ACTA CHEMICA SCANDINAVICA 19 (1965) 375–382

The Conformation of Casein in Aqueous Solution*

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Examination of light scattering data for α-casein solutions at pH 6.5 in ionic strength 0.1 phosphate-NaCl buffer supported earlier light scattering findings in cacodylate buffer that certain casein components exist in solution in the form of a random coil. Hydrodynamic data taken from the literature were interpreted in terms of the Mandelkern and Flory model for an impenetrable effective hydrodynamic sphere and agreement between theory and results for casein was observed. The physical properties of casein were viewed in terms of its amino acid composition. The optical rotatory properties of whole casein solutions in pH 6.5 phosphate buffer and in 6.6 M urea and for β-casein in pH 6.5 phosphate buffer were consistent with the view that the proteins were not helical in these solvents.

INTRODUCTION

Previous light scattering studies [1] have indicated that the aggregates of most casein fractions exist in solution in the form of a random coil, except β-casein which appeared as a compact sphere in pH 6.5, ionic strength 0.066 sodium cacodylate buffer. This shape is in conflict with the rod shape suggested by the high axial ratios previously reported for casein.[2–6] Since cacodylate buffer was not used in the hydrodynamic studies, it was considered desirable to examine a typical casein fraction in phosphate buffer (which was used for hydrodynamic measurements) to determine if the lack of agreement between light scattering and hydrodynamic shapes was due to a buffer ion effect.

Jirgensons [7] determined values of λ_c for casein at pH 9, but it is not possible to predict what this value would be at pH 6.5 in view of the pH dependent aggregation effects known for casein and the pH dependence of the specific rotation reported by Golub and Pickett.[8] Therefore, casein solutions

* Article 1162 from the Department of Dairy Technology, The Ohio State University. Supported partially by a grant from the *National Institutes of Health.*

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were also examined by optical rotatory dispersion at pH 6.5 in phosphate buffer and in 6.6 M urea.

Finally, consideration must be given to the earlier hydrodynamic findings in view of the current results.

EXPERIMENTAL METHODS

Light scattering. The procedure used in this study has been given.[1]

Optical rotatory dispersion. The optical rotatory experiments were performed with a Rudolph Recording Spectropolarimeter with a xenon arc lamp as the light source. The wavelength was varied from 707 to 287 mμ and a 0.50 mm slit width was used. The samples of about 1 % protein concentration were examined in 5 cm polarimeter tubes supplied by the manufacturer. Measurements were made at room temperature (24° $\pm$ 1°C). This temperature was used in view of the insensitivity of optical rotation of casein to temperature within the range 20°—60°C as reported by Golub and Pickett.[8]

Protein concentration. Protein concentration was determined for the optical rotation experiments by measuring the absorption of appropriate dilutions at 280 mμ and comparing with a standard curve which was prepared from the same material. The volume of liquid required to dilute 4.0 g of urea to 10 ml was observed and the protein concentration for the studies with urea was obtained from this information. The protein concentration for the light scattering trials was determined by the Biuret method as previously described.[1]

Preparation and clarification of samples. Stock casein solutions of approximately 1 % were made by dissolving the material in 0.1 ionic strength phosphate buffer containing 0.08 M NaCl, and the pH adjusted to 6.5 with 10 % NaOH. Following overnight dialysis against the same buffer, the samples were centrifuged in a Beckman Model L centrifuge at 30 000 rpm in a No. 40 rotor (average of 59 000 *g*) at 4°C for one h. The upper 3/4 portions of the tubes were carefully decanted for examination by optical rotatory dispersion. The clarification technique used for light scattering has been given.[1]

Preparation of samples. Whole casein was prepared by acid precipitation and α-casein as described by Warner.[9] β-Casein, isolated by the urea fractionation procedure of Hipp *et al.*,[10] was supplied by B. Lindqvist, Mjölkcentralen, Stockholm. Upon ultracentrifugation in a Beckman Model E ultracentrifuge at 20°C, this material showed one fast peak (apparently aggregated material) and a major symmetrical peak with $S_{20,\mathrm{w}} = 1.00$.

