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Sedimentation, Viscosity and Light Scattering Studies of Bovine Fibrinogen at Various Ionic Strengths[1]

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The influence of ionic strength on the sedimentation, viscosity and light scattering behavior of two preparations of bovine fibrinogen was studied. The concentration dependence of the sedimentation increased with increase in ionic strength of the solutions. The sedimentation constants at infinite dilution, however, converged to a single value for each preparation: namely, 7.38 S for PBF and 8.04 S for ABF. The intrinsic viscosity of both preparations of fibrinogen increased with increase in ionic strength of the solutions. The influence of ionic strength on the partial specific volume of PBF was insignificant; however, in the case of ABF, the influence was noticeable. Estimates of the molecular weights from viscosity, sedimentation and partial specific volume data yielded an average value of 253,500. The average length of the molecule was 538 Å. and the average width was 32 Å. The weight average molecular weight determined by the method of light scattering for PBF and ABF in solutions of 0.1 M averaged 245,000 and in solutions of 0.5 M averaged 389,000. The influence of ionic strength on sedimentation, viscosity and light scattering properties of bovine fibrinogen was discussed in relation to hydration, shape of the molecules and the presence of impurities.

Introduction

Physicochemical studies on bovine fibrinogen have yielded valuable information concerning the properties and behavior of this protein. Holmberg,[2] from sedimentation–diffusion data, first reported a value of 700,000 for the molecular weight of bovine fibrinogen. Shulman[3] in a more recent sedimentation–diffusion determination reported a value of 333,000 for the molecular weight. The number-average molecular weights by osmotic pressure measurements have yielded values between 400,000 and 500,000.[4–7] The weight-average molecular weights by the method of light scattering have ranged from 340,000 to 407,000.[8–10] Various workers[11–13] have reported sedimentation studies on bovine fibrinogen. In a recent review, Scheraga and Laskowski[14] summarize the molecular parameters of bovine fibrinogen. In most cases, the criterion of purity was clottability. Scheraga, *et al.*,[15] have reported that denaturation of bovine fibrinogen with 6 M urea caused neither splitting nor aggregation of the native protein but did result in swelling of the molecule. Fitzgerald, Schneider and Waugh[10] indicated that bovine fibrinogen retained its size and shape independently of protein concentration and ionic strength in phosphate buffers to pH 10.8. At higher values of pH, the molecule dissociated into subunits or fragments.

Notwithstanding the many physicochemical studies on bovine fibrinogen discussed above, no study has yet been reported on the effects of ionic strength (μ) on the physical properties of bovine fibrinogen near its isoelectric point. It is for this reason that a study of this kind was undertaken and the results are now reported.

Experimental

Two samples of bovine fibrinogen were used in this study. The first sample was prepared from a preparation of bovine fibrinogen (Lot #C–0505) obtained from the Pentex Corporation. The clottable protein in this preparation was reported by the manufacturer to be 66% of the total protein. A quantity of 200 g. of this fibrinogen was submitted to reprecipitation according to the procedure of Morrison, *et al.*[16] The purified material was dissolved in 1500 ml. of 0.3 M NaCl, shell-frozen and lyophilized. This sample is designated PBF.

The second sample was prepared from bovine fibrinogen (Lot #S–3804) supplied by Armour Laboratories. The clottable protein in this preparation was reported by the manufacturer to be 73.7% of the total protein. A two-gram and a fifteen-gram sample of this preparation were submitted to purification according to the procedure of Laki.[17] After purification, the fibrinogen was dissolved in 0.3 M KCl and stored in the refrigerator. In order to prevent any loss of clottability that might be due to the drying process, the purified fibrinogen was not lyophilized. This sample of fibrinogen is designated ABF.

The method for determining the clottability of the fibrinogen preparations, both PBF and ABF, was similar to that of Morrison.[18]

Solutions of both samples of bovine fibrinogen, PBF and ABF, in NaCl of the appropriate ionic strength were prepared for the sedimentation, viscosity, partial specific volume and light scattering studies. In the case of PBF, appropriate amounts were suspended in 30 to 35 ml. of 0.1, 0.2, 0.3 and 0.5 M NaCl. These protein solutions were

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dialyzed against two liters of the respective NaCl solutions at 5° for two days. During this period, the dialysate was changed twice. In the case of ABF, 40 to 45 ml. of the stock solution were dialyzed against 0.1 and 0.5 *M* NaCl in a manner similar to that employed for PBF. After dialysis, the insoluble material was removed by centrifugation at 10,000 r.p.m. using the Servall angle centrifuge, Model SS-1. The *p*H values of the dialysate and the protein solutions were determined with a Beckman *p*H meter and found to average around 6.3.

