

Immunological Approach to Location of κ -Casein in the Casein Micelle by Electron Microscopy

R. J. CARROLL and H. M. FARRELL, JR.
Agricultural Research Service, US Department of Agriculture
Eastern Regional Research Center
Philadelphia, PA 19118

ABSTRACT

The colloidal stability of skim milk is due to κ -casein in casein micelles; thus, the location of this protein within these complexes has been the subject of speculation. Various models have placed κ -casein at the surface, in the interior, or distributed uniformly in the casein micelle. By a ferritin-labeled double-antibody technique coupled with electron microscopy, location of κ -casein has been related to casein micelle size. Casein micelles with κ -casein located predominantly at the periphery of the micelle have diameters of $1420 \pm 320 \text{ \AA}$ whereas micelles with κ -casein located uniformly throughout have diameters of $920 \pm 220 \text{ \AA}$. These results are in accord with the inverse relationship between micelle size and κ -casein content and support the model of Slattery (19).

INTRODUCTION

Major proteins of bovine milk are α_s -, β -, and κ -caseins, which occur in spherical colloidal complexes called casein micelles. A unified conceptualization of the structure of the casein micelle is lacking, although much chemical and physical information is available. Factors contributing to the structure and stability of casein micelles have been reviewed (4, 16, 24). Although conflicting theories of casein micelle structure exist, all authors agree that κ -casein plays the most crucial role in the stability of the casein micelle (5). Unresolved, however, is the location of κ -casein within the micelles in such a way as to explain their physical and chemical properties. One of the earlier models, proposed by Waugh (24), was called the coat-core model. This model placed κ -casein totally on the periphery of the micelle, the coat, and β -

and α_s -caseins in the interior, the core. Other workers hypothesized that κ -casein is located primarily in the center of the micelle (13) or distributed throughout (6, 12, 15). The model of Slattery (17, 19) proposed a submicelle theory with a predominantly peripheral location of κ -casein.

Several investigators have attempted to use electron microscopy to determine the location of κ -casein in the micelles through the use of heavy metal tags, which have been coupled to antibodies (13) or lectins (8) or produced by chemical reactions specific for the carbohydrate portion of κ -casein (9). Results of these experiments, for the most part, have been inconclusive because two of the research groups (8, 13) were unable to detect sufficient κ -casein in the micelles, although serum κ -casein was observed, whereas the third group's results (9) were indefinite because of a peculiar time-dependency for their reaction. Weigand and Russo (26) published a double-antibody technique using ferritin label to locate murine mammary tumor virus. We adapted this procedure, which uses "underfixation" to achieve maximum immunological reactivity and attempted to locate κ -casein in ultrathin sections of casein micelles.

MATERIALS AND METHODS

Micelles

Fresh warm bovine milk was obtained locally and the cream separated by low-speed centrifugation at 25°C . Casein micelles of skim milk were fixed: a) by the standard method (1) in 2% glutaraldehyde in .07 M phosphate buffer at pH 6.6 (15 min), or b) for antibody tagging for 15 min in .05% glutaraldehyde + 1% paraformaldehyde in .07 M phosphate buffer at pH 6.6 (24). In both cases, 2 ml of skim milk was added to 10 ml of fixative. The fixed micelles were recovered by centrifugation at $100,000 \times g$ for 20 min, dehydrated in serial dilutions of

ethyl alcohol, and embedded in epon-araldite resin.

Preparation of Antibodies and Caseins

Whole caseins were fractionated on a DEAE-cellulose column as described by Woychik et al. (25), and various casein fractions were collected and identified by gel electrophoresis.

κ -Casein purified by the method of McKenzie and Wake (11) served as the antigen; antibodies specific for κ -casein were produced in rabbits by Cappel Laboratories, Cochraneville, PA.¹ Goat anti-rabbit IgG heavy and light-chain antibodies conjugated to ferritin also were obtained from Cappel Laboratories.

Radial immunodiffusion (RID) assays were by the general method described by Guidry and Pearson (7) but modified as in (3). The standard proteins in the assay were dissolved and diluted in buffer (50 mM tris, 4 mM citrate pH 9.0, 5 M urea, and 10 mM dithiothreitol).

