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Equilibrium Casein Micelle Systems*

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ABSTRACT: A casein micelle consists of a core of essentially insoluble α_s- and/or β-caseinates stabilized by a coat layer containing κ-casein. In a system, cores occur in a variety of sizes, thus producing a micelle distribution. Ultracentrifugation is used to characterize reconstituted micelle systems containing α_s- and κ-caseins with respect to the weight fractions of total protein in particles of different size. At final environmental conditions of 37°, pH 6.6, a monovalent ionic strength of 0.05 and 0.012 M calcium chloride, it is concluded that a system is in equilibrium when a particular micelle distribution is present and that the distribution is determined by the system weight ratio of α_s-:κ-casein. (A) Micelle systems develop spontaneously as calcium chloride is increased progressively from zero. Using initial weight ratios, R_I, from 20 to 3, the micelle radius of the maximum weight fraction decreases as R_I is decreased, respectively, from ~425 to ~36 nm. Concomitantly, the concentration of nonmicellar protein (including coat) slowly increases; at 8 mg/ml of α_s-casein, from 0.15 to 0.4 mg per ml. (B) Colloid particles of Ca–α_s-caseinate having radii >440 nm can be stabilized as micelles at original size, or spontaneously transformed into micelles of smaller size, by adding appropriate amounts of κ-casein. (C) After mixing aliquots of micelle systems at different R_I, spontaneous rearrangement leads to a size distribution characteristic of the weight ratio of the mixture. (D) A micelle system at $R_I = 4$ can stabilize as micelles all α_s-casein added to give $R_S = 8.4$. The required κ-casein comes mainly from preexisting micelles. For equilibrium systems, population surface area and κ-casein content yield 3400 Å^2/κ-casein monomer of 20,000. Thus, κ-casein is present as a monolayer at the micelle surface. An examination of system characteristics suggests that micelle surface free energy must be low ($\ll$0.2 erg/cm^2) and must increase as micelle size decreases. Coalescence and subdivision reactions by which micelle sizes are rearranged are considered to be related to micelle surface and core free energies, and to occur through an exchange of core polymers.

Casein micelles of bovine milk are nearly spherical and exist in a distribution of sizes. Average radii, by electron microscopy, range from about 10 to 400 nm, with over 95% of the micelle mass in particles from 30 to 150 nm (Nitschmann, 1949; Hostettler and Imhof, 1951; Dyachenko *et al.*, 1955; Knoop and Wortman, 1960; Adachi, 1963; Saito and Hashimoto, 1964; Rose and Colvin, 1966; Carroll *et al.*, 1968). Size distributions obtained by reconstituting with calcium and other divalent cations have been shown (Adachi, 1963) to resemble closely size distributions found in milk. Casein micelles may be obtained using κ-casein and α_s-casein (Zittle, 1961; Noble and Waugh, 1965; Waugh and Noble, 1965), β-casein (Zittle and Walter, 1963; Dresdner, 1965), or mixtures of the two (Dresdner, 1965). We have chosen to work mainly with κ- and α_s-caseins since their near equivalence of extinction coefficients simplifies analytical procedures.

Our objectives here are to reveal environmental conditions under which micelle systems are at equilibrium, to show that the equilibrium size distribution is determined by the weight ratio of α_s-:κ-casein, and that the micelle population surface area is proportional to the total κ-casein.

At 37° and $CaCl_2$ concentrations yielding micelles (~0.02 M), solubilities of Ca–α_s- and Ca–β-caseinates are less than 0.2 mg/ml, while Ca–κ-caseinate is soluble (Waugh, 1958, 1961; McKenzie, 1967). An examination of monomer and noncovalent polymer characteristics has shown that the longest dimension of either α_s- or β-casein monomers is near 10 nm (Waugh *et al.*, 1970). Usually, colloid particles which are large compared to their constituent monomers and whose major component is insoluble will not form spontaneously, and if formed are unstable as a result of surface free energy (Verwey and Overbeek, 1948; Stauff, 1960; Lange, 1967). There is eventually a bulk separation of phases.

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Materials and Methods

Chemicals, the removal of impurities from urea and dialysis tubing, the preparation of solubilized milk, and first cycle casein have been described previously (Waugh *et al.*, 1962; Noble and Waugh, 1965).

Milk was obtained from individual Guernsey cows typed as β-casein A and α_s-casein B. α_s- and β-caseins were obtained by the procedure of Waugh *et al.* (1970). Pure κ-casein was obtained by a modification (Talbot and Waugh, 1970) of the procedure of McKenzie and Wake (1961). α_s-Casein at approximately 40 mg/ml was dialyzed against distilled water at 2° for 24 hr. κ-Casein was dialyzed against 0.07 M KCl. These stock solutions were stored at −15°.

Absorbance and Extinction Coefficients. In 0.05 M sodium citrate at pH 7.0 (standard diluent) the $E^{1\%}_{1\text{ cm},280\text{ nm}}$ values are 10.0 for α_s- and κ-caseins and 4.7 for β-casein (Waugh *et al.*, 1970). Observed absorbances were corrected by subtracting 1.7 times the apparent absorbance at λ 320 nm. One absorbance unit is the amount of protein per milliliter required to give unit corrected absorbance for 1-cm path.

Ionic Strength. In what follows the symbol "I" is used for the ionic strength contribution due to monovalent ions, and calcium chloride concentrations are specified separately.

Addition of Calcium. Calcium was usually brought to a predetermined concentration by dialysis of protein solutions against large volumes of buffer. In instances a single aliquot of a solution containing $CaCl_2$ and monovalent ions was added with rapid mixing (Noble and Waugh, 1965). The added volume was 0.11 of the volume of the protein solution.

Weight Ratios. The weight ratio of a system is (α_s- plus β-): κ-casein = R. Subscripts indicate particular applications: R_I, a ratio established before micelle formation is initiated, and R_S, a ratio established after $CaCl_2$ has been introduced at some prior point of an experiment, for example, if two micelle systems established at different R_I are mixed.

Coalescent and Particulate Pellets. Sufficient calcium precipitates α_s-casein alone. Particles spontaneously *coalesce*, and on gentle contrifugation form a pellet of bulk phase. In contrast, pellets obtained by ultracentrifuging micelle systems are *particulate*. They redisperse spontaneously in their supernatants (Waugh and Noble, 1965). Micelles may be sufficiently large so that they settle in quiescent systems, giving a micelle *sediment*.

Colloidal Ca–α_s-Caseinate. Spherical colloidal particles of Ca–α_s-caseinate, about 1 μ in diameter by electron microscopy are formed at low I and low calcium chloride concentrations (*e.g.*, $I = 5 \times 10^{-4}$, 0.0045 M $CaCl_2$). Such colloids are unstable: particles coalesce irreversibly on centrifugation, and the introduction of 0.01 M $CaCl_2$ leads to immediate precipitation.

