Internal Structure of Casein Micelles from Bovine Milk

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Abstract

The internal structure of casein micelles from bovine milk has been reinvestigated. Observations from electron microscopy, X-ray diffraction, and enzymatic digestion of the micelles followed by centrifugation suggested that electron-dense particles in the micelles are probably granules of colloidal calcium phosphate and not protein aggregates. These granules are embedded in a surrounding matrix of caseins to form the micelle. The foci of formation of the calcium phosphate granules may be either the ester phosphate groups or the ϵ-amino groups of lysine.

The internal structure of casein micelles is not easily seen in the electron microscope, but Shimmin and Hill (12) have published photographs indicating the presence of electron-dense, approximately spherical particles, about 100 A in diameter, embedded in the matrix within the micelle. They interpret these photographs as evidence for the presence of electron-dense protein aggregates of about 300,000 molecular weight in the micelles, but do not discuss the composition of the matrix. However, the presence of colloidal calcium phosphate in casein micelles (9), the high electron-density of the particles, and comparison of Shimmin and Hill's electron micrographs with those from calcifying collagen (3) suggest that the electron-dense particles may be composed of calcium phosphate, embedded in a matrix of protein. We have, therefore, attempted to identify the material which forms the electron-dense particles by a) X-ray diffraction studies of casein micelles with and without colloidal calcium phosphate, b) comparative electron microscopy of casein micelles with and without colloidal calcium phosphate, and c) enzymatic digestion of the protein components of the micelles, sedimentation of the remaining particles, and analysis of the resulting pellet.[1]

Materials and Methods

For examination by X-ray diffraction, samples of fresh skimmilk and of milk from which colloidal calcium phosphate had been removed (see below) were added drop by drop to liquid nitrogen. The frozen pellets were then dried under vacuum and the friable, powdery residues packed as firmly as possible into 1-mm glass capillary tubes especially manufactured for X-ray diffraction. Wide-angle X-ray diffraction patterns were obtained from the samples with a Hilger-Watts microfocus instrument and a North American Philips micro-camera. With nickel-filtered copper Kα radiation, the exposure times were 18-23.5 hr at 40 kv and 350-400 μa.

For electron microscopy, the micelles in fresh skimmilk from a Holstein herd were fixed by osmium tetroxide and progressively dehydrated, according to the method of Shimmin and Hill (12) (in some tests the crystal of osmium tetroxide was put directly into the milk). Following dehydration, aliquots of the sample were embedded in methacrylate, in Epon, or in Araldite by standard methods (7). Sections were cut with a glass knife on a Cambridge ultramicrotome (A. F. Huxley pattern) and supported on holey carbon films for examination in the electron microscope. The instrument used was a Philips Model 100 C.

Colloidal calcium phosphate was removed from milk by the method of Pyne and McGann (10) and micelles of this milk were prepared for examination in the electron microscope, as above.

In a further test, sections of milk micelles were prepared for electron microscopy, then the grids carrying these sections were immersed in a phosphate-citrate buffer, pH 6.0, for 1 or 2 hr (0.01 M in phosphate; 0.003 M in citrate ion) and rinsed in distilled water. This procedure should dissolve exposed calcium phosphate, but it is important to remember that it will not dissolve calcium phosphate which is wholly surrounded by the embedding medium nor will it dissolve protein impregnated with the embedding medium.

To obtain protein-free calcium phosphate particles from milk, fresh normal skimmilk was digested by papain and alkaline phosphatase as follows: Bacterial alkaline phosphatase (0.2 ml, Worthington Biochemical Corporation BAP-C6137) and a suspension of crystals of papain (1 ml, Nutritional Biochemical Corporation 1-196R) were added to 25 ml of fresh

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[1] The possibility that these particles were osmium-stained fat was excluded by analyses of freeze-dried and ethanol-dried micelles; less than 0.1% fat was present.

skimmilk. To the mixture, 0.5 g of cysteine hydrochloride was added gradually, while maintaining the original pH of the milk, in order to activate the papain. The complete suspension was then placed in a Visking cellulose dialysis sac and dialyzed for 3 hr at 35 C against 1 liter of the same skimmilk, to maintain the pH and reduce the concentration of products of digestion which might have reduced the activity of the enzymes. After digestion, the sample was centrifuged overnight at 105,000 *g* (Spinco no. 40 head, 40,000 rpm) and the pellet collected for examination in the electron microscope and for an ash determination.

