

CASEIN INTERACTIONS AS STUDIED BY GEL CHROMATOGRAPHY AND
ULTRACENTRIFUGATION

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SUMMARY

Gel chromatographic and ultracentrifugal methods were employed to demonstrate structural differences in the soluble casein complexes obtained by several pathways. In solutions containing the purified κ - and α_{s1} -casein fractions or whole acid casein at pH 6.85, I 0.26, 30 °C, an equilibrium mixture is established which promotes the formation of a unit weight ratio complex with a Stokes radius of 114 Å and M_r 700 000. At the low protein concentrations obtained by zonal gel chromatography, the complex dissociates to yield free κ -casein.

Whole casein isolated by high speed centrifugation and freed of calcium at pH 6.85 (first cycle casein) is shown to be an associating system with the polymeric species in rapid equilibrium. First cycle casein dissociates at low protein concentrations to a unit complex with a diameter of about 100 Å and M_r approx. 270 000. The unit complex is shown by gel electrophoresis to contain κ -, α_{s1} - and β -caseins.

The mild preparative methods employed in obtaining whole casein by centrifugation at pH 6.6 probably yield complexes more representative of their native quaternary structure than the casein complexes discussed previously. Our results, therefore, support a model of the native casein micelle built up from a 100-Å-diameter complex containing all the casein fractions.

INTRODUCTION

The casein micelles of cow's milk are large, globular aggregates ranging in size from $3 \cdot 10^6$ – $3 \cdot 10^9$ daltons¹. Elucidation of the structure of the micelle has proven to be a very difficult problem since it requires one to position correctly an enormous number of subunits of α_s -, β - and κ -casein. In addition, the function of ionic calcium and colloidal calcium phosphate in the maintenance of micelle structure must be clarified.

The models of the casein micelle which have been proposed²⁻⁷ are based partly on light scattering and ultracentrifugal studies of the self-association of the caseins

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and their complexes in calcium free media. Another model, derived entirely from electron microscopic investigations has also been published⁸. The studies referred to, and others, report a variety of sedimentation coefficients, obtained at relatively high protein concentrations, for the casein complexes. These reports also contain conflicting observations concerning the stoichiometry of complex formation. A full discussion of this subject is given by McKenzie⁹.

It was felt that reexamination of these observations at low protein concentrations was needed in order to more precisely characterize the soluble casein complexes. To this end the technique of gel chromatography combined with sedimentation velocity and equilibrium measurements was employed.

MATERIALS

Proteins with known Stokes radii were used for calibrating gel chromatographic columns. Bovine thyroglobulin, γ -globulin, catalase, ferritin, and glutamic dehydrogenase were all obtained from Sigma*. Human hemoglobin was purchased from Calbiochem and fibrinogen from Nutritional Biochemical Co. Southern bean mosaic virus was kindly supplied by Dr W. Haller, National Bureau of Standards, Gaithersburg, Maryland.

Biogel A-15, a beaded form of agarose, 100–200 mesh, was obtained from Bio-Rad Laboratories.

The major component of κ -casein B, which is free of carbohydrate, was prepared by Dr J. H. Woychik and Dr H. M. Farrell Jr of this laboratory by DEAE-chromatography in the presence of 3 M urea and 2-mercaptoethanol at pH 7.0. α_{s1} -Casein B was prepared in the same manner by Dr R. M. Parry Jr. Both casein fractions were essentially pure as shown by polyacrylamide-urea gel electrophoresis in the presence of 2-mercaptoethanol.

Whole acid casein was prepared from skim milk by titrating the milk to pH 4.6 at 30 °C, washing the flocculent precipitate several times with distilled water and redissolving it at pH 7.5. This process was repeated several times followed by lyophilization.

What is essentially first cycle casein¹⁰ was prepared from fresh skim milk by centrifuging down the micelles at $167\,000 \times g$, 18 °C, 45 min. The casein pellets at the bottom of the centrifuge tubes were drained and redispersed in buffer containing 0.05 M EDTA for the removal of divalent cations bound to the casein. The almost clear solution was then dialyzed against pH 6.85 buffer for 48 h before use. All experiments reported here were carried out in 0.05 M sodium phosphate buffer, 0.15 M NaCl, pH 6.85, I 0.26. Sodium azide (0.003 M) was added as a preservative.

METHODS

Laurent and Killander¹¹ showed that the elution position of a solute, when passed through a column of porous gel particles, is a function of its Stokes radius. They proposed a linear relationship between $(-\log K_{av})^{1/2}$ and a , the Stokes radius of a solute.

