The Hydrodynamic Properties of Bovine Serum Albumin Monomer and Dimer*

P. G. Squire,† P. Moser,‡ and C. T. O'Konski§

ABSTRACT: Highly purified preparations of defatted bovine serum albumin monomer and dimer were prepared by gel filtration on Sephadex G-100. Sedimentation equilibrium experiments demonstrated that the monomer fraction was homogeneous with respect to mass and had an anhydrous molecular weight of 66,700 ± 400. Sedimentation velocity experiments showed that the concentration dependence of the sedimentation coefficient is dependent upon ionic strength but the value at infinite dilution is not. From this and other studies it was concluded that conformation of the isoionic monomer is not dependent upon ionic strength. After considering the methods of combining various kinds of data on hydrodynamic properties of rigid macromolecules to calculate shape parameters, a new set of functions, the γ functions, was defined. They are especially useful in calculating axial ratios of macromolecules. This is discussed and illustrated with data from the above experiments and from rotary diffusion constants reported earlier. Comparison of our calculated axial ratios for bovine serum albumin with values taken from or based upon earlier literature leads to a choice of 3.0–3.5 as the best available value. It is concluded that hydrated bovine serum albumin monomer can be represented adequately as a prolate ellipsoid of revolution with major axis $2a$ = 140 Å and minor axes $2b$ = 40 Å. Within experimental limitations, this is not inconsistent with Bloomfield's linear aggregate of three spheres. Data on the dimer are consistent with a side-to-side aggregation of such a monomer, with about 50% overlap.

Oncley (1943) and Scheraga and Mandelkern (1953) have shown that by combining the results from several types of hydrodynamic experiments, the shape and hydration of proteins may be independently determined. To this end, three parameters, β, δ, and, more recently (Scheraga, 1961), μ, have been defined in terms of experimentally measured quantities, and values have been tabulated for various axial ratios. The paramter β is rather insensitive to shape, and it has been pointed out (Schachman, 1959) that because of this insensitivity, it may find its greatest use as a method of combining sedimentation velocity and viscosity data for the calculation of molecular weights. On the other hand, δ and μ utilize the rotary diffusion coefficient and, as a consequence of the fact that rotational motion is much more dependent upon shape than is translational motion, these quantities are much more sensitive to axial ratio.

In considering the best possible ways to obtain macromolecular shape from a combination of electric birefringence and dielectric relaxation times with sedimentation data, we arrived at a new set of three parameters for the general ellipsoid, γ_i ($i = a, b, c$) sensitive to shape alone. One of these is related to the parameter μ defined earlier by Scheraga (1961), $\gamma_a = 1/\mu^3$. The μ parameter apparently has not been used to date. Application of the γ functions requires accurate experimental values for the buoyancy-corrected molecular weight, $M(1 - \bar{v}\rho)$, the sedimentation coefficient, s, and the rotary diffusion coefficients, θ_i. Considerations relative to the evaluation of the values of θ_i have been discussed elsewhere (Moser *et al.*, 1966); here we consider the accuracy of determination of the parameters s and $M(1 - \bar{v}\rho)$ and we include a discussion of the use of the γ_i. Values are reported for bovine serum albumin and are combined with the rotary diffusion coefficients reported earlier (Moser *et al.*, 1966) to give our best estimate of the dimensions of the equivalent ellipsoid of revolution for hydrated bovine serum albumin. These conclusions are in close agreement with the ones derived from Kerr effect and dielectric relaxation data alone (Moser *et al.*, 1966).

The uncertainty in the molecular weight of bovine serum albumin has been summarized by Phelps and Putnam (1960) as follows: "Altogether the fact that the molecular weight of . . . bovine serum albumin cannot be stated much better than as being within the range of 65,000–70,000 is a serious reflection on the present status of methods for the determination of molecular weight

* From the Hormone Research Laboratory and the Department of Chemistry, University of California, Berkeley, California 94720. *Received June 17, 1968.* This is paper XIX of the series "Electric Properties of Macromolecules." This work was supported in part by National Science Foundation Grant GB-4226, awarded to P. G. S., a stipend from the Stiftung für Stipendium auf dem Gebiete der Chemie, awarded to P. M., and U. S. Public Health Service Research Grant GM-12082, awarded to C. T. O. Presented at the 9th Annual Meeting of the Biophysical Society, San Francisco, Calif., Feb 1965, Abstract FD6.

† Hormone Research Laboratory, University of California, Berkeley, Calif. Present address: Department of Biochemistry, Colorado State University, Fort Collins, Colo.

‡ Department of Chemistry, University of California, Berkeley, Calif. Present address: Eidg. Technische Hochschule, Laboratorium für Molekularbiologie, Zurich, Switzerland.

§ Department of Chemistry, University of California, Berkeley, Calif.

TABLE I: Values of the Frictional Ratio for Translation and Rotation around the Major and Minor Axes of Prolate and Oblate Ellipsoids of Revolution and Corresponding Values of the γ Functions.

