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## Evaluation by Sedimentation Field-Flow Fractionation of the Susceptibility of Nonfat Dry Milk Proteins to Aggregation

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### Abstract

The proteins isolated from nonfat dry milk (NDM) which had been prepared with a high-temperature treatment (85°C for 30 min) were shown by sedimentation field-flow fractionation (SdFFF) to be highly susceptible to aggregation, with the formation of particles much larger than typical casein micelles (in addition to some micelles). The proteins of medium- and low-temperature NDMs, especially the latter, were less susceptible to such aggregation; caseins isolated from untreated milk do not aggregate under the same experimental conditions. The demonstration of such differences in susceptibility requires careful choice of SdFFF operation parameters, especially initial field strength and sample protein concentration, and the use of a second carrier to disperse or dissolve the aggregates and elute the redissolved protein from the separation chamber. Suitable operation parameters are provided.

### INTRODUCTION

The predominant proteins in milk are the caseins. These proteins, which have molecular weights around 24,000 daltons, exist in milk mainly as large colloidal  $\text{Ca}^{2+}$ -caseinate complexes (1). The complexes are known as casein micelles, although it is recognized that they sometimes include some noncasein proteins. Milk proteins have been extensively investigated at the molecular and micellar levels of organization. Because of the widespread use of nonfat dry milk (NDM) in a variety of food products, and problems encountered with some NDM, the proteins of NDM have received considerable attention (2-4), including the effects of the various processing treatments used in manufacturing it. The effects of heat treatments on the protein micelles of reconstituted NDM are nevertheless not well understood.

Casein micelles have diameters in the range 0.1 to 0.5  $\mu\text{m}$  (5). This is in the size range where sedimentation field-flow fractionation (SdFFF) can operate effectively (6), whereas other techniques for measuring particle size, such as chromatography and sedimentation equilibrium, are less effective (7) or less convenient. In this study we show that the susceptibility of the proteins of NDMs to the formation of very large aggregates (presumably aggregates of micelles) can be determined by carefully controlled application of SdFFF. The size distribution of the casein micelles present is obtained simultaneously.

## EXPERIMENTAL

Disodium ethylenediamine tetraacetate (EDTA) dihydrate (ACS grade) and the detergent FL-70 were purchased from Fisher Scientific Co., Fair Lawn, New Jersey. Piperazine-*N,N'*-bis(2-ethane sulfonic acid) (PIPES) and its disodium salt were from Sigma Chemical Co., St. Louis, Missouri. KCl and sucrose were reagent grade, J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Casein (Na caseinate) was prepared by isoelectric precipitation from the milk of a Holstein cow as follows: Phenyl methyl sulfonyl fluoride (PMSF) was added to the fresh milk, and it was stored at 5°C. On the following day, initial skimming was done by centrifuging at 4000 g and 5°C to remove the fat globules. One liter of the once-skimmed milk was dialyzed against 18 L 10 mM EDTA for 24 h at 5°C, skimmed at 10,000 g for 10 min, and then dialyzed against 18 L water. The caseins were precipitated from the skim milk at room temperature by addition of HCl to pH 4.6. The precipitate was washed at the same pH, suspended in H<sub>2</sub>O, and redissolved by adding NaOH to pH 7.0. The solution was centrifuged at 10,000 g (at  $r_{\text{max}}$ ) for 30 min (4°C) and the upper fat-containing layer was discarded. The caseins were reprecipitated and redissolved as before, the final pH being adjusted to 7.2. The resulting solution was skimmed at 75,000 g and 5°C and filtered through several layers of fine cheesecloth. Ethanol was added to the solution to a concentration of ca. 5% (v/v) and it was lyophilized.

The three NDM powders were prepared as previously described (3) from pooled milk. They differed only with respect to the temperature of the heat treatment used: 63 (low-), 74 (medium-), or 85°C (high-temperature); the duration of the treatment was 30 min in each case. The three NDMs are designated NDM 63, NDM 74, and NDM 85, respectively.

All solutions used for the preparation and FFF of micelle suspensions had a final pH of 6.75 (25 ± 1°C). Their compositions are given in Table 1.

TABLE 1  
Composition of Solutions Used for Preparation and Sedimentation Field-Flow Fractionation of Micelle Suspensions

Component	Concentration of components (mM) in solution					
	S	I	Carrier	PiKES	PiK	PiKE
PIPES	25	25	25	25	50	50
KCl	80	80	80	80	160	160
EDTA	0	0	0	25	0	50
Sucrose	300	300	300	300	0	0
CaCl <sub>2</sub>	0	40	20	0	0	0
pH <sup>a</sup>	6.75	6.75	6.75	6.75	6.75	6.75

<sup>a</sup>Final pH at 25 ± 1°C.