* Reference to brand or firm name does not constitute recommendation by the U.S. Department of Agriculture over any other similar products not mentioned.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e = peak elution volume of a solute, V_0 = void volume of the column, and V_t = bed volume of the column. Siegel and Monty¹² extended the technique by showing how the molecular weight of a solute could be calculated from its Stokes radius and its sedimentation coefficient. This approach was taken in the present study.

Agarose gel particles (Biogel A-15), previously equilibrated with buffer for 24 h, were packed into columns of 2.5 cm diameter to lengths of about 82–95 cm. After further equilibration under downward flow for another 24 h the void volume (V_0) was determined with Blue Dextran 2000 and the bed volume (V_t) was calculated from the column dimensions. Flow rates varying from 24–27 ml/h were maintained by a Mariotte bottle serving as the buffer reservoir. The columns were calibrated with proteins of known Stokes radii taken from the data given by Ackers¹³. Samples were applied to the column in 1.0-ml volumes containing 1–10 mg/ml of protein. The column effluent was collected in 3.5–4.5-ml fractions and these were assayed for protein content by estimating their absorbancies at 280 nm or 230 nm in the case of runs at the lowest concentrations.

Sedimentation velocity experiments were carried out in a Spinco Model E ultracentrifuge using a 12-mm 4° sector cell at 59 780 rev./min and 30 °C. $s_{20,w}^0$ were obtained from runs at four protein concentrations.

Equilibrium centrifugation was performed according to the meniscus depletion method of Yphantis¹⁴ at a rotor speed of 9945 rev./min, 27.5 °C.

The partial specific volume in all molecular weight calculations of the casein fraction or their complexes was taken as 0.73 ml/g, since variations in this parameter for the casein fractions range only from 0.728 ml/g for α_{s1} -casein to 0.741 for β -casein¹⁵.

Polyacrylamide urea gel electrophoresis was carried out according to Wake and Baldwin¹⁶.

RESULTS AND DISCUSSION

The results of calibrating Biogel A-15 columns are shown in Fig. 1. A straight line is obtained by plotting a function of the elution ($-\log K_{av}$)^{1/2} against a , the Stokes radius of a number of well-characterized proteins. It must be noted that no effect of solute concentration on elution volume could be detected indicating operation of the column at close to ideal conditions.

In Fig. 2 the elution patterns for κ -casein over a range of initial concentrations are presented. It is observed that the elution position of κ -casein also shows no concentration dependence. Furthermore, the symmetry of the κ -casein peak strengthens the conclusion that κ -casein is a stable aggregate over the range of concentrations studied. From a function of its peak elution volume ($-\log K_{av}$)^{1/2}, the Stokes radius of the aggregate can be interpolated from the calibration curve shown in Fig. 1. It was found to be approx. 120 Å. The sedimentation coefficient of κ -casein was estimated from schlieren patterns obtained over the concentration range of 10–3 mg/ml. κ -Casein sedimented as a single, slightly asymmetrical peak. A plot of its sedimentation coefficients against concentration was linear, with a negative slope and gave an $s_{20,w}^0$ of 20.5 S. The molecular weight calculated for κ -casein from these two parameters by the equation given by Siegel and Monty¹² was approx. 10^6 .

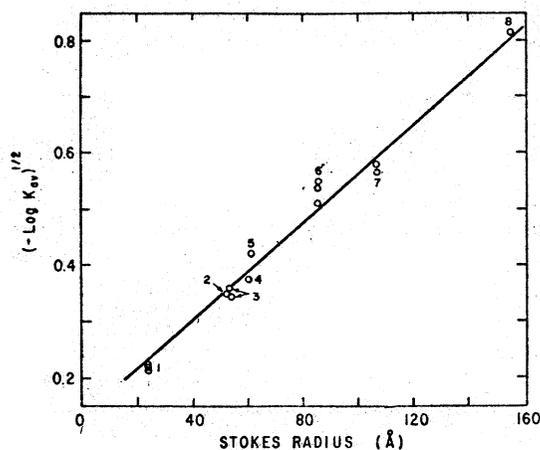


Fig. 1. Calibration of agarose (Biogel A-15) columns with proteins of known Stokes radii. 1, hemoglobin; 2, catalase; 3, γ -globulin; 4, ferritin; 5, glutamic dehydrogenase; 6, bovine thyroglobulin; 7, fibrinogen; 8, Southern bean mosaic virus. Protein was applied to the columns in 1 ml aliquots containing 1–10 mg/ml. All chromatographic experiments were preceded by dialysis of the sample for 24 h against eluting buffer system.

