

Correlation of Refined Models for Casein Submicelles with Electron Microscopic Studies of Casein

To enhance understanding of the milk protein system, an energy minimized three-dimensional (3-D) model of a putative casein submicelle has been constructed using monomeric, κ -, α_{s1} - and β -casein 3-D models. Docking of one κ - and four α_{s1} -casein molecules produced a framework structure whose external portion is composed of the hydrophilic domains of α_{s1} - and κ - and whose central portion contains two large hydrophobic cavities. Preformed symmetric and asymmetric dimers (formed by docking the hydrophobic C terminal regions of two β -casein molecular models) could easily be placed into the two central cavities on the κ -, α_{s1} - framework yielding two plausible energy minimized 3-D structures for submicellar casein. To test these two 3-D structures, theoretical computer generated topographical models were compared to experimental electron microscopic data. Good comparisons of the shapes of the 3-D models with those of the images generated by electron microscopy were achieved for both the symmetric and asymmetric submicelle models.

Introduction

The caseins of milk form a unique biocolloid (the casein micelle) to efficiently transport calcium, phosphate and protein from the mammary gland to the neonate (1-3). The concept that these casein micelles are composed of submicelles has developed from both physical-chemical data and from observations on micelle bioassembly by electron microscopy (1-3). To aid in further elucidation of the structure of the casein micelle, previous reports from this laboratory have produced three-dimensional models refined via energy minimization techniques for κ -casein (4), α_{s1} -casein (5) and β -casein (6). Using these models of monomeric caseins, it was possible to build an energy minimized submicelle structure minimally composed of one κ -casein with four α_{s1} -caseins and four β -caseins via plausible docking sites consistent with solution physical-chemical, biochemical and chemical information (7,8). In the construction of this model, β -casein monomers were added in two ways to complete the minimum submicellar structure. Both of these structures (7,8) were in agreement with the global secondary structure determined via Raman and Fourier transform infrared (FTIR) studies and with data from small-angle X-ray scattering (SAXS).

Comparisons of the putative submicelle models with experimental data can be used to test and improve these working models. In this work, high resolution

transmission electron microscopy (TEM) results for reduced and carboxyl methylated whole casein will be compared with Van der Waals dot surfaces calculated for two energy minimized submicelle 3-D structures using a density of 1.0 dot/0.01 nm². Such comparisons will be used to further test and refine the working models of the casein submicelles.

Methods and Materials

Materials

Whole casein typed α_{s1} -BB, β -AA, κ -AA was prepared as previously described (9). The casein was reduced and alkylated in 8 mol/L urea essentially by the method of Schechter *et al.* (10). Samples, however, were dialyzed exhaustively in the dark at 4°C for 3 d against distilled, deionized water. The samples were adjusted to pH 7.0, centrifuged at 20°C, 100,000 × g for 20 min to remove aggregated material and lyophilized. Amino acid analysis showed >96% conversion to S-carboxymethyl cysteine. Polyacrylamide gel electrophoresis confirmed the absence of polymeric κ -casein bands (11). Samples of the reduced carboxyl-methylated (RCM) casein were prepared for electron microscopy by dissolving the casein in PIPES-KCl buffer (25 mmol/L piperazine-N-N-bis (2-ethanesulfonic acid) pH 6.75 made up to 80 mmol/L KCl). The samples were made up to 30 to 35 mg/mL and were passed through 0.45 μ m filters. The filtrates were adjusted to 25 mg/mL with filtered buffer and equilibrated at 37°C for 30 min.

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Electron microscopy of reduced carboxymethylated whole casein

Thin support films of amorphous carbon were evaporated on strips of cleaved mica and mounted on 400 mesh copper grids. Aliquots (10 μ L) of RCM casein in buffered solution were placed on freshly prepared support films for 30–60 s over a water bath at 37°C, then the sample-side of the grid was washed with a controlled stream of 10 to 15 drops of buffered solution from a disposable Pasteur pipette containing 1% glutaraldehyde at 37°C. This was done to physically stabilize the composition of monomers in the form of submicelles and to trap the equilibrium structures, while reducing the protein concentration to produce a discontinuous monolayer of submicelles. The adsorbed submicelles were washed with a similar controlled stream of 5 to 10 drops of 20 g/L uranyl acetate solution at 37°C for negative staining. Excess uranyl acetate solution was adsorbed from the grid surface into Whatman #1 filter paper, and grids were allowed to air-dry at room temperature.

