Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Chernoff, A. J., and Liu, J. C. (1961), *Blood 17*, 54.
Cuppy, D., and Crevasse, L. (1963), *Anal. Biochem. 5*, 462.
Dalziel, K. (1957), *Acta Chem. Scand. 11*, 1706.
Davis, E. J. (1964), *Ann. N.Y. Acad. Sci. 221*, 1404.
Dempsey, W. B. (1965), *J. Bacteriol. 90*, 431.
Dempsey, W. B. (1969), *J. Bacteriol. 97*, 182.
Ellman, G. (1959), *Arch. Biochem. Biophys. 82*, 70.
Herbst, R. M., and Engel, L. L. (1934), *J. Biol. Chem. 107*, 505.
Kalyankar, G. D., and Snell, E. E. (1962), *Biochemistry 1*, 594.
Martinez-Carrion, M., and Jenkins, W. T. (1965), *J. Biol. Chem. 240*, 3547.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Peterson, E. A., and Sober, G. A. (1962), *Methods Enzymol. 5*, 3.
Sizer, I. W., and Jenkins, W. T. (1962), *Methods Enzymol. 5*, 677.
Snyder, F., and Godfrey, P. J. (1961), *Lipid Res. 2*, 195.
Tiselius, A., Hjertén, S., and Levin, Ö. (1956), *Arch. Biochem. Biophys. 65*, 132.
Uyeda, K., Kurooka, S. (1970), *J. Biol. Chem. 245*, 3315.
Yphantis, D. (1964), *Biochemistry 3*, 297.

Inelastic Light-Scattering Study of the Size Distribution of Bovine Milk Casein Micelles*

S. H. C. Lin, R. K. Dewan, V. A. Bloomfield,† and C. V. Morr

ABSTRACT: We have used the technique of inelastic laser light scattering to measure the diffusion coefficients, and thereby the hydrodynamic radii, of fractionated casein micelles from bovine milk The micelles were fractionated by rate-zone centrifugation in sucrose density gradients. Electron microscopic examination of the fractionated material, and the good fit of the experimental light-scattering spectrum to that expected for a monodisperse suspension of spheres, indicated that the fractions were effectively monodisperse. From measurements on various fractions a cumulative distribution curve of the weight fraction of micelles with radii less than or equal to R was constructed. This distribution had about 80% of the casein in micelles with radii between 500 to 1000 Å, and 95% between 400 and 2200 Å, with a most probable radius of about 800 Å. These radii, determined for micelles in solution, are two to three times larger than those found by other workers using electron microscopy on apparently comparable micellar preparations.

The shape and size distribution of bovine milk casein micelles have been studied by a number of workers with the electron microscope (Nitschmann, 1949; Hostettler and Imhof, 1952; Knoop and Wortmann, 1960; Saito and Hashimoto, 1964; Rose and Colvin, 1966; Carroll *et al.*, 1968). From these studies it can be concluded that casein micelles are strongly polydispersed, roughly spherical in shape, and that their diameters range from 400 to 3000 Å with an average size of about 800 Å. In all these investigations, different techniques for preparation and fixation of the micelles and the shadowing of the specimen were used. In interpreting electron micrographs the possibility is ever present of being misled by artifacts produced by a particular preparative method. Moreover, it is extremely difficult to count small micelles and discriminate them from the background materials on the grid (Carroll *et al.*, 1968). In addition, the limitation of studying only dehydrated micelles under the electron microscope may give a size distribution which no longer represents that in the native milk. Size measurements of casein micelles dispersed in simulated milk ultrafiltrate (SMUF)[1] (Jenness and Koops, 1962), as in the present study, should more correctly reflect their actual size distribution in milk.

Morr *et al.* (1971) described a rate-zone ultracentrifugation technique for fractionating skim milk casein micelles in sucrose gradients. Casein micelles were separated from soluble caseins and the micellar caseins were further separated into a number of rather uniform size fractions. The reduced polydispersity of these fractions has made it possible to determine the average size of the micelles in each fraction in a solvent system similar to milk, thereby permitting a reconstruction of the size distribution curve.

