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SOME FEATURES OF THE ASSOCIATION OF β-CASEIN

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SUMMARY

The temperature-dependent association of β-casein has been investigated by ultracentrifugation and viscometry. At 4° β-casein exists as a monomer with a minimum molecular weight of about 25000. At 8.5° and 13.5° thread-like polymers are formed, that are interlinked firmly. The rate of association appears to be quite low. At 8.5° the degree of polymerization amounts to about 22; at 13.5° it certainly must be considerably higher. The value of the second virial coefficient of the polymers as deduced from Archibald's method is $7.80 \cdot 10^{-6}$ (g/100 ml)$^{-1}$, which is comparable to the values expected for rod-like or coiled polymers. The poor equilibration of the system is discussed.

INTRODUCTION

Heterogeneous casein constitutes about 80% of the milk proteins[1,2]. β-Casein, the association of which is dealt with in the present paper, is one of its principal components. Different methods for the isolation of β-casein have been described[3-5] and by electrophoresis and sedimentation it is concluded to be a homogeneous protein with a minimum molecular weight of about 25000 (ref. 6).

From previous investigations of VON HIPPEL AND WAUGH[7] and of SULLIVAN *et al.*[6], it is known that β-casein shows a strong tendency to associate with rising temperature. At low temperature, *e.g.* 4°, the protein exists in solution as a monomer. HAWLER[8] studied the influence of the ionic strength on the light-scattering of β-casein solutions. From his observations HAWLER concluded that the rate of association is low and the association only partially reversible.

The present investigation was carried out at 8.5° and 13.5°; at these temperatures considerable association is observed. It was undertaken in order to get some insight into the configurational characteristics and the degree of polymerization of the β-casein polymers. Moreover, association now appears to be quite a common phenomenon in protein solutions[8-13]. It is therefore of general interest to compare β-casein with other associating proteins.

Association of proteins can be studied conveniently by ultracentrifugation as applied in this study. From the sedimentation pattern the sedimentation coefficients and the relative amounts of monomer and polymers can be evaluated. The recent development of the so-called Archibald method[14] permits a reliable estimate of the weight average molecular weight. Further, as a consequence of the different

sedimentation velocities of monomers and polymers additional information can be gained regarding the rate of association. As will be shown, with β-casein this rate appears to be quite small as compared to the rate of separation of the sedimenting species.

The intrinsic viscosities of the β-casein monomer and polymers have been determined by complementary viscosity measurements.

MATERIAL AND METHODS

β-Casein was prepared by the urea method of HIPP *et al.*[4] with slight modifications from the alcohol method[4] and WARNER's isoelectric precipitation[3]. The following procedure proved to be most succesful.

The casein fraction soluble in 1.7 M urea was prepared according to HIPP *et al.*[4] and used as the starting material for the isolation of purified β-cascin. By free electrophoresis this fraction was found to contain α-, β- and γ-casein in varying proportions. The pH of its aqueous urea solution was lowered to 4.7 by addition of 0.1 N HCl and $(NH_4)_2SO_4$ was added to a final concentration of 1.6 M. The precipitate formed was filtered off and washed with distilled water. It was redissolved at pH 7.0 by addition of 0.1 N NaOH. The protein concentration was adjusted to 0.5% and the temperature of the solution lowered to 2°. Thereafter α-casein was precipitated by bringing the pH to 4.5 and the precipitate was discarded. The filtrate was warmed up to 33° and the pH raised to 4.9. The precipitate that formed, consisted mainly of β-casein; γ-casein remaining in solution. The purity of this β-casein was checked by free electrophoresis. If necessary, it was redissolved and freed from residual α-casein by a repeated cycle of precipitations at 2° and 33°. The final product was desalted by dialysis, lyophilized and stored in the cold. The β-casein thus prepared was found to be homogeneous by prolonged free electrophoresis and by sedimentation at 4°. By starch-gel electrophoresis in a buffer containing 7 M urea only very minor impurities could be detected[15].