Images of submicellar structures in randomly selected fields on grids were recorded photographically, at instrumental magnifications of 88,000 \times using a Zeiss Model 10 B electron microscope (Thornwood, NY) operating at 80 KV, and 97,000 \times using a Philips Model CM12 scanning-transmission electron microscope (Rahway, NJ) operating at 60 KV.

Selected images of submicelles in photographic negatives were digitized at a resolution of 15 pixels/nm using a Series 68 television camera (DAGE-MTI, Inc., Michigan City, IA) and sequentially processed using the following steps provided by ImagePro Plus 2.0 software (Media Cybernetics, Silver Spring, MD): (1) brightness was inverted to produce a positive image; (2) low-pass filtered with a 3 \times 3 kernel to reduce the intensity of high spatial frequency components above the effective limit of resolution, of \sim 2.5 nm; and (3) dynamic range of gray scale was maximized by applying a linear histogram equalization function. Finally, images made with a video printer (Seikosha America Inc., Mahwah, NJ) were photographically copied and enlarged.

Molecular modeling techniques

All complex aggregate structures employed the energy minimized casein monomer models which were previously reported by this laboratory (4–6). Aggregates were constructed using a docking procedure on an Evans and Sutherland (St. Louis, MO) PS390 interactive computer graphics display driven by Sybyl molecular modeling software (Tripos, St. Louis, MO) on a Silicon Graphics (Mountainview, CA) W-4D35 processor. The aggregate structures were energy minimized with and without water as previously described (7,8).

Results and Discussion

Energy minimized casein complexes from molecular modeling

The 3-D models used in these experiments were tested

using: (1) derived global secondary structure results from spectroscopic experiments; (2) biochemical and chemical modification results; and (3) SAXS results (7,8). The models exhibiting asymmetric and symmetric arrangements of β -casein dimers are shown in **Figs 1** and **2** respectively. SAXS directly measures geometric parameters such as the radius of gyration of the particle, as well as its volume, its surface area and its electron density. Although the overall shape of the particle can be calculated from these geometric parameters, the specific distribution of voids and surface deformations cannot be detected because of the relatively low resolution of the SAXS experiments which are the average structure of many particles. While these parameters were not specifically calculated in these studies (7,8), the results showed agreement between the calculated and experimental SAXS profiles and were in an acceptable range. The SAXS comparison supported the concept that such geometric parameters derived from experimental SAXS profiles were in agreement with those generated from the 3-D submicellar structure. However, another method is needed which can examine individual particles so as to compare experimental topography with that of the 3-D models.

Comparison with electron microscopy

Traditional methods such as X-ray crystallography and high resolution multi-dimensional proton nuclear magnetic resonance (DNMR) are not possible for the casein system because no individual or submicellar casein sample has been crystallized. In addition, multi-dimensional NMR has been used successfully only on small proteins containing approximately 150 residues or less. Since submicellar casein consists of up to ten monomers of about 200 residues each, any two-dimensional NMR spectrum would contain highly overlapped bands which could not be resolved into individual components even utilizing maximum-entropy Fourier deconvolution techniques.

