

Abstract

The casein complexes (casein micelles) of human skim milk were evaluated by electron microscopy. Fourteen samples of human milk were obtained from seven donors whose stage of lactation varied from 1 to 23 months. Compositional and biochemical parameters were measured to evaluate the nature of these samples. For mature human milks, 2-13 months of lactation, average percentage of solids-not-fat was found to be 8.32 ± 0.16 . Protein compositions of the skim milks were studied by the Coomassie blue dye binding method and by SDS-gel electrophoresis; total protein and casein were found to be 0.62 ± 0.18 and 0.29 ± 0.02 percent, respectively. The lysozyme and lactoferrin contents were also determined. The total casein of the skim milks appeared to increase slightly as lactation progressed; an exception occurred in milks obtained from one donor at 22 and 23 months postpartum which exhibited decreased casein and elevated lysozyme and lactoferrin contents. The overall morphology of the casein micelles was evaluated by electron microscopy using platinum shadowing, negative staining, and thin sectioning methods. Platinum shadowing was largely unsuccessful, but negative staining showed a discrete substructure for human casein micelles. Average corrected diameters of 43.0 nm from the area and 47.5 nm from circumference measurements were obtained from analysis of fixed and pelleted human milk micelles.

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Introduction

In all species so far examined the major phosphoproteins of milk occur as colloidal aggregates referred to as casein micelles (Jenness, 1974). These complexes also contain the majority of the inorganic calcium and phosphate of milk (Farrell and Thompson 1974). The ultrastructure of the casein micelles of human milk has not been definitively characterized. The first report of electron microscopy of bovine casein micelles was in 1949 by Nitschmann, who fixed the micelles with formaldehyde and used gold shadowing for contrast. Later, Huth (1957) employed osmium staining of human casein micelles and reported an average diameter of 30 nm. D'Agostini and Calapaj (1958), using the same procedure as Nitschmann, found average diameters of 49 nm, while in a later study Calapaj (1962) found diameters of 75 ± 4 nm for human milk micelles. Ultrathin sections of methacrylate embedded human milk micelles were studied by Knoop and Wortmann (1960, 1967) who measured 2740 micelles and determined a mean diameter of 42 nm. Recently, Rüegg and Blanc (1982) examined human milk micelles from samples at partum and followed lactation for 6 months. By freeze fracture techniques, they found a trend toward increased micelle size with increased lactation and reported diameters between 11-55 nm for volume/surface mean diameter and 16-88 nm for volume moment mean diameter.

The intent of this paper is to evaluate the morphology and to determine size distributions of casein micelles with minimal disturbance of the protein fraction of human milk using the techniques of electron microscopy. Our observations on the casein micelles of human milk using a variety of sample preparation techniques and the problems encountered with the preparation of whole milk and air-dried casein micelles are reported. In the study of human milks, it is important to know that the composition of the samples used for electron microscopy represent normal mature milk and fall within certain limits. Analysis of biochemical parameters of the samples used to characterize the milks are also reported.

Materials and Methods

Freshly drawn milk samples were obtained from multiparous individuals whose stage of lactation ranged from 1 to 23 months. The donors were asked to take the samples during mid-nursing period; milks were maintained at 5°C and received at the laboratory within 24 h. However, four milks were frozen for 4 days and thawed upon receipt. The pH of each sample was determined, and the whole milk was separated by centrifuging in the cold at 1500 X g for 15 min. After samples for microscopy were taken, the skim milk fraction was weighed and lyophilized, and the solids-not-fat content was determined gravimetrically.

Biochemical Determinations

Discontinuous gel electrophoresis in the presence of sodium dodecyl sulfate, and gel densitometry were carried out as previously described by Basch *et al.* (1985), except that the human β -casein band was identified by co-electrophoresis with an authentic standard (Greenberg and Groves, 1984). Protein quantitation of the dried milks was by the Coomassie blue dye binding method as adapted to bovine milk by Douglas *et al.* (1981). Anti-human lactoferrin was purchased from Miles Scientific, Naperville, IL¹ and the purified standard human lactoferrin was the gift of Merton Groves of this laboratory. Lactoferrin content was determined by single radial immunodiffusion as previously described (Douglas *et al.*, 1981). Lysozyme activity was determined spectrophotometrically as detailed by Shugar (1952).

Electron microscopy

Thin sectioning. The method of Salyaev (1968) involved microencapsulation of the whole human milk in 2 and 4% agar gels. The microcapsules containing the fat globules and casein micelles were fixed for 2 h in 2% glutaraldehyde in water adjusted to the pH of the samples, which ranged from pH 6.4 to 7.6. They were washed 3X with water, followed by postfixation overnight in 1% OsO₄ in water at the same pH. The samples were washed with water and were dehydrated through increasing acetone concentrations and embedded in Spurr resin (Spurr, 1969). Sections were stained with uranyl acetate and lead citrate. Sections (~60 nm) were prepared on a LKB Ultratome IV.