Procedure for Immunohistochemistry and Electron Microscopy

The following solutions are required: 1) 10% H₂O₂; 2) HBS (hepes buffered saline) 50 mM hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) in .9% NaCl adjusted to pH 7.2; 3) 1% bovine serum albumin (BSA) in HBS; 4) apoferritin 1 mg/ml in solution 3; 5) 1° Ab—rabbit anti- κ -casein diluted with solution 3 about 1:500; 6) 2° Ab—the IgG fraction of ferritin-conjugated goat anti-rabbit IgG heavy and light-chain diluted in HBS to an A₄₂₀ between .2 and .8; 7) 2% glutaraldehyde in HBS; and 8) 2% uranyl acetate in H₂O.

All solutions were passed through .22- μ m millipore filters. Ultrathin sections of casein micelles were made with an LKB-IV ultramicrotome and collected on cleaned nickel grids. Reactions were carried out on grids immersed in drops of the solutions on layered wax in petri dishes. Sections on the grids were treated as follows: 1) immerse grids in 10% H₂O₂ for 20 min to remove partially the resin and expose more available sites on the micelles for reaction; 2) wash in HBS (20 drops); 3) immerse in 1% BSA in HBS for 10 min (wetting

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

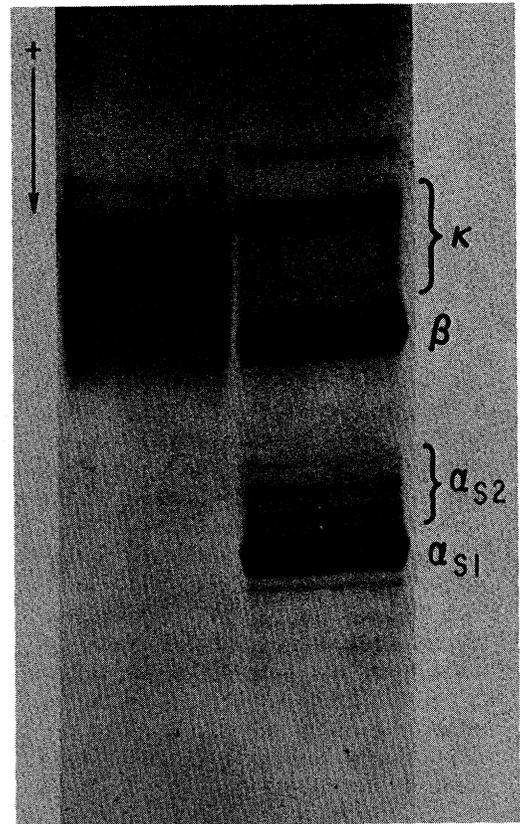


Figure 1. Urea gel electrophoresis of left lane, purified κ -casein; right lane, whole casein.

agent); 4) immerse in 1° Ab (rabbit anti- κ -casein) for 90 min at 37°C; 5) wash in 1% HBS (20 drops); 6) immerse in 1% BSA in HBS for 10 min; 7) immerse in apoferritin in HBS with 1% BSA for 10 min to tie-up reactive sites on resin and to increase specificity of the ferritin-labeled antibody; 8) demagnetize tweezers and grids at this step to minimize any magnetic influence in the ferritin-tagged antibodies; 9) immerse in 2° Ab (goat anti-rabbit IgG conjugated to ferritin) for 120 min at 37°C; 10) wash in 1% HBS (20 drops); 11) fix in 1% glutaraldehyde in HBS for 15 min; 12) wash in HBS (20 drops); 13) wash in water (20 drops); 14) stain in 2% uranyl acetate for 15 min; 15) wash in water (20 drops). The sections were examined in a Zeiss 10B electron microscope operating at 60 kV.

Experimental Design

The reaction of the ferritin-labeled 2° Ab (goat-anti-rabbit) with the 1° Ab (rabbit anti- κ) was demonstrated by electron microscopy. Section micelles were treated in the following fashion: a) No. 1° Ab, No. 2° Ab; b) 1° Ab, No. 2° Ab; c) No. 1° Ab, 2° Ab; d) 1° Ab, 2° Ab.