Electron Microscopy. Systems were fixed by the addition of an equal volume of 1% formaldehyde adjusted to pH 7.0. They were then diluted tenfold with water and a drop immediately applied to a parloidin-coated grid. Electron micrographs were taken on an RCA EMU 3B, which was generously made available by Professor Cecil Hall.

Centrifugation. The objective is to characterize a micelle system. To do so it is assumed that the system contains particles in a set of discrete sizes referred to as classes. The classes include micelles and smaller nonmicellar association products, such as coat, which will mainly appear in the supernatant of a suitable ultracentrifugation. Particles in the ith class have a sedimentation coefficient, s_i, and a radius, r_i, and represent a fraction, f_i, of the total protein mass, W. The f_i are the quantities to be derived. The fraction of total mass in a particular class, f_iW, which is removed by a particular centrifugation step is $\phi_{i,d}$. The fraction of W so removed is F_d. The subscript "d" specifies the centrifugation step and includes ω, the rotor angular velocity; t, the time; and x_m and x_b, the radial distances to meniscus and cell base. The F_d are determined experimentally.

The particle sedimentation coefficient is defined by

$$\omega^2 s_i dt = dx/x \tag{1}$$

For a sector-shaped cell, eq 1 yields an exact expression for the $\phi_{i,d}$. The swinging-bucket rotor employed in this work holds cylinders, and some mass flow along the walls to the base of the tube is expected. It was known that this is compensated (to an unknown extent) by the formation of a viscous layer, just above the pellet, in which particle velocity is decreased. It was also observed that pellets have sharp centripetal surfaces. We have tested (see Centrifugation of Fresh Skim Milk) and used the approximation for which it is assumed that the tube has constant cross section and that $\phi_{i,d}$ is given by the ratio of the distance between the meniscus and the centripetal micelle boundary to the total length of solution in the tube. Then by eq 1

$$\phi_{i,d} = \left(\frac{x_m}{x_b - x_m}\right)_d (e^{\omega_d^2 s_i t_d} - 1);\ 0 \leq \phi_{i,d} \leq 1 \tag{2}$$

and for a population of n classes

$$F_d = \left(\frac{x_m}{x_b - x_m}\right)_d \sum_{i=1}^{n} (e^{\omega_d^2 s_i t_d} - 1) f_i \tag{3}$$

where the restriction $0 \leq \phi_{i,d} \leq 1$ holds for each class.

Micelles are nearly spherical. The coat-core model (Waugh and Noble, 1965) requires that the total micelle population surface area be determined by the amount of adsorbed κ-casein. Let the solvated micelle radius, r_i, include an increment Δ due to κ-casein. Since α_s- and κ-caseins have essentially equal density, it follows that

$$s_i = \frac{2\rho_p(1 - \bar{v}\rho_s)}{9\eta r_i}\left[\frac{(r_i - \Delta)^3}{1 + Q_\alpha} + \frac{r_i^3 - (r_i - \Delta)^3}{1 + Q_\kappa}\right] \tag{4}$$

and

$$\frac{\text{total surface area}}{\text{total κ-casein}} = \frac{3(1 + R)}{\rho_p} \sum_{i=1}^{n} \frac{f_i(r_i - (\Delta/2))^2}{\dfrac{(r_i - \Delta)^3}{1 + Q_\alpha} + \dfrac{r_i^3 - (r_i - \Delta)^3}{1 + Q_\kappa}} \tag{5}$$

In eq 4 and 5, ρ_p is the protein density (approximately $(\bar{v})^{-1}$), $\bar{v}$ is the protein partial specific volume, ρ_s is the solution density, η is the solution viscosity, Q_α and Q_κ are the solvation of α_s- and κ-caseins, respectively, in cubic centimeters of solvent per cubic centimeters of protein, and R is the system weight ratio. We take $\bar{v}$ = 0.728 g/cm³ (McMeekin *et al.*, 1949; McKenzie and Wake, 1959), ρ_p = 1.37 g/cm³, ρ_s = 1.0 g/cm³, η = 0.0069 P at 37°, and Q_α = 2.3 (Noble and Waugh, 1965; Creamer and Waugh, 1966). Q_κ is taken as 3.0 from an extrapolation of the data of Waugh and Noble (1965). A value of Δ = 2.85 nm is obtained from the average area of Table III and eq 5; the average area per gram of

TABLE I: Centrifugation of Skim Milk.[a]

Min	Milk I	Milk II	Micelle Class	s_i (10^{-2} S)	r_i (nm)	f_i Milk I	f_i Milk II
2	0.13	0.105	1	75	144	0.02	0.01
4	0.26	0.21	2	37	102	0.03	0.02
8	0.52	0.42	3	19	72	0.16	0.11
12	0.72	0.59	5	12	59	0.21	0.13
16	0.85	0.72	5	9.3	51	0.16	0.10
20	0.94	0.83	6	7.5	46	0.14	0.08
24	1.00	0.93	7	6.2	42	0.28	0.17
28	1.00	1.00	8	5.3	39		0.38
$10^{-6} \times$ cm²/g of κ-casein						8.9	10.0

[a] Conditions are 37°, 25 krpm, $x_m = 5.2$ cm, $x_b = 9.6$ cm, and centrifugation times given in column 1. Columns 2 and 3 refer to two milks and give F_d, the fraction of the total micelle absorbance removed by centrifugation. Column 4 gives the micelle class (*i.e.*, micelles just completely removed by the corresponding centrifugation), and columns 5 and 6 give, respectively, the class sedimentation coefficient (Svedbergs) and radius. Columns 7 and 8, give, for the two milks, f_i, the fractions of total micellar protein in the micelle classes.

κ-casein multiplied by Δ should equal $\bar{v}(1 + Q_\kappa) = 2.91$ g/cm³.

Calculations were made using either an Olivetti-Underwood Programma 101 or an IBM Model 360 computer. Procedures for statistical analysis are given in Bennett and Franklin (1963). The standard deviation (std dev) will be given as such, or in per cent of the average value of the measurement. Averages are indicated by a bar.

Assay centrifugation was carried out for 1 min at 3800 rpm in an International Model CL centrifuge maintained at 37°. A 1-ml aliquot gave $x_m = 12.0$ cm and $x_b = 13.3$ cm. Assay centrifugation removes all particles having $s_i = >108{,}000$ S by eq 2, thus $r_i = >545$ nm by eq 4. The fraction of W appearing in the pellet after assay centrifugation will be referred to as F_a.

Preparative ultracentrifugations were carried out using polyethylene tubes, a Spinco Model L ultracentrifuge, and the SW 39 rotor controlled at 37° (Waugh *et al.*, 1962; Noble and Waugh, 1965). A small correction for tube end curvature gives $x_b = 9.6$ cm.

Analytical ultracentrifugations were carried out using the Spinco Model E equipped with schlieren optics.

