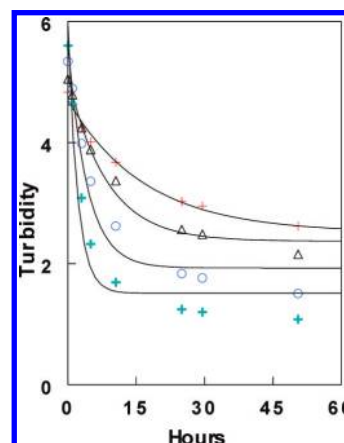


**Figure 7.** Turbidity of skim milk at 25 °C as a function of time for four different LF additions. The LF additions were (from top to bottom): (red +) 0.5%, (Δ) 1%, (○) 2%, and (green +) 4%. Lines are the fit to eq 8.

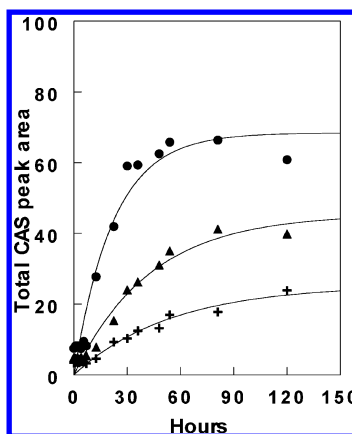


**Figure 8.** Total caseins in the supernatant of skim milk to which (●) 2%, (▲) 1%, and (+) 0.5% LF were added.

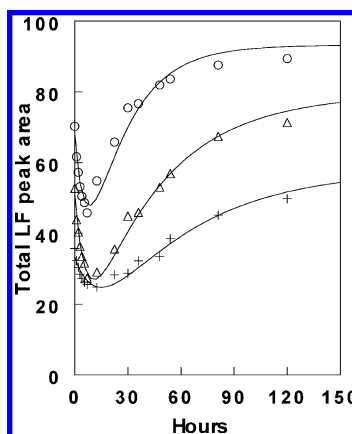


**Figure 9.** Total LF content in supernatant of skim milk to which (○) 2%, (Δ) 1%, and (+) 0.5% LF were added. Data fitted to eq 10 using the kinetics of casein increase in eq 9.

we saw that the disintegration process is first order in LF. We therefore fitted the casein peak areas in Figure 8 to

$$\text{casein}_{\text{peak area}} = a_1(1 - e^{-k_s[\text{LF}]t}) \quad (9)$$

**Table 2. Fitted Coefficients of Eq 9 for the Evolution of Supernatant Casein Peak Area as a Function of Time**

| coeff | [LF] |      |      |
|-------|------|------|------|
|       | 0.5% | 1%   | 2%   |
| $a_1$ | 25.4 | 45.5 | 68.4 |
| $k_s$ | 293  | 187  | 182  |

The determined constants are given in Table 2 for each LF concentration.

The binding of LF is considered a first-order process and the release of casein is accompanied by a proportional amount of LF. We fitted the LF data to

$$LF_{\text{peak area}} = a_0 e^{-k_1 t} + a_2 (1 - e^{-k_s [LF] t}) \quad (10)$$

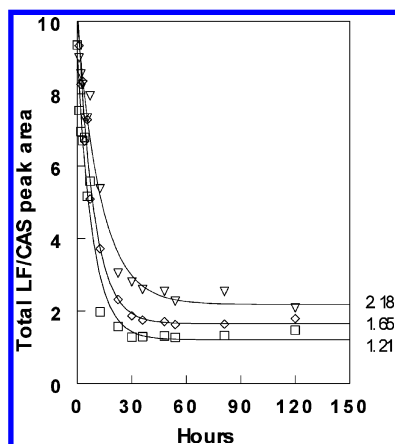
where we used the result of the casein peak area for the second part of the equation. In other words, we used the same kinetics. The determined constants are given in Table 3. The first

**Table 3. Fitted Coefficients of Eq 10 for the Evolution of Supernatant LF Peak Area on Adding LF to Casein Micelles**

| coeff | [LF]   |       |       |
|-------|--------|-------|-------|
|       | 0.5%   | 1%    | 2%    |
| $a_0$ | 33.1   | 50.9  | 67.9  |
| $k_1$ | 0.0751 | 0.158 | 0.157 |
| $a_2$ | 58.1   | 79.3  | 93.2  |

exponential accounts for the decreases of LF (conforms to eq 2) while the second accounts for the increase in LF concentration (conforms to eq 8).