With this in mind, transmission electron microscopy experiments (TEM) were carried out on whole casein under submicellar conditions for comparison with the 3-D models of the casein submicelles with respect to predicted outlines and surface topology of the individual particles. Although many investigators have previously reported electron micrographs of whole casein under submicellar conditions (12–14) these studies did not take into account the possibility of inter-particle association of the κ -casein through disulfide bonds. In a recent report, Groves *et al.* (11) have shown that κ -casein in whole casein exists as a distribution of polymers ranging from tetramers to octamers caused by intermolecular disulfide bonds. These polymeric κ -casein species could, of course, confuse the interpretation of electron micrographs of whole casein under submicellar conditions for two reasons. First, disulfide bonds could lead to a distribution of larger submicellar particles; second, κ -casein-only polymers which would not be associated with α_s - and β -caseins in

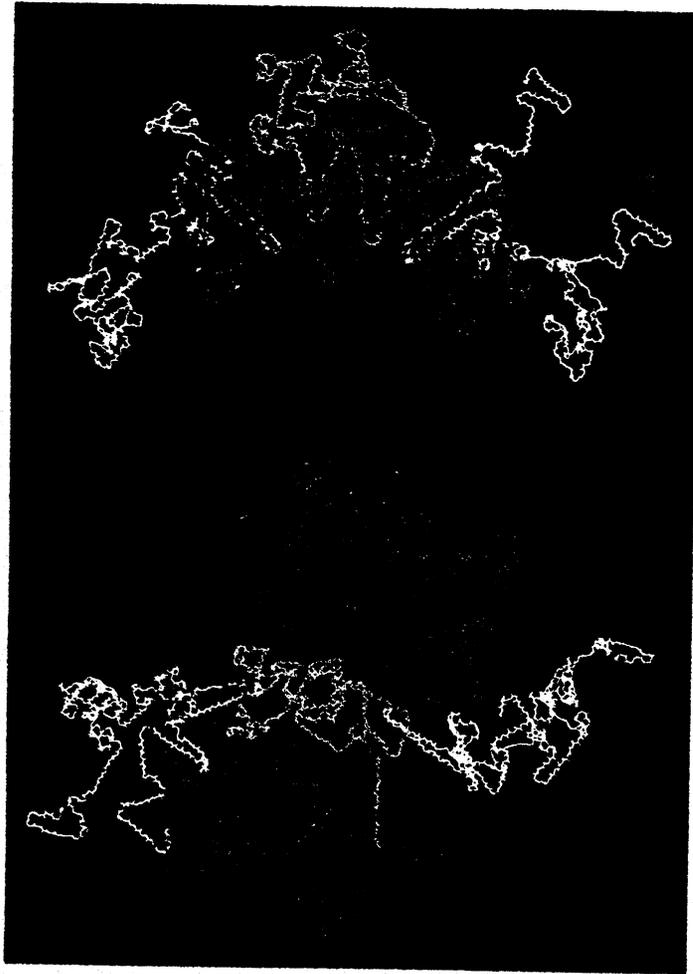


Fig. 1 Energy minimized casein asymmetric submicelle structure, i.e. one α -casein B, four α_1 -casein B and two β -casein A² asymmetric dimers. Orthogonal views of protein backbones without side chains; α -casein B in cyan, α_1 -casein B in red and white; β -casein A² backbone coloured in magenta. Views are identical with the framework structure of **Fig. 5a**.