For observations on the size and shape of whole micelles in individual milks, the samples of fresh skimmilk were diluted 1 vol to 50 of 0.01 M $CaCl_2$ and glass microscope slides dipped into the suspension. After drying in a vertical position on filter paper, the surface of the slide covered with the dried residues was dipped into 0.4% formvar in ethylene dichloride and the film allowed to dry. The formvar films were stripped from the slide to the surface of 10% aqueous ammonium hydroxide, the solution under the film replaced by distilled water until the pH was reduced to 6, the films removed, mounted, and shadowed by standard techniques (7).

Results

X-ray diffraction. X-ray diffraction patterns obtained from the normal skimmilk sample and the colloidal-phosphate-free sample after exposure for 18 and 23.5 hr, respectively, are shown in Figure 1. Two (perhaps three) faint reflections are discernible in the pattern from the normal skimmilk sample which are not present in the pattern from the colloidal-phosphate-free milk. However, the greater part of the scattering from both samples is clearly due to amorphous material, such as the protein of the micelles or the lactose of the milk serum. Colloidal calcium phosphate is present to the extent of only 4 to 6% of the casein micelles (2), therefore represents less than 2% of the dried material in these samples. In addition, fresh, hydrated (uncalcined) calcium phosphate or apatite is known to give diffuse X-ray reflections (1). Lack of a distinct difference between the pattern for normal milk and colloidal-phosphate-free milk probably results from these factors, and therefore gives no indication of the crystalline form or size of the colloidal calcium phosphate in the micelles of normal milk.

Electron microscopy of sections of milk micelles. Casein micelles were readily observed in all milk preparations studied, but they all had ill-defined edges, particularly in very thin sections (Figure 2, bottom), and only a moderate electron-density. Close examination of micelles embedded in Epon or Araldite gave some evidence of an internal structure consisting of electron-dense particles and a less dense matrix, but contrast between particles and matrix was not great, and the size range of the particles was such that they blend into the normal granulation of an enlarged photograph. However, particles approaching 100 A in diameter were observed (arrows in Figure 2). No such particles were observed in micelles embedded in methacrylate.

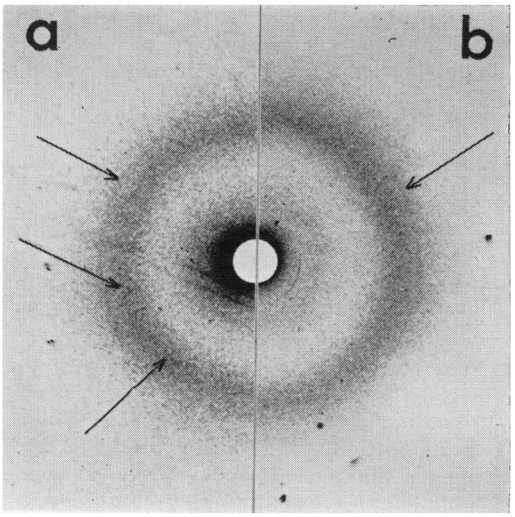

FIG. 1. Debye-Scherrer X-ray diffraction powder diagrams of a) lyophilized normal skimmilk, exposure 18 hr; b) lyophilized colloidal-phosphate-free skimmilk, exposure 23.5 hr.

Examination of micelles from which the calcium phosphate had been removed failed to give unequivocal evidence concerning the existence or nature of the electron-dense particles. Contrast between the micelles and background was less in these electron micrographs (Figure 3) than in those of normal milk (Figure 2), and the edges of the micelles were even less distinct. Granulation (electron-dense particles?) was much less pronounced in these micelles than in those of normal milk, but was present to a greater extent than can be explained on the basis of residual colloidal calcium phosphate.