So these results confirm that the release of LF and casein are intimately related and suggest that LF forms rather strong complexes with the caseins in skim milk. We calculated the ratio of the peak areas of LF and casein and plotted that against time (Figure 10). The data show that the ratio (just after the

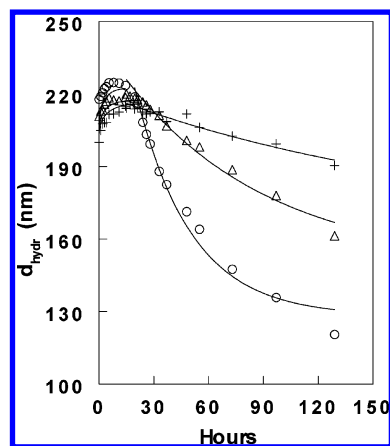
**Figure 10.** Ratio of measured peak areas of LF and caseins in supernatant from milks to which ( $\square$ ) 2%, ( $\diamond$ ) 1%, and ( $\nabla$ ) 0.5% LF were added.

initial adsorption of the LF) soon relaxes to a constant value, long before the final level of casein is reached.

Data were fitted to  $a_0 e^{-a_2 t} + a_2$  assuming the system relaxes to an equilibrium LF/casein ratio and clearly the  $LF_{\text{tot}}/casein_{\text{tot}}$  ratio soon converges to a constant value. The values are indicated on the right-hand side of Figure 10. It is emphasized

that these are peak area ratios and not molar ratios. The values are in line with mass action law equilibrium as the lower LF concentration has the higher plateau value. Caseins are bound to the casein micelles and to the LF–casein complex. Therefore, the concentration of caseins in the supernatant is determined by the amount of complex. At low LF concentration ( $[LF] + [CA]/[CA]$ ) is higher.

**DLS Measurements.** During the same experiment we also measured the hydrodynamic particle size of the CM, which showed that the micelle size initially increased over the first ~10 h and then decreased, with the effects being more pronounced at higher LF concentrations (Figure 11). In order

**Figure 11.** Measured hydrodynamic radius of CM in milks to which ( $\circ$ ) 2%, ( $\triangle$ ) 1%, and ( $+$ ) 0.5% LF were added. Curves calculated from kinetics data in Figure 9.

to interpret this measurement, we assumed that the radius would grow with the cube root of the LF mass and that the kinetics would be the same as above where we analyzed the kinetics of the adsorption of LF to the CM. After the initial growth/swelling of the micelles they disintegrate. We infer that the casein micelles must swell because the DLS radius increases, but turbidity goes down immediately. What is measured in DLS is the (remnants of) casein micelles and not the formed coacervate particles as they contribute too little to the scattering intensity. Prefactors were adjusted to obtain the right starting level. The calculations represent the data quite well (see lines in Figure 11).

**Summary.** The following (kinetic) picture emerges. On adding LF to CM, the LF adsorbs to the CM. As a result, the LF concentration diminishes in the supernatant of centrifuged CM. This is a relatively fast process, time scale hours. The initial adsorption equilibrium can be described quite well with a Langmuir type adsorption.

As an (almost) subsequent process the CM disintegrate, which follows from the initial observation that the skim milk became more translucent. The kinetics and time scale of the disintegration process are about 10 times slower than the adsorption process. Therefore, by evaluating at the right time scale both processes could be quantified. From protein gel electrophoresis we could determine both the kinetics of adsorption and disintegration. The kinetics of the disintegration of the CM as a function of temperature leads to an activation enthalpy of 52 kJ/mol. In addition, it is shown that the ratio of LF/casein in the supernatant was constant after the initial adsorption but LF and casein levels both increased in time.

We measured the apparent hydrodynamic radius of the CM. It seems reasonable that initial adsorption will be on the periphery of the CM. Therefore, the radius of the micelle would grow as the cube root of the LF mass adsorbed to the CM. Also on disintegration we assumed that it would be proportional to the cube root of the mass disintegrating as measured using transmission measurements. Therefore, we used the adsorption kinetics rate constant as determined from eq 2 and the disintegration rate constant as determined from eq 8 and Figure 7 in order to calculate the rise and fall of the CM diameter. We adjusted the prefactors so as to get the right level (i.e., particle size); however, the kinetics were determined independently. We note that all casein micelles appear to disintegrate at the same rate, rather than a subsequent disintegration of the different casein micelles.

The picture of LF binding to caseins was further corroborated and investigated in a separate paper where we titrated one protein into the other at pH 6.5. These results show that LF can form (electrostatic) complexes with the various caseins, i.e.,  $\kappa$ -,  $\alpha$ -, and  $\beta$ -casein. From the temperature (in)dependence and salt dependence we concluded that the process is mainly entropy driven as predicted.<sup>8,10–12</sup>

Interestingly, although LZ was also found to adsorb to the CM, we did not observe a disintegration of the CM under similar conditions where LF caused the micelles to disintegrate. The reason for the different behavior of LF and LZ maybe in the size of the proteins. Large proteins are capable of over or under charging on the adsorption of casein polymer for entropical reasons. As a result, LF is capable of forming stable (charge stabilized) complexes, but LZ apparently is not. The reorganization of the LF–casein complexes leads to a slow disintegration. This will be studied in a separate paper.

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