Human skim milk (0.8 ml) was added to 1 ml of 2% glutaraldehyde and fixed for 30 min. The sample was centrifuged at 96,000 X g for 45 min at 30°C to obtain a pellet. The pellet was cut into small pieces, washed 3X with water, and postfixated with 1% OsO₄. The samples were embedded and sectioned as above. Size distributions of the casein micelles were obtained on micrographs of

sectioned micelles at 148,500X. In the pelleted fractions, few micelles with uncorrected diameters below 30 nm were observed. Only micelles with distinct peripheries were sized. The circumferences of individual micelles were traced using a Houston Instruments digitizing pad. Approximately 45 points per micelle were recorded. The error in measuring a circumference was ~1-2%. The outlines of 2150 micelles were traced. From these traces, diameters were calculated both from the measured circumferences and from the accumulated surface area by point to point triangulation with a chosen center. Frequency distribution of the diameters were then obtained using procedure frequency of the Statistical Analysis System (SAS) (1982).

Shadowing. Preparation of human casein micelles was carried out according to the method of Carroll *et al.* (1968). The skim milk (0.8 ml) was fixed for 15 min in 1 ml of 2% glutaraldehyde in water at the original pH of each sample. The sample was diluted in water, spread on freshly cleaved mica, air dried, shadowed with platinum, and backed with carbon. The film was scored lightly, floated onto a water surface, and picked up on copper grids.

Negative staining. Human skim milk was fixed in 2% glutaraldehyde for 15 min, diluted ~1:100 in water and negative stained with 1% phosphotungstic acid (PTA) at pH 7.0. All observations were carried out on a Zeiss 10-B electron microscope at either 60 or 100 kV.

Results

A series of 14 milk samples were obtained from seven donors, whose stage of lactation ranged from 1 month to 23 months (Table 1). The 1, 2, and 3 month samples are from donor P.W.; the 4, 5, 6, and 8 month samples are from a second donor (C.S.); the 22 and 23 month samples are from donor B.B.W. (Table 1). The pH of the samples varied from 6.4-7.6. One set of four samples which had been kept for 4 days in a home freezer gave severe flocculation upon thawing. Schöch and Matthaehi (1983) have reported recently that flocculation of frozen human milks can be minimized by

Table 1 Milk samples

Donors	Months of lactation
C.S.	4 ^a , 5, 6, 8
D.K.	7
B.W.	9, 10 ^a
B.B.W.	22, 23 ^a
P.W.	1 ^a , 2, 3
D.P.	13
R.W.	6 ^b

^aThese samples were kept 4 days in a home freezer.

^bThis sample was frozen for 6 months.

¹Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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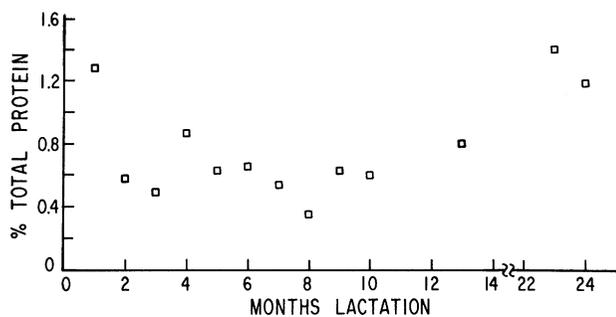


Fig. 1. Percent total protein (W/W) of human skim milks obtained at various stages of lactation.

gently shaking the milks while thawing under cold running water.

The total protein and solids-not-fat contents of the skim milks are plotted as a function of the time of lactation in Figs. 1 and 2. No direct correlation of solids-not-fat with time is observed. The total protein content as measured by the dye binding method was elevated in the first month of lactation in agreement with the data of Ruegg and Blanc (1982). Samples after prolonged lactation (22 and 23 months) also showed elevated protein contents. The frozen samples, after being skimmed, showed no unusual compositional trends.

