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Abstract

The size distribution of casein micelles in camel milk has been determined by electron microscopy. Individual and pooled samples were cryo-fixed by rapid freezing and freeze-fractured. Electron micrographs of the freeze-fracture replica revealed a relatively broad size distribution, with an average micelle diameter around 280 nm in the volume distribution curve. The distribution was significantly broader than that of the particles of cow's or human milk and showed a greater number of large particles. The submicelles were also somewhat larger than those observed in cow's and human milk (approx. 15, 10 and 7 nm, respectively). The average values for the gross composition of camel milk were similar to those of cow's milk. Partition of mineral salts between the serum and micellar phase of camel milk was studied by means of ultrafiltration. The proportion of soluble forms of the minerals expressed as percentage of their total concentrations were 33% for calcium, 69% for magnesium, 52% for phosphorus and 60% for citrate.

Introduction

According to FAO statistics, there are 17 million camels in the world, of which 12.2 million are in Africa and 4.8 million in Asia (22). The camel is a potentially important source of milk. Indeed, in some countries hosting large camel populations, camel milk is one of the main components of the human diet. Milk production varying between 1,800 and 12,700 kg during a lactation period between 9 and 18 months has been reported (13). Information on the characteristics of camel milk is limited. Data available show, however, significant differences between cow and camel milk proteins in properties such as electrophoretic mobility, molecular size (8) and rennet coagulation (7).

While a considerable amount of data is available on micellar casein of bovine milk, very little is known about casein micelles of camel milk. Ali and Robinson (2) have analyzed the size distribution of casein micelles in six samples of camel milk. They determined a number average diameter of 160 nm on electron micrographs of ultra-thin sections. This value, however, overestimates the true mean, because particles with diameters smaller than 14 nm could not be measured. It was therefore considered useful to determine the complete size distribution of casein micelles in camel milk by using freeze-fracture replica of cryo-fixed samples and to compare it to that observed in milk of other species. The freeze-fracture technique allows counting and sizing of the smallest casein micelles including submicelles. Other basic data on the chemical composition of camel milk are also given.

Materials and Methods

Milk samples

Camel milk samples were taken at Ngare Ndare Camel Farm which is situated just north of the equator in Kenya's Laikipia District, at an altitude of 1,730 to 1,890 m above sea level. The animals of indigenous breed (Camelus dromedarius) were all fed exclusively by grazing. The milk samples A and B were collected from 10 individual camels, on two different occasions. On each occasion, the 10 milk samples were pooled, kept refrigerated, and transported to our laboratory within 36 hours. Upon arrival, the milk samples were skimmed, freeze-dried and stored in sealed plastic bags until analysis. Two individual fresh milk samples (numbers 52 and 56) were also used for the analysis. For these samples the time

Table 1. Average chemical composition of camel and cow's milk

Component	unit	camel milk		cow[a]
		x̄	s_x	x̄
Dry matter	g/100 g	12.2	0.7	13
Protein[c]	g/100 g	3.11	0.29	3.5
Total N	mg/100 g	418	21	431[b]
Casein N	% of TN	76		76[b]
Non-casein N	% of TN	24		24[b]
Non-protein N	% of TN	6.7		5.5[b]
Lactose	g/100 g	5.24	0.37	4.6
Fat	g/100 g	3.15	0.32	3.8
Ash	g/100 g	0.80	0.04	0.72
Calcium total	mg/100 ml	157	9	117
Calcium dissolved	% of total	33		32
Magnesium total	mg/100 ml	8.3	0.8	11
Magnesium dissolved	% of total	69		66
Phosphorus total	mg/100 ml	104	4	66
Phosphorus dissolved	% of total	52		53
Citrate total[d]	mg/100 ml	177	3	175
Citrate dissolved	% of total	60		92

[a] Walstra and Jenness (20); [b] Jenness and Patton (12); [c] N x 6.38; [d] as citric acid.

elapsed between collection and examination was 36 hours.

Chemical analysis

Total solids, fat, protein, lactose and ash were determined according to AOAC standard methods (4).

The nitrogen distribution in the milk was determined by the procedure of Aschaffenburg and Drewry (5). The following N-fractions were determined: total protein nitrogen (TN), non-casein nitrogen (NCN) and non-protein nitrogen (NPN), soluble in 12% trichloracetic acid. The amount of casein nitrogen (CN) was calculated by difference.