Results

Centrifugation of Fresh Skim Milk. To determine the applicability of eq 2, an examination was first made of centrifugates of skim milk. For constant x_m and x_b, eq 2 and 3 predict that equal removal of particles from a distribution will be obtained when $\int \omega^2 dt$ has a particular value, independent either of ω or t (*i.e.*, centrifugation at 5 krpm for 40 min should give the same F_d as centrifugation at 10 krpm for 10 min). To test this, the following sequence of three equivalent pairs of (krpm, min) were examined: (5, 40) and (10, 10), (5, 20) and (10, 5) , and (10, 20) and (20, 5). A volume of 5.2 ml gave $x_m = 5.2$ cm and $x_b = 9.6$ cm. Each ultracentrifugation, carried out at 37°, gave a triplicate. Each pellet was drained of supernatant and dissolved in 3.0 ml of buffer containing 4.5 M urea and 0.1 M sodium citrate at pH 7.0. Absorbance was determined using two aliquots. Centrifugations of equivalent pairs were carried out on two different milks, and in three instances sequences were repeated. The average standard deviation of the single absorbance determination, 6.4%, accounts for the variance between averages within a triplicate, std dev = 3.9%, to ≥75% confidence limits. The equivalent pairs of centrifugations of the sequences gave an average std dev of 4.9%. By F test to ≥75% confidence limits, variance between the averages of triplicates accounts for variance between the equivalent pairs of centrifugations. It is concluded that constant $\omega^2 t$ produces constant removal and that eq 2 is a reasonable approximation. Evidently, inaccessible experimental conditions such as large ω and small t can be replaced by small ω and large t, and results can be referred to a single ω.

Of the proteins present in milk micelles, β-casein is the only significant component which has a low extinction coefficient. According to Rose and Colvin (1966), micelles of different size contain the same fractional content of β-casein; therefore, the absorbance contributed to a pellet by micelles of different size will be proportional to micelle mass.

Micelle distributions have been determined by ultracentrifugation. It was observed that pellet absorbances become a constant maximum after centrifugation at 25 krpm for times greater than 24–32 min, depending on the milk lot. Pellet absorbances were converted to F_d using the average maximum, and thus neglecting the small fraction of total casein protein in the supernatant.

As an approximation to distribution, milk is considered to contain micelles in eight classes: those just completely removed at 25 krpm at 2, 4, 8, 12, 16, 20, 24, and 28 min. The micelle class, class sedimentation coefficient, and radius by eq 4 are given in Table I. In order to obtain the f_i of columns 7 and 8, a series of calculations based on eq 3 was used. Here, $x_m/(x_b - x_m) = 1.18$ and $\omega = 9.42 \times 10^6$. For example, for milk II

$$F_8 = 1.0 = f_8 + \sum_{i=1}^{7} f_i \tag{6}$$

$$F_7 = 0.93 = 1.18(e^{\omega^2 d_8 s t_d} - 1)f_8 + \sum_{i=1}^{7} f_i \tag{7}$$

TABLE II: Characteristics of Differential Centrifugation.[a]

Centrifugation							
krpm	min	Vol (ml)	x_m[b] (cm)	C_d[c]	"d" or "i"[d]	s_i (10^{-2} S_i)	r_i (nm)
3.8	1	4.8	8.3	1.66	1	4963	1169
5	5	4.5	5.6	1.40	2	655	425
10	10	4.2	5.9	1.59	3	74	143
20	20	3.9	6.2	1.82	4	8.3	48.5
30	30	3.6	6.6	2.20	5	2.1	24.8
Final supernatant					6	0.8	15.5

[a] The mixed supernatant of one centrifugation contributes the material to be centrifuged at the next. [b] x_b is 13.3 cm for the first centrifugation (International CL), and 9.6 cm for the others (Spinco Model L). [c] $C_d = [x_m/(x_b - x_m)]_d$. [d] The subscripts in this column are used as follows: for F_d, 1–5 designate fractions of the total initial protein in the pellet of the corresponding centrifugation step, and F_6 that in the final supernatant; for s_i (and r_i), 1–5 specify the sedimentation coefficient (and radius) of a micelle class just completely removed at the corresponding centrifugation step, and s_6 (and r_6) values for particles in the final supernatant. s_6 was obtained from experimental data.

Combination of these equations and substitution of ω_d, t_d, and $s_8 = 532 \times 10^{-13}$ yields $f_8 = 0.383$. The sequence of f_i were obtained by successive applications of eq 3. The last row of Table I gives cm^2/g of κ-casein for the total micelle population surface area, calculated by eq 5 using the f_i of Table I and $R = 5.7$ (Waugh and von Hippel, 1956; Sullivan *et al.*, 1959; Marier *et al.*, 1963; Rose and Colvin, 1966).

The calculated distributions of Table I are in agreement with the electron microscopic studies of Saito and Hashimoto (1964) and of Rose and Colvin (1966). There is a variation in micelle distribution between milks, but in each milk 95% of the micelle mass is in particles of radius 30–120 nm.

Effects of Ionic Strength and pH on Path Dependency of Micelle Formation. At pH 7.1 and $I = 0.075$, using mixtures of α_s- and κ-caseins, Noble and Waugh (1965) observed that coalescent pellets of Ca-α_s-caseinate form at $CaCl_2$ concentrations below those required for complete micelle formation. Systems were found to be strongly path dependent: F_a was found to depend on the way in which $CaCl_2$ was added (single aliquot *vs.* incremental addition) as well as on final calcium concentration. Path dependency has been reexamined at 37° using single aliquot addition with rapid mixing to mixtures of α_s- and κ-caseins, to first cycle casein or to solubilized skim milk. $CaCl_2$ dependency of F_a, and the characteristics of pellets are found to be strongly dependent on I. Figure 1 is typical and refers to first cycle casein. At $I = 0.035$ and 0.055 (the maximum I for $F_a = 0$) Ca-α_s-caseinate does not appear; progressively above 0.005 M $CaCl_2$ systems increase in turbidity, finally to give full micelle development. In agreement with previous results (Noble and Waugh, 1965), at $I = 0.075$, pellets of Ca-α_s-caseinate appear between 0.005 and 0.007 M $CaCl_2$. Above 0.007 M $CaCl_2$, F_a decreases and pellets become particulate. Maximum F_a increases in magnitude and in calcium concentration range as I is increased above 0.075. pH has little effect. As it is decreased from 7.1 to 6.3, maximum F_a decreases but slightly. An important consequence of these results is that, at $I \leq 0.055$, $CaCl_2$ can be introduced by dialysis and thus can be brought to known solution concentrations, without encountering the bias introduced by passing through a $CaCl_2$ concentration range where core formation (Ca-α_s-caseinate precipitation) precedes coat formation.