Sedimentation was studied with a Phywe air-driven ultracentrifuge[16]. Apparent molecular weights were determined by the Archibald method before the plateau-region in the cell had disappeared[17,18]. At the bottom of the sedimentation-cell the determinations proved to be unreliable as a consequence of the disappearance of a sharp interface with accumulation of the sediment. The molecular weights recorded therefore originate from the meniscus of the liquid column only. Measurements of the sedimentation patterns were made on the photographic plates enlarged 20 × in a photographic enlarger. The concentrations at the meniscus were evaluated by means of the equations of KLAINER AND KEGELES[17], using the technique proposed by GINSBURG *et al.*[18]. The initial protein concentration was determined with a Meyerhoff synthetic-boundary cell[19].

The β-casein was dissolved in barbiturate buffer of pH 7.50 and adjusted with NaCl to an ionic strength of 0.20. Prior to sedimentation the solutions were stored for at least 24 h in the sedimentation-cell at the measuring temperature. Also the rotor and the vacuum-chamber of the ultracentrifuge were forecooled. In this way the sedimentation behaviour was found to be roughly reproducible, irrespective of the previous treatment of the samples.

The buoyancy-term $(1 - \bar{v}\rho)$, $\bar{v}$ partial specific volume of the protein, ρ density

of the solution was evaluated by the method outlined by KRAEMER[20]. Its value was found to be 0.268 ± 0.002.

The viscosity measurements were carried out in Ubbelohde viscometers with outflow times of about 200 sec and at the same temperatures as applied in the ultracentrifuge. The solutions were equilibrated in the same manner as described for the sedimentation measurements. They were previously filtered through a G-3 glass filter.

RESULTS

In accordance with previous authors[6,7], at 4° β-casein sediments as a single peak with a sedimentation coefficient of 1.50 S. At 8.5° and at concentrations exceeding about 0.15 g/100 ml a rapidly migrating peak appears, as is shown in Fig. 1. The sedimentation coefficient of this rapid peak is extremely concentration-dependent. Similar results were obtained at 13.5°. The influence of the concentration on the sedimentation coefficients of monomer and polymers at 8.5° and 13.5° is represented in Fig. 2. The sedimentation coefficients of the polymers at infinite dilution were obtained by plotting $1/s_{20,w}$ *versus* concentration. By the method of least squares the data at 8.5° were fitted by

$$1/s_{20,w} = 8.79 \cdot 10^{-2} + 4.75 \cdot 10^{-2}C$$

and at 13.5° by

$$1/s_{20,w} = 2.83 \cdot 10^{-2} + 27.50 \cdot 10^{-2}C - 11.92 \cdot 10^{-2}C^2$$

The corresponding values of $s_{20,w}$ at infinite dilution are given in Table II.

The Archibald measurements were incorporated into the plot proposed by TRAUTMAN[21] in which the quantity $RT\partial c/\partial x/\omega^2 x$ measured at various rotor speeds at

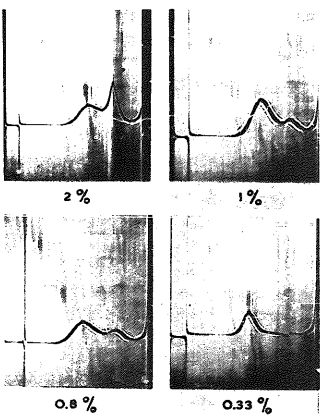

Fig. 1. Typical sedimentation patterns of β-casein solutions at 8.5°. Experimental conditions: barbiturate buffer (pH 7.50; I 0.20); Phywe air-driven ultracentrifuge; synthetic boundary cell at 45000 rev./min. Upper left: 2%, after 100 min; upper right: 1%, after 65 min; lower left: 0.8%, after 80 min; lower right: 0.33%, after 25 min.

the meniscus is plotted against the concentration decrease at the meniscus, Δc_{men}. From the slope of this plot various kinds of molecular weights can be calculated, as was demonstrated by TRAUTMAN[21] and YPHANTIS[22]. Typical plots for β-casein

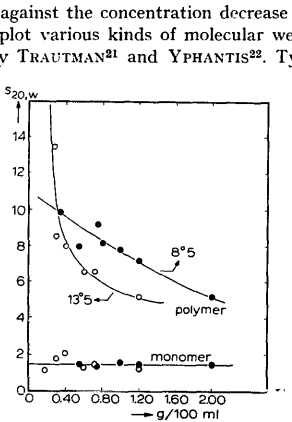

Fig. 2. Concentration-dependence of the sedimentation coefficients of β-casein monomers and polymers. Full circles: experiments at 8.5°; open circles: experiments at 13.5°. Further experimental details as in Fig. 1.

at 8.5° are given in Fig. 3. Also included in this figure is the linear Trautman plot for the β-casein monomer at 4°, which is characteristic for a homogeneous substance. The downward curvatures of the plots at 8.5° indicate the more rapid sedimentation of the polymer as compared to the monomer, which leads to a sharp decrease of the average molecular weight at the meniscus as the sedimentation proceeds. Two points should be noted in this respect.