In recent years, the technique of inelastic light scattering has been widely used to measure the diffusion constants and hydrodynamic radii of biological macromolecules (Dubin *et al.*, 1967, 1970; Cummins *et al.*, 1969; Rimai *et al.*, 1970; Ford *et al.*, 1969). This method measures the spectrum of laser light inelastically scattered from a solution of macromolecules undergoing brownian motion. It is ideally suited for the study of dilute solution of micelles, whose scattered light spectrum has high intensity and spectral width conveniently in the audiofrequency range.

* From the Department of Food Science and Industries (S. H. C. L. and C. V. M.), and Department of Biochemistry (R. K. D. and V. A. B), University of Minnesota, St. Paul, Minnesota 55101. *Received July 16, 1971.* This research was supported in part by grants from the National Institutes of Health, A. P. Sloan Foundation, the Graduate School of the University of Minnesota, and the Minnesota Agricultural Experiment Station.

† Alfred P. Sloan Foundation Fellow.

[1] SMUF is a salt solution (pH 6.6) containing calcium, magnesium, potassium, sodium, phosphate, citrate, carbonate, sulfate, and chloride, which simulates milk ultrafiltrate.

TABLE I: Centrifugal Conditions for Fractionating Casein Micelles in Sucrose Gradients.[a]

Condition	Rpm	Min	Ml of Skim Milk
A	5,000	5	0.5
B	5,000	20	0.4
C	10,000	30	0.3
D	20,000	40	0.3
E	25,000	120	0.5

[a] IEC B-35 preparative ultracentrifuge, SB-269 swinging-bucket rotor. Maximum distance from center of rotation, 15 cm.

Materials and Methods

Size Fractionation. A detailed account of the preliminary steps in the fractionation procedure, *viz.*, preparation of skim milk, formation of sucrose gradients in SMUF, sample layering on the gradients, centrifugation, gradient removal, and monitoring, has been given by Morr *et al.* (1971). Linear 8-ml sucrose gradients, 15–25% (w/w), in 12 × 96 mm centrifuge tubes were prepared in SMUF to minimize changes in casein micelles during fractionation.

Skim milk casein micelles were fractionated at 25° according to different centrifugation conditions listed in Table I. The conditions were chosen to obtain monodispersed fractions covering the widest possible micelle range. Four replicate gradients were used in each centrifugation condition and each centrifuged gradient was carefully removed from the centrifuge tube by gentle suction from a water aspirator and collected in eight fractions. The first fraction was collected from the top of the gradient (7.5–6.0 cm height in the centrifuge tube) while the next six fractions (2–7) were collected as 1-cm fractions from 6.0 cm down to the bottom of the tube. The pellet was designated as fraction 8. Corresponding fractions from the replicate gradients were combined and dialyzed against SMUF to remove sucrose. The refractive index of the dialyzed fraction was measured by an Abbe refractometer for determining the residual sucrose concentration which ranged from 0.5 to 1.25%. The viscosity was read from the calibration tables (Handbook of Biochemistry, 1968), relating the concentration of sucrose to its viscosity.

Protein concentration in terms of absorbance units at 278 nm (1-cm path length) was determined with a Beckman DB spectrophotometer. Turbidity contributed by residual lipid material and casein micelles was removed by extraction with chloroform and by addition of 0.032 M EDTA solution to break up the micelles, respectively. Total casein in milk was obtained from the difference of absorbance units between skim milk and isoelectric whey (pH 4.6). The percentage of total casein in each of the gradient fractions was calculated from these absorbance data. Skim milk normally yields a total of about 40 absorbance units of which 27 units are contributed by the caseins and the balance by whey proteins, peptides, and amino acids.

Electron Microscopic Examination. The procedure was adapted from Morr *et al.* (1966) and Carroll *et al.* (1968) with much simplification for the present study. Skim milk and micelle fractions were fixed with 1% glutaraldehyde for 15 min and then dialyzed against water to remove salts and excess glutaraldehyde. Samples were diluted with water and sprayed onto Formvar-coated copper grids. A Philips 300 electron microscope was operated at 60 kV to obtain micrographs with a direct magnification of 6840×.

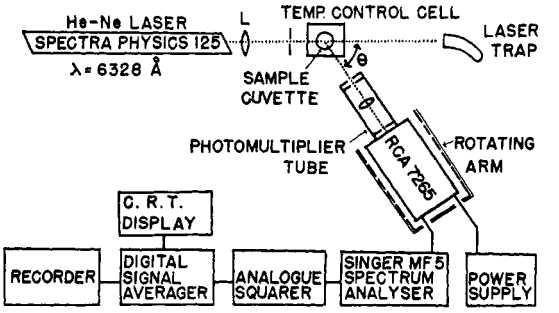

FIGURE 1: Block diagram of laser self-beat spectrometer.