For the sedimentation determinations, the concentrations of the protein solutions were determined from a curve of refractive index increment *vs.* concentration. A dipping refractometer was used. The initial concentrations of the fibrinogen solutions were determined by evaporating 2-ml. quantities of the protein solutions and solvents to constant weight in an oven at 110°. Concentrations calculated for successive dilutions of the initial protein solution and refractive index increment values completed the curve.

The concentrations of the protein solutions for the light scattering determinations together with the respective refractive index increments were obtained with the Brice–Phoenix[19] Differential Refractometer using light at 436 mμ. The concentration of the protein in the initial solution was obtained by evaporation to dryness of the appropriate solutions as in the case of the sedimentation studies.

The Spinco analytical ultracentrifuge was used at a speed of 59,780 r.p.m. The temperature at each determination was near 20°. Sedimentation determinations were made on at least eleven different concentrations of protein at each ionic strength. The concentrations (c) of the protein ranged from 0.02 to a maximum of 3.59 g. per 100 ml. for PBF in 0.5 *M* NaCl. Maximal concentrations at lower ionic strengths were less. The maximal concentrations for ABF were also less. The method of interpretation has previously been described.[20] Sedimentation constants (S_{20}) were corrected to water as solvent at 20° and expressed as Svedberg units (S). The equations of the regression lines for S_{20} on c were calculated by the method of least squares.

The method for the determinations of viscosity and density was that used previously.[20] Concentrations of PBF ranged from 0.04 to a maximum of 0.97 g. per 100 ml. in 0.5 *M* NaCl. Maximal concentrations of PBF at lower ionic strengths were less. The highest concentration of ABF in 0.5 *M* NaCl was 1.64 g. per 100 ml. In order to determine the influence of the heavy component on the viscosity of the fibrinogen, a preparation of ABF was first dissolved in and dialyzed against 0.1 *M* NaCl. Insoluble material was removed by centrifugation. The solution was divided into two equal volumes, and viscosity measurements were immediately carried out on one volume. The ionic strength of the second volume was increased to 0.5 *M* NaCl by dialysis against three changes of 0.5 *M* NaCl. Viscosity measurements then were made on this solution. Equations of the regression lines for the reciprocal of the relative viscosity ($1/\eta_r$) *vs.* c were calculated by the method of least squares.

The procedure for the determination of partial specific volume has been described.[21]

The Aminco-Microphotometer[22] was used for the light scattering measurements. The round cell of 3.69 cm. inside diameter was used. The cell was positioned in the instrument by having a square glass plate cemented to the bottom of the cell which fitted into a square slot in the cell holder. The cell was calibrated with Ludox (du Pont) at a wave length of 436 mμ by a procedure similar to those[23-25] previously described. The cell constant for the 90° setting was found to be 0.0808. The linearity of the instrument at 0° was tested using K_2CrO_4 solutions ranging in concentration from 1×10^{-4} to 10×10^{-4} *M*. The linearity of the instrument at 90° was tested using fluorescein solutions at concentrations ranging from 30 to 480 g. $\times 10^{-6}$ per l. The Rayleigh ratio (R_{90}) for pure benzene was found to be 33.9×10^{-6} cm.$^{-1}$ corrected for water. The depolarization value for benzene was found to be 0.40. A sample of crystalline bovine albumin (Armour) was found to have a molecular weight of 77,000, a value in agreement with those found by others.[25] This sample of albumin did have a small amount of a heavy component appearing in the sedimentation diagram when examined on the ultracentrifuge. The dissymmetry method[26] was used to calculate the molecular weights of the fibrinogens. The correction factor for dissymmetry, assuming fibrinogen to be a rod, was obtained from the tables of Doty and Steiner.[27] The depolarization correction was applied according to the method of Debye.[28]

The solutions for light scattering were rendered dust-free by filtering with pressure through a coarse Seitz pad having a pore size of one micron. The pad was washed thoroughly with distilled water, followed by citrate buffer to remove calcium and finally solvent for the proteins. The solutions were filtered directly into the light scattering cell, covered to prevent contamination with dust. At least seven serial dilutions of the initial protein were made at each ionic strength. The concentrations ranged from 0.05 to 2 g. per 100 ml. After dilution, the solutions were filtered again through the Seitz pad before light scattering measurements were made. The concentration was always determined following filtration. Depolarization measurements were made at each protein concentration.