Firstly, the steep initial slope of the Trautman plots makes a reliable extra-

TABLE I

APPARENT MOLECULAR WEIGHTS IN β-CASEIN SOLUTIONS OF DIFFERENT CONCENTRATIONS AT 4° AND 8.5°

Protein concn. (g/100 ml)	*Temperature °C*	$M_m \times 10^{-3}$	$\bar{M}_{app} \times 10^{-3}$	$M_p' \times 10^{-3}$**
1.00	4	24.9 ± 1	—	—
0.19	8.5	25.9 ± 2	70 ± 15	—
0.33	8.5	26.1 ± 2	98 ± 7	—
0.50	8.5	24.4 ± 2	112 ± 7	460
0.60	8.5	24.7 ± 2	102 ± 7	360
0.75	8.5	28.8 ± 2	116 ± 7	—
0.80	8.5	35.9 ± 2	90 ± 7	242
1.00	8.5	*	111 ± 7	278
1.20	8.5	27.7 ± 2	117 ± 7	244
1.50	8.5	23.4 ± 2	102 ± 7	192
2.00	8.5	*	112 ± 15	190

* Measurements limited to low speeds of rotation; final slope of Trautman plot not known.
** Calculated from $\bar{M}_{app}$ and the areas of the sedimentation peaks.

polation to the value of $(RT\partial c/\partial x/\omega^2 x)_{men}$ at $\Delta c_{men} = 0$ rather difficult. As a consequence, the initial apparent molecular weights derived from this quantity have a probable error of about 12 %. At 0.19 and 2.00 g/100 ml the error was estimated to

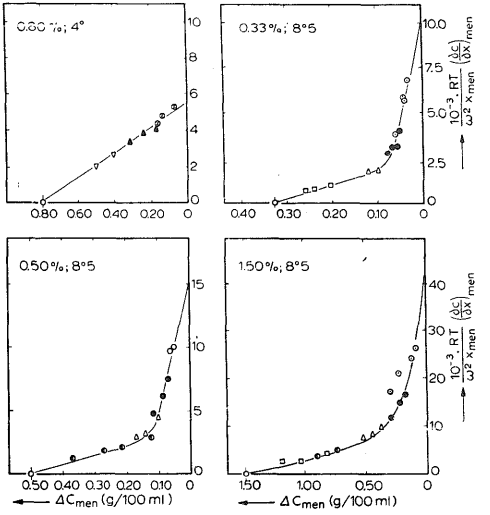

Fig. 3. Typical Trautman plots for β-casein at 4° and 8.5°. Upper left: 1 %, 4°; upper right: 0.33 %, 8.5°; lower left: 0.5 %, 8.5°; lower right: 1.5 %, 8.5°. Experimental details, see Fig. 1. ○, 10000 rev./min; ⊙, 12000 rev./min; ●, 15000 rev./min; ⊘, 18000 rev./min; △, 21000 rev. per min; ▲, 27000 rev./min; ◐, 33000 rev./min; □, 36000 rev./min; ▽, 42000 rev./min.

be twice as much due to the greater inaccuracy in the extrapolation of the concentration gradients to $(\partial c/\partial x)_{men}$ at such extreme concentrations[23]. At 13.5° even the initial slope of the Trautman plots was so steep, that extrapolation was not feasible, as is clearly demonstrated in Fig. 4. The molecular weights of the β-casein polymers derived from the Archibald measurements are limited therefore to the lower measuring temperature of 8.5°. They are collected in Fig. 5 and Column 4 of Table I.

The second remarkable point concerns the final slopes of the Trautman plots, which are constant and in nearly all cases correspond to the molecular weight of the β-casein monomer. The molecular weights of the monomer, which are derived from these final slopes are also included in Table I, Column 3.