Solutions of low-, medium-, and high-heat NDM proteins were prepared as follows. A 7.08-g sample of each NDM powder was dissolved in solvent PiKE, and the resulting solution (A1) was made up to a volume of 50 mL with the same solvent. A1 was dialyzed extensively at 5°C against solution PiK, and filtered through an 8-μm Millipore filter. Only the barest trace of residue was visible on the filter, and the filtrate was clear. Water, 2 M sucrose, and solvent S were added to an aliquot of the filtrate to yield a solution (B1) having the composition of solvent S and a protein concentration  $c_p$  of 13.5–18.0 g/L.

Micelle formation was induced at room temperature (24 ± 1°C) by the addition, to an aliquot of B1, of an equal volume of solution I, while stirring vigorously, yielding a micelle suspension (C1) having the composition of the carrier solution, with a protein concentration of 6.8–9.0 g/L. (In some early experiments, B1 was first diluted with solution S to the desired protein concentration, and a small volume of 2 M CaCl<sub>2</sub> added to yield the micelle suspension C1 having the same composition as that described above. In all experiments, samples to be compared were prepared in identical fashion.) After incubation for the desired time interval, the incubated suspension (D1) was subjected to SdFFF.

The SdFFF system was similar to that previously described (6). The dimensions of the channel were 92.8 × 2.00 × 0.0254 cm; the radius was 16 cm. The accumulation (outer) wall of the channel was lined with Kapton Polyimide Temp-R-Tape K-250 (CHR Industries, New Haven, Connecticut) to minimize adsorption. Samples were injected with a Rheodyne #7120 injection valve inserted into the Carrier line just upstream from the point of entry into the fractionator. The sample was run-in for 60 s. This was followed by relaxation. The pump was a Waters 6000A HPLC pump. All runs were made at room temperature (24.5 ± 1.5°C). The field program

consisted of two or three phases: (1) a constant field phase, where the field ( $G$ ) was held constant for the interval  $t_c$  min at its initial value  $G_0$  (rotational velocity  $\omega_0'$ ), (2) an exponential decay phase with a field decay constant  $\tau_g$  (corresponding to a rotational velocity decay constant  $\tau_\omega = 2\tau_g$ ), and usually (3) a hold phase where the rotational velocity ( $\omega'$ ) was held constant at a very low value  $\omega'_{\text{Hold}}$  beginning at time  $t_{\text{Hold}}$  when the decaying rotational velocity of Phase 2 reached the preselected value  $\omega'_{\text{Hold}}$ . Unless otherwise stated, the operational parameters for FFF included a flow rate  $f = 1.00$  mL/min, a relaxation time  $t_{\text{rx}} = 10.0$  min,  $t_c = \tau_\omega = 10.0$  min, and  $\omega'_{\text{Hold}} = 60$  rpm. The flow-through absorbance detector was usually set at a wavelength  $\lambda' = 280$  nm and a sensitivity of 0.04 AUFS. The detector signal was, unless otherwise stated, amplified 10-fold and sent through an autoranging scale expander, with the decimal portion of the amplified signal being sent to a recorder set for a range of 0–1 V.

Effective particle mass,  $M_{\text{eff}}$  (g/mol particles), was calculated from elution (retention) time  $t_e$  with the following equations (6).

For the constant field phase  $t_e \leq t_c$  ( $G = G_0$ ),

$$\lambda[\coth(1/2\lambda) - 2\lambda] = t_0/6t_e \quad (1a)$$

$$M_{\text{eff}} = R_0 T / \lambda w G_0 \quad (1b)$$

For the decay phase  $t_c \leq t_e \leq t_{\text{Hold}}$ ,

$$M_{\text{eff}} = \frac{6R_0 T}{w G_0 t_0} \{t_c + \tau_g [e^{(t_e - t_c)/\tau_g} - 1]\} \quad (2)$$

For the hold phase  $t_{\text{Hold}} \leq t_e$ ,

$$M_{\text{eff}} = \frac{6R_0 T}{w G_0 t_0} \left\{ t_c + \tau_g [e^{(t_{\text{Hold}} - t_c)/\tau_g} - 1] + \left( \frac{\omega_0'}{\omega'_{\text{Hold}}} \right)^2 (t_e - t_{\text{Hold}}) \right\} \quad (3)$$