Laser Self-Beat Spectrometer. A schematic diagram of the self-beat spectrometer is shown in Figure 1. Plane-polarized monochromatic light (λ_0 = 6328 Å) provided by a Spectra-Physics Model 125 CW He-Ne laser (~50 mW) was focused on to a cell containing the scattering solution. A constant temperature of 25 ± 0.1° was achieved by mounting the cell in a cell holder maintained at 25°.

Light scattered at an angle θ, after passing through a pin hole, was collected by a lens and imaged on a pin hole at the face of an RCA-7265 photomultiplier tube. The two pin holes and the collecting lens were mounted in a blackened metallic circular tube which screwed on to the surface of the photomultiplier tube. Proper choice of interchangeable pin holes allowed variation of the angular acceptance as well as the illuminated area of the surface of the photpmultiplier. The laser source, temperature control cell holder, collecting optics, and the photomultiplier tube were all placed on a heavy steel table supported by kinetic air mounts to isolate them from building vibrations.

The phototube output was analyzed with a Singer spectrum analyzer (Model MF-5) with AL-2 audiofrequency plug in (20–35,000 Hz). To measure the power spectral density of the photomultiplier current the analyzer output was squared and averaged by an analog squarer and a Fabri-Tek digital signal averager. This spectrum was examined on a Tektronix oscilloscope and was simultaneously recorded on a strip-chart recorder. Approximately 5 min were required to record a scattering spectrum at 1 sec/spectral scan, and several 100 scans to average out random noise. Experimental curves were fitted to theoretical Lorentzian line shapes with a Hewlett-Packard calculator and plotter.

The apparatus was standardized by examining 910- and 1700-Å diameter polystyrene latex spheres obtained from the Bioproducts Department of Dow Chemical Co. Dilute suspensions of these latex spheres were made at different concentrations in deionized water and were filtered with a Millipore filter (pore size 0.45 μ). Diffusion coefficients determined for these reference spheres with the present equipment were found to be within ±2% of values calculated by the Einstein–Stokes equation.

It has been demonstrated theoretically (Pecora, 1964; Cummins *et al.*, 1969) and experimentally (Dubin *et al.*, 1967) that the self-beat power spectrum of the photocurrent due to scattering from a monodisperse suspension of spherical particles has a single Lorenzian shape and is given by

$$S_1(\Delta\nu)\alpha(K^2D/\pi)/[(\Delta\nu)^2 + (K^2D/\pi)^2] \quad (1)$$

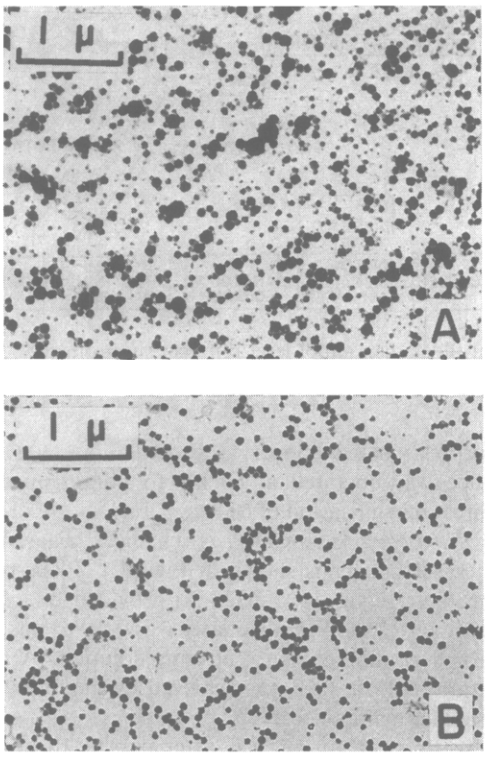

FIGURE 2: Electron micrographs of casein micelles: skim milk (A); and micelle fraction D4 of Table II (B).