Sections prepared for electron microscopy, then washed in phosphate-citrate buffer (cf. Methods) appeared quite similar (Figure 4) to those of normal casein micelles (Figure 2). However, close examination of these photographs reveals the presence of both electron-

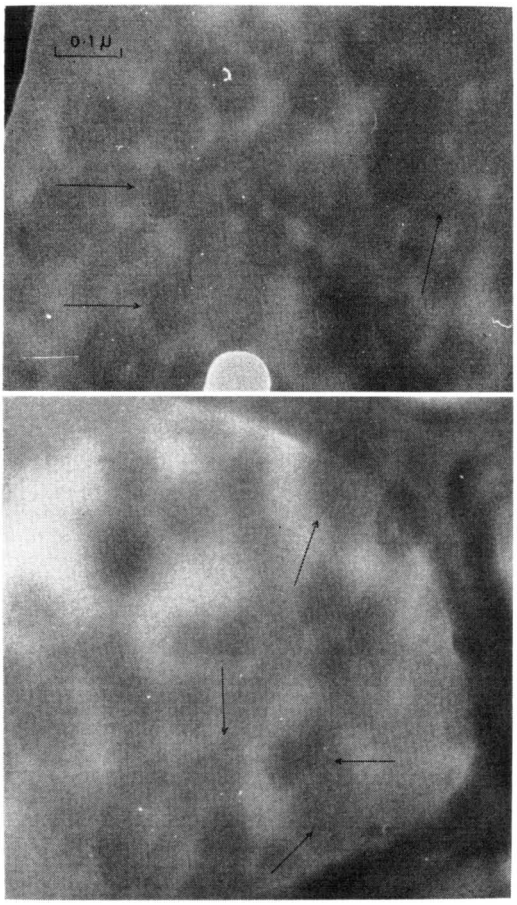

FIG. 2. Thin sections of different thickness of normal milk micelles fixed in osmium tetroxide and embedded in Epon. Note the small electron-dense particles within the micelles, the lack of a definite boundary to the micelles, and the electron-density of the matrix surrounding the small particles. Note also that the relative contrast of the particles to the matrix is no better in the lower photograph (thinner section) than in the upper photograph (thicker section). The arrows point to groups of particularly distinct particles.

dense particles (Figure 4, *a* arrows) and of spots having less electron-density than the surrounding matrix (Figure 4, *b* arrows). This suggests that some exposed, particulate material, presumably the electron-dense particles, has been dissolved by the buffer, leaving empty spaces in the embedding medium (Epon).

Since the insoluble calcium phosphate content of milk can be increased by raising the pH of the milk, it appeared possible that some of the calcium phosphate thus formed might appear as a surface deposit on the micelles. The micelles from milks adjusted to pH 8.0 with sodium or calcium hydroxide were, therefore, fixed in osmium tetroxide, according to the

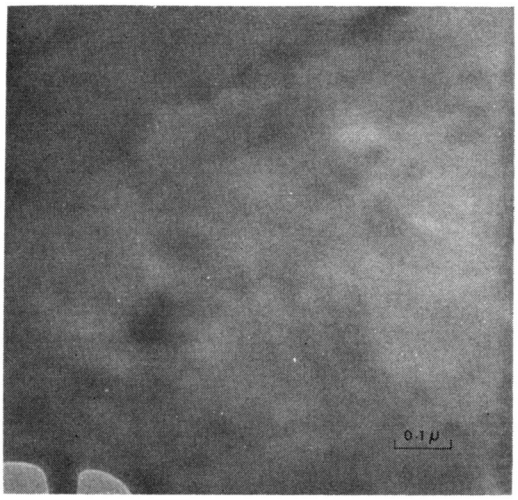

FIG. 3. A thin section of Epon-embedded colloidal phosphate-free milk micelles. Note the lack of contrast of the micelles as compared to those in Figure 2, and the more uniform distribution of the electron-scattering power of the material within the micelles.

method of Shimmin and Hill (12), embedded in Epon, and examined. The electron micrographs showed no evidence of any deposit on the micelle surfaces and no evidence for the site of deposition of the additional calcium phosphate.

