

J. Cerbulis

The action of several dispersing agents on casein micelles was studied. In whole milk, 89.1 to 93.3% of the total casein was in sedimentable micelles. When whole milk was dialyzed against distilled water, only 71.3% of casein was in centrifugate; dialyzed against phosphate only 22% of the casein was in centrifugate. Milk, dialyzed against a chelating agent such as ethylenediaminetetraacetate, gave 2% of the casein in centrifugate, but dialysis of this demineralized milk (by chelating agent) against 0.1M CaCl₂ solution, gave 63.2% of the casein in centrifugate. When whole milk was dialyzed against 4M urea, 5.7% of casein was in centrifugate but when urea contained CaCl₂, the centrifugate frac-

tion was 43.7%. Treatment of milk with dodecyl sulfate and deoxycholate gave 2% centrifugate. The presence of CaCl₂ in dialysis solution caused CMEP (chloroform-methanol extractable proteins) to migrate from the micelles to the nonmicellar casein fraction. The chelation decreased the lipid content of casein fractions from homogenized pasteurized milk considerably. All treatments decreased the micelle content in milk, but no treatment gave lipid-free centrifugate or supernatant casein fractions. Milk total lipid fraction, obtained with chloroform-methanol (2 to 1), contained calcium and magnesium.

Electron-dense particles of colloidal calcium phosphate reportedly are embedded in a surrounding matrix of casein to form micelles (Rose and Colvin, 1966) which are formed only if α_s -casein and κ -casein are present simultaneously (Waugh, 1961). β -Casein is incorporated into the micelle by a secondary specific interaction (Waugh, 1961). Calcium and hydrogen bonds are two of the major factors responsible for maintaining the stable structure of the casein micelle. The micelles are dispersed by urea, alkali, and calcium-complexing agents (Beeby and Kumet, 1959).

In previous studies from this laboratory, the lipid content and the composition of these lipids from casein micelles and other fractions of milk were determined in relation to the ultracentrifugation of skim milk (Cerbulis, 1968). The present report extends these studies.

The term micelles, as used here, refers to the polymeric casein units which can be sedimented from fresh milk or commercial homogenized milk by ultracentrifugation (60 minutes at 78,500 g. and 25° C.). The supernatant casein refers to the smaller polymeric casein units which are not sedimented by ultracentrifugation and are obtained by precipitation at pH 4.6 (von Hippel and Waugh, 1955). The centrifugate, as used here, refers to the denser material separated by ultracentrifugation from treated milk samples with dispersing agents.

It was desirable to investigate the role of calcium and hydrogen bonds in the binding of lipids to micelles. In addition to determining the distribution of casein and lipids in unheated milk, such determinations were also made on whole milk samples treated with phosphate, urea, chelating agent,

detergents, or simply dialyzed against distilled water. Inorganic cations were determined in the total lipid fraction obtained from the whole milk by atomic absorption spectrophotometry.

EXPERIMENTAL

Milk. Fresh pooled Holstein milk was cooled immediately to 2° C. on the farm, then transported to the laboratory and used the same morning. Commercial homogenized pasteurized vitamin D-enriched milk was obtained from a local distributor.

Preparation of Milk Fractions. The cream was obtained by the separation of the whole milk at 33° C. by a laboratory separator (De Laval, Model 100). The milk samples (treated and untreated) and cream were centrifuged in a Beckman Model L4 Ultracentrifuge equipped with a No. 30 rotor for 60 minutes at 25° C. and 30,000 r.p.m. as described by Cerbulis (1968). Ultracentrifugation gave three layers: compact cream layer, Supernatant I, and pellet (Micelles I). Dialysis was performed at 2° C.

Treatment of Milk Samples.

1. Fresh whole milk samples (500 ml.) were dialyzed against 5 liters outer solution for 3 days as follows:

Dialyzed against distilled water, changing water three times daily. Dialyzed against 0.1M sodium phosphate, pH 6.6, changing phosphate solution daily.

Dialyzed against 4M urea solution, changing urea solution daily.

Dialyzed against 4M urea solution, containing 0.1M CaCl₂, changing solution daily.

Dialyzed against 3% disodium ethylenediaminetetraacetate, 0.1M sodium acetate, pH 6.4, changing buffer solution daily.