Gel electrophoretic patterns of skim milk from donors at 3, 7, 9, and 22 months lactation are shown in Fig. 3. Standards for molecular weight calibration on these sodium dodecyl sulfate gels and a sample of bovine milk are included for comparison. Of special note in the 22 month samples was the high albumin, immunoglobulin, lactoferrin, and lysozyme contents. Both the lactoferrin and lysozyme concentrations of all the milks sampled were determined and are presented in Table 2. An increased lysozyme content does not necessarily mean an increase in the lactoferrin content, but the 8 month sample from the same donor as the 4, 5, and 6 month samples showed significant increases in both. Milks at 22 and 23 months showed a comparatively high lysozyme and lactoferrin content, indicating a probable infection. The four frozen samples showed no unusual protein distribution. No bands comparable to the major bovine α_{s1} -casein were observed, however, for purified caseins minor bands in this region can be visualized. The band in human milks comparable to bovine κ -casein was not distinct. In similar gels, purified human κ -casein migrates in the region above β -casein with a molecular weight of $\sim 37,000$ (Brignon *et al.*, 1985). The human κ -casein bands were not visualized at the concentrations employed here. Acid precipitation of the caseins showed no clear cut separations of whey and casein as was achieved for bovine milks and demonstrated by electrophoresis (Basch *et al.*, 1985). In samples such as those obtained at 8, 22, and 23 months, where the lysozyme

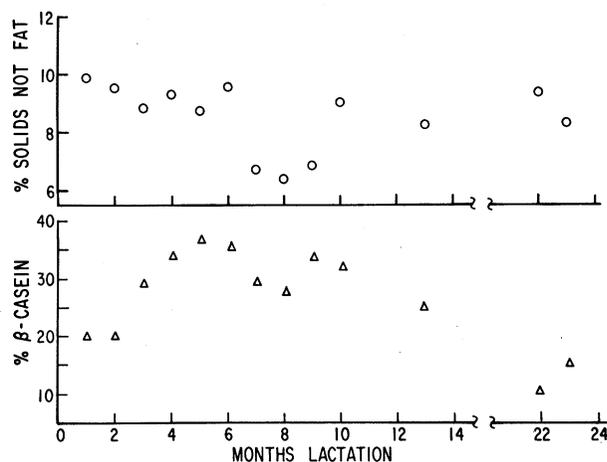


Fig. 2. Percent solids-not-fat (W/W) of human skim milks obtained at various stages of lactation; the β -casein content (% of total protein) was obtained by densitometry.

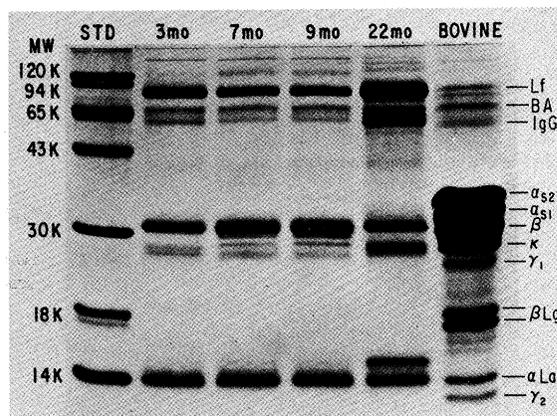


Fig. 3. Sodium dodecyl sulfate gel electrophoresis pattern of human skim milk from donors at 3, 7, 9, and 22 months. Bovine skim milk proteins on right for reference. Abbreviations are: lactoferrin LF, bovine serum albumin BA, heavy chain immunoglobulin IgG, β -lactoglobulin β -Lg, and α -lactalbumin α -La; the various caseins α_{s2} , α_{s1} , β , κ , γ_1 , and γ_2 . For the 22 month sample note the increased lactoferrin, albumin and immunoglobulin contents; the new band appearing just above α -La is lysozyme.

and lactoferrin contents were high, these proteins partially co-precipitated with the caseins. In the case of the whole human milks then, only the β -casein band could be clearly defined as a casein, and since it accounts for most of the stainable casein bands, all other bands were considered whey (noncasein) protein. The β -casein content was therefore calculated by densitometry and is shown in Fig. 2. The percent β -casein is lower initially, rises to a relatively constant level and decreases upon prolonged lactation. The 22 and 23 month samples showed decreased

Table 2 Lactoferrin and lysozyme contents of human skim milks

Months of lactation	Lysozyme units/mg protein	Lactoferrin mg/100 mg protein ^a
1	16	16.4
2	260	16.8
3	296	14.0
4	319	10.3
5	52	11.4
6	96	10.0
7	525	11.8
8	1 004	23.3
9	273	15.0
10	333	19.3
13	51	20.7
22	1 130	35.1
23	1 600	36.5
Average ^b	320 ± 281	15.2 ± 4.6

^a Assay by radial immunodiffusion

^b Average for mature milks (2-13 months).

casein with elevated total protein, lysozyme, and lactoferrin levels.

In Table 3, the average values obtained for the mature human skim milk composition are compared with similar averages reported by Blanc (1981). The results of this study compare favorably. The data for the 1, 22, and 23 month milks were not included in these averages.