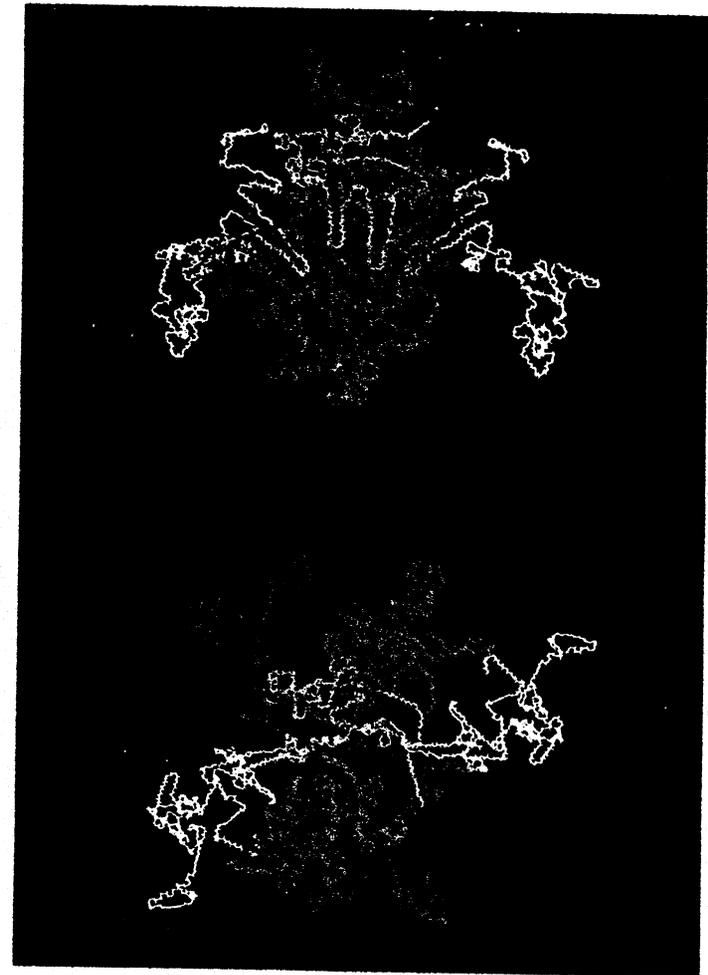


Fig. 2 Energy minimized casein symmetric submicelle structure, i.e. one α -casein, four α_1 -casein B and two β -casein symmetric dimers. Orthogonal views of backbones without side chains; α -casein B in cyan, α_1 -casein B in red and white and β -casein A² in magenta.

the correct stoichiometry could appear to be submicelles. To avoid these problems, whole casein was modified with iodoacetic acid as described in the Methods section: the resulting S-carboxyl methyl α -casein within whole casein would not be capable of undergoing sulphhydryl-disulfide interchange. In addition, during this procedure the stoichiometry of α - α_{s1} - α_{s2} - β -casein of 1:4:1:4 should be randomized. The procedure of Schechter *et al.* (10) uses 8 mol/L urea but was modified to include slow (3 d) dialysis at 4°C in place of a column. This dialysis procedure was expected to randomize the monomers to yield a more uniform submicellar caseinate preparation.

The lyophilized RCM whole casein sample was suspended in 0.025 mol/L PIPES pH 6.75 with 0.08 mol/L KCl and processed for TEM as described in Methods and Materials. A typical example of the total field of view is shown in Fig. 3. A large number of particles with diameters from 15 to 20 nm were found, in agreement with the static and dynamic light scattering results (9) which yielded a Stokes radius of approximately 9.0 nm for RCM casein submicelles. Few larger particles, possibly due to nonfixative associated cross-links, occur in the total field. The overall size of these particles are of that noted in previous studies of casein submicelles (12-14). Most previous studies have indicated a cauliflower-like appearance for both micelles and submicelles, with the greatest detail for submicelles being shown in the topographical images of Kimura *et al.*

(12). The question here is whether or not the 3-D models built from the monomeric caseins, as described above, could give rise to the observed topographical details. The following procedures were devised to compare the 3-D models with TEM representations at comparable scales without losing the detail of the models, but also without overextending the resolution provided by the TEM samples.

To insure that no preconceived notion of the nature of the 3-D submicellar model could influence the choice of the casein particles selected for comparison, 200 submicellar structures were selected from 6 total fields (such as Fig. 3) by an electron microscopist who had not observed the completed 3-D structures. These 200 pictures were enlarged twice over that of Fig. 3 and 15 particles were selected which were thought to be similar to the predicted 3-D submicellar structures by one of the authors (H.M.F.). A montage of these 15 images is shown in Fig. 4. The submicellar particles display at least three topographical shapes: ellipsoidal, circular and rhomboidal.