Estimates of the molecular weights and dimensions were made from the sedimentation, viscosity and partial specific volume data. The method of calculation was that used by Lauffer[29] for the molecular parameters of tobacco mosaic virus, and Koenig, *et al.*,[30] for the molecular parameters of bovine serum albumin. The molecule was assumed to be a prolate ellipsoid of revolution, unhydrated.

Results

Sedimentation findings revealed that both preparations of fibrinogen, PBF and ABF, contained in addition to the main component, small amounts of lighter and heavier components as impurities. These impurities were evident especially at ionic strengths from 0.2 to 0.5 *M* NaCl, Fig. 1. At an ionic strength of 0.1 *M* NaCl, the light and heavy impurities were essentially absent, Fig. 2. Sedimentation diagrams for PBF and ABF were similar, although figures for PBF only are shown. Figure 3 presents plots of the sedimentation data *vs.* concentration for the two preparations of fibrinogen at the various ionic strengths. The equations for the lines of regression and the correlation coefficients are also listed. In the case of PBF, sedimentation constants at concentrations above 1.66 g./100 ml. are not shown. All data, however, were used in the calculation of the equations. The concentration scale was chosen to emphasize values at the lower concentrations. There is a progressive increase in the slopes of the regression lines with ionic strength for both preparations of fibrinogen. The intercepts of the regression lines seem to converge to nearly a single value for PBF and to another single value for ABF.

The viscosity data including the regression lines for $1/\eta_r$ on c, the correlation coefficient, and the standard error of the slope (σ) are presented in Fig. 4. The weight intrinsic viscosities are equivalent to the slopes of the regression lines. A progressive increase in weight intrinsic viscosity is observed with increase in ionic strength for both preparations of fibrinogen. The last two lines in Fig. 4 represent

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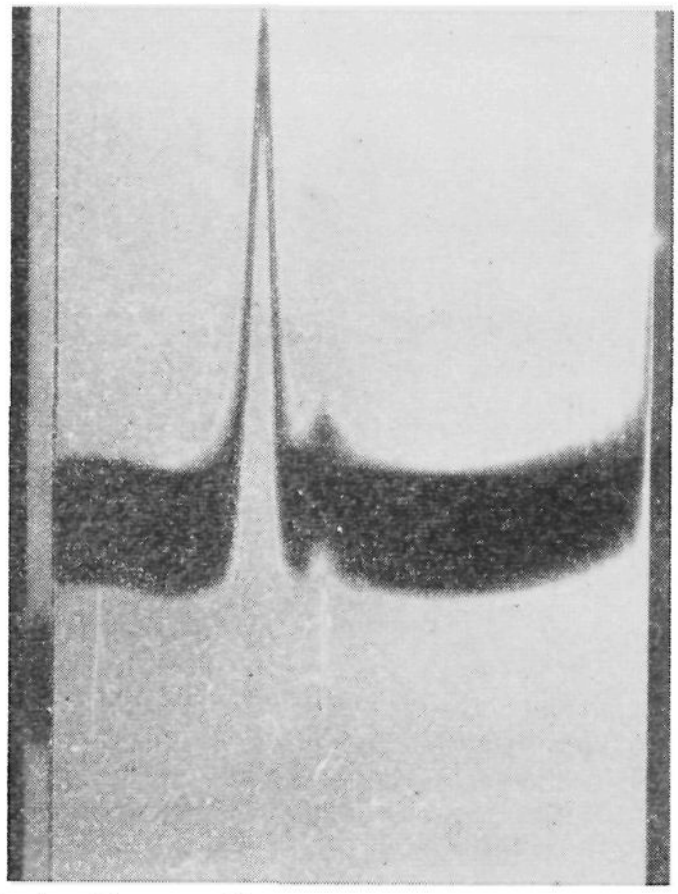

Fig. 1.—Ultracentrifuge diagram of bovine fibrinogen in 0.5 *M* NaCl, 1.08% concentration, 59,780 r.p.m., 20°. Sedimentation is from left to right. The equation of the light component is $S_{20} = 1.10 + 0.21c$. The equation of the heavy component is $S_{20} = 9.60 - 0.73c$.