Buffer. The buffer used in this study, unless stated otherwise, was pH 6.5, ionic strength 0.1, sodium phosphate buffer containing 0.08 M NaCl.

EXPERIMENTAL RESULTS

Light scattering. Previous light scattering studies with cacodylate buffer as the solvent showed α-casein to be typical of the casein fractions studied, with the exception of β-casein. α-Casein's behaviour was examined in phosphate buffer at an ionic strength of 0.1 and 0.05. The samples were examined at 30°C after holding for 30 min at this temperature in order to attain equilibrium as previously described.[1] After this time, a complete light scattering envelope was taken.

The results for the experiment with an ionic strength of 0.1 are presented (Fig. 1) in the form of a Zimm plot.[11] A definite concentration dependency was observed in this case in contrast to the behaviour of the same protein in cacodylate buffer at the same temperature, where no concentration relationship was observed.* The Z-average radius of gyration and interaction coefficient,

* The observed behavior in the present case would be found with a concentration dependent associating process.

calculated from the information given in Fig. 1, were 645 Å and -3.79×10^5, respectively. Polydispersity in the system is indicated by the curvature of the $Kc/R\Theta$ lines with increasing angle. However, the order of magnitude of the weight average molecular weight was the same as previously observed, being 1.49×10^6 in phosphate buffer and 6.17×10^6 in cacodylate buffer. Although the ionic strength was 0.066 for cacodylate and 0.1 for phosphate buffer, the difference in molecular weight does not appear to be entirely an ionic strength effect since the molecular weight was 2.1×10^6 in 0.05 ionic strength phosphate buffer.

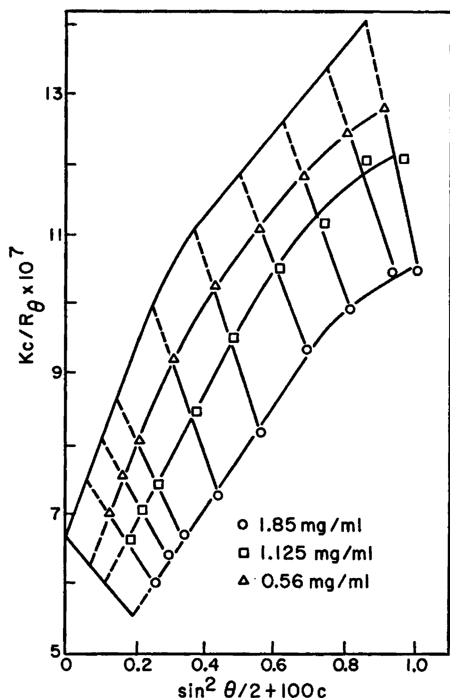

Fig. 1. Zimm plot of α-casein at 30°C in pH 6.5, 0.10 ionic strength phosphate-NaCl buffer.

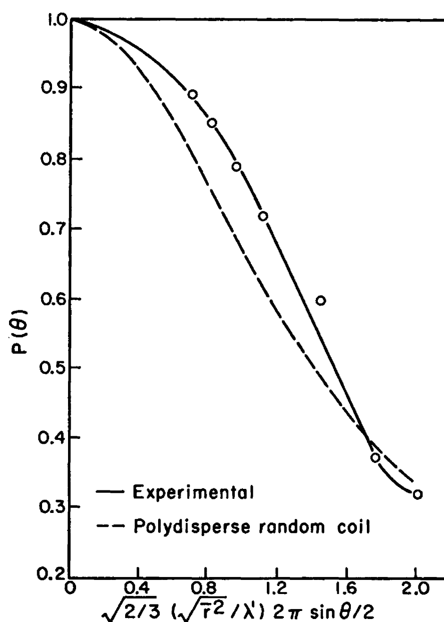

Fig. 2. Variation of the particle scattering factor with angle for α-casein at 30°C in pH 6.5, 0.05 ionic strength phosphate-NaCl buffer and a polydisperse random coil model.