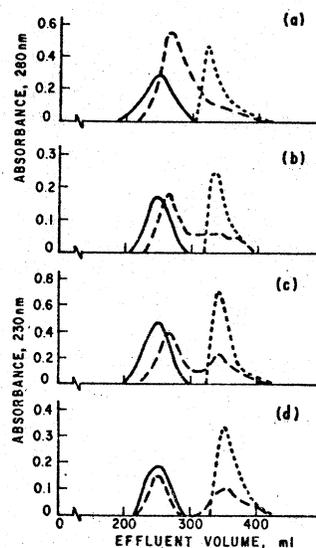


Fig. 2. Gel chromatography of κ -casein (—), α_{s1} -casein (-----), and a 1:1 weight ratio mixture of $\kappa + \alpha_{s1}$ -casein (- - -). (a) 10 mg/ml, (b) 5 mg/ml, (c) 2 mg/ml, (d) 1 mg/ml. The concentrations given here refer to the total protein concentration applied to the column in a sample volume of 1 ml. $V_0 = 167$ ml, $V_t = 402$ ml.

As shown in Fig. 2, α_{s1} -casein is eluted as a single peak with a skewed trailing edge, its peak elution volume steadily increasing with decreasing concentration. This type of concentration dependence of the molecular size of α_{s1} -casein is characteristic of a self-associating system in which the polymeric species are rapidly reequilibrating relative to the time of the chromatographic experiment.

Velocity ultracentrifugation of a 1:1 weight ratio mixture of κ - and α_{s1} -casein gave a single, slightly asymmetrical peak in the concentration range from 10–3 mg/ml. From schlieren patterns at four protein concentrations, the $s_{20,w}^0$ of the $\kappa + \alpha_{s1}$ complex was calculated to be 14.9 S. Evidently, complex formation is favored in this concentration range.

Some difficulties were encountered when a 1:1 weight ratio mixture of κ - and α_{s1} -caseins was gel chromatographed in order to obtain the Stokes radius of the complex. The elution patterns obtained are shown in Fig. 2. In the highest concentration experiment, Fig. 2a, a single peak, with a skewed trailing edge, is eluted at a slightly greater volume than κ -casein, indicating a reduction in Stokes radius for the $\kappa + \alpha_{s1}$ complex relative to κ -casein itself. However, without further experiments at several concentrations, the Stokes radius of the complex cannot be calculated from its peak elution volume since the skewed trailing edge of the boundary suggests that the complex is dissociating due to dilution of the initial protein zone as it migrates through the column. If the interacting casein aggregates are in rapid equilibrium relative to the

time of the chromatographic experiment, no region in the elution profile can be correlated with a specific component in the equilibrium mixture applied to the column. It was necessary then to establish what type of equilibrium actually existed in this system of interacting proteins. Gel chromatography of the 1:1 weight ratio mixture of $\kappa + \alpha_{s1}$ -casein was then performed at three lower concentrations. The elution patterns obtained from these experiments are shown in Figs 2b, 2c and 2d. Progressive dissociation of the complex occurs as the concentration is reduced, since the asymmetry of the boundary is accentuated and a discrete peak develops at the trailing edge. In the experiment at the lowest concentration, Fig. 2d, the complex is completely dissociated since two peaks are eluted coincidentally with κ - and α_{s1} -caseins when the latter are chromatographed separately at the same total concentration. From this experiment we conclude that the rates of complex formation and dissociation are sufficiently slow so that re-equilibration during the chromatographic experiment is not significant. It should be possible then to correlate peaks in the elution profile with individual reacting species. The slow peak which develops at the trailing edge of the reaction boundary is, evidently, α_{s1} -casein since this peak manifests the same concentration dependence of its elution volume as pure α_{s1} -casein. As the loading concentration is reduced, the fast peak is eluted at a constant volume while the area under this peak continuously decreases relatively to the area under the slow peak. An obvious conclusion is that the 1:1 weight ratio complex is dissociating with a concomitant increase in free α_{s1} -casein. However, a peak does not appear at the elution position of pure κ -casein during complex dissociation until the process is completed and κ - and α_{s1} -caseins are regenerated (Fig. 2d). This might be explained if one realizes that as α_{s1} -casein is slowly released from the $\kappa + \alpha_{s1}$ complex, a series of smaller complexes containing subaggregates of κ - and α_{s1} -casein could result. These complexes would be eluted between the 1:1 weight ratio complex and the slow peak at the trailing edge of the boundary which has been shown to represent pure α_{s1} -casein. In Figs 2b and 2c this situation is most evident.