At the meniscus concentrations corresponding to the constant final slopes of the Trautman plots, obviously no polymer is left at the meniscus. These meniscus concentrations considerably exceed the value of 0.15 g/100 ml, below which the polymer had also disappeared from the sedimentation patterns of solutions equilibrated overnight. Hence it appears that the rate of re-equilibration of the association during sedimentation is quite small as compared to the rate of separation of monomers and polymers.

This behaviour should be compared with the associations of rennin[11], β-lactoglobulin[12] and flagellin[13], that have been shown to be rapid. In the case of α-chymotrypsin[10] the rate of association was found to be not very large, though GILBERT's theory for the sedimentation of rapidly associating macromolecules[24,25] could still be applied. This theory predicts that given rapid re-equilibration, the area of the slow peak in the sedimentation pattern should remain constant on varying the protein concen-

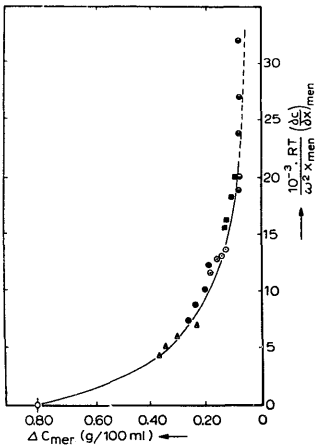

Fig. 4. Trautman plot for β-casein at 13.5°. Protein concentration, 0.80 g/100 ml. Experimental details, see Fig. 1. ◒, 6000 rev./min; ■, 18000 rev./min; ⊙, 12000 rev./min; ●, 15000 rev./min; △, 21000 rev./min.

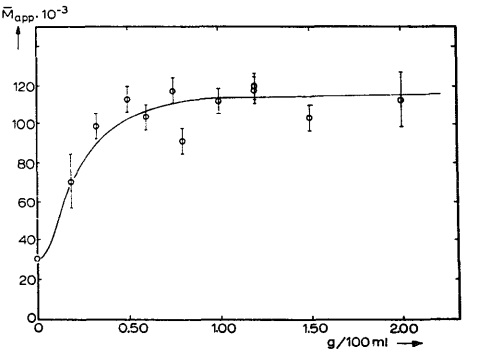

Fig. 5. Apparent weight average molecular weights *versus* concentration for β-casein solutions at 8.5°. Points: extrapolated values at zero time of sedimentation. Experimental details as in Fig. 1.

tration[24,25,12]. Naturally in the opposite case, this area should increase with concentration. Fig. 6 shows the latter to occur with β-casein at 8.5° and 13.5°. This is further evidence that the rate of re-equilibration during sedimentation is quite slow. As was mentioned above, HAWLER[8], investigating the influence of salt on the light-scattering of β-casein, also ascribed his findings to slow equilibration.

Graphs of the reduced viscosity *versus* the concentration for β-casein solutions at 4°, 8.5° and 13.5° are given in Fig. 7. As was found by SULLIVAN *et al.*[6], the viscosities decrease with rising temperature, indicating that the intrinsic viscosities of the β-casein polymers are lower than that of the monomer. This suggests that some folding or coiling of the polymers occurs as their molecular weight increases, though an alternative explanation of decreased hydration could be given as well[26].

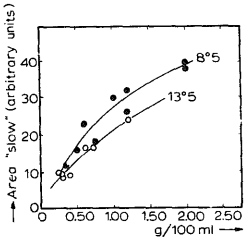

Fig. 6. Areas under the slow peaks of the sedimentation patterns of β-casein at 8.5° and 13.5°. Full circles: 8.5°; open circles: 13.5°. Further experimental details, see Fig. 1.

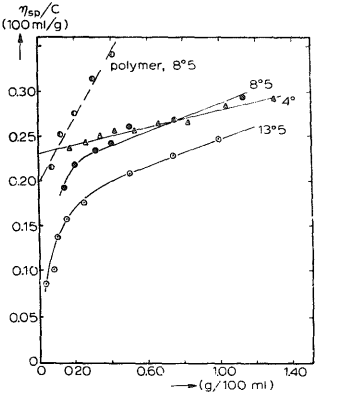

Fig. 7. Reduced-viscosity plots for β-casein at different temperatures. Solvent used as in Fig. 1. △, 4°; ●, 8.5°; ⊙, 13.5°. – – –, calculated reduced viscosity for the polymer at 8.5°.