The particle size  $M_{\text{Prot}}$  (g protein/mol particles) was calculated from  $M_{\text{eff}}$  (Eqs. 1–3) with the equation

$$M_{\text{Prot}} = \frac{M_{\text{eff}}}{1 - \rho \bar{v}} \quad (4)$$

The void-volume peak ( $t_e$  close to  $t_0$ ) consists of nonmicellar casein and whey proteins. The second peak consists of casein micelles. The third peak, i.e., material eluted after the second peak by a micelle dispersing agent or a  $\text{Ca}^{2+}$  chelator, is attributed to protein aggregated into particles much larger than typical micelles.

## RESULTS AND DISCUSSION

The first observation indicating that SdFFF could be utilized to demonstrate aggregation, i.e., the presence of casein-containing particles substantially larger than typical casein micelles, was made during early experiments with casein. When a relatively concentrated solution of casein was subjected to SdFFF 1 day after addition of  $\text{Ca}^{2+}$  (Fig. 1, upper curve), a small nonmicellar casein (void-volume) peak (Peak 1) was seen, followed by a large micelle peak (Peak 2) with a maximum at  $t_e \approx 33$  min ( $M_{\text{Prot}} \approx 5 \times 10^{10}$ ). When an identical run was made a day later (lower curve of

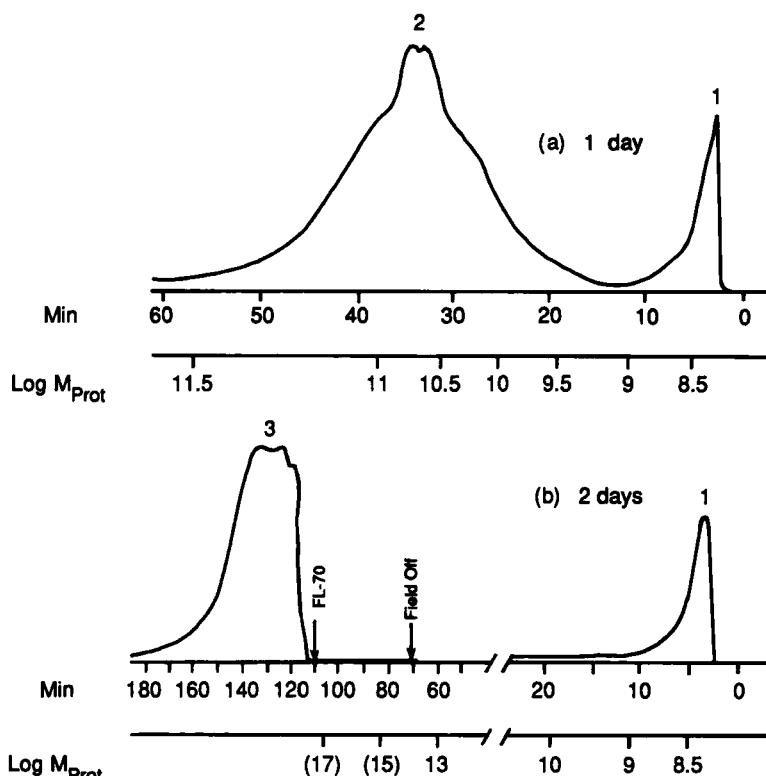


FIG. 1. Sedimentation field-flow fractograms of casein obtained (a) 1 and (b) 2 days after addition of  $\text{CaCl}_2$ . Carrier flow rate ( $f$ ), 1.00 mL/min. Sample: Protein concentration ( $c_p$ ), 25 g/L;  $\text{Ca}^{2+}$  concentration, 20 mM; volume injected ( $V$ ), 100  $\mu\text{L}$ . SdFFF operation parameters: relaxation time ( $t_{\text{rel}}$ ) for (a) 9.4 min, for (b) 8.9 min; constant (initial) field strength ( $G_0$ ), 86.1 g; duration of constant field ( $t_c$ ), 10.0 min; exponential field decay constant ( $\tau_x$ ), 5.0 min; field strength during terminal hold phase ( $G_{\text{Hold}}$ ), for (a) 0.64 g (provided by rotational velocity of rotor  $\omega_{\text{Hold}} = 60$  rpm), for (b) 0 (no hold phase). Detection: wavelength ( $\lambda'$ ), 254 nm; sensitivity ( $S$ ) dial setting, 0.04 AUFS. Recorder range, 0–50 mV.