RESULTS AND DISCUSSION

An alkaline urea gel of the purified κ -casein preparation is shown in Figure 1 along with a whole casein standard. Antibodies raised in rabbits to this preparation did not crossreact with purified fractions of α_{s1} -, β -, γ -, or α_{s2} -caseins as judged by Ouchterlony double-diffusion and immunoelectrophoresis (2). With DEAE-cellulose chromatography in the presence of urea, whole casein was separated as in Figure 2. First peaks eluted after application of

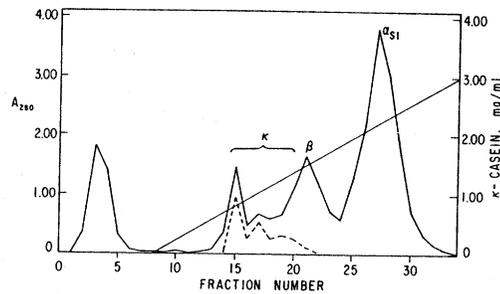


Figure 2. DEAE-Cellulose chromatography showing separation of whole casein (see Figure 1 for gel) in the presence of 4 M urea with a sodium chloride gradient from 0 to .3 M starting after fraction 7 (solid line). The dotted line shows the elution of the glycosylated and nonglycosylated κ -casein peaks as detected by RID. The solid line the A_{280} for the caseins.

the salt gradient represent κ -casein (25), and, furthermore, under these conditions the first

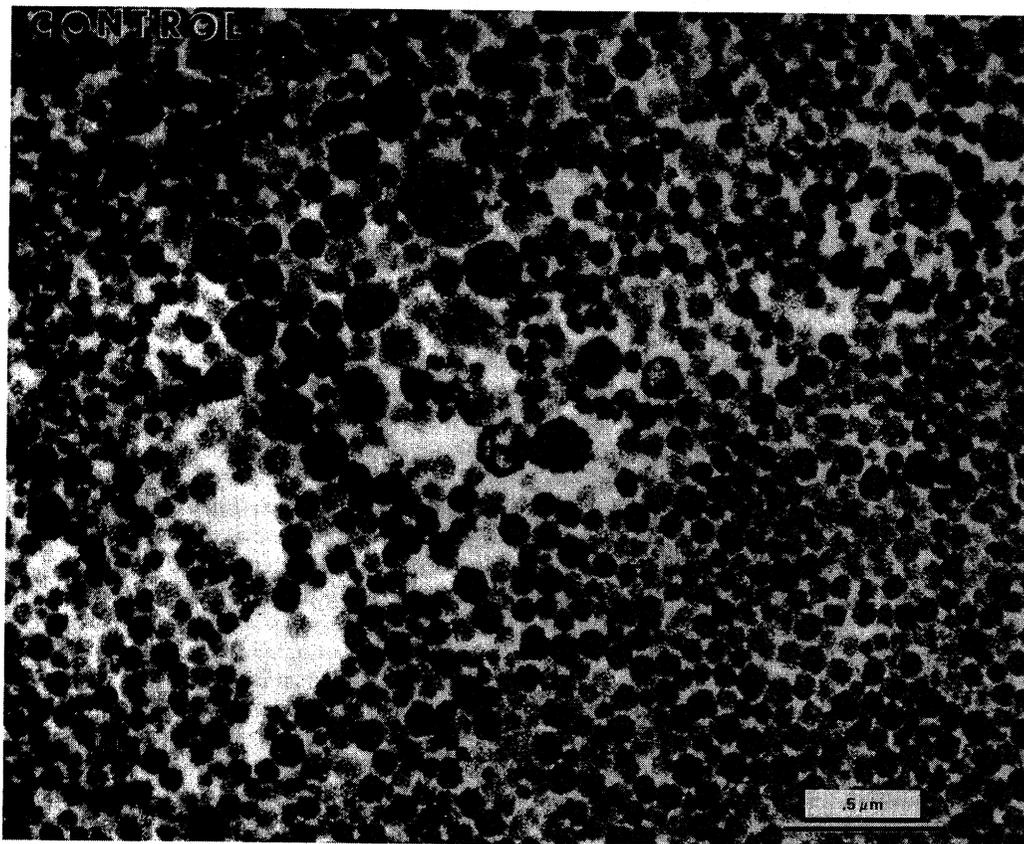


Figure 3. Ultrathin-sectioned casein micelles (conventionally fixed) showing a wide size distribution.