This trend, an inverse relationship between ionic strength and molecular weight of α-casein, was the same as reported by Sullivan *et al.*,[12] but was opposite to that indicated by Halwer.[13] However, Halwer was measuring the increase in turbidity upon the addition of electrolyte rather than molecular weight obtained by extrapolation to zero protein concentration. No difference was noted in the shape of the α-casein particles at 0.05 or 0.1 ionic strength buffer as determined from the variation of the particle scattering factor,

P(Θ), with angle as previously described.[1] The curve given in Fig. 2 for the trial of ionic strength 0.05 was typical. A theoretical curve prepared from the data given by Stacey [14] for a polydisperse random coil is given for comparison. Good agreement between the two curves may be noted. Thus, the results obtained for the shape of α-casein in phosphate buffer are in agreement with those obtained in cacodylate buffer.

Optical rotatory dispersion. Whole casein and β-casein were chosen for examination by optical rotation. As noted earlier, the former was typical of the other casein fractions examined (whole, α-, α_s-, K-, and β-casein) with the exception of β-casein. β-Casein was examined to determine if the differences in light scattering which existed between it and whole casein would also be revealed by another physical measurement. Whole casein was examined in phosphate buffer and phosphate buffer-6.6 M urea, and β-casein was examined in phosphate buffer.

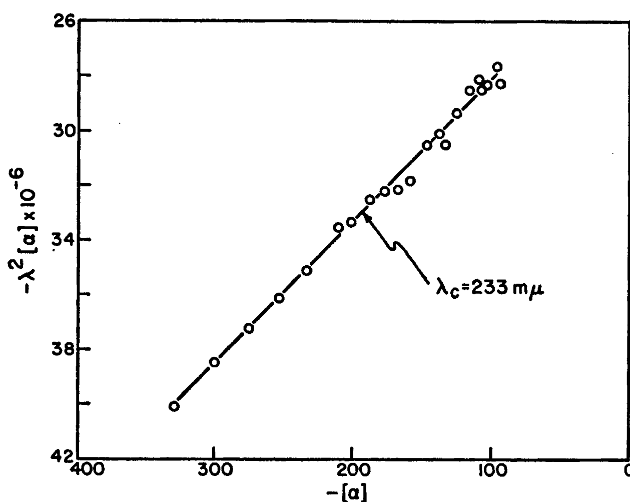

Fig. 3. Yang-Doty plot of optical rotary dispersion data obtained for whole casein in pH 6.5, ionic strength 0.1 phosphate-NaCl buffer.

All three samples were found to obey simple dispersion when plotted by the method utilized by Yang and Doty,[15] but not over the same wavelength range. A plot of $-\lambda^2[\alpha]_\lambda$ *versus* $-[\alpha]_\lambda$ for whole casein in phosphate buffer (Fig. 3) revealed that simple dispersion persists from 537—347 mμ. A similar behavior was observed with β-casein, only the straight line region ranged from 587—337 mμ. However, a straight line was noted with whole casein in urea over the entire range extending from 697 to 307 mμ. The variation observed with these three samples may be related to light scattering interference in the case of β-casein and whole casein in the absence of urea. For the third sample, the urea would be expected to reduce the aggregate size and the light scattering interference would be decreased.

The straight line portion of these curves was used to evaluate the quantity λ_c, which can be used as an index of the helical content of proteins. These

results are summarized in Table 1. The values of λ_c, which ranged from 233 mμ for whole casein to 212 mμ for β-casein, are in the range expected for proteins in the random coil configuration.[16]

Table 1. Optical properties of whole casein in phosphate buffer, β-casein in phosphate buffer, and whole casein in 6.6 M urea.