Fig. 1), the micelle peak was virtually missing; only a barely perceptible, broad Peak 2 was visible (in the region of the break in the figure). No protein was eluted even after the field was turned off at  $t_e = 72$  min, for which  $M_{\text{Prot}} \approx 10^{14}$ . For such particles the diameter  $d \geq 10 \mu\text{m}$ . They would therefore be expected to elute early-on by the steric mechanism. It appeared reasonable to assume that the micelles had grown in size, aggregated, and adhered to the outer (accumulation) wall of the rotor. The separation chamber was therefore eluted with 0.1% FL-70, whereupon a large peak (Peak 3) was obtained, much larger than that caused (in the absence of protein) by the solvent change. The effluent was turbid. In later runs the detergent was replaced by eluants containing EDTA to chelate the  $\text{Ca}^{2+}$  and redissolve the protein.

While casein micelles can be run at much higher initial field strengths than that (86.1 g) used for the sample of Fig. 1 without substantially altering the calculated results, it quickly became apparent that this is not the case for NDM samples. Field-flow fractograms of NDM 63 run, 60 min after initiation of micelle formation, with two programs differing only in  $G_0$  are shown in Fig. 2.  $G_0$  for the lower curve was exactly twice that for the upper curve. The typical micelle peak eluting at ca. 24 min ( $M = 5 \times 10^{10}$ ) in the lower  $G_0$  program (11 g) was badly depleted when  $G_0$  was increased to only 22 g ( $\omega'_0 = 350$  rpm), and the amount of nonmicellar protein (eluting at  $t_e \approx 4$  min) was somewhat reduced. The protein missing from these peaks was eluted by the reagent (PiKES) containing 25 mM EDTA, indicating that the missing protein had aggregated. From the elution (retention) time, the *minimum* particle size of the aggregated material was calculated to be  $3 \times 10^{11}$  (g protein/mol particles). However, we have never seen a third peak, i.e., a postmicellar peak, even after long elution times, except on elution with detergent or EDTA. The protein eluted by these agents therefore very likely derives from protein precipitated on the accumulation wall of the separation chamber, as discussed above in connection with Fig. 1. These results show: 1) that the NDM 63 sample did not contain any aggregated micelles (upper curve), and 2) that short exposure to a mild gravitational (centrifugal) field induced aggregation in the sample (lower curve). Samples of reconstituted micelles made from native casein showed no indication of aggregation even when run at much higher  $G_0$  values (unpublished observations). Most runs on NDM milk were made with a  $G_0$  of 5.5 g ( $\omega'_0 = 175$  rpm).

The dependence of induced aggregation on field strength can be easily explained in terms of the protein concentration at (and near) the accumulation wall of the separation chamber (see below). Reducing the field strength reduces this concentration. Alternatively, the protein concentration ( $c_p$ ) of the sample injected can be reduced. The effectiveness of the