The plots at 8.5° and 13.5° show a remarkable downward curvature, that may also be noticed in the figures presented by VON HIPPEL AND WAUGH[7], SULLIVAN *et al.*[6] and SCHOBER *et al.*[27]. The expectation is, however, that as a consequence of progressive dissociation on dilution, they should bend upwards to the viscosity plot of the monomer. The data of Fig. 7 were checked at different times after dilution of the samples, but they proved to be constant even after 24 h. Evidently they represent equilibrium data. It was further thought that the phenomenon could be due to the adsorption of protein to the glass-filter or the viscometer[28,29]. However, the concentration of the solutions was not changed after passing through the glass-filter. Moreover the curvature was also found with unfiltered solutions. It thus is questionable whether adorption can account for the curvature of the reduced viscosity plots at 8.5° and 13.5°. No explanation of the phenomenon can be given as yet.

CALCULATIONS

The molecular weight of the polymers from the Archibald measurements and the areas of the sedimentation pattern

The apparent molecular weight measured by Archibald's method in polydisperse, non-ideal systems has been considered by KEGELES *et al.*[30] and FUJITA *et al.*[31].

The results presented in Figs. 2 and 3 suggest that non-ideality effects in the present Archibald measurements should originate mainly from the β-casein polymers. The apparent molecular weights were analyzed therefore by assuming ideal behaviour of the monomers. It can easily be shown by following the arguments of KEGELES *et al.*[30], that for this case the apparent molecular weight is given by

$$\bar{M}_{app} = (c_m M_m + c_p M_p^2)/c \qquad (1)$$

where c_m, c_p and c represent the concentrations of monomer and polymer and the total concentration, respectively. M_p' is the apparent molecular weight of the polymer and defined by

$$M_p' = M_p(1 - \partial \ln f_p/\partial \ln c_p) \qquad (2)$$

f_p being the activity coefficient of the polymers. Alternatively one may apply the equivalent expression:

$$1/M_p' = 1/M_p + Bc_p \qquad (3)$$

with B the second virial coefficient as obtained from Archibald's method[30,31].

Values of M_p' were calculated by Eqn. 1 from the values of $\bar{M}_{app}$ given in column 4 of Table I, and assuming the monomer molecular weight to be 24900.

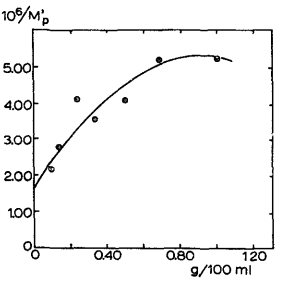

Fig. 8. Extrapolation of the reciprocal apparent molecular weight of the β-casein polymer at 8.5°.

The concentrations of monomer and polymer were derived from the areas under the peaks of the sedimentation patterns correcting for radial dilution and neglecting the JOHNSTON–OGSTON effect[32,14]. The results are collected in Column 5 of Table I and plotted against c_p in Fig. 8. For an extrapolation according to Eqn. 3 the data were fitted by the method of least squares, yielding

$$1/M_p' = 1/5.95 \cdot 10^5 + 7.80 \cdot 10^{-6} c_p - 4.17 \cdot 10^{-6} c_p^2 \qquad (4)$$

Accordingly the polymer molecular weight at 8.5° found by extrapolation to zero

concentration is $5.95 \cdot 10^5$ and the second virial coefficient, B, $7.8 \cdot 10^{-6}$ (g/100 ml)$^{-1}$. These results will be discussed below.

The intrinsic viscosity of the β-casein polymers

The specific viscosity of a solution of monomers and polymers is given by[26,33]:

$$\eta_{sp} = [\eta]_m c_m + [\eta]_p c_p + B_m c_m^2 + B_p c_p^2 + B_{mp} c_m c_p + \ldots \quad (5)$$

where the constants B_m, B_p and B_{mp} account for the mutual interactions of monomers, polymers and monomers and polymers, respectively.