Then the peak at the leading edge of the reaction boundary, if our analysis is correct, can be ascribed to the 1:1 weight ratio $\kappa + \alpha_{s1}$ complex. From its elution volume, which is constant over the entire concentration range until complete dissociation occurs, the Stokes radius of the complex is found to be 114 Å. This value, together with the $s_{20,w}^0$ of 14.9 S, was used to calculate a molecular weight for the complex of approx. 700 000 by the equation given in Siegel and Monty¹². Apparently interaction of the κ - and α_{s1} -casein aggregates leads to dissociation of the κ -casein and the reversible formation of a complex of intermediate size.

The elution pattern of solubilized whole acid casein is shown in Fig. 3. Two peaks were obtained which were identified by gel electrophoresis as predominantly κ -casein in Peak A and a mixture of α_s - and β -caseins in Peak B. The elution position of the peak containing κ -casein correlated well with a molecule of Stokes radius equal to 123 Å. This value is very close to that previously obtained for pure κ -casein. When whole acid casein was chromatographed at a higher concentration, as shown in Fig. 4, two peaks were again eluted. The leading edge of the first peak, A, and the trailing edge of the second peak, B, were identified by gel electrophoresis. Fraction A contained predominantly κ - and α_s -caseins, whereas Fraction B contained α_s - and β -caseins. The elution position of the first peak corresponds to a molecule with a Stokes radius of 115 Å, very close to the value obtained for the $\kappa + \alpha_s$ complex previously

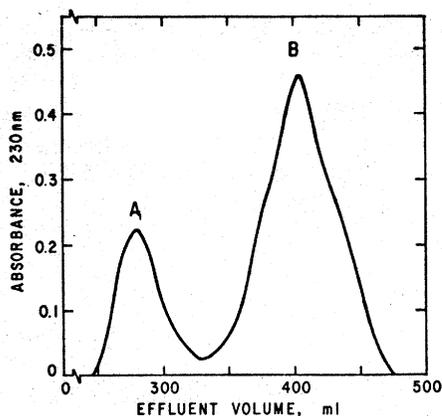


Fig. 3. Gel chromatography of whole acid casein. 10 mg protein in 1 ml was applied to the top of the column. $V_0 = 194$ ml, $V_t = 465$ ml.

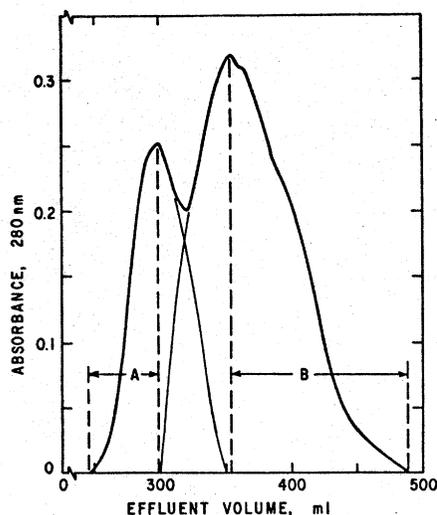


Fig. 4. Gel chromatography of whole acid casein. 40 mg protein in 2 ml was applied to the top of the column. $V_0 = 194$ ml, $V_t = 465$ ml.

described. Apparently whole acid casein at pH 6.85, $I = 0.26$, 30°C , is also an equilibrium mixture of interacting caseins in which the formation of $\kappa + \alpha_s$ -casein complex is favored by increasing concentration. As was observed for the mixture of purified κ - and α_{s1} -caseins, free κ -casein is obtained by dissociation of the complex at low protein concentrations.

When first cycle casein was applied to the gel chromatographic column at a sample concentration of 15 mg/ml, practically all of the casein was obtained in one peak, with a skewed trailing edge, that was eluted at a much larger volume than pure κ -casein. This indicates that κ -casein must be much less self-associated in first cycle casein. As the sample concentration was reduced to 10 mg/ml (Fig. 5), the elution volume of the single peak increased. Decreasing the sample concentration further did not result in any increase in the peak elution volume of the boundary. This limiting elution volume corresponded to a solute with a Stokes radius of approx. 50 \AA . Isolation of the peak and determination of its composition by gel electrophoresis revealed that it contained all of the major casein fractions.