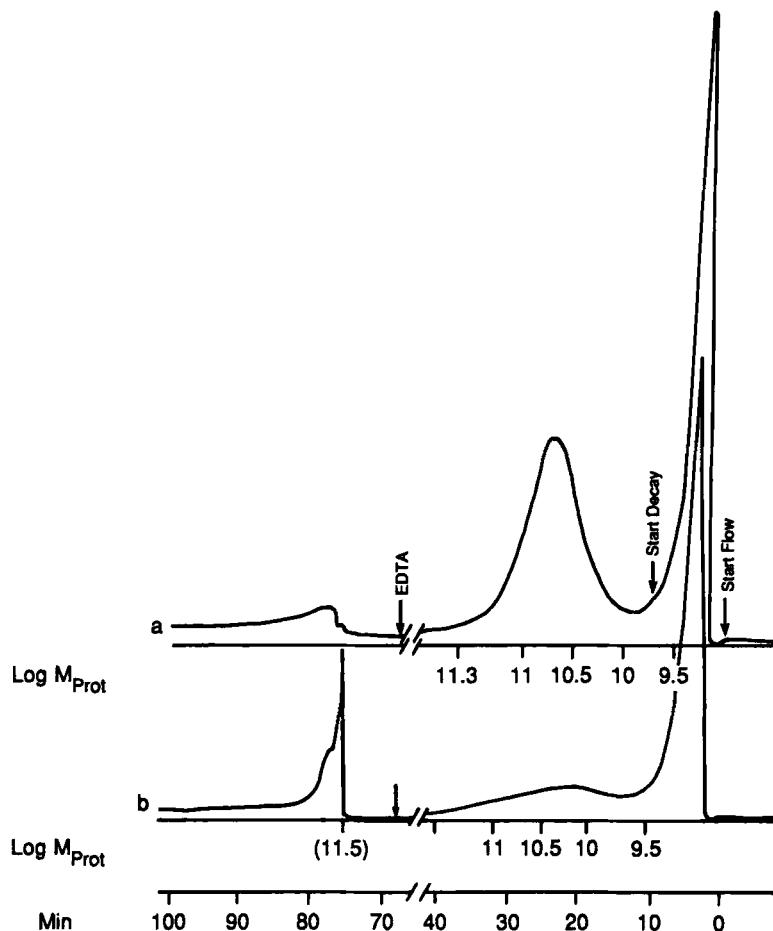


FIG. 2. The effect of the initial field strength  $G_0$  on aggregation of micellar proteins. SdFFF was initiated 60 min after addition of  $\text{Ca}^{2+}$  to the proteins isolated from low-heat NDM. The initial carrier was replaced by PiKES at the point indicated by "EDTA." [The change in apparent absorbance in (a) on elution with PiKES is typical of the solvent change. The EDTA peak in (b), to the extent that it exceeds the corresponding peak in (a), is due to aggregated protein (Ca caseinate) which was redissolved by EDTA.] Parameters: (a)  $G_0 = 11$  g, (b)  $G_0 = 22$  g,  $c_p = 27.2$  g/L;  $t_{rx} = 10.0$  min;  $G_{\text{hold}} = 0.64$  g;  $\lambda' = 280$  nm; signal amplification,  $10 \times$ ; recorder range, 0–1 V. Other parameters as for Fig. 1.

latter strategy is shown in Fig. 3. In Run (a) the micelle suspension (made from the proteins of NDM 63) had a concentration of 27.2 g protein/L, and 100  $\mu\text{L}$  was injected. In Run (b) the protein concentration was reduced (by a factor of 4) to 6.8 g/L and 327  $\mu\text{L}$  (2.22 mg protein) was injected.

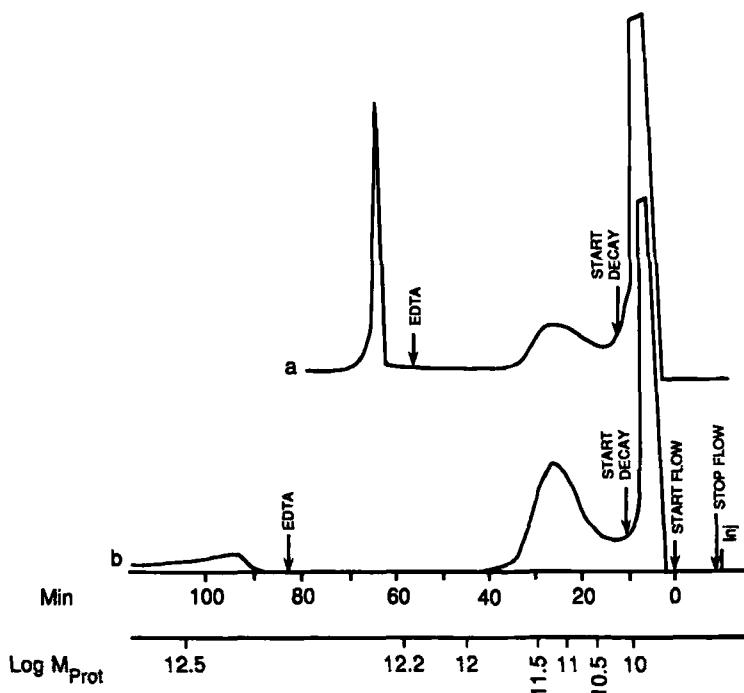


FIG. 3. Effect of sample protein concentration on aggregation of micellar proteins. (a)  $c_p$ , 27.2;  $V$ , 100  $\mu$ L. (b)  $c_p$ , 6.8 g/L;  $V$ , 327  $\mu$ L.  $G_0$ , 5.5 g;  $G_{\text{Hold}}$ , 0.16 g ( $\omega_{\text{Hold}} = 30$  rpm). All other conditions as for Fig. 2.

The reduction of  $c_p$  (with only a slight reduction in the amount of protein injected) completely eliminated aggregation.