As can be seen from the reduced-viscosity plot at 4° (Fig. 7), for the monomer we have to a fair degree

$$(\eta_{sp})_m = [\eta]_m c_m + B_m c_m^2 \quad (6)$$

with $[\eta]_m = 0.23$ and $B_m = 0.049$.
From Eqns. 5 and 6 we obtain

$$\{\eta_{sp} - (\eta_{sp})_m\}/c_p = [\eta]_p + B_p c_p + B_{mp} c_m = [\eta]_p + B_p c_p + (B_{mp}/K_n^3) c_p^{1/n} \quad (7)$$

assuming the equilibrium between monomers and polymers to be given by

$$c_p = K_n^3 c_m^n \quad (8)$$

From Eqn. 7 we see, that a plot of $\{\eta_{sp} - (\eta_{sp})_m\}/c_p$ *vs.* c_p should yield $[\eta]_p$ in the limit of zero polymer concentration.

For an evaluation of $[\eta]_p$ at 8.5° Eqn. 7 was applied to the linear part of the reduced-viscosity plot at this temperature, using the values of $[\eta]_m$ and B_m given above. The concentrations c_m and c_p again were derived from the areas in the sedimentation patterns. As is shown by the dotted line in Fig. 7, $\{\eta_{sp} - (\eta_{sp})_m\}/c_p$ varies linearly with c_p. Apparently the interaction constant B_{mp} in Eqn. (7) is negligibly small. By extrapolation $[\eta]_p$ is found to be 0.20.

At 13.5° no calculation of $[\eta]_p$ was attempted on account of the considerable curvature in the reduced-viscosity plot.

The molecular weight of the polymers from $s_{20,w}$ and $[\eta]_p$

From the values of $s_{20,w}$ and $[\eta]_p$ an estimate of the molecular weight of the polymers can be made with the aid of the well-known equation of SCHERAGA AND MANDELKERN[14]:

$$M_p = 4.090 (s_{20,w})^{3/2} [\eta]_p^{1/2} / (1 - \bar{v}\rho)^{3/2} \quad (9)$$

where $s_{20,w}$ is in Svedbergs and $[\eta]_p$ in 100 ml/g. The application of this equation to the present results at 8.5° leads to a polymer molecular weight of $4.88 \cdot 10^5$. For

TABLE II

HYDRODYNAMIC CHARACTERISTICS AND MOLECULAR WEIGHTS OF β-CASEIN MONOMERS (m) AND POLYMERS (p)

Temperature (°C)	$s_{20,w}$ (Svedbergs)		$M \cdot 10^{-5}$		$[\eta]$ (100 ml/g)		Axial ratio*	
	m	p	(Eqn. 3)	(Eqn. 9)	m	p	m	p
4	1.50	—	—	—	0.23	—	12.2	—
8.5	1.50	11.4	5.95	4.88	—	0.20	—	11
13.5	1.50	35	—	—	—	—	—	—

* Calculated for prolate ellipsoids from $[\eta]$ with $\bar{v} = 0.74$ (ref. 1) and a hydration of 0.2 g/g.

comparison the calculated polymer molecular weights are included in Table II. It can be seen that at 8.5° both methods of calculation lead to molecular weights differing by about 20%. In view of the approximate character of Eqn. 9 and the uncertainty in the extrapolation according to Eqn. 4 this may be considered satisfactory.

If it is assumed that β-casein polymers behave as random coils with restricted draining, the constant in Eqn. 9 should be replaced by 3920 (ref. 14). Accordingly, the molecular weights calculated by this equation would be diminished by about 20%, leading to a worse agreement with the molecular weight derived from the Archibald measurements.

DISCUSSION

Figs. 1 and 2 confirm previous observations of VON HIPPEL AND WAUGH[7] and of SULLIVAN *et al.*[6]. The latter authors state, however, that the lowest temperature at which association is observed, is 15°. The reason for this discrepancy between their and our results is not clear, the more so, because our viscosity measurements at 8.5° agree fairly well with the results of SULLIVAN *et al.* at 8°. Anyhow, β-casein is shown to associate at rising temperatures and in this respect resembles the casein micelles, that occur in milk[2,7]. No explanation of this peculiar temperature influence can be given as yet, though several mechanisms have been suggested in the case of other proteins[9,32]. No doubt the strong association is connected with the extremely high content of apolar side-chains found in β-casein[9].