In order to study the distribution of salts between the dissolved and colloidal phases in milk, it was filtered through a diaflo ultrafiltration membrane (Amicon PM10). The ultrafiltration was carried out under nitrogen at a pressure of 0.35 MPa. In both the original milk and the collected ultrafiltrate the following minerals were determined: calcium and magnesium by atomic absorption spectrophotometry (19), phosphorus by the phosphomolybdate method described in the International Dairy Federation Standard (11) and citrate enzymatically by using a commercially available test kit (Boehringer, Mannheim, West Germany, catalog number 139076).

For amino acid analysis, casein was precipitated from skimmed milk with 0.01 mol/l acetic acid at pH 4.5 - 4.6. The precipitate was washed three times with water and freeze-dried. 20-30 mg of this acid casein were hydrolyzed with 6 mol/l HCl for 24 hours at 110°C under vacuum. The hydrolysate was analyzed on a model Liquimat III amino acid analyzer (Kontron Instruments AG, Zürich) according to the procedure of Amado et al. (3).

Electron microscopy

The reconstituted and fresh skimmed milk samples were cryo-fixed using the propane jet-freezing technique. This technique basically involves the rapid freezing (approximately 10,000 K.s^{-1}) of a very low mass specimen in a jet of liquid propane at 88 K (14, 15). Freeze-fracture replicas were then obtained as described earlier (16). Fourteen to sixteen electron micrographs of each sample were taken at a magnification of approximately 20,000x and the negatives were enlarged 2.6 times for counting and classifying the particles. The total surface area of milk observed for the four samples was 742 micrometers^2. 6,618 particles were counted on this surface. A diameter class width of 20 nm was chosen for the classification of the particles on the prints. A transparent sheet with bars corresponding to the different size classes was placed over the prints. The size class of each particle was found by fitting it into the appropriate diameter range. Particles smaller than about 5 nm in diameter were not considered.

Statistical analysis

Conversion of the observed size distribution of plane sections into real distribution of spherical particles was made using a method proposed by Goldsmith (10). The original FORTRAN program was modified and translated into GW-BASIC for use on MS- DOS microcomputers. Copies of the program are available on request from one of the authors (M.R.). A slice thickness of 5 nm was assumed. Preliminary calculations revealed rather broad size distributions with relatively low frequencies in the larger size classes. The class width was therefore increased from 20 to 40 nm.

Table 2. Amino acid composition of whole casein from camel and cow's milk

Constituent	% amino acid	
	camel	cow[a]
Aspartic acid	7.28	6.52
Threonine	4.87	4.42
Serine	5.39	5.75
Glutamic acid	21.26	20.35
Proline	11.62	10.33
Glycine	0.90	2.27
Alanine	1.98	2.80
Valine	5.43	6.48
Cysteine	0.02	0.65
Methionine	2.70	2.51
Isoleucine	6.23	5.54
Leucine	10.89	8.41
Tyrosine	3.84	5.59
Phenylalanine	4.01	4.73
Lysine	6.53	7.33
Histidine	2.44	2.70
Arginine	4.63	3.62

[a] Alais and Blanc (1); recalculated on a %-basis. Tryptophane was not determined.

The equations used to calculate the various mean diameters (d_n, d_v, d_{vs}, d_{vm}), the distribution width (c_s) and volume fraction (v) are explained in detail elsewhere (16). It should be remembered, that the number average and volume average diameters d_n and d_v are sensitive to shape, errors at both ends of the distribution function, and total number of particles. These values are meaningful only for corresponding symmetrical distribution curves. The weighted mean diameters d_{vm} and d_{vs} are more useful averages for the characterization of the casein micelle distributions. The distribution width c_s corresponds to the coefficient of variation of the surface-weighted distribution (21). Distribution curves from different samples were compared using a standard chi-square test for multiway frequency tables.

Results and Discussion

Chemical composition of camel milk

Table 1 shows data on the chemical composition of the camel milk used for this study. Values for cow's milk from the literature (12, 20) are presented for comparison. In general, the gross composition of camel and cow's milk is similar. The values of CN, NCN and NPN expressed as percentage of the total N appear also to lie in the same ranges.