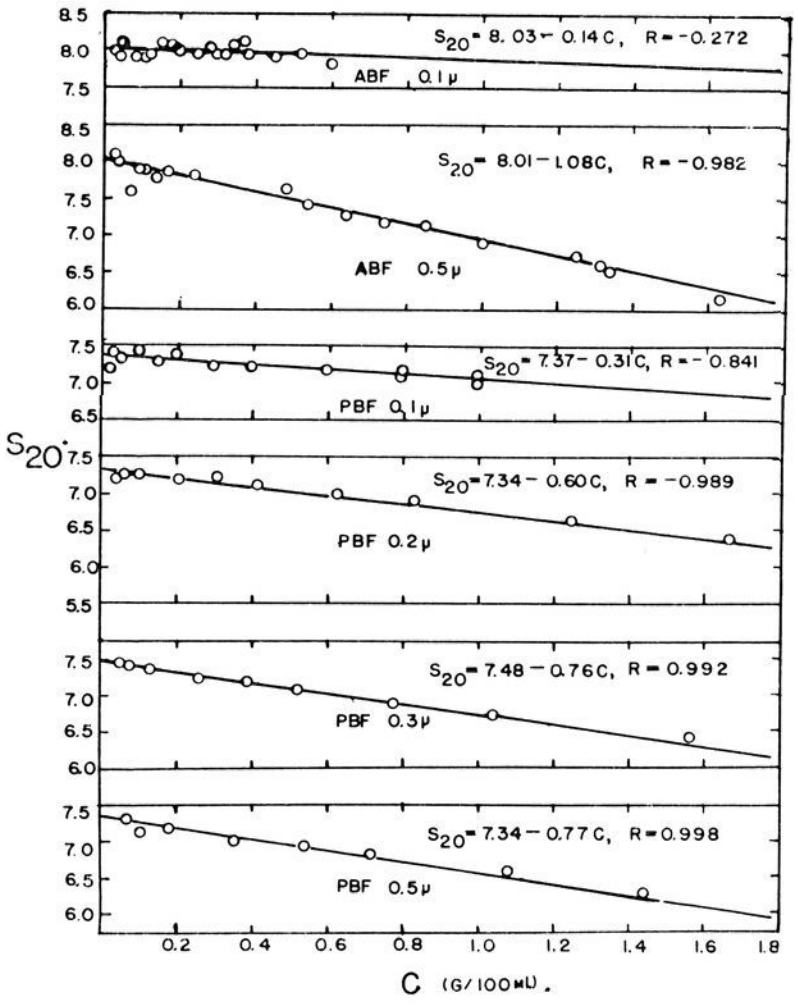

Fig. 3.—Variation of S_{20} with c for PBF and ABF at various ionic strengths of NaCl.