The tremendous concentration-dependence of the sedimentation coefficients of the β-casein polymers (Fig. 2) is quite unusual for proteins. It indicates that the β-casein polymers in solution exist as more or less stiff rods[26] or as interlinked flexible coils[35]. Also the value of $7.80 \cdot 10^{-6}$ (g/100 ml)$^{-1}$ found for the second virial coefficient of the polymers at 8.5° is interesting in this respect. Comparison shows that this value is of the same order as the values found by the same technique for the virial coefficients of polyvinylchloride[30] and polystyrene[31]. In these investigations the polyvinylchloride had a molecular weight of about 50000, whereas the polystyrene had nearly the same molecular weight as the β-casein polymers at 8.5°. These synthetic polymers exist in solution as random coils[26] and it can be shown that for such molecules the virial coefficient is only slightly dependent on the molecular weight[26]. On the other hand, with proteins and especially with globular proteins, the virial coefficients usually appear to be much smaller[23,24]. It would be premature, however, to conclude from this that the β-casein polymers have a randomly coiled structure. For the virial coefficients of randomly coiled and rod-like molecules do not differ very much[26]. Moreover we have seen that the molecular weight calculated from $s_{20,w}$ and $[\eta]_p$ on the basis of the random-coil model is in serious disagreement with that derived from the Archibald measurements. The conception that the polymers are stiff, impenetrable rods is further favoured by the steep slope of $\{(\eta_{sp} - (\eta_{sp})_m\}/c_p$ at 8.5°. We feel therefore, that a definite choice between a randomly coiled and a rod-like structure on the basis of the present experiments cannot be made as yet.

The ideal behaviour of the monomer demonstrated by Figs. 2 and 3 seems to be inconsistent with its high intrinsic viscosity. For comparison we have given in Table II the values of the axial ratios of monomer and polymer as deduced from their intrinsic viscosities. It was assumed that both kinds of molecules could be represented

by prolate ellipsoids of revolution with an equal hydration of 0.2 g/g. It can be seen from Table II that the calculated asymmetry of the monomer is somewhat larger than that of the polymer. The question thus arises, why the monomer, in contrast to the polymers behaves ideally. For instance one might imagine that in the Trautman plot at 4° (Fig. 3) this ideal behaviour is only simulated by the opposite effects of non-ideality and polydispersity[22,23]. Likewise in the plot of $s_{20,w}$ *vs.* c the concentration-dependence of $s_{20,w}$ could be compensated by the formation of lower polymers[14]. Although it would be highly fortuitous that such a complete compensation would occur at all concentrations in both kinds of plots, a further investigation of the homogeneity of the slow peak was considered worthwhile. This can easily be achieved by the boundary-spreading test proposed by BALDWIN[36] for the case where the sedimentation coefficient is concentration-independent. According to this author, for a homogeneous substance the plot of $(S - s)^2$ *vs.* $1/t \exp(\bar{s}\omega^2 t)$ at fixed values of $g^*(S)/g^*(S)_{max}$ should pass through the origin. Here S is an apparent coefficient, that is defined by

$$S \equiv \ln (x/x_0)/\omega^2 t \qquad (10)$$

where x and x_0 are the respective distances from the centre of rotation of some point in the boundary considered and the meniscus; ω is the angular velocity and t the time of sedimentation. $\bar{s}$ is the mean sedimentation coefficient of the boundary and $g^*(S)$ the apparent distribution of sedimentation coefficients. BALDWIN's test was applied to the trailing edge of the slow peak of the sedimentation pattern. Fig. 9 shows clearly that the plots at fixed values of $g^*(S)/g^*(S)_{max}$ pass indeed through the origin, suggesting homogeneity of the slow peak. Consequently, we conclude that the ideal behaviour of the slow component cannot be due to the compensating effects of non-ideality and polydispersity, but that it is real. A possible explanation of this paradoxical ideality might be that we have assumed too low a hydration of the

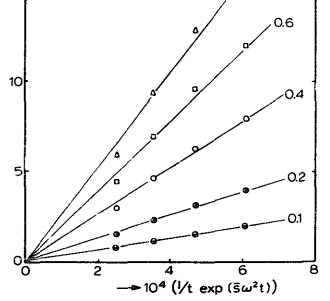

Fig. 9. BALDWIN's boundary-spreading test applied to the slow peak of the sedimentation pattern. The number at each plot indicates the fixed value of $g^*(S)/g^*(S)_{max}$. Protein concentration, 0.81 g/100 ml.

monomer as compared with the polymer, thus overestimating its axial ratio. It is worthwhile to mention that the higly asymmetrical fibrinogen molecules also show nearly ideal behaviour[37].