Morphological evaluation of selected human milk samples was carried out by electron microscopy. Minimum manipulation of the milks was desirable in order to obtain reproducible electron micrographs to provide reliable size distribution data. The first method of choice in this case was the microencapsulation method of Salyaev (1968) in which whole milk is entrapped in an agar microcapsule and carried intact through fixation, dehydration, and embedding. No centrifugation steps are required and this method has been successfully used to prepare bovine milks for electron microscopy. Sectioned whole milk obtained by the microencapsulation procedure is shown in Fig. 4. Only fat globules and cell fragments associated with it are observed. Some of the membrane surrounding the fat globule appears to be missing, and few casein micelles are observed. Even at higher magnifications than that in Fig. 4, no casein micelles were observed, although some micelles were found entrapped in the agar wall of the microcapsule. Apparently, the casein micelles were not retained by the agar, only the much larger fat globules remained. The smaller casein micelles of human milk may have been lost through the pores of the agar microcapsule. Increasing the agar content of the microcapsule to 4% did not retain intact casein micelles. In

Table 3 Mature human milk composition (g/100 g milk)

	Blanc (1981)	This study ^a
Solids-not-fat	8.50	8.32 ± 1.16
Protein	0.90	0.62 ± 0.18 ^b
Casein	0.25	0.29 ± 0.02 ^c

^a 2-13 month data

^b Protein by Coomassie blue dye binding

^c Casein by densitometry considering only the β -casein fraction and reported as the fraction of total protein applied to the gel.

contrast to Fig. 4, frozen milk samples were highly disorganized. Little or no fat globule membrane remained intact and phase separation apparently occurred within the fat droplets.

Shadowing of the casein micelles was carried out according to the method of Carroll et al. (1968). The results obtained were disappointing; the micelles from human milk appeared collapsed as evidenced by lack of shadows. In an attempt to determine the reason for lack of shadows, bovine and human milk casein micelles were prepared and shadowed at the same time (Fig. 5). Relatively good results were obtained with the bovine micelles (Fig. 5a), while the human micelles appeared flattened with few shadows (Fig. 5b).

Negative staining with phosphotungstic acid was employed by Calapaj (1962) to determine size distribution of human milk micelles. Examples of negative stained human casein micelles are presented in Fig. 6. Nonuniform spread of the stain or excessive staining could lead to inaccurate size measurements. But the fine structure can be resolved, and a submicellar structure of the micelle as theorized for bovine casein (Farrell and Thompson, 1974) can be observed (Fig. 6, insert).

An example of a thin section of pelleted human milk micelles (8 months lactation) is shown in Fig. 7. The average diameters obtained as described in Materials and Methods are given in Table 4. Figure 8 shows a histogram of the observed values as derived from the area calculation. In this case the average diameter was found to be 42.4 nm (standard deviation = 8.9 nm), and the micelles ranged in size from 20 to 104 nm. Over 95% of the micelles were found to fall between 28 and 60 nm. A similar distribution was formed for the diameter calculated from the circumference except the mean was slightly larger, 47.3 nm (standard deviation = 11.7 nm). In addition, various moments of each distribution were calculated as described by Rügge and Blanc (1982); the average diameters are

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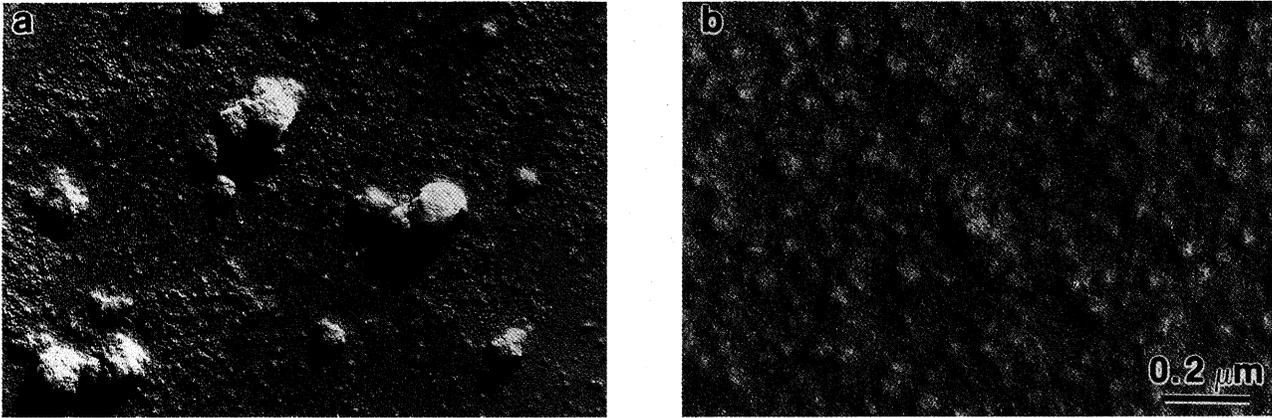


Fig. 5. Bovine (a) and human (b) casein micelles fixed in glutaraldehyde and then shadowed at the same time. The human casein micelles are much smaller, collapsed and flattened compared with the bovine micelles. (Sample shown (b): 12 months.)