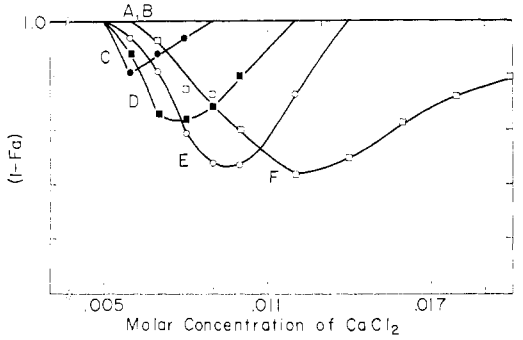

FIGURE 1: Effects of ionic strength on micelle formation at pH 6.9. $CaCl_2$ was added in single aliquots to first cycle casein at 9 A/ml. Systems were then incubated for 75 min and assay centrifuged, and F_a is the fraction of total absorbance removed. Conditions are 0.01 M imidazole and KCl concentrations as follows: (A) 0.03 M; (B) 0.05 M (these remain at $F_a \simeq 0$); (C) ●, 0.07 M; (D) ■, 0.1 M; (E) ○, 0.15 M; and (F) □, 0.2 M.

A *standard dialysis* has been used to reduce the possibility of sample deterioration and to permit a more effective equilibration of micelle population. Samples were kept at pH 6.6 but were first dialyzed for 7 hr at 2° against 7×10^{-4} M $CaCl_2$ and 2×10^{-4} M sodium cacodylate, and subsequently for 16 hr at 37° against 0.012 M $CaCl_2$, 0.04 M NaCl, and 0.001 M sodium cacodylate. When $CaCl_2$ was introduced by single aliquot addition, higher total concentrations were used to compensate for calcium binding.

Micelle Distributions at Different Initial α_s-:κ-Casein Weight Ratios, R_I. After standard dialysis, systems up to $R_I = 20$ at 8 mg/ml α_s-casein are free of micelle sediment. At $R_I = 25$ sediment appears after a few minutes quiescence, and a large particulate pellet is produced by assay centrifugation. As R_I is increased above 25, the amounts of sediment and particulate pellet increase.

Aliquots of systems up to $R_I = 20$ were differentially centrifuged using the sequence given in Table II. This procedure was chosen to conserve material. In it, the supernatant of one step is decanted and mixed. Two 0.1-ml aliquots are taken for absorbance determination and a standard volume of the

TABLE III: Distributions of Particles in Micelle Systems at Different Initial Weight Ratios, R_I, of α_s-:κ-Caseins.[a]

R_I	No.[a]	Concn[b] of α_s-Casein	$\bar{f}_1$	$\bar{f}_2$	$\bar{f}_3$	$\bar{f}_4$	$\bar{f}_5$	$\bar{f}_6$	Area[c]
20	10	8	*0.20*	*0.44*	*0.22*	0.08	0.02	0.04	11.2
			0.11	0.06	0.10	0.03	0.05	0.03	2.7
7	3	7.7	0.06	*0.13*	*0.32*	*0.39*	0.04	0.06	8.8
			0.03	0.06	0.04	0.10	0.05	0.03	1.1
5	3	15–20	0.01	0.04	*0.14*	*0.44*	*0.30*	0.07	10.9
			0.02	0.02	0.02	0.06	0.11	0.03	0.6
4	6	12–10	0.01	0.04	0.06	*0.41*	*0.43*	0.05	10.2
			0.02	0.04	0.04	0.13	0.17	0.03	0.1
3	3	9	0.02	0.05	0.04	*0.49*	*0.48*	0.00	8.1
			0.04	0.06	0.03	0.05	0.03	0.01	0.0

[a] The $\bar{f}_i$'s are average weight fractions of total protein in particles of the ith class. Class s_i and r_i are given in Table II. Under each $\bar{f}_i$ are recorded the values of $\bar{f}_i$ and the standard deviations of $\bar{f}_i$. Major $\bar{f}_i$'s are in italic type. [b] Number of individual experiments. [c] α_s-Casein concentration in milligrams per milliliter. [d] 10^{-6} cm^2 per g of κ-casein.

remainder is centrifuged at the next step. Thus, the volume progressively decreases and x_m increases.

The measured sequence of F_d is used to calculate micelle size distribution[1]. For the steps of Table II

$$\phi_{i,d} = c_d(e^{\omega^2 d s_i t d} - 1);\ 0 \leq \phi_{i,d} \leq 1 \tag{8}$$

Except for step 1, an aliquot to be centrifuged has had removed at prior steps different fractions, each up to unity, of the particles in each assigned class. The fraction of W removed at a centrifugation step, taking prior removal into account, is then

$$F_d = \sum_{i=1}^{6} f_i \phi_{i,d} \prod_{j=0}^{d-1} (1 - \phi_{i,j}) \tag{9}$$

The fraction of W in the supernatant *after* the dth centrifugation step is given by

$$\left(1 - \sum_{j=0}^{d} F_j\right) = \sum_{i=1}^{6} f_i \prod_{j=0}^{d} (1 - \phi_{i,j}) \tag{10}$$

In using eq 9 and 10 for calculations, all $\phi_{i,0}$ are zero; and since the maximum $\phi_{i,d}$ is unity, calculated $\phi_{i,d} > 1$ are taken as unity. Thus, progressively, micelle classes cease to contribute to the series of F_d, and only class 6 particles appear in the final supernatant. Applying eq 10 to the final supernatant, in which case

$$\left(1 - \sum_{j=0}^{5} F_j\right) = F_6$$

leads to

$$f_6 = F_6 \Big/ \prod_{j=0}^{5} (1 - \phi_{6,j}) \tag{11}$$

which gives $f_6 = F_6/0.606$. Six sequential applications of eq 10 yield the f_i.

[1] To conserve space, values of F_d are not given.

Table III gives the $\bar{f}_i$ distribution at different R_I. As R_I is decreased, average micelle size decreases. For each R_I, over 80% of the protein is distributed in two or three major $\bar{f}_i$, which are underlined. This, of course, is also a characteristic of the F_d. The total micelle surface area per gram of κ-casein was obtained by using the $\bar{f}_i$ and eq 5.

In all experiments F_6 (final supernatant) is always small ($\sim$0.03) and is the only F_d which has this characteristic. In separate experiments systems at different R_I, keeping α_s-casein concentration constant, and systems at $R_I = 7$ at different total protein concentrations were examined. After standard dialysis, 3.6 ml of each system was centrifuged at 37° for 30 min at 30 krpm. Duplicate supernatant aliquots of 1.2 ml were diluted to 3.0 ml for absorbance determination. Results for constant 8 mg/ml of α_s-casein are given in Figure 2. It is evident that as R_I is decreased the supernatant protein increases, from 0.15 mg/ml at $R_I = 20$ to 0.4 mg per ml at $R_I = 3$. At constant $R_I = 7$, it was found that the supernatant protein increases only from 0.17 to 0.22 mg per ml when α_s-casein is increased from 7 to 21 mg per ml.