Here the photocurrent frequency is $\Delta\nu = \nu - \nu_0$, where ν_0 is the frequency of the incident laser light and ν is one of the frequency components of the scattered light; $S_1(\Delta\nu)$ is the power spectral density, D is the diffusion coefficient, and

$$K = (4\pi n/\lambda_0) \sin(\theta/2) \quad (2)$$

Here λ_0 is the wavelength of the incident light under vacuum, θ is the scattering angle, and n is the refractive index of the solution. The half-width at half-height of the Lorentzian given by eq 1 is

$$\Delta\nu_{1/2} = K^2 D/\pi \quad (3)$$

According to the Einstein–Stokes relation for spheres

$$D = kT/6\pi\eta R \quad (4)$$

where k is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, and R is the radius of the sphere. Thus the following relation is obtained

$$\Delta\nu_{1/2} = 8kTn^2 \sin^2(\theta/2)/3\lambda_0^2\eta R \quad (5)$$

which permits computation of the radius of spherical particles from the value of $\Delta\nu_{1/2}$ obtained from the experimental Lorentzian curve. The uncertainty in $\Delta\nu_{1/2}$ is ±2%.

Results

Casein micelle fractions obtained from skim milk by rate-zone ultracentrifugation appeared rather uniform in size, when examined by electron microscopy. Figure 2 shows micrographs of fractionated and unfractionated micelles. By using the various centrifugal conditions given in Table I, proceeding from A to E, fractions containing successively small casein micelles were collected. Since residual lipid material in skim milk was concentrated in the top gradient fraction and casein micelle fractionation was incomplete in the upper portion of condition A and B gradients, these fractions were not studied by inelastic light scattering. Gradient fractions examined for scattering included the fourth and sixth fractions obtained from centrifugal conditions A and B, and the second, fourth, and sixth fractions from conditions C, D, and E. Similarly the seventh and eighth fractions in all conditions were not examined because they contained large micelles which were not likely to be uniform in size.

Fractionated micelles were stable during the period of study (typically several hours at room temperature); and no variation in size was observed when the same micelle fraction was examined on the first day or on the second day after fractionation was completed, with refrigeration overnight. Use of SMUF as a dispersing solvent stabilizes the micelles against breakdown.

A typical scattering spectrum for a casein micelle fraction obtained at a scattering angle (θ) of 90° along with a calculated Lorentzian curve is shown in Figure 3.

In order to construct the size distribution curve of casein micelles in milk, the radii, the casein concentration of chosen micelle fractions and the total amount of casein added to the gradients were determined. Cumulative weight distributions $W(R)$ (see Appendix, eq A8) were obtained by summing the weight fraction of micelles with radius less than or equal to R and plotting the sum *vs.* R. About 95% of the total caseins were recovered between the smallest and largest micelle fractions with radii ranging from 400 to 2200 Å. The data from a pooled milk sample are reported in Table II and the size distributions for three different pooled milk sampes are shown by the points in Figure 4. The solid lines in Figure 4 represent the best fit to the data by a Schulz–Zimm distribution (see Discussion).

Comparative studies indicated that the apparent radii of

TABLE II: Diffusion Coefficients and Radii of Micelle Fractions Obtained under Different Centrifugal Conditions as Determined by Inelastic Light-Scattering Technique.[a]

Fraction[a]	Cumulative Casein Wt Distribution	D (cm²/sec) × 10^8	Radius (Å)
E4	0.026	4.63	541
E6	0.104	4.21	592
D2	0.046	4.49	555
D4	0.256	3.23	767
D6	0.439	2.98	828
C2	0.440	3.0	822
C4	0.715	2.47	999
C6	0.807	2.08	1179
B4	0.880	1.88	1293
A4	0.951	1.12	2165
A6	0.979	0.79	3075

[a] Estimated uncertainties in D and R are ±2%. [b] The letter represents the centrifugal condition (Table I) and the number denotes the fraction number for that condition.

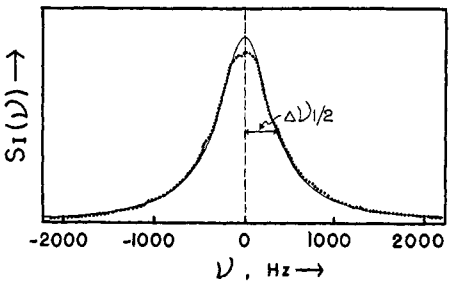

FIGURE 3: Inelastic light-scattering spectrum of casein micelle fraction D4 of Table II obtained by rate-zone ultracentrifugation: experimental spectrum (· · · · · · · · ·); calculated Lorentzian (——); $\Delta\nu_{1/2}$ = 359 Hz.