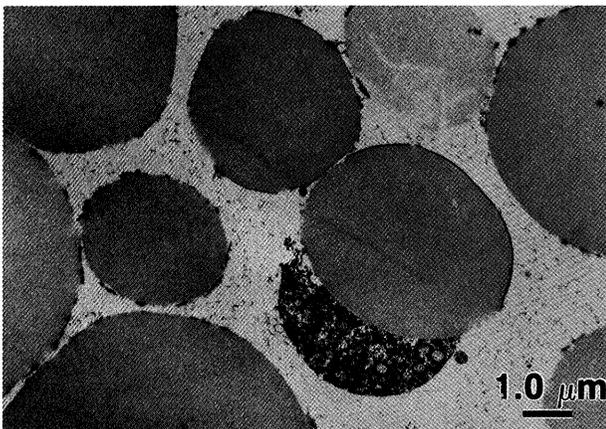


Fig. 4. Whole human milk prepared by micro-encapsulation method. Sections show fat globules without intact membrane and some cell fragments. No casein micelles are observed. (Sample shown: 13 months.)

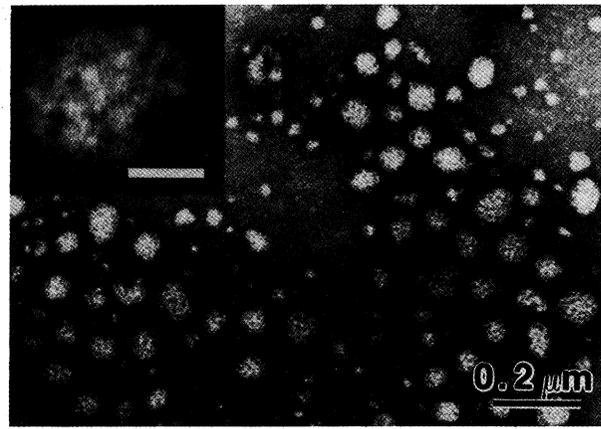


Fig. 6. Negative stained (1% PTA) human casein micelles showing a wide range of sizes and their spherical nature. Insert at higher magnification, the submicellar structure is clearly resolved. Bar = 30 nm. (Sample shown: 8 months.)

given in Table 4. The data from all of the size distribution measurements were also corrected using the method of Goldsmith (1967) which takes into account the relationship between micelle size and the section thickness. The correction changed the observed micelle diameters by less than 1 nm in each case (Table 4).

Discussion

Analysis of average compositional data of the human milk samples have indicated good agreement with literature values. However, milks obtained at 22 and 23 months showed high albumin, immunoglobulin, lysozyme and lactoferrin levels, while the 1 month sample with its elevated protein content seemed closer to colostrum than to mature milk. The 8 month sample, which yielded exceptional structural information, had an elevated lysozyme level, but its other compositional

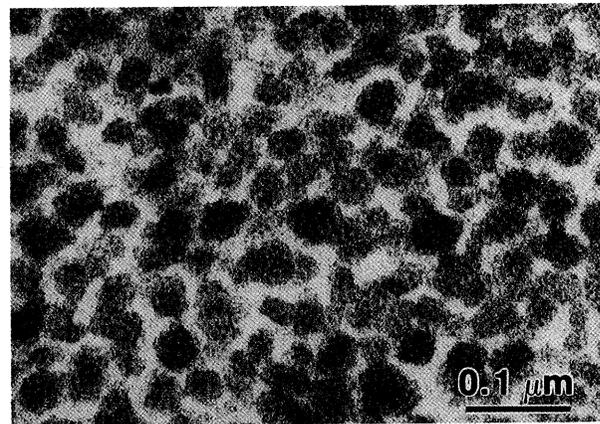


Fig. 7. Ultrathin section of human milk casein micelles. (Sample shown: 6 months.)

Table 4 Average diameters in nm for human milk casein micelles.

Distribution moments ^a	From Area ^b		From Circumference ^c	
	Observed	Corrected ^d	Observed	Corrected ^d
Number	42.4	43.0	47.3	47.5
Average-d _n	s.d.8.9		s.d.11.7	
Volume	44.4	44.9	50.5	50.4
Average-d _v				
Volume/Surface	46.6	46.9	54.2	53.8
Average-d _{vs}				
Volume moment	49.8	49.9	59.8	59.1
Average-d _{vm}				

^aThe various distribution moments calculated are defined by Rügge and Blanc (1982)

^bDiameters calculated from tabulated areas

^cDiameters calculated from tabulated circumferences

^dEach distribution was corrected for section thickness by the method of Goldsmith (1967)

data were well within 2 standard deviations of the means (compare Table 3 and Figs. 1 and 2). The milks were characterized to insure representative samples for observation with the electron microscope.