Although small-angle X-ray scattering techniques have demonstrated the relative uniformity of casein submicelles, other experiments have cast doubt on older considerations that the submicelle may be a unique and stable structure (1,2). The irregularity of the particles observed at a resolution of 2.5 nm in Fig. 4 reinforces this concept. Thus the possibility of finding groups of submicellar particles arranged in a semi-crystalline fashion seems remote. Similarly, if submicelles are composed of nonidentical subunits, then a direct 3-D reconstruction may not be achievable. The possible relationships between the 3-D models presented above and the submicelles observed in projection by TEM were explored using images processed to maximize the range of contrast and reduce spatial frequency variations in intensity. Selected particles of the montage of Fig. 4 were then photographically enlarged to bring the scale up to the low resolution images presented by the modeling package graphics. To do this, Van der Waals surfaces of the asymmetric and symmetric 3-D energy minimized submicelle structures were calculated with a density of 1 dot/0.01 nm², which when projected on a black background yields a low resolution model capable of comparison with the photographically enhanced representations of the TEM. This approach seems justified, since in work with crystalline proteins whose overall dimensions are known, negative staining with uranyl salts can achieve a high TEM resolution of up to 3.0 nm (15), thus the 3-D models can be considered to be of low resolution if in agreement with TEM. Particle images were analysed by Fourier transforms and compared with particle-free backgrounds. These comparisons demonstrated the maximum resolution to be at least 3 nm for all of the particles used for enhancement. As the average submicelle particle size is between 15 and 20 nm, reflections in the Fourier ring correlations as 1/10 and 1/7.5 nm⁻¹ can be taken to be characteristic for proteins (such as catalase, MW = 232,000) observed in freeze-substitution experiments (16). Although the enhanced representations and

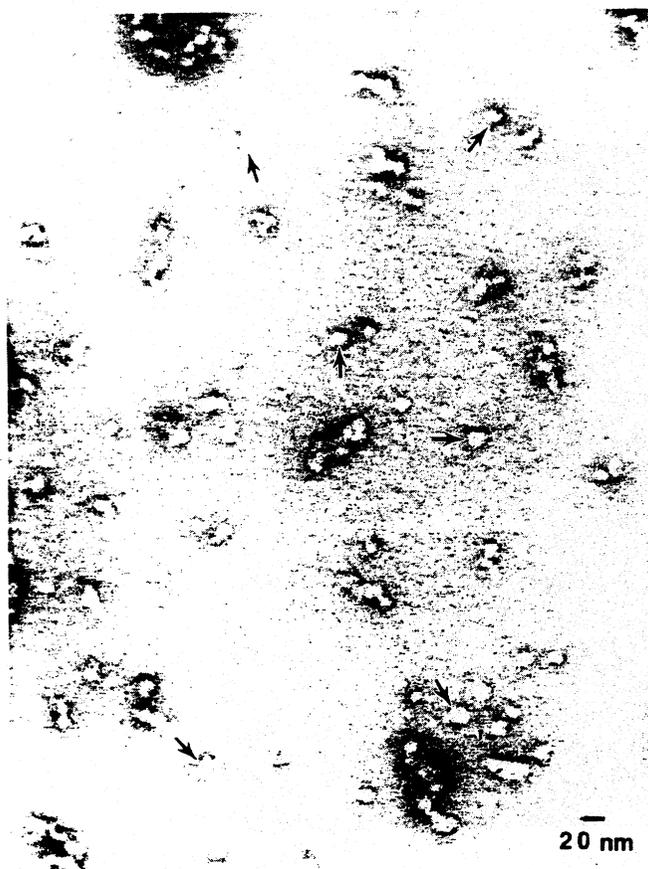


Fig. 3 Negatively stained submicelles of modified whole casein. Most single particles have diameters ranging from 15-20 nm and some asymmetry in shape; bar = 20 nm



Fig. 4 Selected images of submicelles, chosen from a sample of nearly 200 single particles representing three types of profiles: ellipsoidal (top row), circular (middle row) and rhomboidal (bottom row); bar = 10 nm