Another milk sample, after dialysis against chelating agent, was dialyzed further for 3 days against distilled water, then dialyzed against 0.1M CaCl₂ solution, pH 6.4, for 3 days, changing the solution daily. All samples under this section were ultracentrifuged.

2. Detergent treatments (final levels of added agents):

To the fresh milk was added 1 mg. per ml. sodium dodecyl sulfate and 2 mg. per ml. sodium deoxycholate, stirred for 4 hours, and then ultracentrifuged.

To the fresh milk was added 10 mg. per ml. sodium dodecyl sulfate and 20 mg. per ml. sodium deoxycholate, stirred for 4 hours, and then ultracentrifuged.

3. CaCl₂ Treatment of Supernatant I (See Preparation of Milk Fractions):

CaCl₂ was added to the Supernatant I as indicated below, then ultracentrifuged to obtain Micelles II (centrifugate) and Supernatant II.

Ten millimoles CaCl₂ per liter was added to the Supernatant I from fresh milk.

Ten millimoles CaCl₂ per liter was added to the Supernatant I from commercial homogenized pasteurized milk.

A 415-ml. milk sample was used for each centrifugation.

Lipid Extraction and Separation. Micelle and centrifugate fractions were suspended in water, dialyzed against distilled water for 2 days, then freeze-dried. Supernatant casein fractions were dialyzed against distilled water for 2 days, then adjusted to pH 4.6 with HCl and the precipitate recovered by filtration and freeze-dried. Casein fractions obtained from dialysis experiments against distilled water were freeze-dried without second dialysis. The lipids were extracted from the casein with chloroform-methanol (2 to 1, v./v.) as described previously (Cerbulis, 1967; Cerbulis, 1968). All lipid extracts were analyzed by thin-layer chromatography (TLC) and some were also separated on the silicic acid column as described previously (Cerbulis, 1968).

Determination of Cations in Lipid Fraction. Glass distilled solvents were used for the experiments for the determination of cations. Lipid fractions were washed with distilled water.

Total lipid fraction of whole milk was obtained in two ways: Whole milk was freeze-dried and then the milk powder was extracted with chloroform-methanol (2 to 1) as described previously (Cerbulis, 1967); fluid whole milk was extracted directly with chloroform-methanol as described by Cerbulis (1969) and the resulting chloroform layer was used for analysis.

The lipid sample was ashed in a platinum crucible in an electric muffle furnace at 800° C. for 24 hours. The ash obtained was dissolved in 1N HCl and assayed for cationic composition with a Perkin-Elmer 303 atomic absorption spectrophotometer.

Polyacrylamide Gel Electrophoresis. This was described previously by Cerbulis and Custer (1967).

RESULTS AND DISCUSSION

Distribution of Casein. Ford *et al.* (1955) had reported that 5 to 10% of the total casein in cow's milk was in the nonmicellar form and 90 to 95% in micellar form. Present research showed that micellar casein comprised 89.1 to 93.3% of the total casein in the fresh Holstein milk and 81% in cream and 80.9% in the fresh goat milk (Tables I and II).

Homogenization and pasteurization decreased the amount of sedimentable micellar casein in commercial milk to 63 to 83% of the total casein (Tables I and II).

Removal of calcium and other multivalent cations from milk by chelating agent dispersed the micelles. Only 1.1 to

2% of the total casein was in centrifugate form; the pellet was colored brownish red and polyacrylamide gel electrophoresis showed that the α_s -casein and β -casein were the major components. A considerable part of the material was nonmigrating. Apparently calcium and other multivalent cations are important bonding forces in casein micelles. On subsequent treatment of the demineralized milk with CaCl₂, 63.2% of the total casein reverted to the centrifugate form.

Addition of 10 mmoles per liter CaCl₂ to Supernatant I of fresh milk and of commercial milk gave the additional centrifugate content of 8.2% and 13.3% (Micelles II, Centrifugate), respectively (Table II). Still 1.0% and 3.5% of casein remained in supernatant. Bohren and Wenner (1961) stated that the supernatant casein was sedimented quantitatively by a second centrifugation after the addition of 60 mmoles of CaCl₂ per liter to the Supernatant I.

Dialysis against 4M urea permitted 5.7% of the casein to remain in centrifugate form, whereas dialysis against urea-CaCl₂ resulted in 43.7% remaining in the centrifugate form. In the dialysis against urea, some calcium and other multivalent cations were removed from the milk and the low value for sedimentable casein (5.7%) was a result of loss of calcium and hydrogen bond splitting.