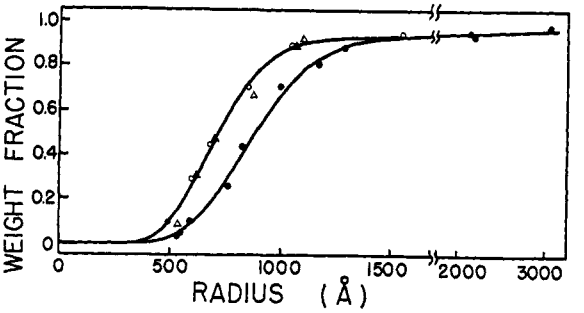

FIGURE 4: Size distribution of casein micelles in milk by inelastic light scattering: milk sample I with y = 0.0158, z = 11.0 (●); milk samples II and III with y = 0.0195, z = 11.0 (○ and △). y and z represent the parameters of Schulz–Zimm distribution (see Appendix).

glutaraldehyde-fixed micelles appeared to be about two to three times large by inelastic light scattering than by electron microscopy. Scattering measurements also revealed that the radii of small and large size casein micelles were reduced to about one-third of their original value when suspended in 50% sucrose solution. The shrinkage effect was found to be entirely reversed by resuspending the micelles in SMUF (Dewan *et al.*, 1971, unpublished data).

Discussion

The technique of inelastic light scattering has been widely used to study the diffusion constants and hydrodynamic radii of biological macromolecules. It is conveniently applicable to homogeneous macromolecular systems but for polydisperse systems, with broader distribution of molecular weights, the interpretation of data may be complicated and difficult. Native casein micelles in milk are highly polydispersed and it is therefore necessary to fractionate them into a number of relatively monodisperse fractions before the inelastic light-scattering technique can be used to study their size characteristics. Previous work (Morr *et al.*, 1971), as well as the present study, has shown that rate-zone ultracentrifugation can be utilized to fractionate polydisperse casein micelles into nearly monodisperse fractions suitable for inelastic light-scattering measurements. Electron microscopy has confirmed that reasonably monodisperse casein micelle fractions are obtained by rate-zone ultracentrifugation. The relatively close fit of the experimental scattering curves for casein micelle fractions to single Lorentzians confirms the essential monodispersity of the fractions and substantiates the practicability of rate-zone ultracentrifugation for the inelastic light-scattering study.

Casein micelle size distributions have been reported by a number of workers (Nitschmann, 1949; Rose and Colvin, 1966; Carroll *et al.*, 1968). Their data, which were dependent upon the restricted observations of casein micelles with the electron microscope, provide number *vs.* size class relationships. It is obvious that numerous micelles, which were too small to be distinguished from the background materials, and a few large micelles as well, may not be included in the size determination. In electron microscopy a very minor fraction of the total micelle population is examined, which reduces the accuracy of the method in size determinations. In addition micelles in the dehydrated state appear much smaller in size than native micelles in SMUF. The technique of inelastic light scattering, which is able to study the size of casein micelles in solution, overcomes these inherent difficulties. Micelles having radii less than 400 Å are too small, while those larger than 3000 Å are not present in sufficient numbers to be studied by inelastic light scattering. Nevertheless weight distributions rather than number distributions were determined with good reliability, since the weight fractions of both very large and very small micelles are small ($\leq$0.05). This made it reasonable to extrapolate the $W(R)$ *vs.* R curve to low and high R once the central portion of the distribution curve had been constructed.

In order to characterize quantitatively the distribution of micellar sizes, we fit the weight distribution curves to a two-parameter distribution of the form proposed by Schulz (1939) and Zimm (1948). The properties of this distribution are summarized in the Appendix. It is characterized by the parameters z, which represents the reciprocal of the relative variance (eq A4 and A6), $1/z = (\bar{R}^2/\bar{R}^2) - 1$, and y which together with z gives the number-average radius (eq A3) $\bar{R} = z/y$.