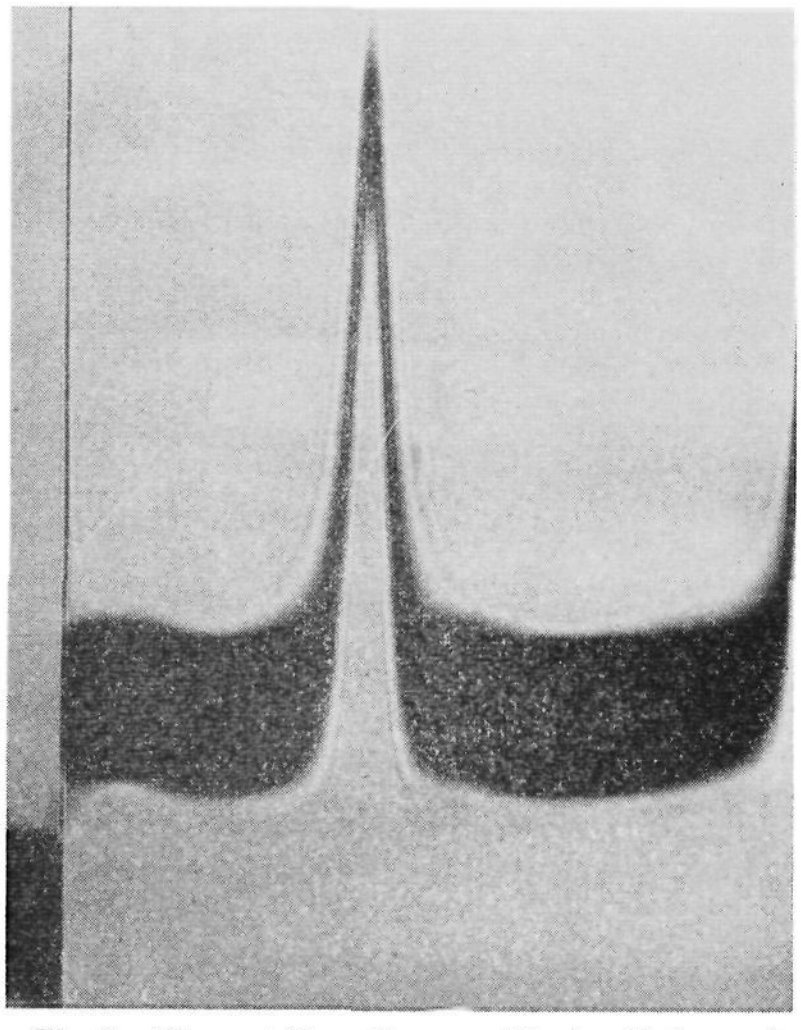

Fig. 2.—Ultracentrifuge diagram of bovine fibrinogen in 0.1 *M* NaCl, 1.00% concentration, 59,780 r.p.m., 20°. Sedimentation is from left to right.

viscosity data for the preparation of ABF dissolved first in 0.1 *M* NaCl and then after the ionic strength had been increased to 0.5 *M* NaCl.

The maximal amounts of PBF which dissolved in 0.1, 0.2, 0.3 and 0.5 *M* NaCl were 1.00, 2.10, 2.60, and 3.60 g. per 100 ml., respectively. No solubility behavior was observed for ABF since the final product was obtained in solution.

The partial specific volume (an average of three values in the case of PBF and seven values in the case of ABF) for each ionic strength are listed in Table I. The partial specific volume for PBF remained essentially constant for the ionic strengths studied. There was a difference in the partial specific volumes for ABF at ionic strengths of 0.1 and 0.5. The volume intrinsic viscosities and estimates of the molecular parameters from viscosity, sedimentation and partial specific volume data are also given in Table I.

Table II summarizes the data obtained from light scattering measurements. The turbidities of the protein solutions were corrected for the turbidity of the solvent (about 3×10^{-5} cm.$^{-1}$ at 90°). The equations of the regression lines for Hc/τ *vs.* c are given. The dissymmetry and depolarization values are listed for each ionic strength studied. The molecular weights as calculated from the reciprocals of the intercepts of the Hc/τ *vs.* c regression lines also are listed. The experiments indicated by a ionic strength of 0.1 to 0.5 refer to those in which the fibrinogen was first dissolved in and dialyzed against 0.1 *M* NaCl, the insoluble material removed by centrifugation, and the ionic strength subsequently increased to 0.5 by dialysis.

The clottability of the lyophilized sample of PBF was 23.7%. The clottabilities of the samples obtained from the purification of the 2-g. and the 15-g. quantities of ABF by the Laki procedure were

TABLE I

MOLECULAR PARAMETERS OF BOVINE FIBRINOGEN CALCULATED FROM SEDIMENTATION, VISCOSITY AND PARTIAL SPECIFIC VOLUME DATA

Ionic strength μ	Partial specific vol. V	Volume intrinsic viscosity, $[\eta]_v$[a]	Mol. wt.	Mol. dimensions, Å. Diameter	Length	Axial ratio
		Fibrinogen, PBF				
0.1 M	0.731 ± 0.002	26.40	229,000	32.4	504.8	15.58
.2 M	.723 ± .001	28.49	222,000	31.5	514.4	16.34
.3 M	.729 ± .004	29.77	241,000	32.1	540.0	16.80
.5 M	.737 ± .003	31.89	253,000	32.3	567.2	17.57
			Av. 236,000	32.1	531.6	16.57
		Fibrinogen, ABF				
0.1 M	0.740 ± 0.003	26.08	274,000	34.6	535.5	15.46
.5 M	.717 ± .004	30.82	252,000	32.2	552.8	17.18
			Av. 263,000	33.4	544.2	16.32

[a] $[\eta]_v = ([\eta]_w/V)100$, where $[\eta]_w$ is the weight intrinsic viscosity.

89.4 and 82.0%, respectively. These values of clottability for PBF and ABF were obtained when the clot was allowed to stand at room temperature for two hours. By allowing the clot to stand 16 hours with refrigeration, the Laki purification of ABF gave clottability values of 95%. There was always some heavy component present in the purified ABF. When the product was dialyzed against 0.1 M NaCl, the clottability of the material remaining in solution was slightly less and there was no heavy component. The values for clottability do not include the customary 3% added to correct for the liberation of a peptide and a carbohydrate during the clotting process. The clottability values in Table II for ABF are for the same preparation at the ionic strength indicated when the clot was allowed to stand two hours at room temperature.