Systems at $R_I = 3.0$–1.0 and κ-casein, after standard dialysis, were examined in the analytical ultracentrifuge. The results are given in Table IV. For all systems, protein is present essentially in a single broad peak skewed to have a sharp trailing edge. The data of Table IV and eq 2 yield a

TABLE IV: Sedimentation Patterns at 37° and pH 6.6 of Systems at Low $R_I = \alpha_s$-:κ-Casein.[a]

R_I	1	1.5	2.0	2.5	3.0
α_s-Casein (mg/ml)	6.0	7.2	8.0	8.6	11.3
s_{37} (S)	80	115	150	225	300
$\bar{r}$ (nm)	15.5	18.4	21.0	25.5	29.4

[a] The rows give, respectively, R_I, the α_s-casein concentration, the sedimentation coefficient of the maximum ordinate of the peak, and the particle radius corresponding to s by eq 4.

TABLE V: Stabilization and Transformation of Colloidal Ca–α_s-Caseinate.[a]

Sample	R_S	F_a	Sample	R_S	F_a	Sample	R_S	F_a
Colloid	∞	0.68	3	48	0.90	7	4.8	0.30
Control	∞	0.98	4	19	0.74	8	1.9	0.02
1	191	0.93	5	14	0.62			
2	95	0.92	6	9.6	0.55			

[a] R_S is the system weight ratio of α_s-:κ-casein after addition of κ-casein. F_a is the fraction of the total protein in a pellet after assay centrifugation.

result in good agreement with Figure 2. Pure κ-casein has $s_{37} \simeq 30$ S.

Stabilization and Size Transformation of Colloidal Ca–α_s-Caseinate by Added κ-Casein. Colloid containing 7.1 mg/ml of α_s-casein was formed at 37°, pH 7.0, and $I = 5 \times 10^{-4}$ by single aliquot addition of $CaCl_2$ to give 0.0045 M. Aliquots of systems were assay centrifuged and F_a determined. Results are given in Table V. The colloid gives $F_a = 0.68$. The pellet is coalesced, and, as indicated above, contains all particles having $r_i \geq 545$ nm. For the remaining samples, the ionic environment was altered to $I = 0.03$, 0.022 M $CaCl_2$, pH 6.6, and 37° by mixing 1.0 ml of colloid and 0.2 ml of a solution containing 0.108 M $CaCl_2$ and 0.18 M NaCl. The control of Table V refers to an alteration without added κ-casein, in which case $F_a = 0.98$ (coalesced pellet) and the supernatant protein is due to Ca–α_s-caseinate solubility. For samples 1 through 8 κ-casein was present in the added salt solution to give system R_S of 191–1.9. One hour after addition, F_a was determined. In all cases, pellets were particulate.

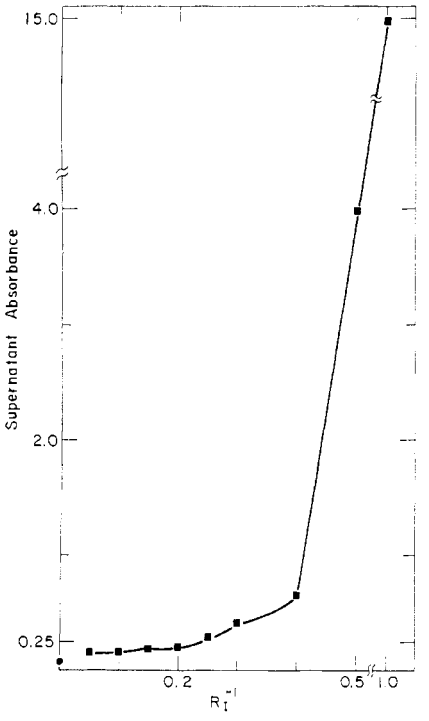

FIGURE 2: Supernatant protein after centrifugation at 30 krpm for 30 min of systems at different R_I. Conditions at 37° are 0.012 M $CaCl_2$–0.04 M NaCl–0.001 M sodium cacodylate and pH 6.6. The solubility of Ca–α_s-caseinate is indicated by the dot at $R_I^{-1} = 0$.

From relative diffusion coefficients it is expected that the most rapid reactions (ion–protein) convert colloid particles into Ca–α_s-caseinate cores which would spontaneously coalesce. Intermediate in rate are interactions of κ-casein with cores, and least rapid are interactions between core particles themselves. For R_S of 191 through 48 some micelle sediment appears after 1 min. For $R_S = 48$, this and $F_a = 0.9$, which is greater than 0.68 (colloid), show that significant coalescence of cores occurs before coating is sufficient to prevent its occurrence. At $R_S = 19$, $F_a = 0.74$, nearly that of the colloid. Evidently, coating is sufficiently rapid to preserve cores essentially at original size.

As R_S is decreased below 19, F_a progressively decreases. There is also a progressive decrease in turbidity (apparent absorbance at 360 nm). Evidently, κ-casein sufficiently in excess of that required for immediate size stabilization produces a decrease in average size (size transformation). In other experiments, a value of $R_S \simeq 19$ for original size stabilization was obtained for colloid distributions like that described above. However, this R_S decreased as colloid particles were established at smaller average original size by lowering the $CaCl_2$ concentration at colloid formation.

Systems which transformed were found to have a time dependency of turbidity. Fifteen equivalent systems at 37°, pH 6.6, $I = 0.05$, 0.019 M $CaCl_2$, and $R_S = 8$ were prepared and F_a determined for each between 10 and 300 min. The observed change in $\bar{F}_a$ during the first 10 min was more than 2.5 times as great as the change in the next 5 hr.

Transformation in Systems Obtained by Mixing $R_I = 20$ Systems with κ-Casein and Other R_I Systems. Systems at 37° and $R_S = 7$ were obtained by mixing, after standard dialysis, aliquots of a micelle system at $R_I = 20$ (no micelle sediment) with appropriate aliquots of κ-casein or systems at $R_I = 1$, 2, 3, 4, or 5. κ-Casein was at 14–17 mg/ml. The other systems were as follows: $R_I = 20$ had 0.4 mg/ml of κ-casein; $R_I = 5$, 4, and 3 had 3 mg/ml of κ-casein; $R_I = 2$ had 4 mg/ml of κ-casein; $R_I = 1$ had 8 mg/ml of κ-casein. After mixing, systems were incubated for 4 hr at 37°. Aliquots of systems before mixing and after mixing plus incubation were differentially centrifuged (Table II). Assuming that particles are stable during incubation, predicted F_d were obtained from the mass ratio of the two systems mixed and their individual F_d[1]. Sets of predicted and observed F_d were converted to predicted and observed f_i using the procedure described above. For added κ-casein and $R_I = 1$ or 2, differential centrifugations are not available. The data of Table IV suggest that κ-casein or $R_I = 1$ contribute only to f_6, and of its protein, $R_I = 2$ contributes 0.55 to f_5 and 0.45 to f_6. The number of experiments performed, their variances, and the t test were used to calculate the level of significance of the difference

TABLE VI: Results of Studies of Transformation Obtained by Mixing a Micelle System at $R_I = 20$ with Other Systems (Column 1) to Give a Final System Weight Ratio of $R_S = 7$.