The average values for the solids-not-fat and the protein composition were comparable to the results calculated from the data of Rügge and Blanc (1982) (Table 3). It was also observed that at prolonged lactation a lower casein content was correlated with higher lysozyme and lactoferrin contents (Table 2 and Fig. 2, 22 and 23 month samples). No evidence of bacteria was observed in these milks by electron microscopy, more samples would be needed to prove this conclusively, but few humans lactate for 22-23 months.

The determination of the ultrastructure of the human milks requires selection of the most appropriate method to adequately preserve both skim milk and fat globule fractions. The use of Salyaev's (1968) method was selected in order to preserve the lipid-protein relationships in human milk. This method was adapted by Henstra and Schmidt (1970) for bovine milks; unfortunately, the casein micelles of human milk were apparently lost during processing. The fat globules with their larger sizes were retained and are shown in Fig. 4.

The second method of choice was that of Carroll *et al.* (1968). This procedure conducted on skim milk does not require pelleting of the micelles and all sizes of micelles are uniformly dispersed. This method should give a reliable size distribution of the human casein micelles. As can be seen (Fig. 5a), the bovine casein micelles gave good shadows, while the human casein micelles (Fig. 5b) appear collapsed and flattened, as judged by the ratio of shadow length to measured diameters. In reviewing the literature, Rügge and Blanc (1982) noted that β -casein is the major protein of the human casein micelle. This is

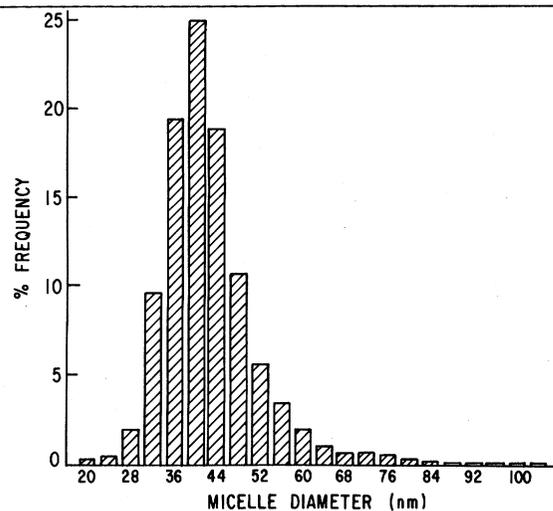


Fig. 8. Histogram displaying adjusted size measurements as obtained from sectioned human milk micelles. (Figure 7.)

confirmed by examination of the gel electrophoresis patterns; little or no α -caseins are found in the skim milks analyzed. Human milk also has a substantially lowered total inorganic calcium + phosphate level (Holt and Jenness, 1984), when compared to most species so far examined. The collapse of human milk casein micelles upon drying may be explained by their lack of the α -caseins with high calcium affinity, as well as their reduced colloidal calcium-phosphate content. Both of these factors could contribute to a three-dimensional structure which is less stable to drying than that of the bovine casein micelles. Even the introduction of glutaraldehyde crosslinking was not enough to prevent their collapse.

Examination of the fine structure observed (Fig. 6) for negative stained human casein micelles suggests that they, like their

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bovine and rat counterparts, may be assembled through the aggregation of submicellar particles (Farrell and Thompson, 1974).

Measurements of human milk casein micelles were made on sections from milks at 8 months lactation (Fig. 7). The program used to measure the sizes of the thin sectioned micelles allows for the calculation of their diameters either from the length of the circumferences or from the accumulated surface areas. Diameters calculated from the circumferences were somewhat greater than those calculated from surface areas. If the surfaces of the micelles are somewhat rough (as would be anticipated based on Figure 6), then larger apparent diameters based on circumferences would be expected. However, it is difficult to predict which method is more accurate since both values are in reasonable agreement. The Goldsmith correction for section thickness has little effect on the average diameter, probably because the majority of the human milk micelles are contained within the 60 nm section thickness. The observed average diameters are also only slightly altered when volume, volume surface and volume moments are calculated (Table 4). This differs significantly from the responses of the freeze fracture data obtained by Rüegg and Blanc (1982), who reported micelle sizes ranging from 8-14 nm for d_n and 16-88 nm for d_{vm} . The insensitivity of our data to these calculations may be due to the fact that the centrifugal force used resulted in an uneven distribution of the micelles. In the pelleted fractions, few particles with uncorrected diameters below 30 nm were observed, because smaller micelles and submicelles would not have been sedimented.