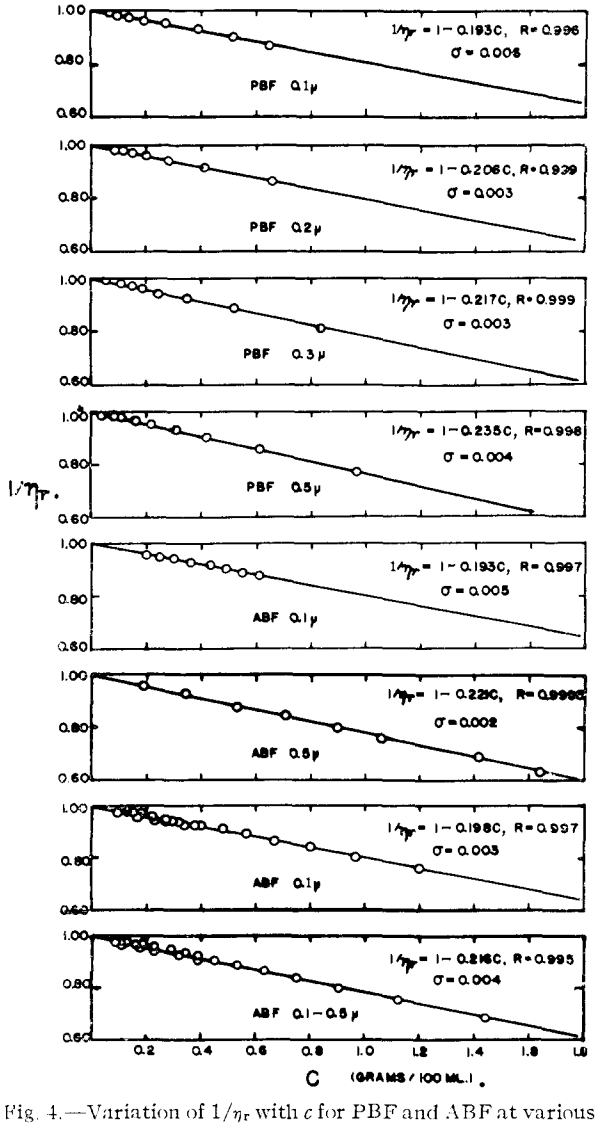

Fig. 4.—Variation of $1/\eta_r$ with c for PBF and ABF at various ionic strengths of NaCl.

Discussion

The light scattering data indicate an influence of ionic strength on the value of the molecular weight. At ionic strengths of 0.3 and 0.5, the values of molecular weight for PBF and ABF are in agreement with values reported in the literature.[7-10,14] It should be emphasized that most of the molecular weights reported in the literature were determined in solutions having ionic strengths of 0.35 to 0.45. The molecular weights determined at the lower ionic strength by light scattering are lower than those determined at higher ionic strengths. These values are in agreement with the molecular weights estimated from sedimentation, viscosity and partial specific volume data. The dissymmetries at the lower ionic strengths are somewhat lower than values reported in the literature.[10,14] In every determination of the molecular weight by light scattering, sedimentation analyses were made on various dilutions of the protein solutions. At the higher ionic strengths, there was always a component heavier than fibrinogen present in the diagram. At the lower ionic strengths, the heavy component decreased and was entirely absent at an ionic strength of 0.1. It is believed, therefore, that the higher molecular weight at an ionic strength of 0.5 and the lower molecular weight at an ionic strength of 0.1 are due to the difference in the amount of the heavy component in solution. Since the method of light scattering gives the weight-average molecular weight, the presence of a heavier and larger component would influence the values obtained.

TABLE II

LIGHT SCATTERING PARAMETERS FOR BOVINE FIBRINOGEN AT VARIOUS IONIC STRENGTHS

	Clottability, %	$\Delta n/c$ 436 mμ	Depolarization pu	Dissymmetry $I_{45°}/I_{135°}$	Regression line[a]	Correlation coefficient	Mol. wt.
0.1	92.6 (ABF)	0.1933	0.004	1.08	$Hc/\tau = 0.42-0.43c$	0.970	236,000
.1	23.7 (PBF)	.1940	.004	1.10	$Hc/\tau = .40-.24c$	.996	249,000 ± 8,000[b]
.2	23.7 (PBF)	.1935	.003	1.14	$Hc/\tau = .35-.24c$	.993	290,000
.3	23.7 (PBF)	.1987	.003	1.14	$Hc/\tau = .30-.12c$	.920	333,000 ± 16,500[c]
.5	23.7 (PBF)	.1800	.002	1.20	$Hc/\tau = .25-.06c$	.938	398,000
.5	82.9 (ABF)	.1939	.005	1.13	$Hc/\tau = .26-.02c$	.616	379,000
.1–0.5	79.3 (ABF)	.1900	.004	1.08	$Hc/\tau = .40-.18c$	.937	249,000
.1–.5	23.7 (PBF)	.2135	.005	1.11	$Hc/\tau = .41-.13c$	.975	245,000