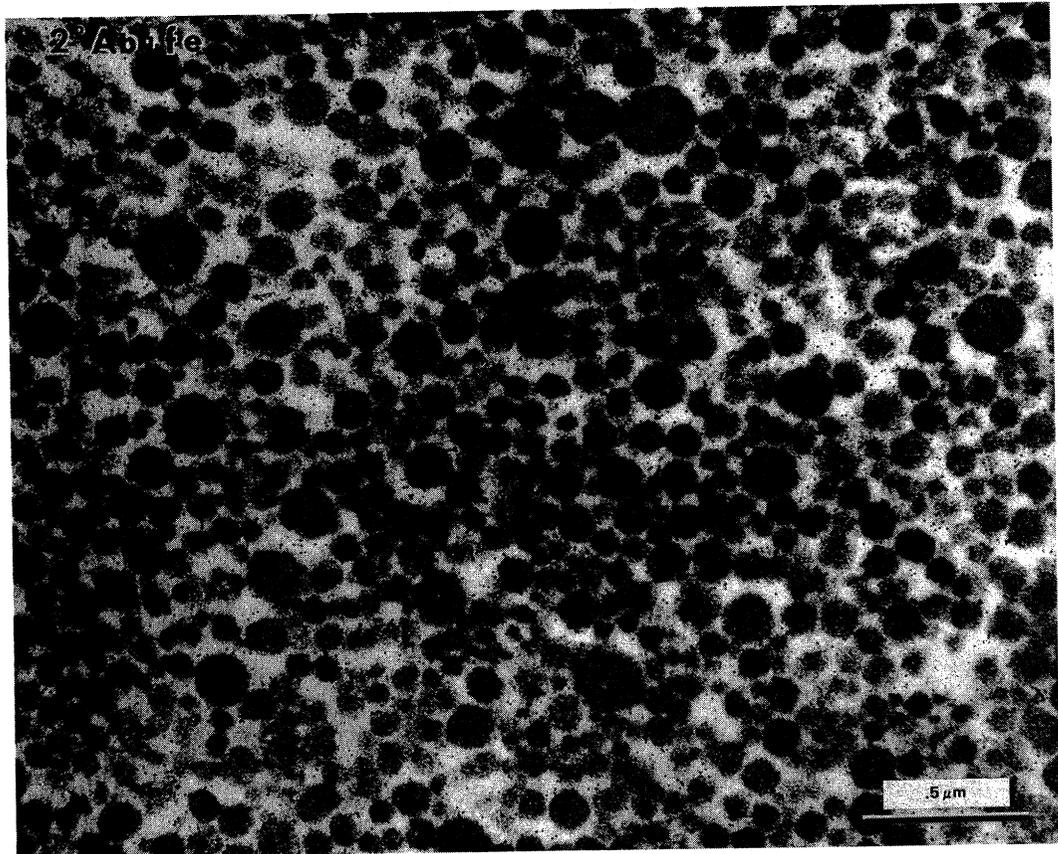


Figure 4. Ultrathin-sectioned casein micelles (underfixed) reacted with secondary antibody (2° Ab) only. Some nonspecific binding is observed.

peak of κ -casein contains predominately nonglycosylated protein whereas successive peaks contain various glycosylated forms of the κ -casein (18, 22, 25). Radial immunodiffusion (RID) studies of each fraction showed that the antiserum used in this work reacts quantitatively with both glycosylated and nonglycosylated κ -caseins, as the profile by immunoassay agrees with the expected elution profile of κ -casein (25). The rabbit anti-bovine κ -casein was used without further purification and is termed the primary antibody (1° Ab).

Ferritin-labeled IgG fraction of goat anti-rabbit heavy and light chain IgG, which was obtained commercially, was passed through a 450-nm filter and chromatographed in HBS on Sephadex S-300; a heterogeneous profile was obtained. The peaks eluting at the void volume and near the tail were eliminated and the mid-

dle portions pooled. These fractions were examined in the electron microscope to show that when spread out they yielded a uniform nonaggregated preparation. Also, these fractions by double immunodiffusion contained both goat IgG and horse ferritin. These pooled fractions then were passed through sterile 450-nm filters into sterile tubes and stored at 5°C . This preparation of ferritin-conjugated goat anti-rabbit IgG is referred to as secondary antibody (2° Ab).

Ultrathin sections of conventionally fixed casein micelles were observed in the electron microscope (Figure 3) and, in general, were similar to those reported by Shimmin and Hill (20). Structural differences were small between these standard micelles and micelles obtained by the fixation method of Weigand and Russo (26). Sections were roughly 600 \AA thick, and