Attempts to isolate the electron-dense particles. If caseinate micelles consist of electron-dense particles of colloidal calcium phosphate,

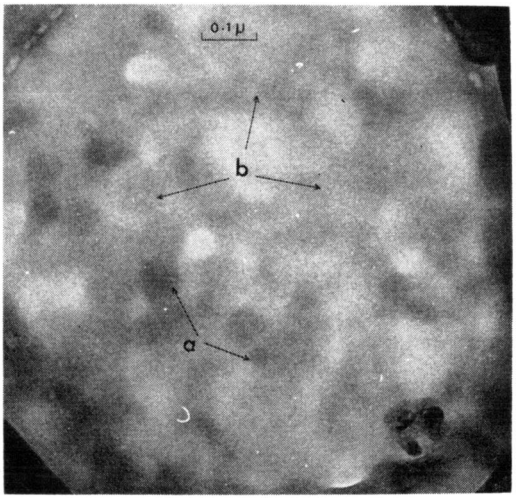

FIG. 4. A thin section of Epon-embedded, normal milk micelles immersed in phosphate-citrate buffer for 2 hr, then rinsed with distilled water. Note the decrease in average contrast of the micelles, some remaining electron-dense particles a, and many apparent holes in the micelles b.

dispersed in a matrix of protein, digestion of the casein by suitable enzymes, without changing other conditions of the suspension, should release the particles and permit their sedimentation. Accordingly, the casein was digested by papain and bacterial alkaline phosphatase (to hydrolyse ester phosphate groups that might link colloidal calcium phosphate to peptide chains and thus hinder its sedimentation) and the mixture centrifuged. The pellet had a clear, gelatinous appearance similar to that of freshly prepared tricalcium phosphate. After washing once in cold 0.1 M calcium chloride, it had an ash content of 49.3% and the ash had a phosphate ($PO_4^{\equiv}$) content of 52.3%. Electron micrographs of a dispersion of the unwashed pellet showed that it was grossly heterogeneous (Figure 5), but that electron-dense particles of about 100 A diameter could be distinguished occasionally. Since extensive consolidation of the gel probably occurred during sedimentation, the presence of even a few granules resembling those in milk may be significant.

Electron microscopy of whole milks. Photographs of shadowed replicas of whole micelles from two different milks are shown in Figure 6. These two photographs illustrate the wide differences which have been observed in the external form and surface of milk micelles from different cows.

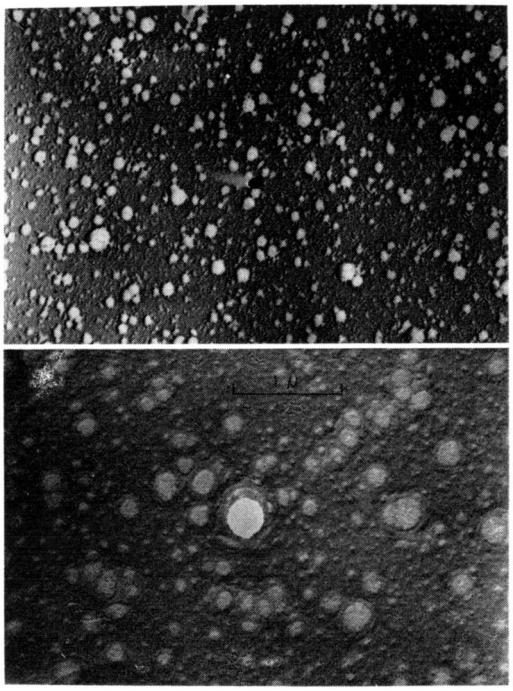

FIG. 6. Photographs of replicas of whole micelles from two different milks. Note the contrast between these micelles in apparent height and firmness and the presence of an outer fringe of less electron-dense material around the micelles of one milk.

Discussion and Conclusions

Although our electron micrographs of micelles from milk appear quite different from those published by Shimmin and Hill (12), they confirm the observation that caseinate micelles contain some electron-dense particles embedded in a less dense matrix. We are inclined to attribute the less well-defined edges of the micelles observed by us, as compared to those by Shimmin and Hill (12), to a variation in the fine structure of the milks. Even wider variations have been observed when milks from individual cows were studied at lower magnification (Figure 6). The smaller size and lesser electron-density, relative to the matrix, of the particles observed by us may also result from differences in the conditions existing in different milks. Variations in composition between milks are often quite marked, and if such differences exist during the period when calcium phosphate is precipitating they could markedly affect the physical parameters of a sensitive colloidal system. However, the differences between our own and Shimmin and Hill's (12) photographs could also be caused by unrecognized differences in the fixing, dehydrating, and photographic techniques. Conclusions in this regard must await the examination of a number of individual milks under a variety of conditions.