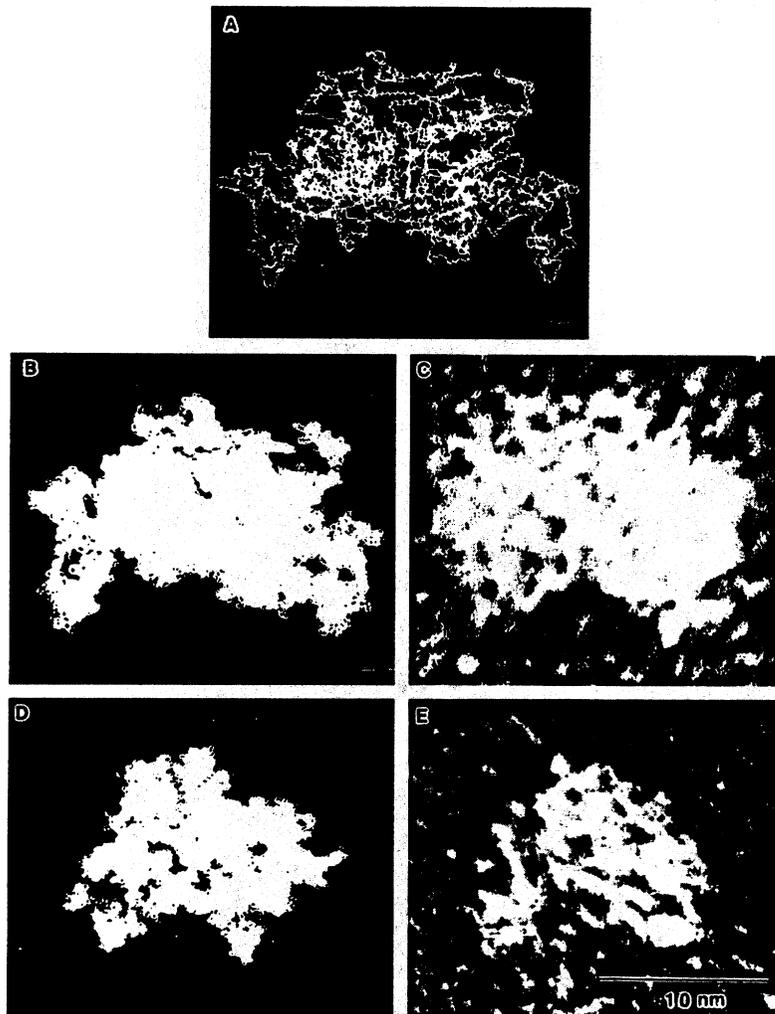


Fig. 5 Comparison of matched shape and dimensions of asymmetric submicelle 3-D model with photographically enlarged image enhanced representations of submicelles. (TEM bar = 10 nm; Molecular Model 1/2 axis = 5 nm). (a) backbone structure for asymmetric model: (b) Van der Waals dot surface of model: (c) enlargement of image enhanced micrograph; (d) Van der Waals of (a) rotated 90° about y-axis: (e) enlargement of image enhanced representation of the submicelle particle

the low resolution models both show greater detail, the following comparisons can be regarded as safe only with respect to overall high resolution detail of size, shape, and rugosity. When taken together with SAXS data, the TEM data point to open, highly hydrated structures for the casein submicelles. With all of these qualifications in mind, the following comparisons are made to invite further research and experimentation. The actual 3-D orientation of the submicellar particle on the thin amorphous carbon grid could not be determined. It is assumed that such orientation could be of a pseudo-random character. This necessitated performing rotation of the 3-D models in the computer for comparison with the micrographs rather than the opposite. The best images for comparison are shown in **Figs 5** and **6** for the asymmetric and **Fig. 7** for the symmetric 3-D models. All images compared in each TEM and 3-D model are at the same scale in each figure.