System Added	No.[a]	Line[b]	Particle Class[c] 1	2	3	4	5	6	Area[d]
κ-Casein	7	$\bar{f}_i$	0.08	0.39	0.23	0.12	0.07	0.11	8.1
		$\delta\bar{f}_i$	−0.13 (95)	−0.02	+0.06 (75)	+0.05 (75)	+0.06 (75)	+0.02 (75)	
		$\Delta\bar{f}_i$	−0.02	−0.26	+0.09	+0.27	−0.03	−0.05	
$R_I = 1$	1	$\bar{f}_i$	0.03	0.23	0.23	0.19	0.22	0.10	11.5
		$\delta\bar{f}_i$	−0.15	−0.06	−0.01	+0.10	+0.21	−0.09	
		$\Delta\bar{f}_i$	+0.03	−0.10	+0.09	+0.20	−0.18	−0.04	
$R_I = 2$	1	$\bar{f}_i$	0.04	0.30	0.15	0.20	0.21	0.10	11.2
		$\delta\bar{f}_i$	−0.12	+0.04	−0.06	+0.12	+0.05	−0.03	
		$\Delta\bar{f}_i$	+0.02	−0.17	+0.17	+0.19	−0.17	−0.04	
$R_I = 3$	4	$\bar{f}_i$	0.05	0.32	0.16	0.27	0.16	0.04	9.2
		$\delta\bar{f}_i$	−0.10 (90)	+0.01	+0.07 (75)	+0.05 (75)	−0.04	+0.01	
		$\Delta\bar{f}_i$	+0.01	−0.19	+0.16	+0.12	−0.12	+0.02	
$R_I = 4$	6	$\bar{f}_i$	0.03	0.28	0.11	0.40	0.11	0.07	10.3
		$\delta\bar{f}_i$	−0.06 (90)	+0.03	−0.02	+0.16 (99)	−0.12 (95)	+0.01	
		$\Delta\bar{f}_i$	+0.03	−0.15	+0.21	−0.01	−0.07	−0.01	
$R_I = 5$	3	$\bar{f}_i$	0.03	0.13	0.29	0.36	0.15	0.04	10.1
		$\delta\bar{f}_i$	0	−0.06	+0.09	+0.05 (75)	−0.05 (75)	−0.03 (75)	
		$\Delta\bar{f}_i$	+0.03	0	+0.03	+0.03	−0.11	+0.02	
$\bar{f}_i$ for $R_I = 7$			0.06	0.13	0.32	0.39	0.04	0.06	8.8

[a] Number of independent experiments. [b] The first line for each added system gives observed $\bar{f}_i$. The second line gives $\delta\bar{f}_i$, which is observed $\bar{f}_i$ minus predicted $\bar{f}_i$. $\delta\bar{f}_i$ is negative if $\bar{f}_i$ decreases during 4-hr incubation. Values in parentheses indicate the confidence limits for $\delta\bar{f}_i$ if they are 0.75 or more. Confidence limits were not calculated for the single experiments using $R_I = 1$ and 2. The third line gives $\Delta\bar{f}_i$, the further change in observed $\bar{f}_i$ required to give $\bar{f}_i$ for $R_I = 7$, which are given in the last line of this table and in Table III. [c] Particle class characteristics are recorded in Table II. [d] Area in 10^{-6} cm^2 per g of κ-casein.

between predicted and observed $\bar{f}_i$. The results are summarized in Table VI. The average areas given in the last column were obtained using the observed f_i for each experiment and eq 5, and averaging the results for each group. Electron micrographs obtained on systems at times during incubation showed the presence of spherical micelles in a distribution of sizes, and verified that the average micelle size decreases.

An examination of the $\overline{\delta f_i}$ of Table VI leads to the following, which includes results concerning the δf_i: (1) $\overline{\delta f_1}$ are found always to be negative if significant, and to decrease as the R_I of the added system increases; (2) $\overline{\delta f_2}$ appear to be not significant. However, $\delta f_2 = -0.44$ ($\delta f_1 + 0.1$), significant at 95% confidence limits. Evidently, f_1 and f_2 are both decreasing, but a sufficiently large decrease in f_1 can give rise to a net increase in f_2; (3) $\overline{\delta f_3}$ increase if significant changes are observed; (4) all $\overline{\delta f_4}$ increase significantly. It is noted that $\bar{f}_4$ is the maximum $\bar{f}_i$ for $R_I = 7$; and (5) after 4-hr incubation, all systems have approached but not attained the distribution for $R_I = 7$. Most $\bar{f}_1$ and $\bar{f}_6$ are close to values for $R_I = 7$, but $\bar{f}_2$ and $\bar{f}_5$ are too high, and $\bar{f}_3$ and $\bar{f}_4$ are too low.

In all cases the smallest and largest micelles of the predicted distribution disappear, and there appear micelles for the maximum $\bar{f}_i$ of $R_I = 7$. Given sufficient time, it is expected that all systems of Table VI would reach the distribution for $R_I = 7$.

Stabilization of Added α_s-Casein by a Micelle System. A micelle system at 37°, pH 6.6, and $R_I = 4$, and containing 18 mg/ml of α_s-casein, 0.04 M KCl, 0.018 M $CaCl_2$, and 0.005 M sodium cacodylate, was established by single aliquot addition of $CaCl_2$. To a 2.6-ml aliquot were added, alternately at 37° with gentle mixing, 0.1-ml aliquots either of α_s-casein at 40 mg/ml, $I = 0.003$, and pH 7, or of a buffer containing 0.04 M $CaCl_2$, 0.08 M KCl, and 0.01 M sodium cacodylate at pH 6.6. The ionic environment and pH were thus maintained. A total of 1.3 ml each of buffer and α_s-casein solution gave final $R_S = 8.4$. Just after the last addition of buffer, systems were differentially centrifuged (Table II). The results are given in Table VII. If water replaced the addition of α_s-casein, the F_d's of the micelle system at $R_I = 4$ were unchanged. From Table VII it is evident that none of the added α_s-casein forms a precipitate, almost half appears in F_1, and a large fraction in F_3. The observed F_d were converted to f_i. These and eq 5 give a value of 12.9×10^{-6} cm^2/g of κ-casein.

The κ-casein required to stabilize added α_s-casein as micelles comes mainly from micelles in classes 4 and 5 of the R_I system, and not from class 6. This was shown by ultracentrifuging the initial micelle system and testing the supernatant, as just described, for stabilization of added α_s-casein.