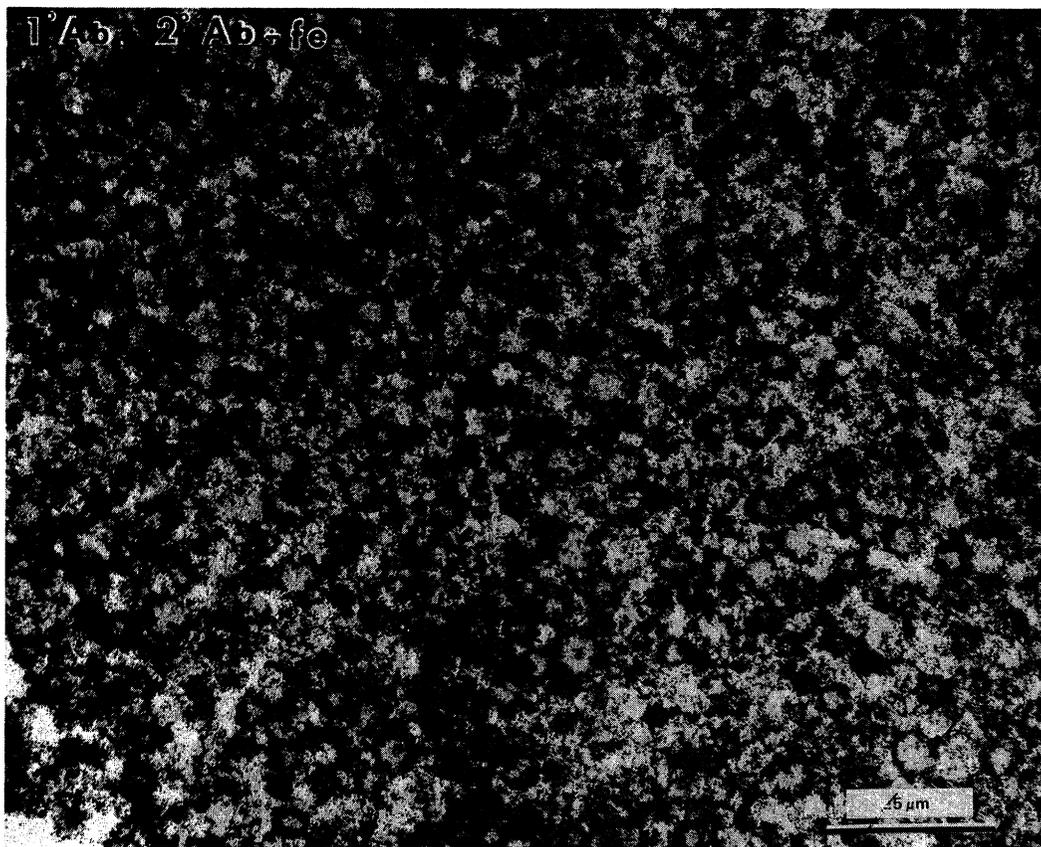


Figure 5. Ultrathin-sectioned casein micelles (underfixed) treated with the primary antibody (1° Ab) followed by the secondary antibody (2° Ab) increased density of the label is observed.

measured sizes of micelles ranged from $\sim 300 \text{ \AA}$ to $\sim 2500 \text{ \AA}$, which agrees with the overall size distribution of casein micelles (1).

Figure 4 shows results of one of the experiments on "underfixed" micelles. The 2° Ab was applied without the primary antibody; all other steps were carried out. Distribution of ferritin is sparse over the entire section. This could be considered the background and shows minimal nonspecific binding of the 2° Ab by itself; chromatographic purification of 2° Ab was necessary to achieve high quality controls. Another control used 1° Ab alone; because no electron-dense marker was attached to the 1° Ab, this control was completely negative.

Results when both 1° and 2° Ab were reacted with sectioned micelles are in Figures 5 and 6. As evidenced by the tremendous increase of deposition of the electron-dense ferritin

marker, the reaction appeared to work well. Close inspection of the micrographs revealed micelles with ferritin over the entire cross-section; however, many micelles have ferritin deposited only at the periphery of the micelles or between micelles. These observations of the labeled micelles allowed us subjectively to place the micelles into two easily distinguishable classes: 1) uniformly and 2) peripherally stained micelles. Diameters of the micelles in these two classes then were measured in a single blind experiment (total counted = 200). Those with ferritin deposited over the entire cross-sectional area had a diameter of $920 \pm 220 \text{ \AA}$ whereas micelles with the ferritin label only at the periphery, giving a doughnut-like appearance, had a diameter of $1460 \pm 320 \text{ \AA}$. Sectioned micelles are shown at higher magnification in Figure 6, which depicts the micelle-