Property	Whole casein in phosphate buffer	β-Casein in phosphate buffer	Whole casein in 6.6 M urea
λc	233 mμ	212 mμ	218 mμ
$[\alpha]_D$	−81	−91	−97
$[\alpha]_{467}$	−138	−154	−169
Conc. of protein %	1.06	0.950	0.763

Jirgensons [7] obtained values for λ_c of 218 and 223 for β-casein at pH 9.2 and α-casein at pH 8.9, respectively. Also given in Table 1 are the experimental values of $[\alpha]$ obtained at 589 mμ and 467 mμ. The latter are given so as to position the straight line used to calculate λ_c for the three samples. The value of $[\alpha]_{589}$ is given to permit comparison with the results obtained at the D line of sodium. The corresponding values from the literature for α-casein and β-casein were −90 and −140 degrees, respectively, under the same conditions as given above [16] and −101 for whole casein at pH 6.9.[17] The latter value was given by McMeekin, who also found the optical rotation of casein to remain unchanged by heating in solution. He noted a slight increase in the value for the rotation which was obtained in the presence of 5 M guanidine hydrochloride. This increase was reversible, and the values returned to the original point was then guanidine was removed.

Golub and Pickett [8] reported that optical rotation observed at 546 mμ with whole casein, obtained by acid precipitation, varied greatly with pH. The value of $[\alpha]_{546}$ increased from a low of about −120 at pH 6 to a maximum at pH 12 of approximately −155. This effect could explain at least part of the differences in results obtained in this study and those previously noted.

Attempts were made to fit the experimental data from 687−317 mμ to the equation of Moffit and Yang [18] for complex dispersion. This equation is

$$[\alpha]\lambda = \left(\frac{100}{M_0}\right)\left(\frac{n^2+2}{3}\right)\left[\frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2}+\frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right]$$

where M_0 is the average residue weight which was calculated from the data of Gordon *et al.*[19] to be 117 for whole casein and 115 for β-casein, n is the refractive index of the solvent, which was 1.333 for phosphate buffer when determined as previously described,[1] λ is the wavelength, $[\alpha]\lambda$ the specific rotation at the wavelength λ, and λ_0, a_0, and b_0 are constants which are determined by graphic means. The constant b_0 can be used as an index of the helical content of the protein when $\lambda_0 = 212$ mμ. Tanford [20] has found that the values of the constant a_0 as well as the specific rotation are influenced by the solvent.

When λ_0 was set at 212 mμ, values of b_0 equal to −97, −53, and 0 and of a_0 equal to−455, −530, and −604 were calculated for whole casein in phos-

phate buffer, β-casein in phosphate buffer, and whole casein in 6.6 M urea, respectively. Values of $\pm$ 100 for b_0 can be consistent with a random coil conformation according to Tanford *et al.*[21] However, this does not eliminate the possibility that casein has equal portions of both the α- and β-structure.

Finally when the data for whole casein in phosphate buffer were examined in the manner recently described by Schecter and Blout,[22] values of H_{225} and H_{193} of 38 and 15 corresponding to 38 and 15 % α-helix, respectively, were obtained. This poor agreement could be typical of proteins such as β-lactoglobulin which exhibit a certain amount of order other than helices.

Infrared flow dichroism: Studies were conducted with 4.3 % whole casein in phosphate buffer solutions of D_2O at both pD 6.5 and 12. The measurements were made in a Perkin-Elmer 421 Grating Spectrophotometer with a calcium fluoride cell. The flow dichroism mechanism had been previously tested with a poly-γ-benzyl-glutamate sample of molecular weight 135 000 and a rotary diffusion constant of 21 200 was obtained in agreement with the expected value of 21 240.[23]

Maxima were observed at 1450, 1550, and 1635 cm^{-1}, but dichroism was not observed. However, it was only possible to say that the rotary diffusion coefficient was greater than 50 000 employing a flow rate of 100 000 cm^{-1} and the observed noise level of the instrument. This was adequate to support the random coil shape for the casein aggregate, but further elucidation of the monomer shape was not possible.

DISCUSSION

The samples used in this study at pH 6.5 in phosphate buffer were casein aggregates as evidenced by the magnitude of the molecular weight obtained by light scattering. The information leading to the particle shape provided by the light scattering data cannot be interpreted in a manner other than a random coil. This conformation of the aggregate is supported by the results obtained at pH 6.5 from the infrared flow dichroism studies.