Discussion

Waugh and Noble (1965) propose that casein micelles consist of variable size cores of Ca–α_s- and/or Ca–β-caseinates stabilized by a coat layer, of uniform thickness, containing κ-casein. As $CaCl_2$ is introduced into a calcium-free system, molecules progressively interact according to their specificities. Ca–κ-caseinate becomes distributed over the surfaces of core polymers and their association products (Waugh *et al.*, 1970), and as final conditions are approached,

TABLE VII: Stabilization of Added α_s-Casein by a Micelle System at $R_I = 4$ at a Final Weight Ratio of $R_S = 8.4$ α_s-:κ-Casein.

	Ppt	F_1[a]	F_2	F_3	F_4	F_5	F_6
$R_I = 4$, observed		0.00	0.01	0.14	0.46	0.34	0.05
$R_S = 8.4$, observed[b]	0.00	0.23	0.05	0.21	0.29	0.17	0.05
$R_S = 8.4$, predicted	0.47	0.00	0.01	0.07	0.24	0.18	0.03
δF_d	−0.47	+0.23	+0.04	+0.14	+0.05	−0.01	+0.02

[a] F_d are weight fractions of initial protein present in the pellet of a centrifugation step (Table II), and that in the final supernatant (F_6). [b] The second row records the observed F_d after the addition of α_s-casein and buffer. The third row records predicted F_d, calculated on the assumptions that added α_s-casein precipitates, and that the micelle system remains at the F_d distribution of the first row. The last row gives the δF_d obtained by difference.

uncoated core surfaces interact until adequate surface coverage by κ-casein limits further reduction in total micelle surface area.

Under environmental conditions of 37°, pH 6.6, and approximately $I = 0.04$ and 0.015 M $CaCl_2$, it is concluded that systems containing α_s- and κ-caseins are in equilibrium when particular micelle distributions are present. This conclusion is drawn from the following. Micelles develop spontaneously during slow (or rapid) introduction of $CaCl_2$ into calcium-free systems (Table III); Ca-α_s-caseinate colloid can be stabilized at original size, and size transformed, by addition of κ-casein (Table V); after mixing micelle systems of different R_I, the micelle distribution shifts toward that characteristic of the weight ratio of the mixture (Table VI); and micelle systems can stabilize added α_s-casein as micelles (Table VII).

According to Table III, κ-casein covers an average area of 10.2×10^6 cm^2/g. A monomer weight of 20,000 (Swaisgood and Brunner, 1963; Kalan and Woychik, 1965; Woychik *et al.*, 1966; Pujolle *et al.*, 1966; Talbot and Waugh, 1970) then gives an average solvated monomer area of 3400 Å^2. For $\bar{v} = 0.728$ and a κ-casein solvation of $Q_\kappa = 3$ ml of water/ml of protein, the average solvated thickness is $\Delta = 2.85$ nm. It is concluded that κ-casein is present at the micelle surface as a monolayer.[2]

For a particular micelle size, the Gibbs and Langmuir equations (Stauff, 1960; Lange, 1967) lead to the equation of state[3]

$$\gamma_{0,i} - \gamma_i = \frac{kT}{X_m} \ln\left(\frac{X_i}{x_i - X_m}\right) \tag{12}$$

where $\gamma_{0,i}$ and γ_i are, respectively, the surface free energies per unit area of an uncoated core and of the corresponding micelle at radius r_i, k is Boltzmann's constant, and T is temperature. X_i is the micelle surface area per κ-casein monomer, and X_m this area at surface saturation. The fraction of the surface covered, θ, is X_m/X_i. Taking $X_m = 3.4 \times 10^{-13}$ cm^2/monomer at 37° yields $kT/X_m = 0.13$ erg/cm^2. Equation 12 then suggests that if $(\gamma_{0,i} - \gamma_i) \geq 0.5$ erg/cm^2, $\theta \geq 0.98$. From this it is assumed that κ-casein monomers are closely packed in the coat layer of micelles.

A stable micelle population is in equilibrium with a solution coat activity, a_e. The results of ultracentrifugation (Table IV) show that free coat, which may be taken either as κ-casein or as a unit monomer association product of κ-casein and α_s-casein, is expected to appear essentially in the final supernatant. The supernatant will also contain at least α_s-casein monomers and particles (which may be micelles) having $r_i \leq 25$ nm. According to Figure 2, as R_I is decreased from 20 to 3, final supernatant protein remains low, but increases slowly, from 0.15 to 0.4 mg per ml.[4] This is interpreted to show that a_e increases as R_I decreases, thus as average micelle size decreases.

A preliminary approach to the free-energy changes involved in establishing an equilibrium distribution is made by examining, in a closed system, the subdivision of an initial single large micelle to produce, at constant core volume, an equilibrium population of smaller uniform micelles. Using the subscripts "q" and "p" to refer to the initial and final systems yields

$$\Omega_p = 4\pi r_q^3 r_p^{-1} \tag{13}$$

$$a_p V = C - \frac{\Omega_p}{X_m N} \tag{14}$$

$$\Delta F = \gamma_p \Omega_p - \gamma_q \Omega_q + CNkT \ln \frac{a_p}{a_q} \tag{15}$$

where Ω is the total micelle surface area, a is the solution coat concentration (activity), V is the system volume, C is the total moles of coat, N is Avogadros number, and ΔF is the change in system free energy on subdivision.

Equation 13, 14, and 15 yield

$$\Delta F = 4\pi r_q^3\left(\frac{\gamma_p}{r_p} - \frac{\gamma_q}{r_q}\right) + CNkT \ln\left[\frac{(CX_m N r_p - 4\pi r_q^3) r_q}{(CX_m N r_q - 4\pi r_q^3) r_p}\right] \tag{16}$$

For a maximum or minimum in system free energy $d(\Delta F)/dr = 0$. Differentiating eq 16 yields

$$\frac{d\gamma_p}{dr_p} = \frac{\gamma_p}{r_p} + \frac{CNkT}{4\pi r_q^3 - CX_m N \gamma_p} \tag{17}$$

[2] It is noted that, when used in eq 5, $\Delta = 2.85$ nm yields values of κ-casein content *vs.* micelle size in agreement with the results obtained by Sullivan *et al.* (1959).

[3] Equation 12 is derived on the assumption that solutions are ideal, surfaces are planar with fixed sites, and adsorbed molecules are immobile. It is thus not strictly applicable. However, it is considered to be useful in approximating the extent of surface coverage.

[4] Below $R_I \simeq 2.5$, final supernatant protein increases rapidly. This results from the fact that system $\bar{s}_{37}$ (Table IV) rapidly decreases, and increasing fractions of the population, including micelles, have $s_i <$ 210 S.

For a system at $R_I = 10$, 37° and 3 g of κ-casein/l., using $Q_\alpha = 2$ yields a total core volume of 66.6 ml, $CNkT = 3.867 \times 10^6$ and $CX_mN = 3.07 \times 10^7$. Using these values in eq 16, and assuming that γ_p must be positive, it can easily be shown that, independent of r_p, γ_p is less than 0.2 erg/cm². Corresponding values of r_p and maximum values for γ_p, in eq 17, show that for a minimum in free energy, $d\gamma/dr$ is always negative. The latter conclusion is considered to be consistent with the experimental result that as R_I is decreased, average micelle size of a population decreases and equilibrium coat activity increases.