The above experiments established conditions which are suitable for SdFFF of reconstituted micelle suspensions made from low-temperature NDM proteins, including  $G_0$  and  $c_p$  values which do not induce aggregation. The question then arises whether these conditions can be utilized to evaluate the effect of enhanced heat treatment on the susceptibility of the NDM proteins to aggregation. This question was answered in the affirmative by the data illustrated in Figure 4; the protein exposed to the high-temperature treatment (85°C) aggregated (b) whereas the medium-temperature (74°C) (a) and low-temperature (63°C)-treated proteins (not shown) did not. Whether the protein eluted by the EDTA-containing reagent (PiKES) was already aggregated before the NDM 85 micelle suspension was subjected to SdFFF or whether aggregation occurred while the protein particles were under the influence of the gravitational (centrifugal) field cannot be determined from the figure (Fig. 4b). Now, prior

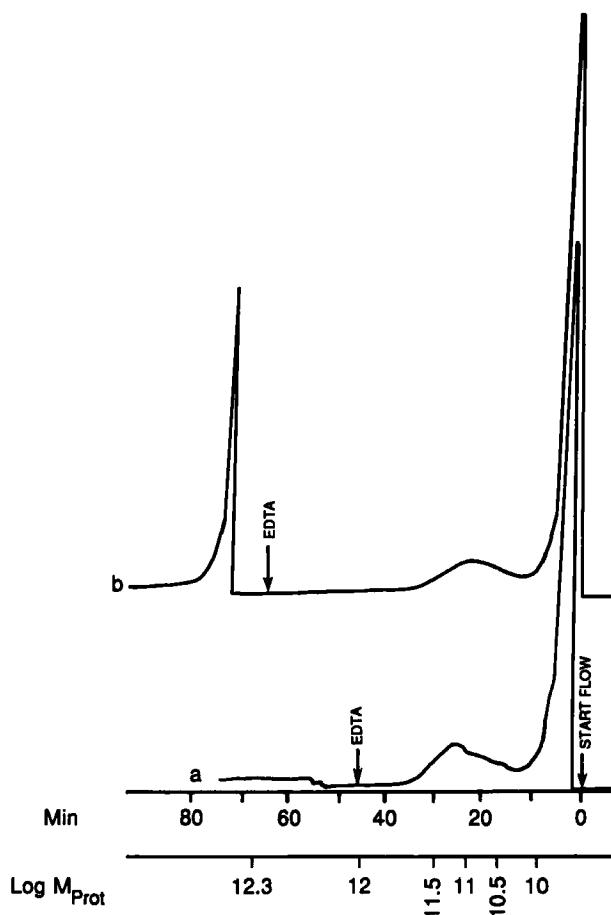


FIG. 4. Comparison of the susceptibility to aggregation of the proteins of NDM 85 (b) to those of NDM 74 (a).  $c_p$ , 6.8 g/L;  $V$ , 100  $\mu$ L. All other conditions as for Fig. 3.

to the addition of  $\text{Ca}^{2+}$  (solution I in Table 1), the solution of the NDM 85 protein (in solvent S) was completely clear, as were the solutions of the NDM 63 and NDM 74 proteins (see Experimental section), indicating the absence of casein micelles and larger particles. If there were aggregates in the NDM 85 sample prior to injection into the fractionator, they must have formed during the 1-h incubation period after addition of  $\text{Ca}^{2+}$ . However, regardless of when the aggregates formed, before or after sample injection, the greater susceptibility of NDM 85 proteins to aggregation in the presence of  $\text{Ca}^{2+}$  is clear.