However, it is considered that there is also good evidence to consider the random coil shape for casein "monomers". A recent model by Mandelkern and Flory described by Yang [24] and Scheraga [25] assumes the random coil to be represented by a non-draining sphere. By employing this relationship and the data of von Hippel and Waugh,[26] values for the required constant were found to be 2.25×10^6 or 2.72×10^6 for the casein "monomer" depending upon the choice of the viscosity. These values are in agreement with that of 2.1×10^6 predicted and 2.5×10^6 generally observed for flexible polymers; Ref. 25, p. 28. It is impossible to choose between the random coil and a prolate ellipsoid of axial ratio of about 15 (for the average viscosity) on the basis of hydrodynamic data alone due to the identity of the form derived by Mandelkern and Flory and the β constant of Scheraga [25] (as pointed out by the author). However, the dependence of the intrinsic viscosity of casein to the degree of aggregation [2,3,26] indicates that an effective hydrodynamic spherical shape, definitely not rod-like structure, represents the aggregate as well as the "monomer". This may not be true for β-casein.[12]

One cannot argue that casein should exist as a random coil due to its apparent low helical content; *e.g.*, myoglobin exists in a compact form despite its high helical content. However, one can consider the fact that λ_c is nearly the same at various degrees of aggregation as evidence, in addition to the viscosity data, that a large change in conformation does not occur upon changing the pH from pH 9 to 6.5.

The fact that casein resembles denatured proteins was pointed out by Halwer[13] and McMeekin,[17] but all previous hydrodynamic measurements have yielded high axial ratios for casein,[2-6] even though the globular shape and never rods was observed by electron microscopy.[27,28] That denatured proteins may resemble random coils has been emphasized by Schachman.[29] It is suggested here that the random coil form should be considered in future examination of the various casein fractions. One might also be suspicious of the possible existence of the random coil form for other proteins with an axial ratio of about 15. The necessity to exercise caution when using cylindrical *hydrodynamic models* to represent the shape of casein particles when interpreting other properties[4,30,31] must be emphasized.

Waugh and von Hippel[6] noted that it was necessary to assume a hydration of 0.8 to 1.0 g water/g protein in order to reconcile their viscosity and sedimentation and diffusion data, and Plomley *et al.*[5] assumed a hydration of 0.4 g water/g protein for their calculations. In view of the recent examination of methods for examining particle shape[24,25,29] assumptions of this type no longer appear to be necessary.

The optical rotation results obtained in this study in the presence and absence of urea at pH 6.5 and the findings of Jirgensons,[7] at pH 9 suggest a non-helical form. It is not difficult to understand why casein might exist in solution in the absence of the α-helix due to its large content of proline. This view is supported by the statement of Doty: "It appears that a single proline residue will break up the helical configuration in its vicinity and the affect may extend to as many as 20 residues in the aqueous solution."[32] Certainly casein qualifies as a protein with a large quantity of proline.[33] This high content of proline could easily be responsible for the large negative rotation of β-casein[16] in an analogous manner to that of collagen.[34]

It is considered that further physical chemical experimentation must be done before the shape of the casein components in solution can be positively described. This is especially true in view of the infrared analysis by Shigorin and Zubov,[35] which found globular casein in the α-form. Presumably, they were working with dried films and not with aqueous solutions.

Finally, if one accepts the casein molecule in the random coil form, it is not difficult to understand the aggregation tendencies exhibited by this protein in comparison with globular proteins. It could be that the normal hydrophobic forces which contribute to helical structure are not able to exert their influence due to the rigidity imposed by the proline residues. Therefore, these hydrophobic forces are manifested in intermolecular reactions. The temperature dependent changes in aggregation noted with several casein fractions[1] is consistent with this view.

Acknowledgements. In addition to the hosts at the sponsoring institutions, the author would like to express appreciation to Mr. M. Zeppezauer for assisting with the optical rotatory dispersion measurements, Miss M. Lundberg for aiding with the ultracentrifuge experiments, and Dr. W. L. Courchene for making the infrared flow dichroism measurements and calculations.

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Received November 20, 1964.