This, however, raises an important question regarding the definition of a casein micelle. The casein micelles of most species are thought to be assembled from rather spherical submicelles (Farrell and Thompson 1974 and Schmidt 1982). For bovine casein these have been estimated to have an upper limiting size of 18 nm (Pepper and Farrell 1982); Rüegg and Blanc (1982) estimated them to range from 6 to 12 nm for human caseins. Thus casein micelles constructed from at least four submicelles of 10 nm each should have apparent diameters of at least 20 nm. Structures below this size might be considered to be non-micellar casein. Historically, one view of micellar casein has been considered to be that casein sedimented at 100,000 X g for half an hour or more. Thus the data given here could be considered valid for micellar casein based on method of isolation and particle diameters greater than those expected for a minimum spherical micelle composed of four submicelles. Admittedly the frequency distributions obtained are somewhat skewed.

In summary, compositional data were obtained on milks from donors at various stages of lactation up to 23 months. Several of the milks exhibited "high" lactoferrin and

lysozyme as well as immunoglobulin levels. All the other milk samples fell within close limits. Attempts to observe whole milks were unsuccessful because of the loss of casein micelles. In addition, the use of shadowed micelles was unsuccessful due to collapse of the human milk micelles upon drying. Negatively stained human milk micelles show a distinct submicellar structure. Sectioned micelles were measured and a histogram developed which showed an uncorrected size distribution of human milk casein micelles ranging from 20-104 nm, with an average size of 42.4 nm for milk obtained at 8 months; this value was increased by only 0.6 nm by the correction of Goldsmith (1967). These results fall in the mid-range of previously published size distributions obtained by a variety of electron microscopic procedures.

Acknowledgments

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References

- Basch JJ, Douglas FW, Procino LG, Holsinger VH, Farrell HM Jr. (1985) Quantitation of caseins and whey proteins of processed milks and whey protein concentrates. *J. Dairy Sci.*, 67, 23-31.
- Blanc B. (1981) Biochemical aspects of human milk—comparison with bovine milk. *World Rev. Nutr. Diet.*, 36, 1-89.
- Brignon G, Chtourou A, Ribadeau-Dumas B. (1985) Preparation and amino acid sequence of human k-casein. *FEBS Lett.*, 188, 48-54.
- Calapaj GG. (1962) Ricerche al microscopio elettronico sulla polidispersione della caseina de latte umano e bovine. (Electron microscope study of the polydispersed caseins of human and bovine milk.) *Boll. Ist. Sier Oter Milan*, 41, 276-291.
- Carroll RJ, Thompson MP, Nutting GG. (1968) Glutaraldehyde fixation of casein micelles for electron microscopy. *J. Dairy Sci.*, 51, 1903-1908.
- D'Agostini BA, Calapaj GG. (1958) Ricerche al m.e. sulla caseina del latte di alcune specie animali. (Electron microscope study of the caseins of the milks of several animal species.) *Acta Med. Vet.*, 4, 9-15.
- Douglas FW Jr, Greenberg R, Farrell, HM Jr, Edmondson LF. (1981) Effects of ultra-high-temperature pasteurization on milk proteins. *Ag. Food Chem.* 29, 11-15.

Farrell HM Jr, Thompson MP. (1974) Physical equilibria proteins, in : Fundamentals of Dairy Chemistry, 2nd Ed, B.H. Webb, A.H. Johnson, J.A. Alford (eds.), The AVI Publishing Co., Westport, Connecticut, 442-473.

Goldsmith PL. (1967) The calculation of true particle size distributions from the sizes observed in a thin slice. Brit. J. Appl. Phys., 18, 813-830.

Greenberg R, Groves ML. (1984) Plasmin cleaves human β -casein. Biochem. Biophys. Res. Comm., 125, 463-468.

Henstra S, Schmidt DG. (1970) Ultradünnschnitte aus Milch mit Hilfe der Mikrokapsel-Methode. (Ultra thin sections of milk by the means of the microencapsulation method.) Naturwissenschaften, 57 (5), 247.

Holt C, Jenness R. (1984) Interrelationships of constituents and partition of salts in milk samples from eight species. Comp. Biochem. Physiol., 77A, 275-282.

Huth E. (1957) Elektronenoptische Untersuchungen zur Kolloidstruktur von Säuglingnahrungen. (Electron optical investigations of the colloidal structure of infant foods.) Kinderheilkunde, 105, 247-249.

Jenness R. (1974) The composition of milk, in : Lactation—A Comprehensive Treatise, B.L. Larson, V.R. Smith (eds.), Academic Press, New York, 3-37.