For all three of the samples in Figure 4, z = 11, assuming that α = 3; *i.e.*, that the molecular weight is proportional to R^3. Two of them had y = 0.0195 Å^{-1}, while the third had y = 0.0158 Å^{-1}. The reason for these different values of y, which were measured on pooled milk samples obtained on different days, is not known. However, the identical values of z indicate that the sharpness of the distribution remains constant.

The present study has shown that about 80% of the total micelles by weight have radii ranging from 500 to 1000 Å (Figure 4). It is difficult to compare these weight distribution values with number distribution values obtained from electron microscopic examination, since weight distribution places more emphasis upon the larger micelles than the smaller ones. Though the number of small micelles may be quite large, they will contribute substantially less to the weight distribution than to the number distribution. Conversion of number distribution values obtained by others using electron microscopy to weight distribution, by assuming uniform density, does not completely reconcile these differences. The number distribution values by electron microscopy still favor small radii, and greater breadth of the distribution for casein micelles than does the weight distribution determined here by inelastic light scattering. For example, we have fit the number distribution obtained electron microscopically by Nitschmann (1949) to a Schulz–Zimm distribution, obtaining a best fit with z = 6 and y = 0.0101 Å^{-1}.

The most probable weight fraction, as indicated by the inflection point in the distribution curves in Figure 4, was observed to center around a radius of about 800 Å. In electron microscopic studies, radii of about 400–500 Å (Nitschmann, 1949), less than 125 Å (Rose and Colvin, 1966) and 500 to 850

Å (Carroll *et al.*, 1968) have been reported to comprise about 58, 35–64, and 75% of the micelles, respectively. Although great variations between milk samples as well as various preparation and counting techniques could account for some variation among these data, the micelle size observed by the electron microscope was usually much smaller than that observed by inelastic light scattering. It is tempting and plausible to attribute this to shrinkage of the micelles on the electron microscope grid, which is not prevented by glutaraldehyde fixation. In this connection it should be noted that the electron microscopic radius of MS2 bacteriophage is 125 Å, while that determined by inelastic light scattering in solution is 205 Å (French *et al.*, 1969). This measurement of macromolecular sizes under native conditions represents one of the major advantages of the inelastic light-scattering technique.

Although it appears that the Schulz–Zimm distribution, with appropriate choices of y and z, provides a good fit to the data, the relevance of this fit to the mechanism of formation of casein micelles must remain a matter of speculation. The distribution was derived (Schulz, 1939) according to the following considerations. It is imagined that a population of polymer chains exists with a "most probable" (Flory, 1953) distribution of sizes, equivalent to the distribution (A1) with $z = 1$. These polymer chains are then taken z at a time and combined into "superpolymers." This suggests that casein micelles may be formed from preexisting subaggregates of casein monomers, which is in general accord with recent speculations on casein micelle structure (Morr, 1967; Waugh, 1967; Rose, 1969). However, most of these models presume that the subaggregates have a definite fixed size; and it must also be kept in mind that the existence of three chemically distinct species of casein will complicate the analysis. Nevertheless, it is interesting to observe that casein micelles occupy an intermediate position between the fixed size characteristic of most other biologically functional aggregates (ribosomes, viruses, multisubunit enzymes, etc.) and the very broad size distribution characteristic of most synthetic polymers (Peebles, 1971). The association of preexisting subaggregates provides an attractive way to explain the intermediate character of the size distribution of casein micelles.

Appendix

In order to summarize succinctly the parameters of the observed cumulative distribution functions for casein micelles, we fit the observations to a function of the form first proposed by Schulz (1939) and Zimm (1948). If $f(R)\mathrm{d}R$ is the fraction of micelles with radius between R and $R + \mathrm{d}R$, then the Schulz–Zimm distribution is of the form

$$f(R)\mathrm{d}R = \frac{R^{z-1}y^z e^{-yR}}{\Gamma(z)}\mathrm{d}R \tag{A1}$$

$\Gamma(z)$ is the Γ function of z (Abramowitz and Stegun, 1965), and equals $(z - 1)!$ for integral values of z. Using the standard integral

$$\Gamma(z) = \int_0^\infty t^{z-1}e^{-t}\mathrm{d}t$$

average value of the nth power of R is

$$\bar{R^n} = \int_0^\infty R^n f(R)\mathrm{d}R = \frac{\Gamma(z+n)}{y^n\Gamma(z)} \tag{A2}$$