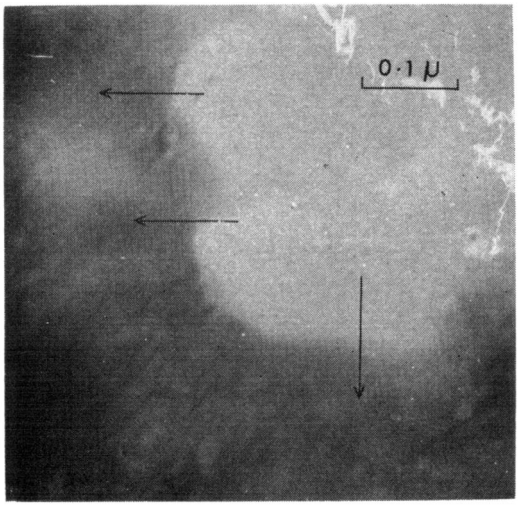

FIG. 5. Dispersion of the gelatinous pellet found in the centrifuge tube, after digestion of the casein. Note the granules of electron-dense material in some places (arrows).

An unequivocal conclusion as to the composi-

tion of the electron-dense particles cannot be reached at present, but it appears reasonable to assume that they are granules of calcium phosphate and not protein aggregates. Evidence for this assumption is found in the lesser density and decreased granulation of micelles from which colloidal calcium phosphate had been removed (Figure 3), the appearance of electron transparent spots in sections washed in buffer to dissolve exposed calcium phosphate (Figure 4), and the sedimentation of a calcium phosphate (containing a few electron-dense particles) after extensive enzymatic digestion of the protein (Figure 5). This assumption is also supported by the similarities between the particles observed in milk and those of calcium phosphate granules in calcifying collagen, bone and teeth (3), and in mitochondria (4). On the other hand, the presence of some electron-dense particles in our electron micrographs of micelles from which calcium phosphate had been removed (Figure 3) suggests that some of the granulation arises from another source or is an artifact.

On theoretical grounds, the two most probable loci for precipitation of calcium phosphate within a casein micelle appear to be the ester phosphate groups (11) and the ϵ-amino groups of lysine (3, 5). If the ester phosphate groups add calcium and phosphate to form colloidal calcium phosphate, it must be assumed that, apart from possible steric effects caused by folding of the molecule, all such groups [five per mole in β-casein (8), 12 per mole in α_{s1}-casein (6)] will do so more or less equally. If so, the resultant colloidal particles, which constitute only 4 to 6% of the micelles (2), will be too small for observation by electron microscopy. Such particles would, however, contribute to the over-all electron-density of the micelles.

Evidence concerning the relation between ϵ-amino groups and calcium phosphate particles in collagen and bone (3) suggests that several ϵ-amino groups must be suitably oriented before they can act as a crystallization center. There are numerous lysine residues in casein [12 per mole in β-casein (8), 17 per mole in α_{s1}-casein (6)], but they are probably randomly dispersed within the micelle and, therefore, would form few loci for calcium phosphate precipitation. Consequently, if ϵ-amino groups form the loci for precipitation, relatively few particles should form and these could be sufficiently large for observation by electron microscopy.

If we assume that the electron-dense particles observed by Shimmin and Hill (12) are calcium phosphate, then their results strongly suggest that only a limited number of loci have induced calcium precipitation. Our own results, on the other hand, are subject to either interpretation. Some fairly large particles of calcium phosphate were probably observed (and remained intact after enzymic digestion of the protein), but interpreting all of the granulation in these photographs as being caused by particulate calcium phosphate is not prudent at present. There also seems to have been an over-all loss of electron-density, conformable with a loss of highly dispersed calcium phosphate, in the micelles from which calcium phosphate was removed.

Acknowledgments

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