Knoop E, Wortmann A. (1960) Zur Größenverteilung der Caseinteilchen in Kuhmilch, Ziegenmilch und Frauenmilch. (The size distribution of casein particles in cow, goat, and human milk.) Milchwissenschaft, 15, 273-281.

Knoop E, Wortmann A. (1967) Das Auftreten von Kasein-Micellen im Kolostrum der Frau, der Kuh und der Schweines. (The occurrence of casein micelles in human, bovine, and porcine colostrum.) Milchwissenschaft, 22, 198-205.

Nitschmann H. (1949) Elektronenmikroskopische Größenbestimmung der Calcium-caseinatteilchen in Kuhmilch. (Electron microscopic size determination of calcium-casein particles in cow's milk.) Helv. Chim. Acta, 32, 1258-1264.

Pepper L, Farrell HM Jr. (1982) Interactions leading to formation of casein micelles. J. Dairy Sci. 65, 2259-2266.

Rüegg M, Blanc B. (1982) Structure and properties of the particulate constituents of human milk. A review. Food Microstruct., 1, 25-47.

Salyaev RK. (1968) A method of fixation and embedding of liquid and fragile materials in agar microcapsulae, in : Proc. 4th Europ. Regional Conf. Electron Microsc., Rome, D. S. Bocciarelli (ed.), Tipografia Polialotta Vaticana Rome, II, 37-38.

SAS Users Guide: Basics, (1982), SAS Institute, Inc., Box 8000, Cary, NC 27511 923 pp.

Schmidt DG. (1982) Association of caseins and casein micelle structure, In: Developments In Dairy Chemistry-1, P.F. Fox (ed.). Applied Science Pub., Essex, England, 61-86.

Schöch G, Matthaei S. (1983) Tiefgefrieren von Frauenmilch. (Deepfreezing of human milk.) DMW, 108, 49, 1894-1895.

Shugar D. (1952) Measurement of lysozyme activity. Biochim. Biophys. Acta 8, 302-308.

Spurr AR. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res., 26, 31-43.

Discussion with Reviewers

W. Buchheim: Which step of your preparatory treatment may have caused the loss of fat globular membrane material (Fig. 4)?

D. N. Holcomb: Does the agar encapsulation lead to any "extraneous" matter which might be visible in the micrograph?

W. Buchheim: On Fig. 4 there are numerous small particles visible between the fat globules which are of thread-like appearance. How do you interpret these structures?

Authors: These three questions are inter-related, and should be answered jointly. The "extraneous"-thread like material seen in Fig. 4 could have several origins. It could be from the encapsulating material as suggested by Dr. Holcomb. It could be artifactual; Dr. Schmidt at NIZO has some convincing evidence that some thread like structures as visualized by electron microscopy can be artifacts. The material could also represent shed fat globule membrane. Patton and coworkers (J. Dairy Sci. 63:697-700, 1980) have shown that for goat's milk, a significant amount of cholesterol and phospholipid are shed into the skim milk phase upon standing for 24 hr at 4°C; similar changes were not observed in cow's milk. They took this as evidence for fragility of the goat fat globule membrane. Our observations (Fig. 4) show little continuous membrane for human milk fat globules. A large portion of the bilayer may have been shed simply by standing overnight in the cold. Portions of the interfacial "fuzzy coat" (Freudenstein et al., Exptl. Cell Res. 118, 277-294, 1979) appear to be missing; it is possible that the thread like material may originate as part of this layer. The electron density of the

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extraneous material appears to be more like that of the "fuzzy" coat than the agar capsule, the casein micelles or the lipid bilayer. However all the sources could contribute to the material observed in Fig. 4.

W. Buchheim: The number frequency distribution of casein particles (Fig. 8) exhibit a relative maximum at 42 nm. Do you believe that the decreasing number of smaller particles reflects the true situation in human milks?

C. Holt: Many studies by electron microscopy of the bovine casein micelle have revealed a large number fraction of particles in the smallest size class yet this work on human milk reveals few such micelles. Is there a real difference here?

Authors: As discussed in the manuscript, it would have been preferable to have conducted the micelle size distribution on whole or skim milks. However, our best results were obtained on micelles harvested by centrifugation. It is possible that some of the smaller micelles could have been excluded by this process, but where to draw the line between micellar and non-micellar caseins may be problematic for human milks.

Additional References

Freudenstein C, Keenan TW, Eigel WN, Sasaki M, Stadler J, Franke WW. (1979) Preparation and characterization of the inner coat membrane associated with fat globule membranes from bovine and human milk. *Exptl. Cell Res.* 118, 277-294.

Patton S, Long C, Sokka T. (1980) Effect of storing milk on cholesterol and phospholipid of skim milk. *J. Dairy Sci.* 63, 697-700.