Further using the fact that $\Gamma(z + 1) = z\Gamma(z)$, we find that the arithmetic mean radius is

$$\bar{R} = z/y \tag{A3}$$

and the variance of R is

$$\mathrm{var}(R) = \overline{(R - \bar{R})^2} = \bar{R^2} - (\bar{R})^2 \tag{A4}$$
$$= z/y^2$$

Thus the two parameters y and z which characterize the Schulz–Zimm distribution are related to its mean and variance by

$$y = \bar{R}/\mathrm{var}(R) \tag{A5}$$

$$z = (\bar{R})^2/\mathrm{var}(R) \tag{A6}$$

We see from eq 6 that the larger z, the more sharply peaked the distribution.

$f(R)\mathrm{d}R$ is the quantity observed by electron microscopic counting of micelles in particular size ranges. The inelastic light-scattering studies described in this paper give a somewhat different distribution function. This is $w(R)\mathrm{d}R$, the weight fraction of casein contained in micelles with radius R and $R + \mathrm{d}R$. To connect $w(R)$ and $f(R)$, we assume that micellar radius and molecular weight M are connected by a power law relation

$$M(R) = AR^\alpha \tag{A7}$$

where A is a constant independent of R. Then

$$w(R)\mathrm{d}R = \frac{M(R)f(R)\mathrm{d}R}{\int_0^\infty M(R)f(R)\mathrm{d}R}$$
$$= \frac{R^{\alpha+z-1}y^z e^{-yR}\mathrm{d}R}{\int_0^\infty R^{\alpha+z-1}y^z e^{-yR}\mathrm{d}R}$$
$$= \frac{R^{z+\alpha-1}y^{z+\alpha}e^{-yR}\mathrm{d}R}{\Gamma(z+\alpha)} \tag{A8}$$

If it is assumed that the casein micelles are spherical and of constant density, then $\alpha = 3$.

In this paper we have determined the cumulative weight distribution $W(R)$, which equals the fraction of total casein contained in micelles with radius less than or equal to R. Mathematically

$$W(R) = \int_0^R w(R')\mathrm{d}R' \tag{A9}$$

If z and α are integers, it is possible to integrate eq A9 in closed form

$$W(R; y, z+\alpha) = 1 - \left[1 + yR + \frac{(yR)^2}{2!} + \ldots + \frac{(yR)^{z+\alpha-1}}{(z+\alpha-1)!}\right]\exp(-yR) \tag{A10}$$

It is to this equation that the cumulative distribution curves in Figure 4 were fit to obtain values of y and z, assuming $\alpha = 3$.

Acknowledgments

We are grateful to Mrs. Jülyet A. Benbasat for help in analyzing the distribution curves, and to Professor R. Jenness for advice.

References

Abramowitz, M., and Stegun, I. A. (1965), Handbook of Mathematical Functions, New York, N. Y., Dover Publications, Inc., p 253.
Carroll, R. J., Thompson, M. P., and Nutting, G. C. (1968), *J. Dairy Sci. 51*, 1903.
Cummins, H. Z., Carlson, F. D., Herbert, T. J., and Woods, G. (1969), *Biophys. J. 9*, 518.
Dubin, S. B., Benedek, G. B., Bancroft, F. C., and Freifelder, D. (1970), *J. Mol. Biol. 54*, 547.
Dubin, S. B., Lunacek, J. H., and Benedek, G. B. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1164.
Flory, P. J. (1953), Principles of Polymer Chemistry, Ithaca, N. Y., Cornell University Press.
Ford, N. C., Jr., Lee, W., and Karasz, F. E. (1969), *J. Chem. Phys. 50*, 3098.
French, M. J., Angus, J. C., and Walton, A. G. (1969), *Science 163*, 345.
Handbook of Biochemistry (1968), Cleveland, Ohio, Chemical Rubber Co., p J-250.
Hostettler, H., and Imhof, K. (1952), *Landwirt. Jahrb. Schweiz 66*, 307.
Jenness, R., and Koops, J. (1962), *Ned. Melk-Zuiveltijdschr. 16*, 153.
Knoop, E., and Wortmann, A. (1960), *Milchwissenschaft 15*, 273.
Morr, C. V. (1967), *J. Dairy Sci. 50*, 1744.
Morr, C. V., Josephson, R. V., Thomas, E. L., and Frommes, S. P. (1966), *J. Dairy Sci. 49*, 403.
Morr, C. V., Lin, S. H. C., and Josephson, R. V. (1971), *J. Dairy Sci.* (in press).
Nitschmann, H. (1949), *Helv. Chim. Acta 32*, 1258.
Pecora, R. (1964), *J. Chem. Phys. 40*, 1604.
Peebles, L. H., Jr. (1971), Molecular Weight Distribution in Polymers, New York, N. Y., Interscience Publishers.
Rimai, L., Hickmott, J. T., Jr., Cole, T., and Carew, E. B. (1970), *Biophys. J. 10*, 20.
Rose, D. (1969), *Dairy Sci. Abstr. 31*, 171.
Rose, D., and Colvin, J. R. (1966), *J. Dairy Sci. 49*, 1091.
Saito, J., and Hashimoto, Y. (1964), *J. Fac. Agr. Hokkaido Imp. Univ. 54*, 17.
Schulz, G. V. (1939), *Z. Phys. Chem., Abt. B 43*, 25.
Waugh, D. F. (1967), 2nd North-East States Milk Protein Seminar, Ithaca, N. Y., Cornell University, Oct. 26–27.
Zimm, B. H. (1948), *J. Chem. Phys. 16*, 1099.