Concentrations of calcium, magnesium, phosphorus and citrate, along with their partition between the dissolved and colloidal phases are also given in Table 1. As generally reported in the literature (9, 12), about one third of the calcium and phosphate, 75% of magnesium, and 90% of the citrate of fresh cow's milk are present in the serum phase. In camel, milk the distribution of calcium, magnesium and phosphorus is similar. However, the amount of citrate in the serum phase was found to be lower in camel milk.
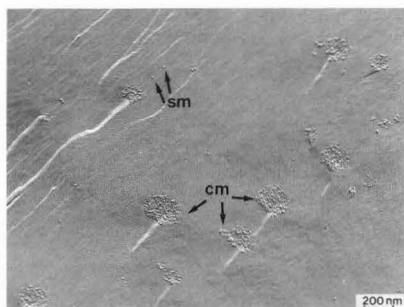

Fig. 1. Freeze-fractured casein micelles in camel milk (cm: casein micelles; sm: submicelles).
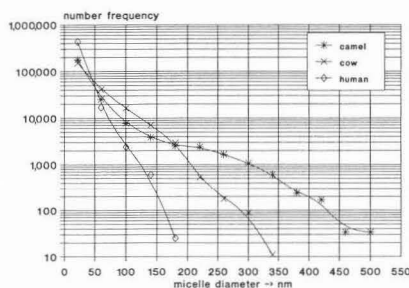

Fig. 2. Number of particles observed in freeze-fractured camel, cow's and human milk. The ordinate is logarithmic and gives the number of particles per mm^2 fractured area and per nm class width.

The amino acid compositions of pooled camel and cow's milk casein are presented in Table 2. A similar pattern can be observed for both species. The most pronounced differences were found for glycine and cysteine, both being significantly lower in camel milk casein.

Size distribution of casein micelles

Fig. 1 shows a typical electron micrograph of casein particles in freeze-fracture replica of camel milk. The mean diameter of the submicelles was on the average 15 nm. This is a rough estimate, because of uncertainties in the technique (plastic deformation of proteins etc.).

The average number of particles observed on such freeze fractured surfaces is shown graphically in Fig. 2. The ordinate gives the normalized frequency of particles per unit area, i.e., the average number of particles per mm^2 fractured area and per nm class width. The distribution is significantly broader than that of cow's or human milk and shows a greater number of large particles.

Table 3. Size distribution of casein micelles in camel milk com pared to cow's milk

Parameter	unit	herd milk A[*]	herd milk B[*]	individual 52[*]	individual 56[*]	pooled data	ranges camel	ranges cow[a]
Average micelle diameter								
d_n , number average	nm	28	28	27	26	27	26- 28	21- 24
d_v , volume average	nm	63	57	51	50	55	50- 63	44- 50
d_{vs}, volume/surface av.	nm	165	131	113	114	129	113-165	90-100
d_{vm}, weight average	nm	288	222	212	237	243	212-288	104-140
Distribution width								
c_s , % of d_{vs}	%	0.5	0.6	0.8	0.9	0.7	0.5-0.9	0.6-1.0
Volume fraction[c], v	%	3.2	2.6	2.4	2.9	2.8	2.4-3.2	2.0-4.0
Submicelles								
d_n , number average	nm	b	b	b	b	15	14- 16	10- 11

[*]A and B: pooled samples, freeze-dried and reconstituted; 52 and 56: fresh samples.

[a] From Rüegg et al. (16) and Schmidt et al. (17,18);   [b] 14-16 nm;   [c] calculated from size distribution
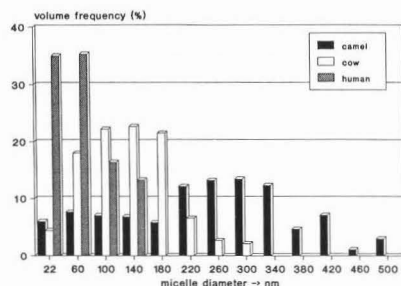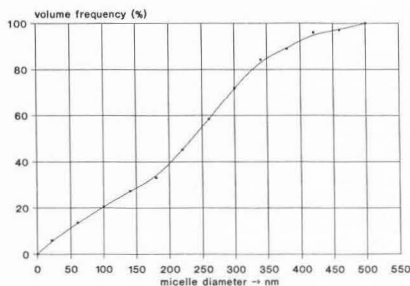

Fig. 3 (at left). Size distribution of casein particles in camel milk compared to cow's and human milk (volume frequency histogram).

Fig. 4 (to the right). Cumulative particle volume distribution of casein micelles in camel milk (pooled data from two individual and two herd milks).

The differences between the distribution curves of the two individual camel milks and the herd milk samples were most pronounced in the diameter range of about 200 to 500 nm. However, the differences were statistically not significant.