Though polymers of a low degree of polymerization thus seem to be absent from the slow peak, it may well be that the fast peak is heterogeneous with respect to molecular weight. Here BALDWIN's test fails on account of the extremely large concentration-dependence of the sedimentation coefficient of the fast peak. In case the polymer thus might be heterogeneous, the molecular weights calculated in the previous section represent average values. For instance, it can easily be shown that then the polymer molecular weight calculated by Eqn. 1 is the over the polymers only averaged weight average.

The shape of the sedimentation patterns and the fact that the sedimentation coefficients decrease with concentration over the whole concentration range suggest, however, a very limited distribution of polymer molecular weights.

Evidence has been obtained that the association of β-casein responds only slowly to changes in the concentration. One might wonder whether equilibrium is attained at all. This suspicion is further supported by the large scattering of the experimental points of Figs. 5 and 6 and by the incomplete reversibility observed by HAWLER[8]. In order to investigate whether serious deviations from the equilibrium occur, values of the association constants were calculated at different concentrations. The concentrations c_m and c_p in these calculations again were taken from the areas of the sedimentation patterns. The non-ideality of the polymer was accounted for by[38]:

$$\ln f_p = BM_p c_p \qquad (11)$$

Accordingly Eqn. 8 is modified into

$$K_n = (c_p/M_p) \exp (BM_p c_p)/(c_m/M_m)^n \qquad (12)$$

Values of pK_n calculated with the reasonable values of $n = 20$, 22 and 24 are collected in Table III. It can be seen that especially at the lower concentrations with no value of n pK_n is constant. Three possible explanations can be given for this observation. The first of these is that the experimental errors in c_m and c_p and in particular the neglect of the JOHNSTON–OGSTON effect[14,32] could account for the inconstancy of pK_n. For it is clear from Eqn. 12 that as a consequence of the large value of n, relatively small errors in c_m greatly affect the magnitude of K_n. However, in order to account for the whole inconstancy of pK_n, one has to assume an error of about 100 % in c_m. This seems far too high to be explained by the JOHNSTON–OGSTON effect[14].

The next possibility is that Eqn. 11 does not apply to the β-casein polymers.

TABLE III

VALUES OF pK_n CALCULATED FROM THE AREAS OF THE SEDIMENTATION PATTERNS WITH DIFFERENT VALUES OF THE DEGREE OF POLYMERIZATION (n)

c_{tot} (g/100 ml)	c_m (g/100 ml)	c_p (g/100 ml)	pK_{20}	pK_{22}	pK_{24}
0.50	0.40	0.10	−89.39	−98.95	−108.53
1.00	0.66	0.34	−85.97	−95.17	−104.43
1.50	0.81	0.69	−85.09	−94.16	−103.20
2.00	0.94	1.06	−84.52	−93.61	−102.48

Actually this equation has been severely criticized for non-globular proteins[38]. Again however, it is doubtful whether deviations from Eqn. 11 could explain the inconstancy of pK_n completely.

We are thus left with the third possibility, that the inconstancy of pK_n is due, partially or completely, to the poor equilibration of the system. As a consequence of this one might expect deviations of c_m, c_b and n from the equilibrium values. The data of Table III demonstrate clearly that a small shift of n brings about a tremendous change in pK_n. Consequently the observed inconstancy in pK_n cannot be due to large variations in n. The value of n calculated in the previous section (*i.e.* $n = 20$–24) thus seems fairly well established. This high value is remarkable, since in most protein associations studied hitherto[9, 13] the degrees of association were considerably lower.

At 13.5° the molecular weight determinations failed. From the extrapolated value of $s_{20,w}$ and from the extremely steep initial slope of the Trautman plot the conclusion seems to be justified, however, that the degree of association is considerably higher than at 8.5°.

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