Figure 5 shows a comparison of images generated by the Van der Waals dot surfaces of the 3-D asymmetric submicelle structure at a density of 1 dot/0.01 nm², which matches the photographic enlargement of the image-enhanced representations of the submicelles. In **Fig. 5a** the backbone structure is shown to provide an

orientation of structure in 3-D space. As can be seen, this orientation of the model results in the α -casein portion (i.e. horse and rider) appearing at the top and centre of the structure. Here the calculated Van der Waals image (**Fig. 5b**) and the best representation (**Fig. 5c**) which corresponds to this ellipsoidal Van der Waals surface shows close agreement with respect to size, overall shape, and rugosity of surface. By rotating this Van der Waals image by 90° about the y-axis (image is assumed to be in the x-y plane) the Van der Waals 3-D asymmetric model (**Fig. 5b**) and another TEM representation (**Fig. 5c**) are in agreement. A further photographically enlarged version of the dot surface again rotated 90° and the correspondingly magnified TEM representation is given in **Fig. 6a** and **b**. However, these results do not provide proof of the asymmetric structure being the correct model.

In fact, when the same procedures were applied to the symmetric model (see **Fig. 7**) the agreement is as good as in **Figs 5** and **6**. **Figure 7** shows two examples of the corresponding calculated Van der Waals dot surface for the symmetric model and best photographically enlarged TEM representations. The orientation of the top 3-D model in space is the vertical view shown in **Fig. 2** (bottom); for the symmetric structure, the surface

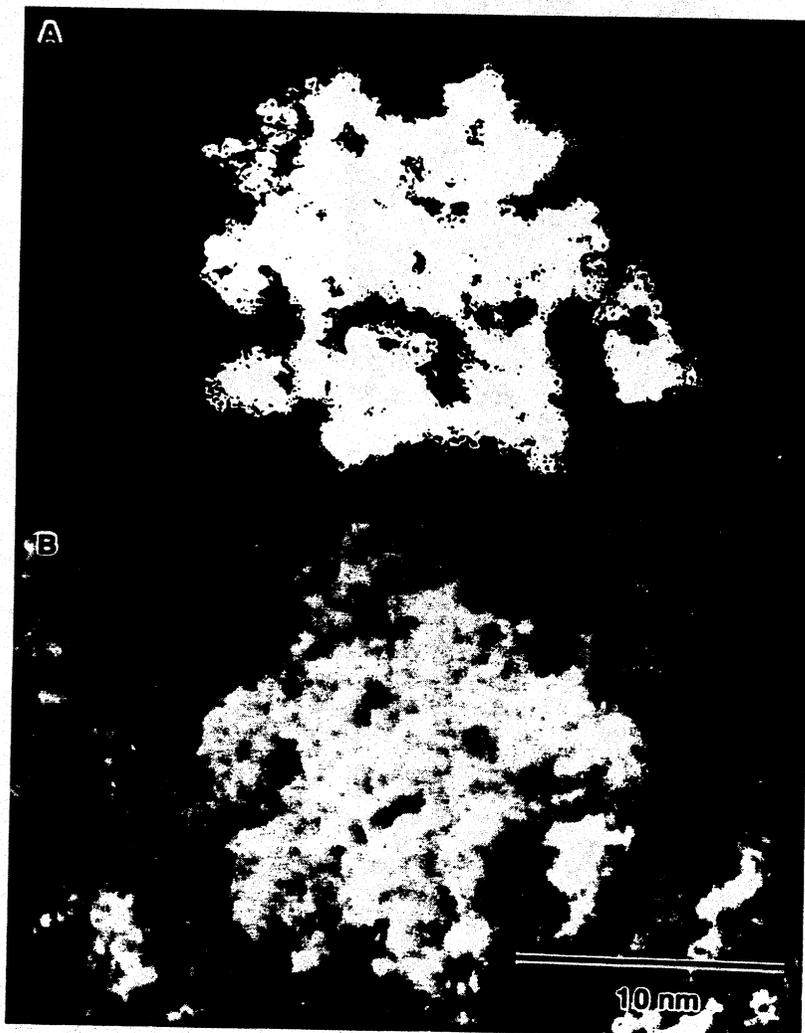


Fig. 6 Asymmetric model produced by a 90° rotation about x-axis of Fig. 5d: (a) Van der Waals model, (b) matching submicelle image showing comparable details of size and shape in profile