The particles in the lowest size class with diameters smaller than 40 nm comprise about 80% of the observed total number of particles but represent only 4-8% of the mass or volume of the casein in camel milk. It is therefore meaningful to consider the weight or volume frequency distribution. Fig. 3 shows the volume frequency of the pooled data of the four milk samples, compared again with the distributions found in cow's and mature human milk (16). The volume distribution curve of casein micelles in camel milk is broad and shows a maximum around 280 nm.

As can be deduced from the cumulative distribution curve in Fig. 4, micelles with diameters between 125 and 310 nm comprise about 50% of the volume or mass of the casein.

Some statistical data derived from the distribution curves, such as mean diameters, width of the distribution, and volume fraction are summarized in Table 3. For comparison, the ranges of the corresponding values for cow's milk are also included.

In earlier investigations, camel milk, after rennet addition, was found to coagulate 2 - 3 times slower than cow's milk. The coagulum obtained was a precipitate in the form of flocks and no homogeneous clot formed (7). The present investigation revealed a relatively broad size distribution of casein micelles in camel milk with a greater number of large micelles

than in cow's milk. The poor rennetability could be related to these differences in the size of casein particles. Coagulation time varies with the micelle size and reaches an optimum in the medium and small size micelles. This appears to be related to the availability of k-casein. The content of k-casein decreases with increasing micelle size (6, 20).

From the results obtained it can be concluded that camel milk casein differs from cow's milk casein in terms of micellar size distribution. However, it would be premature to discuss the impact of this difference in relation to the preparation of products from camel milk. Various biochemical aspects must also be considered and additional studies are necessary to correlate any special feature of product structure with the findings in this investigation.

Acknowledgements

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Discussion with Reviewers

W. Buchheim: Apparently reconstituted (freeze-dried) skim milk was used for electron microscopy work. Is there any danger that freeze-drying might affect size, shape, and distribution of micelles?
P. Resmini: It is written that both fresh and freeze-dried milk samples have been analyzed, but no data are reported concerning these two different products. Freeze-fracturing techniques suggest that the usual freeze-drying of liquid milk may modify the structure of casein micelles, due to the low freezing rate that promotes ice crystal formation inside the micelles, therefore freeze-drying of milk does not seem to be a suitable technique for ultrastructure studies of casein. Please comment.
Authors: The freeze-dried samples were reconstituted to 12.2 % dry matter at 30 – 35°C. There is a certain risk that freezing and thawing or reconstitution of the freeze-drying affects the structure of casein particles. To our knowledge, no statistically significant differences between size distribution in fresh and reconstituted preparations has been reported in the literature and no significant difference was observed in the present investigation.

W. Buchheim: In my opinion, the number and sizes of micelles and non-micellar casein, visible in Fig. 1 contradict the frequency values given in Fig. 2 because the micrograph shows approximately equal number of small particles and cross-sections of large micelles, instead of 100- or 1000-fold.
Authors: Fig. 1 is not a "random picture". A sector has been chosen which shows both large and small micelles. Therefore, the size distribution on this Fig. cannot be used to estimate the real distribution. The area of Fig. 1 represents about 7.9 micrometers^2.

This is only about 1/100th of the total area that has been measured.

W. Buchheim: In case that the amount of non-micellar casein ("submicelles") has been overestimated, some average values (e.g., d_n, d_v, and even d_{vs}) would be too small. According to reviewer's own experience (see e.g., Food Microstructure 5(1), 181-192, (1986)) direct determination of d_{vs} from micrographs (via circumferences and areas of particles) is the best way for testing such possible discrepancies. Authors: The unweighted mean diameter d_n and to some extent the other measures of the mean which are based on the lower moments of the distribution function are sensitive to both ends of the distribution as well as to the total number of the particles counted. The higher the power of the moments, the less is the sensitivity to the uncertainty in the estimation of the smallest particles. d_{vm} is therefore the most robust estimate of the mean diameter. Considering the very broad size distribution of the casein particles in camel milk, the meaning of an "average diameter" should not be overestimated.

W. Buchheim: I have some doubts as to how meaningful size values for so-called submicelles are. Protein molecules are plastically deformed when freeze-fractured, so that we identify primarily only their existence in the plane of cleavage. Slightly modified fracturing and shadowing conditions may influence their apparent size so that measurements of "diameters" and comparisons in different experiments are questionable.

P. Walstra: Conclusions about the size of submicelles are, in my opinion, rather questionable because of the uncertainties in the technique.

Authors: We agree with the reviewers' comment. The diameter of the submicelles is a rough estimate. It has mainly be added for comparison and because of the pronounced difference to that of cow's milk.