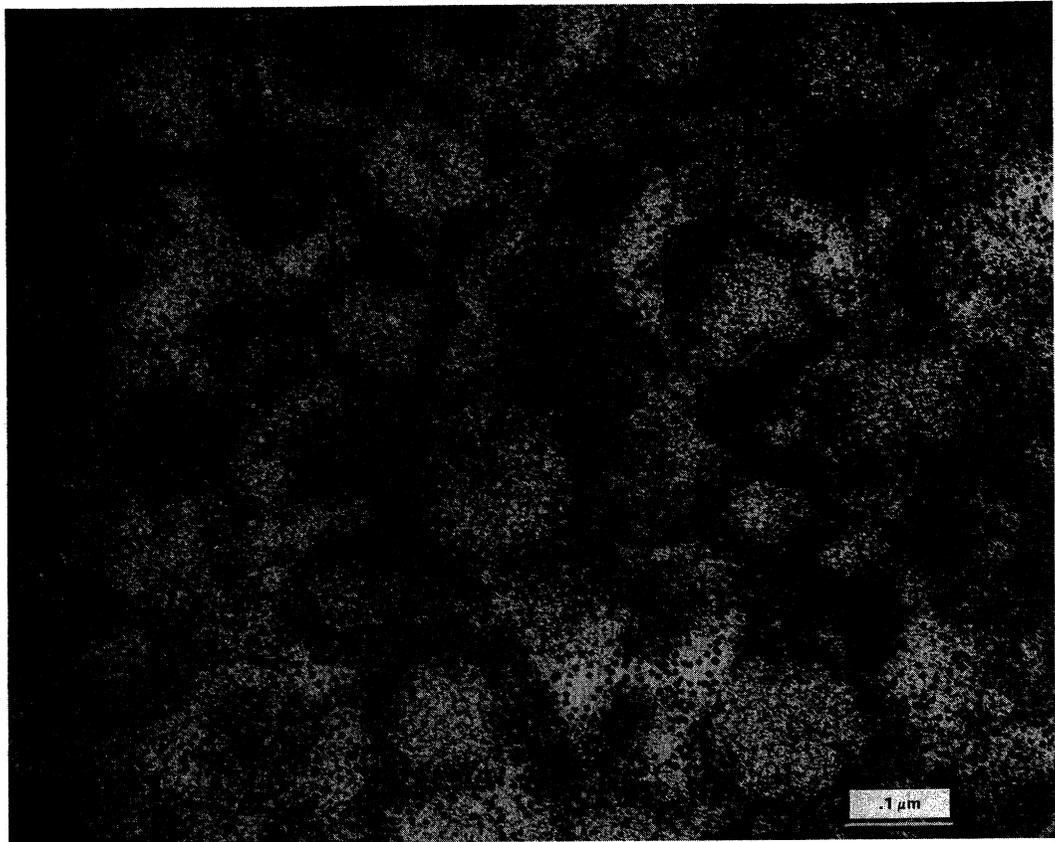


Figure 6. Higher magnification of ferritin-labeled micelles as in Figure 5.

ferritin complex in greater detail. Both types of ferritin-labeled micelles are visible in this micrograph. Results were similar with micelles that were centrifuged first from skim milk at $100,000 \times g$ and resuspended in a simulated milk salt solution.

In the following discussion, it is assumed that the ferritin marker does not penetrate the sections to any great extent; that is, ferritin will react only with exposed surfaces. The control (Figure 4) bears this out. The larger casein micelles in Figures 5 and 6 are definite sections as their average diameter is over two times the section thickness. Here, because ferritin appears primarily at the periphery, κ -casein must occupy a surface site in these casein micelles. For the smaller group, whose average diameter is only 1.5 times the section thickness, the ferritin appears to be uniformly distributed. For

these micelles, the data could reflect equatorial sections of small micelles, tropical sections of mid-sized micelles, or polar caps of large micelles. In the first two cases, uniform staining ought to represent a uniform distribution of κ -casein, and in the last case, peripheral staining of caps would simulate a uniform distribution. It would be unlikely, however, that all of the particles in this class would represent only polar caps. Thus, an argument can be made for uniform distribution of κ -casein in this class of micelles that are sectioned partly and are smaller. This, however, does not imply that casein micelles exist in only two distinct classes. Micelles were, after all, placed into these classes subjectively. Most logically, as shown by McGann et al. (10), there is a smooth function that relates micelle size to κ -casein content and perhaps also to its location within the micelle.

In this discussion we are dealing only with the readily observable extremes.