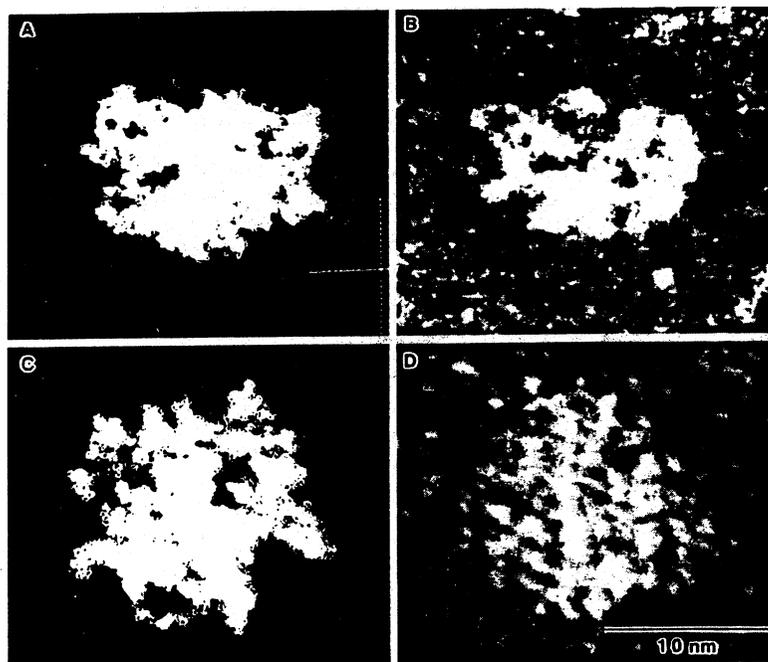


Fig. 7 Comparison of symmetric submicelle 3-D model with photographic enlargement of image enhanced representation of the submicelle. (TEM bar = 10 nm; Molecular Model 1/2 axis = 5 nm). (a) calculated Van der Waals surface and (b) photographic enlargement of image enhanced representation of the submicelle; (c) rotation of (a) 90° about y-axis. Van der Waals model and (d) photographic enlargement of image enhanced representation showing comparable structure: a central ridge in the 3-D electron density model can be matched to a similar central region in the image; both show an overall circular profile. Bar = 10 nm

features shown in this Figure exhibit long ridges 3 to 5 nm in length. Rotation of this model in an oblique direction by 90° yields the image shown in Fig. 7c. This image is more spherical in overall shape but still contains extended ridges. These are somewhat reminiscent of the surface shapes predicted by Kimura *et al.* (12). Here again the overall size, shape and, perhaps, rugosity of the model is in line with the photographically enlarged representations of the TEM images. Whether or not the porosity predicted by the 3-D model occurs cannot be supported by the TEM since the depressions or pores are below resolution. Good correlation, however, between the images generated for the dot surfaces of the model and the TEM appears to exist in at least five different spatial orientations of the models. It should be noted that further orientations of the model showed images which closely resembled those already shown. It appears that a reasonable correlation between the 3-D models and the TEM images exists. Such images show that the submicellar models could give rise to the TEM images, but do not show a distinction between the asymmetric or the symmetric form. Both structures seem to be possible. Future studies involving atomic force or scanning tunneling microscopy may provide an answer to this problem and may further test the predicted 3-D structures. As emphasized in previous papers on the 3-D models of submicellar casein (7,8), it must be kept in mind that these structures represent working models. They are not the final native structures but are presented to stimulate discussion and to be modified as future research unravels the nature of these non-crystallizable proteins. Inspection of a recent drawing

of the casein micelle by Holt (2) demonstrates how structures such as those presented here could be further aggregated into the casein micelle. Continued dialogue and research in this area may stimulate the new concepts necessary to bring together divergent views and to finally produce an accurate micelle model. It is hoped that this work is a further step in that direction.

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