Phosphate treatment also removed some calcium from milk leaving only 22% of the total casein in the centrifugate form. Dialysis of milk against distilled water removed free calcium and other ions from milk and decreased the casein micelle content from approximately 90 to 70%. This phenomenon was explained by Waugh (1961) as the micelles being in rapid equilibrium with their constituent components and complexes in solution.

Detergents were very effective dissociating agents for casein micelles and fat globules. Only 2% of casein remained in centrifugate form after treatment with detergents. Even at low concentrations (1 mg. per ml. sodium dodecyl sulfate) of detergent in milk, free fat appeared on the milk surface during stirring. High concentrations gave a considerable layer of butteroil after ultracentrifugation. None of the other treatments (urea, chelation, phosphate) ever gave free butteroil. Harwalkar and Brunner (1965) had reported that the structural organization of the fat globule membrane depends principally on hydrophobic bonding and that dodecyl sulfate is an effective dissociating agent of fat globule membrane. However, dodecyl sulfate-deoxycholate treatment did not give fat-free casein fractions.

Lipid Content of Casein Fractions. Quantitative data of the lipid content in casein micelles, centrifugates, and supernatant caseins obtained from untreated and treated milk samples are presented in Tables I and II.

Micelles, centrifugates, and supernatant casein fractions from homogenized pasteurized milk contained considerably more lipid than the same fractions from untreated whole milk.

The treatment of milk with phosphate, urea, and chelating agents gave centrifugates of increased lipid content, but the lipid decreased in the supernatant casein fraction. The total amount of lipids removed with casein fractions from treated milk samples, were considerably lower than from untreated milk, particularly in the CaCl₂ treatment. The casein lipid content was decreased by treatment with CaCl₂ or by removal of calcium ion, particularly in homogenized pasteurized milk. No one treatment gave fat-free casein fractions.

Addition of CaCl₂ to the Supernatant I gave additional micelles (Micelles II) (Table II) but these Micelles II contained 10 to 13 times more lipid than Micelles I. The Supernatant Casein II contained still more lipids than Micelles II.

Distribution of Casein in Milk and Lipid and CMEP Content in Casein Fractions

Milk	Milk Treatment	Distribution of Casein				Supernatant Casein		Lipids Removed with Caseins, G./L. Milk
		Micelles, %	Supernatant casein, %	Micelles		Lipids, %	CMEP, %	
				Lipids, %	CMEP, %			
Holstein, whole milk	Untreated	93.3	6.7	0.63	2.25	12.40	0.10	0.93
Goat, whole milk	Untreated	80.9	19.1	0.25	0.60	7.37	0.70	1.16
Commercial homogenized pasteurized whole milk ^a	Untreated	78.0	22.0	5.13	0.52	31.21	0.38	3.91
Holstein, cream	Untreated	81.0	19.0	0.62	0.63	28.82	0.40	1.01 ^b
Holstein, whole milk	Untreated, control	89.1	10.9					
Holstein, whole milk	Dialyzed against chelating agent	1.4	98.6					
Commercial homogenized pasteurized whole milk ^a	Untreated, control	63.0	37.0	5.46	5.21	21.14	1.73	1.80
Commercial homogenized pasteurized whole milk ^a	Dialyzed against chelating agent	1.1	98.9			2.64	1.30	
Holstein, whole milk	Dialyzed against distilled water	71.3	28.7	0.55	0.27	7.40	0.84	0.51
Holstein, whole milk	Dialyzed against phosphate	22.0	78.0	1.10	0.65	1.36	0.13	0.32
Holstein, whole milk	Dialyzed against urea	5.7	94.3	2.08	1.94	1.14	0.28	0.25
Holstein, whole milk	Dialyzed against urea, contg. CaCl ₂	43.7	56.3	1.78	0.09	1.03	1.17	0.13
Holstein, whole milk	Dialyzed against chelating agent	2.0	98.0	0.84	1.64	1.30	0.06	0.34
Holstein, whole milk	Dialyzed against:							
	(1) chelating agent, centrifuged							
	(2) distilled water							
	(3) CaCl ₂	63.2	36.8	0.32	0.33	0.76	0.26	0.11
Holstein, whole milk	1 mg./ml. dodecyl sulfate } 2 mg./ml. deoxycholate }	73.2	26.8	0.60		3.61		0.28
Holstein, whole milk	10 mg./ml. dodecyl sulfate } 20 mg./ml. deoxycholate }	2.0	98.0	2.48		0.65		0.17

^a 3.2% fat content.
^b Calculate to 1 l. cream.