Parry and Carroll (13) attempted to react intact, fixed casein micelles with ferritin conjugated directly to rabbit anti- κ -casein. They found no κ -casein on the surface of the micelles but did detect κ -casein between micelles. The condition used to fix the micelles was 1% glutaraldehyde for 15 min. For red blood cells (23), this type of fixation decreases the ability of the antigen to react with antibody by formation of surface cross-linkages. Presumably one reason for the lack of detection (13) of surface κ -casein would be overfixation. Horrisberger and VonLanthen (8) reported on the use of gold-labeled lectin to locate glycosylated κ -casein in the casein micelle. No evidence was found for glycosylated κ -casein on the surface of the casein micelle, but κ -casein was located on the bridging network interconnecting the micelles. Again the question of fixation time and conditions could play a role here. These workers used .25% glutaraldehyde for 15 h at 20°C. The method employed by us uses a mixture of .05% glutaraldehyde and 1% paraformaldehyde for only 15 min as recommended by Weigand and Russo (26) for best preservation of reactive groups.

Kudo et al. (9) used periodic acid-silver methenamine to stain the carbohydrate groups of κ -casein in thin sections. They reported that after 60 min the stain was uniformly distributed over the entire cross-sectional area of all micelles. However, they also found with only 10 min staining that the silver stain was located at the periphery of the larger micelles (>2200 Å), but on micelles less than 1000 Å the stain was evenly distributed throughout the cross-sectional area. They interpreted their findings to mean that κ -casein is distributed throughout the entire micelle, but the more highly glycosylated molecules of the protein are located on the outer portions of the particle. However, this interpretation would place a considerable amount of glycosylated protein inside of the casein micelle. A problem with their results could be, as admitted by the authors, lactose. Calculations on the recent data of Slattery (18) for the κ -casein content of various sizes of micelles show that Kudo and coworkers (9) may have overestimated the amount of glycosylated κ -casein, and, hence, the trapped lactose would account for the overstaining observed,

especially at their longer reaction times (see Appendix for calculation).

With the antibodies in this study, it has been shown by combination of column chromatography and RID, that all fractions of κ -casein, both with and without carbohydrate, are reactive with the antiserum. By this approach, all types of κ -caseins are tagged and can be located by electron microscopy. Both the silver methenamine (9) and gold lectins (8) would fail to detect nonglycosylated κ -casein, which accounts for approximately half of the protein (18).

Larger micelles have κ -casein predominately on their periphery; the size of these micelles averaged 1420 Å. Based on average size of the casein micelle \sim 1400 Å (1), by this approach, roughly half of the micelle population would contain κ -casein only at the periphery, whereas smaller micelles may have κ -casein more evenly distributed. The inverse relationship between κ -casein content and micelle size, first shown by Sullivan et al. (21), has been confirmed recently by Slattery (18) and by McGann et al. (10). Our results show that the larger micelles, which contain proportionately less κ -casein, have this protein located predominately at the surface for stabilization. Conversely, smaller micelles, which have elevated κ -casein contents, have this protein more evenly distributed. Given that intermediate cases of κ -casein distribution exist, the data here are in accord with previous data on κ -casein content (10, 18, 21). Finally, results of this study are in accord with the role of κ -casein in submicelle interactions (14), and are supportive of the model for casein micelle structure proposed by Slattery and Evard (19).

ACKNOWLEDGMENTS

The authors would like to thank J. K. Farrell for his careful measurement of the casein micelles.

APPENDIX

Following the data reported by Kudo et al. (9), if we assume a casein content of 3%, then .2 ml of skim plus 2.0 ml of fixative, yields 2.72 mg casein/ml, and a further 1:10 dilution of this yields 272 μ g casein/ml. Using averages from Slattery's work (18) (\sim 15% κ -casein, glycosylated at 5%), the figure of 272 μ g casein/ml reduces to 2.05 μ g of protein-bound

carbohydrate/ml. Conversely, Kudo et al. (9) report .880 mg of lactose/ml at this stage. Therefore, their results show more trapped lactose than glycoprotein, accounting for increased positive strains at 60 min for all micelles.