Velocity centrifugation of first cycle casein was attempted in order to estimate the sedimentation coefficient of the unit complex which could then be combined with its Stokes radius by the equation of Siegel and Monty¹² for calculation of its molecular weight. At the lowest solute concentration attempted, 2 mg/ml, a broad peak with a skewed trailing edge was obtained indicating that considerable self-association was occurring even at this concentration. From the gel chromatographic experiments, in which the solute undergoes considerable dilution through boundary spreading, it was realized that lower concentrations must be studied if dissociation to the unit complex was to be observed. Equilibrium centrifugation of first cycle casein was then performed. The result is graphically described in Fig. 6. A plot of $\ln j$ vs r^2 is linear over a

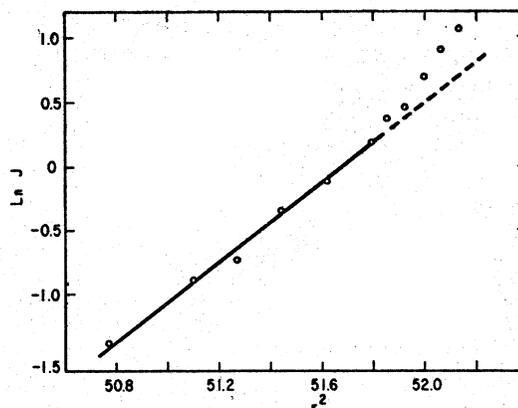
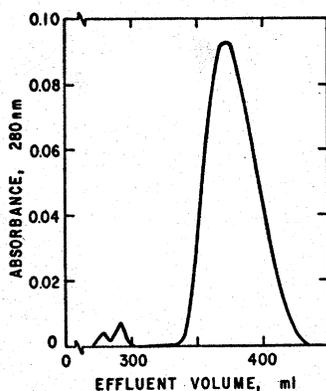


Fig. 5. Gel chromatography of first cycle casein. 10 mg protein in 1 ml was applied to the top of the column. $V_0 = 175$ ml, $V_t = 437$ ml.

Fig. 6. Plot of $\ln j$ against r^2 from equilibrium sedimentation of first cycle casein at a rotor speed of 9 945 rev./min, 27.5 °C and initial protein concentration of 0.25 mg/ml. The molecular weight was calculated according to the method of Yphantis¹⁴.

considerable distance from the meniscus, curving upward toward the cell bottom where the solute concentration increases. That the curvature probably represents inhomogeneity due to a concentration dependent association is indicated by the gel chromatographic experiment on first cycle casein described previously. The slope of the linear portion of the plot is taken to be proportional to the molecular weight of the approx. 100-Å-diameter complex observed by gel chromatography. The molecular weight was found to be approx. 270 000.

The results indicate that first cycle casein is a system of self-associating unit complexes containing α_s -, β - and κ -caseins of approx. 100 Å, M_r approx. 270 000 and perhaps of constant composition. These findings support the model of the casein micelle proposed by Shimmin and Hill⁸ in which the micelle is built up from 100 Å diameter complexes, M_r approx. 300 000, of uniform composition which are cross-linked by calcium and colloidal calcium phosphate. At least two studies^{17,18}, however, indicate that the composition of the unit complex might not be constant since an inverse relationship between micelle size and κ -casein content was demonstrated. Nevertheless, two recent papers employing electron microscopy¹⁹ and light scattering²⁰ give evidence generally supporting the model of Shimmin and Hill⁸.

From the evidence presented here, it is apparent that models of the micelle, based on studies of casein interactions in calcium free media, must be approached with some caution, since complexes formed by these interactions display significant path-dependence. Thus, first cycle casein would be more likely to retain the quarternary structure of the native submicellar aggregates than complexes reconstituted from the purified components or whole acid casein, since the former preparation is obtained by relatively mild chemical and physical methods.

There is some evidence, however, that even first cycle casein has undergone some structural changes during its preparation. Thompson *et al.*²¹ have shown that micelles reconstituted from first cycle casein by dialysis against skim milk are poorly solvated, more uniform in size and larger than the natural micelles of cow milk. McGann and

Pyne²² have, furthermore, indicated that colloidal calcium phosphate probably has a role in maintaining the structural relationships between the casein subunits in the micelle. These, and other questions, are the subjects of continuing investigations of the structure of casein micelles.

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