Structure Studies on Chromatin and Nucleohistones. Thermal Denaturation Profiles Recorded in the Presence of Urea*

Allen T. Ansevin,† Lubomir S. Hnilica, Thomas C. Spelsberg, and Susan L. Kehm

ABSTRACT: Structural information about chromatin and artificial nucleohistones was revealed by plots of the temperature derivative of hyperchromicity *vs.* the temperature (derivative denaturation profiles) from thermal denaturation experiments. Soluble preparations of rat thymus or liver chromatin displayed derivative denaturation profiles with a characteristic pattern of ascending steps when studied in a medium containing 3.6 M urea. Such profiles provide a convenient test for the intactness of chromatin since degraded preparations showed distinctly different patterns, characterized by more sharply defined maxima and minima. Comparisons of denaturation profiles of treated and untreated nucleoproteins in various denaturation media revealed that: (1) profiles of natural chromatin were more complex than those of artificial nucleohistones; (2) less than 10% of the nucleic acid within fresh chromatin denatured as extended stretches of free DNA; (3) both autolysis of chromatin and trypsin digestion exposed DNA and destabilized distinctive regions in the denaturation profile; (4) fixation of chromatin by formaldehyde elevated denaturation temperatures; (5) derivative denaturation profiles appear to reflect physical as well as chemical features of nucleoprotein complexes; (6) a cacodylate buffer containing 3.6 M urea resolves the structure of chromatin better than a buffer containing only 5 mM sodium cacodylate, and reveals the structure of lysine-rich nucleohistones better than 0.25 mM Versenate buffer; (7) spontaneous formation of ammonium cyanate in a urea-containing medium does not produce major artifacts that prevent the interpretation of denaturation profiles; (8) the nucleohistones reconstituted at low ionic strength in this laboratory appeared to have greater structural variety than related complexes reconstituted elsewhere by the gradient dialysis method.

The formation of complexes between basic proteins and DNA is known to increase the thermal stability of DNA (Huang and Bonner, 1962; Allfrey *et al.*, 1964; Ohba, 1966; Huang *et al.*, 1964; Sponar *et al.*, 1967; Olins *et al.*, 1967, 1968). Since the denaturation of DNA, either free or within a complex, is accompanied by an increase in uv optical absorbance, complexes may conveniently be detected by means

* From the Department of Physics (A.T.A.) and from the Department of Biochemistry (L. S. H. and S. L. K.), The University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025, and from the Department of Obstetrics and Gynecology, Vanderbilt University, Nashville, Tennessee (T. C. S.). *Received July 6, 1971.* This research was supported in part by Robert A. Welch Foundation Grants G-290 and G-138, U. S. Public Health Service Grant CA 07746, and U. S. Atomic Energy Commission Contract AT-(40-1)-2832. Programming assistance was provided by the Department of Biomathematics through support received from USPHS Grant No. CA 11430.

† To whom to address correspondence.