REFERENCES

- 1 Carroll, R. J., M. P. Thompson, and G. G. Nutting. 1968. Glutaraldehyde fixation of casein micelles for electron microscopy. *J. Dairy Sci.* 51:1903.
- 2 Crowle, A. J. 1973. Page 226 *in* Immunodiffusion. 2nd ed. Academic Press, New York, NY.
- 3 Douglas, F. W., Jr., R. Greenberg, H. M. Farrell, Jr., and L. F. Edmundson. 1981. Effects of ultra-high-temperature pasteurization on milk proteins. *J. Agric. Food Chem.* 29:11.
- 4 Farrell, H. M., Jr., and M. P. Thompson. 1974. Physical equilibria: proteins. Page 442 *in* Fundamentals of dairy chemistry. 2nd ed. B. H. Webb, A. H. Johnson, and J. A. Alford, ed. Avi Publ. Co., Westport, CT.
- 5 Farrell, H. M., Jr. 1973. Models for casein micelle formation. *J. Dairy Sci.* 56:1195.
- 6 Garnier, J., and B. Ribadeau Dumas. 1970. Structure of the casein micelle, a proposed model. *J. Dairy Res.* 37:493.
- 7 Guidry, A., and R. E. Pearson. 1979. Improved methodology for quantitative determination of serum and milk protein by single radial immunodiffusion. *J. Dairy Sci.* 62:1252.
- 8 Horrisberger, M., and M. VonLathen. 1980. Localization of glycosylated κ -casein in bovine casein micelles by lectin-labelled gold granules. *J. Dairy Res.* 47:185.
- 9 Kudo, S., S. Iwata, and M. Ma. 1979. An electron microscopic study of the location of κ -casein in casein micelles by periodic acid-silver methenamine stain. *J. Dairy Sci.* 62:916.
- 10 McGann, T.C.A., W. J. Donnelly, R. D. Kearney, and W. Buchheim. 1980. Composition and size distribution of bovine casein micelles. *Biochim. Biophys. Acta* 630:261.
- 11 McKenzie, H. A., and R. G. Wake. 1961. An improved method for the isolation of κ -casein. *Biochim. Biophys. Acta* 47:240.
- 12 Morr, C. V. 1967. Effect of oxalate and urea upon centrifugation properties of raw and heated casein micelles. *J. Dairy Sci.* 50:1744.
- 13 Parry, R. M., Jr., and R. J. Carroll. 1969. Location of kappa casein in milk micelles. *Biochim. Biophys. Acta* 194:138.
- 14 Pepper, L., and H. M. Farrell, Jr. 1982. A study of monomer interactions leading to the formation of casein submicelles. *J. Dairy Sci.* 65:2259.
- 15 Rose, D. 1969. A proposed model of micelle structure in bovine milk. *Dairy Sci. Abstr.* 31:171.
- 16 Schmidt, D. G., and T.A.J. Payens. 1976. Colloidal aspects of casein. *In* Surface and colloid science. Vol. 9. E. Matijevic, ed. Wiley and Sons, Inc., New York, NY.
- 17 Slattery, C. W. 1979. A phosphate-induced submicelle-micelle equilibrium in reconstituted casein micelle systems. *J. Dairy Res.* 46:253.
- 18 Slattery, C. W. 1978. Variation in the glycosylation pattern of bovine κ -casein with micelle size and its relationship to a micelle model. *Biochemistry* 17: 1100.
- 19 Slattery, C. W., and R. Evard. 1973. A model for the formation of casein micelles from subunits of variable composition. *Biochim. Biophys. Acta* 317: 529.
- 20 Shimmin, P. D., and R. D. Hill. 1964. An electron microscopic study of internal structure of casein micelle. *J. Dairy Sci.* 31:121.
- 21 Sullivan, R. A., M. M. Fitzpatrick, and E. K. Stanton. 1959. Distribution of kappa casein in skim milk. *Nature* 183:616.
- 22 Swaisgood, H. 1975. Primary sequence of kappa casein. *J. Dairy Sci.* 58:583.
- 23 Tokuyasu, K. T., and S. J. Singer. 1976. Improved procedures for immunoferritin labeling of ultrathin frozen sections. *J. Cell Biol.* 71:894.
- 24 Waugh, D. F. 1971. Formation and structure of casein micelles. Page 3 *in* Milk proteins. Ch. 9. Vol. II. H. A. McKenzie, ed. Academic Press, New York, NY.
- 25 Woychik, J. H., E. B. Kalan, and M. E. Noelken. 1966. Chromatographic isolation and partial characterization of reduced κ -casein components. *Biochemistry* 5:2276.
- 26 Weigand, R. A., and J. Russo. 1977. Immunoferritin labeling of intracellular murine mammary tumor virus after thin sectioning for electron microscopy. Page 378 *in* Proc. 35th Annu. Mtg., Electron Microsc. Soc. Am.