As is the case for other equilibrium micelle systems, enthalpy effects would not in themselves be expected to yield size distributions. It therefore appears that entropy effects must be making contributions. According to Table III, as R_I is decreased the maximum in the distribution of micelle weight fractions shifts to micelles of smaller size. However, if weight fractions are converted to number fractions there are no maxima: for all distributions the number fraction increases as r_i decreases, and in addition as r_i decreases, the ratio of the number of micelles in a smaller size to that in the next larger size appears always to increase. If an entropy increase is attributed entirely to an increase in the number of micelles, it would be expected that micelle surface free energies are well below the maximum of 0.2 erg/cm² calculated above.

Studies of transformation (Tables V, VI, and VII) reveal the existence of coalescence and subdivision reactions by which micelles of a particular size appear or disappear. These must lead to and occur at equilibrium. The rate of a subdividing reaction for a particular micelle size is expected to depend on micelle concentration, and to increase as micelle surface free energy decreases (thus as coat activity increases). The rate of a coalescence reaction, from the theory of von Smoluchowski (see Overbeek, 1952), is expected to depend on the concentrations of the micelles coalescing and to increase as micelle surface free energies increase.

After mixing systems at difference R_I, since a_e increases as R_I is decreased, micelles from the lower R_I system must dissociate coat and those from the higher R_I system must accept coat. It seems likely that transient coat activity will be a maximum shortly after mixing. The $\gamma_i - r_i$ relation and the expected dependencies of transforming reactions then suggest that for some time the most rapid net changes will involve, as observed, micelles at the ends of the initial mixed distribution: coalescence of the smaller micelles from the lower R_I system and subdivision of the larger micelles from the higher R_I system.

Elsewhere (Waugh *et al.*, 1970) it is shown that the cores of micelles are constructed of nearly spherical "core polymers," each containing approximately 30 radially oriented monomers. In cores, these polymers interact at their surfaces; however, the intercore polymer interaction energy is less than that of the monomer interactions involved in core polymer formation. Let the coat activity of an equilibrium system be increased from a_e to a_c, thus producing a decrease in R. Rates of subdivision reactions increase, and rates of coalescence reactions decrease, compared to their value at a_e. At a_c, coat enters a micelle surface to increase surface coat activity and lower surface free energy. The core responds to this introduction: the negative free-energy change in the coat is compensated to some extent by an increase in core free energy, caused by a separation of the surfaces of core polymers. When interactions between a core polymer (or a group of core polymers) and the remainder of the core have been sufficiently reduced, subdivision takes place and a_c decreases. It seems likely that as micelle size increases, the products of its subdivision will be progressively more unequal in size. The energy barriers to a subdivision reaction are evidently those attending the insertion of surface coat and the separation of core polymers at their junctions.

Coat–core models have been accepted by Rose (1965), Payens (1966), and McKenzie (1967). Payens suggests that cores contain random coils of β-casein, to which compact α_s-casein monomers are attached by hydrophobic bonds. Monomer properties and the formation of limited core polymers (Waugh *et al.*, 1970) suggest that this core model is unlikely.

Garnier and Ribadeau-Dumas (1969) present an inherently different model for micelle structure. In this model, small nodes of κ-caseinate are interconnected by constant diameter strands of α_s- and/or β-caseinates. The model is a member of a group which assumes that micelles are large chemical compounds. Previously (Waugh and Noble, 1965), models of this type were considered unlikely on the basis of the phase rule. Without highly restrictive specifications, the model of Garnier and Ribadeau-Dumas will be difficult to apply to account for equilibrium, the spontaneous resuspension of micelle pellets, the results of transformation, and the near constant total micelle surface area per gram of κ-casein. In addition, the model predicts that micelle solvation will increase as R_I is increased; from 14 ml of solvent/ml of protein at $R_I = 14$ to 240 at $R_I = 100$. Experimentally (Waugh and Noble, 1965) micelle solvation is found to decrease as R_I is increased, and to be less than 2.8 at $R_I = 20$.

Parry and Carroll (1969) use electron microscopy in an attempt to locate κ-casein in milk micelles. By using κ-casein combining antibodies, they obtain no evidence for a κ-casein-stabilizing "coat." They propose that 30% of κ-casein, combined with small amounts of α_s- and β-caseins, is in solution. High molecular weight aggregates of the remaining κ-casein are placed at the centers of micelles, and are surrounded by insoluble Ca–α_s- and Ca–β-caseinates. Some of the problems involved in accounting for system characteristics using a model where κ-casein is not at the surface have been discussed by Waugh and Noble (1965). Additional problems are encountered if an attempt is made to account for system equilibria. Critical requirements for revealing surface coat by electron microscpy are that the antibody must be directed against that portion of κ-casein which is exposed (substituent macropeptides) and, after interaction, the complex must remain at the surface and not be removed. In this connection it is interesting that, in the ionic environment of milk micelles, removing micellar κ-casein would not lead to immediate core precipitation (Waugh, 1971).

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Selective Adsorption of Bis(1-anilino-8-naphthalenesulfonate) to the Multiple Forms of Lactic Dehydrogenase*

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ABSTRACT: Each of the five lactic dehydrogenase (LDH) isozymes from beef interacts differently with bis(1-anilino-8-naphthalenesulfonate) (bis(ANS)). The parent enzymes, H_4 and M_4, both have 10–12 primary dye binding sites. The sedimentation coefficient of beef M_4 increases from 7 to 22 S when 12 moles of bis(ANS) is bound per mole of LDH. This change indicates that beef M_4 associates extensively in the presence of the dye. Apparently the tetramer is the associating unit. The association is reversed by the addition of NADH. The sedimentation coefficient of beef H_4 increases from 7 to 7.5 S in solutions of bis(ANS). Circular dichroism and sedimentation measurements show that the overall dye binding to the M subunit is diminished in the hybrid LDH's. The H subunit, in contrast, always binds approximately the quantity of dye predicted from the fluorescence titration of H_4. The bound bis(ANS) molecules undergo local rotation in both M_4 and H_4. This flexibility suggests that the bound dye is located in a mobile medium, possibly near the surface of the LDH molecule.

This paper deals with the binding of bis(1-anilino-8-naphthalenesulfonate) (bis(ANS))[1] to the multiple forms of lactic dehydrogenase (LDH). The purpose of these experiments is to compare the dye binding properties of the parent enzymes, H_4 and M_4 (Cahn *et al.*, 1962), and to investigate subunit interactions in the hybrid LDH's, H_3M, H_2M_2, and M_3H.

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[1] Abbreviations used are: LDH, lactic dehydrogenase; BSA, bovine serum albumin; bis(ANS), bis(1-anilino-8-naphthalenesulfonate); *n*, average number of moles of ligand bound per mole of protein.