[a] $Hc/\tau = 1/M + 2Bc$, where $H = 32\pi^3 n^2 (\Delta n/c)^2/N\lambda^4$, n is refractive index of solvent, $\Delta n/c$ is refractive index increment constant for protein, N is Avogadro's number, λ is wave length in cm. (436 mμ), c is concentration, g./ml., τ is turbidity, M is molecular weight, B is the interaction constant. For convenience $(Hc/\tau) \times 10^5$ was used instead of Hc/τ; therefore M is multiplied by 10^5 to give true value. [b] Three determinations. [c] Two determinations.

In Table II, there is good agreement in the intercepts of the regression lines for the determinations made at an ionic strength of 0.1 and those experiments containing material soluble at an ionic strength of 0.1 and subsequently having the ionic strength increased to 0.5. The slopes are significantly more negative for the lower ionic strengths than for the higher ionic strengths. Actually, the slope is a measure of the interaction of the particles as has been discussed by Stacey.[26] The interaction of the particles is reduced by increasing the ionic strength. In the case of the experiments in which the fibrinogen was first suspended at ionic strength 0.1, the insoluble material removed by centrifugation, and finally the ionic strength increased to 0.5, the interaction is lower than for the experiment maintained at an ionic strength of 0.1 throughout. The interaction is somewhat greater than in the case of the experiment carried out entirely at ionic strength of 0.5, and thus containing the heavy component.

The agreement in the values for molecular weight of PBF and ABF is significant in view of the marked difference in the clottability of the two preparations. The estimation of the molecular weights from sedimentation, viscosity and partial specific volume data is based upon the assumption of an unhydrated prolate ellipsoid of revolution. It would seem that the assumption of an unhydrated prolate ellipsoid of revolution has not resulted in a molecular weight different from that obtained by light scattering. It is therefore reasonable to assume that fibrinogen is a compact molecule and since it is the first plasma protein to be precipitated upon the addition of salt, it is less hydrated than the other plasma proteins.

The intercepts of the regression lines in Fig. 3 for PBF at the various ionic strengths are nearly the same. The same observation can be made for ABF. The intercepts for ABF are slightly higher than those for PBF. A similar difference has been observed by Fitzgerald, Schneider and Waugh.[10] This difference probably can be attributed to a difference in the method for measuring rotor temperature. The sedimentation studies on PBF were made on an earlier model ultracentrifuge, whereas studies on ABF were made on a recent model ultracentrifuge possessing better rotor temperature control.

The progressive increase in slope of the regression lines for S_{20} on c indicates that the dependence of sedimentation constant on concentration is influenced by the ionic strength of the solution. The effect of ionic strength on sedimentation may be due to changes in hydration of the protein molecule. Any change in hydration of the protein molecule with variation in ionic strength may result in alterations in the asymmetry and/or the effective volume of the molecule. Notwithstanding the considerable change in concentration dependence of the sedimentation constant with ionic strength, there is apparently insufficient change in the shape and/or size of the molecule to alter appreciably the sedimentation constant at infinite dilution.

The progressive increase of the intrinsic viscosity with increase in ionic strength is in accord with the sedimentation behavior. This increase in intrinsic viscosity with increase in ionic strength may be explained by a reasoning similar to that used in explaining the sedimentation behavior.

It is conceivable that part of the increase in intrinsic viscosity at the higher ionic strength may be due to an increase in the amount of a component heavier than fibrinogen over and above that dissolving at the lower ionic strengths. The intrinsic viscosities for both PBF and ABF were the same at an ionic strength of 0.1; however, there was a difference in the intrinsic viscosities for PBF and ABF at an ionic strength of 0.5. It is believed that this difference is due to the difference in the amount of the heavier-than-fibrinogen component dissolving in both preparations at this ionic strength. The increase in intrinsic viscosity from 0.198 for ABF dissolved in 0.1 M NaCl to 0.216 after the ionic strength had been increased from 0.1 to 0.5 M indicates that part of the increase in intrinsic viscosity is real and not due entirely to heavy component impurity. At ionic strength 0.1 M NaCl, no heavy component was present when examined on the ultracentrifuge. There was no heavy component present when the ionic strength of the same solution was increased to 0.5. The values of intrinsic viscosity herein reported are somewhat lower than those (0.25 to 0.27) reported by other workers.[3,6,8,14] Every preparation of fibrinogen dissolved at an ionic strength of 0.2 or higher possessed a small amount of a heavier component when examined in the ultracentrifuge.