A possible mechanism of field-induced aggregation emerges from a more detailed examination of the factor referred to above, viz., the protein concentration at the outer (accumulation) wall of the rotor. For any species at equilibrium, as one moves away (radially inwards) from the wall into the channel, the concentration  $c$  falls exponentially from its value  $c_0$  at the wall. The critical parameter of FFF,  $\lambda$ , can be defined (8) as the value of  $l/w$ , where  $l$  is the distance from the wall at which  $c = c_0/e$ , and  $w$  is the width (thickness) of the separation channel, i.e., in SdFFF, the radial distance between the outer and inner walls. It is easily shown that for  $\lambda < 0.2$ , which holds in practice for most retained components,  $c_0 = \langle c \rangle / \lambda$ ,  $\langle c \rangle$  being the average concentration across the channel width. For particles having an effective mass  $M_{\text{eff}}$  of ca.  $10^{10}$ , such as the casein micelles in our fractograms, a field strength of  $5.5 \text{ g}$  ( $\omega' = 175 \text{ rpm}$ ) gives a particle distribution with  $\lambda = 1.8 \times 10^{-2}$  and therefore produces a  $c_0$  of  $55\langle c \rangle$ . If the micelle concentration early in the run, before much dispersion has taken place, is  $4 \text{ g protein/L}$ ,  $c_0 = 220 \text{ g protein/L}$ . Assuming, conservatively (9), that the micelle contains about  $4 \text{ g}$  of trapped solvent/g protein, the micelles occupy all of the space adjacent to the wall; there is no room for free solvent (carrier). A gravitational (centrifugal) field of  $5.5 \text{ g}$  or more and a sample micelle concentration of  $4 \text{ g protein/L}$  or more would therefore be expected to greatly facilitate crosslinking interactions between micelles.

The agent crosslinking two micelles is presumably a denatured whey protein (or a polymer thereof) or serum albumin (BSA) which binds to  $\kappa$ -casein molecules on the surfaces of the two micelles by disulfide linkages (10). Beta-lactoglobulin has a sulphydryl group which is exposed when the protein is denatured; the denaturation temperature ( $T_d$ ) is  $78^\circ\text{C}$ . Serum albumin also has a sulphydryl group, and both proteins have disulfide groups. The  $T_d$  of BSA is  $64^\circ\text{C}$ . Alpha-lactalbumin has disulfide groups but no sulphydryl; its  $T_d$  is  $62^\circ\text{C}$ . [Renaturation of lactalbumin occurs on cooling (10, 11).]

We hypothesize that the following sequence of events applies to our work: During the heat treatment in the preparation of the NDM, whey proteins and serum albumin are denatured. Which whey proteins are denatured depends on the temperature of treatment. Some binding of denatured protein to  $\kappa$ -casein on the surface of the micelle takes place by disulfide linkage. On addition of EDTA (see Experimental section) the micellar structure is destroyed, releasing the caseins. Disulfide linkages between  $\kappa$ -casein and the denatured proteins remain intact, but there is little crosslinking of  $\kappa$ -casein molecules by the denatured proteins. When  $\text{Ca}^{2+}$  is added, micelle formation takes place; complexes of  $\kappa$ -casein and denatured protein are incorporated into the micellar surface. When the micelles are concentrated by the initial centrifugal field at the outer (ac-

cumulation) wall of the channel, the denatured protein on the surface of one micelle can bond by disulfide linkage to the  $\kappa$ -casein of an adjacent micelle. Hydrophobic or van der Waals interactions may also be involved (11). A single micelle can be linked in this way to two or more other micelles. Crosslinking thus leads to polymerization, i.e., aggregation of the micelles.

It was of some interest to determine whether any differences could be detected in the size distributions of very young micelles and aggregates of micelles formed from the proteins of the three NDMs. To minimize the period of exposure to  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$ -free solutions of the NDM proteins (in solvent S) were injected into the fractionator, the carrier being the usual one (20 mM in  $\text{Ca}^{2+}$ ). The period of exposure of protein to  $\text{Ca}^{2+}$  prior to the start of elution was thus 11 min (1 min run-in + 10 min relaxation) as compared to 71 min in most of the experiments cited above. The size distributions of the young micelles (Fig. 5) are broad but distinctly different. Peak maxima were estimated to occur at  $t_e = 15.5, 22$ , and 37.5 min for