Table II. Effect of Addition of CaCl₂ to Supernatant I

	Distribution of Casein, %		Lipids in Casein Fractions, %		CMEP in Casein Fractions, %	
	Fresh milk	Commercial homogenized milk	Fresh milk	Commercial homogenized milk	Fresh milk	Commercial homogenized milk
	Micelles I	90.8	83.2	0.23	2.55	1.30
Micelles II	8.2	13.3	3.05	25.10	0.24	0.23
Supernatant casein II	1.0	3.5	7.71	28.9	1.86	Nil (Traces)
Whey proteins ^a	—	—	1.60	2.29	1.14	0.24

^a Nondialyzable proteins, freeze-dried.

The important findings in this paper and in the previous report (Cerbulis, 1968) are that the lipid (neutral lipid and phospholipids) is bound to casein in a way which is not affected by hydrogen bond splitting agents (dissociating agents), chelating agents, or detergents. This lipid is an intimate component of casein complex. The role of the lipids in the micelles deserves elucidation (Bolcato *et al.*, 1969). Apparently, casein particles in milk are surrounded by neutral lipid and phospholipids. Present results suggest an extended model for casein micelles, originated by Waugh (1967) and modified by Rose (1968) where the fat molecules are attached around the casein units forming clusters.

Homogenization and pasteurization induce the formation of new casein-lipid complexes, but these new complexes are dissociated with chelating agent. Apparently, the newly formed linkages between casein and lipids are different than in original (untreated) casein complexes.

Chloroform-Methanol Extractable Proteins (CMEP). Urea treatment gave 0.94% CMEP in centrifugate and 0.28% in supernatant casein but when urea solution contained CaCl₂, 0.09% of CMEP was in centrifugate and 1.17% in supernatant casein. Presence of CaCl₂ in the urea treatment caused CMEP to migrate to the centrifugate to the supernatant casein fractions. Electrophoresis showed that this fraction contained γ -casein, temperature-sensitive casein, λ -casein fraction, and other minor proteins (Cerbulis and Custer, 1967).

Composition of Lipid Fractions. All lipid fractions, prepared in these experiments, were analyzed by TLC. The composition of lipid fractions was identical with the previous findings (Cerbulis 1967; Cerbulis, 1968). No selective release of lipids from casein was observed. No free phospholipids were found in casein fractions. Free butteroil obtained in detergent experiments did not contain free phospholipids.

Polyvalent Cations in Lipid Extracts. The presence of calcium, magnesium, iron, manganese, copper, and cobalt in the total lipid fractions of whole milk was determined by atomic absorption spectrophotometry on the ash (Table III).

Table III. Results of Assays for Polyvalent Cations in Milk Lipids^a

Cations	Dry Procedure	Wet Procedure
Ca ⁺²	3.68 × 10 ⁻⁷	2.16 × 10 ⁻⁷
Mg ⁺²	Trace	1.67 × 10 ⁻⁷
Fe ⁺³	Trace	Trace
Cu ⁺²	1.32 × 10 ⁻⁶	0.9 × 10 ⁻⁶
Mn ⁺²	0.10 × 10 ⁻⁶	0.13 × 10 ⁻⁶
Co ⁺²	Trace	Trace

^a Milligrams of cations per milligrams of total lipid.

Polyacrylamide Gel Electrophoresis of Casein Fractions. Electrophoresis showed that the micelle, centrifugate, and supernatant casein fractions from all samples of fresh milk and of homogenized milk contained both α -casein and β -casein. CMEP from urea, phosphate, or chelating agents treated milk samples were similar to that of previous report (Cerbulis and Custer, 1967). CMEP fractions from detergent-treated milk contained γ -casein, temperature-sensitive caseins, other minor proteins, and β -caseins.

ACKNOWLEDGMENT

The author is indebted to J. H. Custer for the electrophoresis and M. T. Lukasewycz for the atomic absorption spectrophotometry.

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Received for review February 13, 1969. Accepted May 6, 1969. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.