The difference in the amount of clottable protein in PBF and ABF can be due in part to the fact that the determinations on PBF were made after the sample had been stored in the lyophilized form in the refrigerator at about 5° for several years. The determinations on ABF were made on the fresh preparation since the preparation was never lyophilized. Clottability of fibrinogen does not indicate homogeneity either with regard to sedimentation or with regard to electrophoresis. It would seem that one can obtain a homogeneous preparation of fibrinogen with low clottability. In the case of PBF, the original intent was to purify a quantity of fibrinogen to be stored in the lyophilized form so that a number of studies could be made on the same material. Some clottability is lost during the drying process. Although the clottability of PBF was low, its physical properties are similar to those of ABF. By all standards ABF was a good preparation of fibrinogen. Furthermore, the sedimentation and electrophoretic homogeneities of both PBF and ABF were similarly improved on purification. The results for ABF agree rather well with those of Casassa[31] and Sturtevant *et al.*[32] Both groups of workers found variations in the clottability of fibrinogen samples prepared by purification of commercial bovine fibrinogen.

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Summary

Definite changes in the sedimentation, viscosity and light scattering properties of two preparations of bovine fibrinogen are observed in solutions of NaCl at various ionic strengths.

The changes are discussed in relation to shape, volume, hydration, and interaction of the protein molecules. The influence of the presence of impurities is also discussed.

The molecular parameters were calculated from sedimentation, viscosity and partial specific volume data. The weight-average molecular weights were calculated from light scattering data. The average molecular weight from both sets of data at an ionic strength of 0.1 is 249,000 for both preparations of fibrinogen.

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CHICAGO 11, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY]

Adenosine-3′:5′-phosphoric Acid: A Proof of Structure[1]

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One of the products obtained in the degradation of adenosine-5′-triphosphoric acid (ATP) in aqueous barium hydroxide at 100° was shown conclusively to be adenosine-3′:5′-phosphoric acid, a six-membered cyclic phosphate. This compound is identical with the cofactor for the interconversion of liver phosphorylase–dephosphophosphorylase isolated from natural sources. It also has been found to mediate the adrenocorticotropic hormone activation of adrenal cortical phosphorylase. The proof of structure of adenosine-3′:5′-phosphoric acid was obtained by the use of a variety of enzymatic and chemical degradations, as well as by the direct determination of the molecular weight of the compound by ultracentrifugation. Adenosine-3′:5′-phosphoric acid was prepared also by the action of dicyclohexylcarbodiimide on adenosine-5′-phosphoric acid. A new technique for degrading adenine nucleotides by means of liquid, anhydrous hydrogen fluoride is described.

A new adenine ribonucleotide with unexpected properties has been isolated from the products obtained on degradation of adenosine-5′-triphosphoric acid (ATP) with aqueous barium hydroxide.[4,5] This new substance was assigned a cyclic dinucleotide structure as the result of a preliminary study. Further experiments reported in this paper show conclusively that the original structural assignment is incorrect and that the new nucleotide actually is the monomeric cyclic phosphate, adenosine-3′:5′-phosphoric acid (A-3′:5′-P).

Sutherland, *et al.*, have reported the formation of a heat-stable factor by tissue particles which stimulates the interconversion of liver phosphorylase and the dephosphophosphorylase.[6] Using a variety of criteria, they have demonstrated that this factor and the A-3′:5′-P obtained by the degradation of ATP[4,5] are identical.[7] Haynes has found that A-3′:5′-P serves as an intermediate agent in the induced stimulation of adrenal phosphorylase by adrenocorticotropic hormone (ACTH).[8]

It was readily demonstrated that A-3′:5′-P is an adenine nucleotide. The following observations led to this conclusion: (1) Its ultraviolet absorption spectrum is essentially identical with the spectra of the adenylic acids; (2) deamination by nitrous acid yields a product with the characteristics of an inosine derivative; (3) adenine is obtained on acid hydrolysis; (4) elementary analyses for carbon, hydrogen, nitrogen and phosphorus are those expected for an adenine nucleotide; and, (5) adenosine is slowly formed by the action of *Crotalus adamanteus* venom. All other evidence which was obtained is in agreement with this conclusion.

Configuration of the Anomeric Carbon Atom.—The specific rotation of A-3′:5′-P[5] is the same in sign (negative) and order of magnitude as the spe-

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