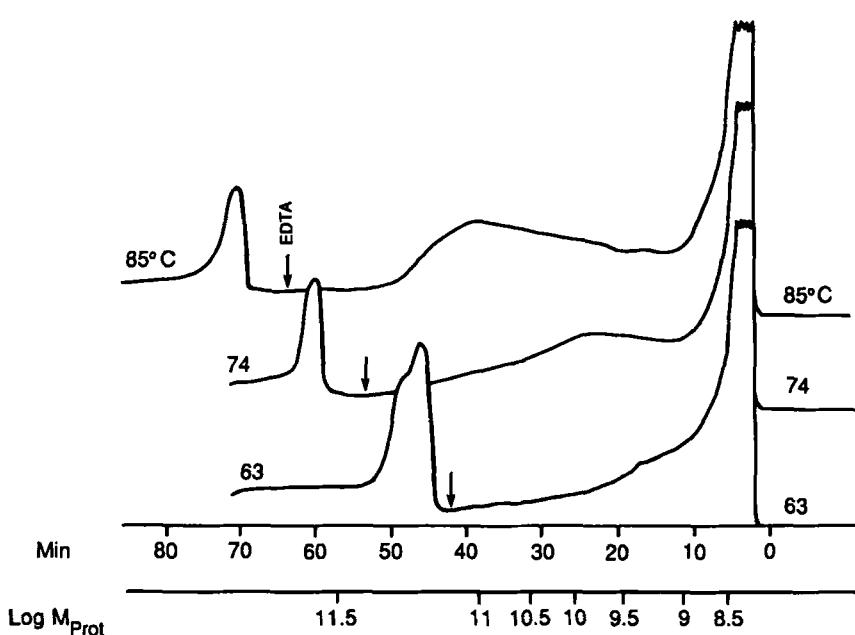


FIG. 5. Fractograms of the proteins of low (63)-, medium (74)-, and high (85°C)-heat NDM which were not preincubated with  $\text{Ca}^{2+}$  prior to injection into the fractionator.  $c_p$ , 18.3 g/L;  $G_0$ , 86.1 g. The initial carrier (Table 1) was replaced by PiKE (Table 1) at the point indicated by "EDTA" (vertical arrow pointing downward). The  $\log M_{\text{Prot}}$  scale does not apply beyond the point where the channel was eluted by EDTA, since protein in particulate form is redissolved by this reagent and elutes with it. All unspecified conditions as for Fig. 2.

NDM 63, NDM 74, and NDM 85, respectively. The corresponding micelle sizes ( $M_{\text{Prot}}$ ) are  $2 \times 10^9$ ,  $6 \times 10^9$ , and  $1 \times 10^{11}$ . The tendency of the high-heat-treated proteins to form much larger particles, on exposure to  $\text{Ca}^{2+}$ , than the low- and medium-heat-treated proteins is thus apparent very early in micelle reconstitution.

The EDTA peaks for NDM 74 and NDM 85 are almost completely solvent transition peaks due to the change from the initial carrier to solvent PiKE. Surprisingly, some protein appears to have been eluted by EDTA from sample NDM 63 (at  $t_c = 46$  min). This may have resulted from premature elution with EDTA.

## CONCLUSION

The susceptibilities of the proteins of various nonfat dry milks to the formation of micellar aggregates can be evaluated, under carefully controlled conditions, by SdFFF. Parameters suitable for such evaluation have been determined; these include a casein concentration of 6.8 g/L and an initial field strength of 5.5 g. Aggregated protein is eluted from the separation chamber by replacing the initial carrier (eluant) with a second carrier containing a reagent (for example, a detergent or EDTA) which dissolves or disperses the aggregates.

## GLOSSARY

$M_{\text{eff}}$	effective particle mass in the medium in which the particle is suspended (g/mol particles)
$M_{\text{Prot}}$	Mass of protein in the particle (g protein/mol particles)
$t_{\text{rlx}}$	duration of relaxation (min)
$t_c$	time (from start of eluant flow) when constant field phase ( $G = G_0$ ) terminates and decay begins (min)
$\tau_w$	exponential decay constant of the rotational velocity of the rotor (min)
$\tau_g$	exponential decay constant of the field $G$ (min)
$t_{\text{Hold}}$	time at which decay phase terminates and hold phase begins (min)
$t_e$	elution (retention) time (min)
$t_0$	value of $t_e$ for unretained particles (min)
$\omega'$	rotational velocity of rotor (rpm)
$\omega'_0$	value of $\omega'$ during constant field phase (rpm)
$\omega'_{\text{Hold}}$	value of $\omega'$ during hold phase (rpm)
$G$	gravitational (centrifugal) field strength (acceleration) (cm/s <sup>2</sup> )
$G_0$	value of $G$ during constant field phase (cm/s <sup>2</sup> )
$G_{\text{Hold}}$	value of $G$ during hold phase (cm/s <sup>2</sup> )

$w$	width (thickness) of the separation channel (radial distance between outer and inner walls) (cm)
$\lambda$	basic retention parameter of FFF (dimensionless)
$\lambda'$	wavelength (nm)
$T$	absolute temperature (Kelvin)
$R_0$	gas constant (erg·Kelvin <sup>-1</sup> ·mol <sup>-1</sup> )
$\rho$	density of the medium (g/cm <sup>3</sup> )
$\bar{v}$	partial specific volume of the protein (cm <sup>3</sup> /g)

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