Prolate						Oblate					
a/b	f/f_e	θ_e/θ_b	$\frac{2\theta_e}{\theta_a+\theta_b}$	γ_a	γ_b	b/a	f/f_e	θ_e/θ_b	$\frac{2\theta_e}{\theta_a+\theta_b}$	γ_a	γ_b
1.0	1.000	1.000	1.000	1.000	1.000	1.0	1.000	1.000	1.000	1.000	1.000
1.2	1.003	1.058	0.992	0.954	1.017	1.2	1.003	0.983	1.030	1.026	0.980
1.4	1.010	1.143	0.999	0.902	1.032	1.4	1.010	0.998	1.074	1.032	0.959
1.6	1.020	1.248	1.014	0.850	1.047	1.6	1.019	1.032	1.128	1.027	0.939
1.8	1.031	1.369	1.031	0.801	1.064	1.8	1.030	1.078	1.189	1.015	0.919
2.0	1.044	1.505	1.050	0.756	1.083	2.0	1.042	1.132	1.226	1.000	0.901
2.5	1.078	1.892	1.095	0.661	1.142	2.5	1.073	1.289	1.435	0.959	0.861
3.0	1.112	2.344	1.134	0.588	1.215	3.0	1.105	1.465	1.626	0.920	0.829
4.0	1.182	3.396	1.190	0.487	1.390	4.0	1.166	1.843	2.026	0.860	0.783
5.0	1.255	4.641	1.226	0.421	1.593	5.0	1.223	2.240	2.437	0.818	0.752
6.0	1.314	6.044	1.250	0.375	1.812	6.0	1.277	2.646	2.853	0.787	0.730
8.0	1.433	9.401	1.279	0.3133	2.303	8.0	1.373	3.471	3.691	0.745	0.701
10.0	1.543	13.37	1.295	0.2748	2.836	10.0	1.458	4.305	4.534	0.719	0.683
15.0	1.784	25.86	1.313	0.2205	4.305	15.0	1.636	6.407	6.649	0.684	0.659
20.0	1.996	41.82	1.321	0.1899	6.012	20.0	1.782	8.519	8.768	0.665	0.646
30.0	2.356	84.93	1.327	0.1562	9.991	30.0	2.020	12.753	13.008	0.646	0.634
40.0	2.668	137.4	1.330	0.1383	14.288	40.0	2.212	16.992	17.25	0.637	0.627
100.0	4.064	695	1.333	0.0968	50.45	100.0	2.934	42.45	42.71	0.620	0.616

of proteins." We agree that the uncertainty in molecular weight exists, but not regarding its cause. Since most preparations previously studied contained appreciable amounts of dimer and higher polymers, it appears that the state of the preparations rather than the methods are the source of the uncertainty. Here we report the preparation of highly purified monomer by gel filtration and a precise determination of the molecular weight. A second uncertainty concerns the use of the sedimentation coefficient, s. High-precision measurements of s *vs.* concentration are in the literature, but the question arises as to whether the conformation of bovine serum albumin is the same in the deionized solutions used in the determination of the θ_t values as it is in the buffer systems used in sedimentation velocity measurements. Experimental data relative to these questions constitute the experimental portion of this paper.

Theory

It is possible to combine the expressions for rotary and translational frictional coefficients into one relation, which defines the γ functions for a rigid hydrodynamic ellipsoid. The three γ functions each correspond to rotary diffusion of one of the principal hydrodynamic axes of rigid molecule around the other two axes. They depend only upon shape, and, as we will show, can be calculated directly from experimental quantities. Since they are obtained from data on solvated macromolecules, the conclusions derived from them regarding shape are strictly applicable only to the solvated system.

In general, the rotary diffusion constants defined by Einstein may be written as

$$\theta_i = kT/\zeta_i$$

where $i = a, b, c$ labels the axis, and ζ_t is the frictional coefficient for rotation *around* the axis i. The symbols k and T have their usual meanings. To see how these rotary diffusion constants may be combined with the translational diffusion coefficient, let us consider a sphere of radius r in a solvent of viscosity η. Its rotary diffusion constant, $\theta = kT/8\pi\eta r^3$, varies inversely as the cube of the radius, whereas the translational diffusion coefficient (eq 1) varies inversely as the radius. Here f is the

$$D = kT/f = kT/6\pi\eta r \tag{1}$$

translational frictional coefficient. In general, for any fixed shape, all of the rotary diffusion constants depend upon dimensions cubed, and the translational diffusion coefficient depends upon dimensions to the first power. Thus the ratio of the rotary diffusion constants to the cube of the translational diffusion coefficient will give a quantity independent of size. Keeping in mind that $D/D_e = f_e/f$, where the subscript e refers to values for the sphere equivalent to the solvated macromolecule, it is convenient to form the ratios as follows

$$\left.\begin{aligned}\gamma_a &= \left(\frac{f}{f_e}\right)^3 \frac{\theta_b+\theta_c}{2\theta_e}\\ \gamma_b &= \left(\frac{f}{f_e}\right)^3 \frac{\theta_a+\theta_c}{2\theta_e}\\ \gamma_c &= \left(\frac{f}{f_e}\right)^3 \frac{\theta_a+\theta_b}{2\theta_e}\end{aligned}\right\} \tag{2a}$$

In a general notation eq 2a may be expressed as

$$\gamma_i = (f^3/2\theta_e f_e)\sum_{j\neq i}\theta_j \qquad (2b)$$

where $i = a, b, c$, and $j = a, b, c$. Here $1/2\theta_e f_e^3$ is a factor which makes the γ's independent of size and equal to unity for a sphere. The combinations of rotary diffusion constants which appear in eq 2a are directly determinable from the well-known relations (Edsall, 1949, 1953) for the dielectric relaxation times

$$\tau_{e_1} = \frac{1}{\theta_b + \theta_c} \quad \tau_{e_2} = \frac{1}{\theta_a + \theta_c} \quad \tau_{e_3} = \frac{1}{\theta_a + \theta_b}$$

For applications of eq 2a and 2b, f can be calculated by means of

$$f = \frac{M(1 - \bar{v}\rho)}{Ns} \qquad (3)$$

where M is the molecular weight, $\bar{v}$ is the partial specific volume of the solute, ρ is the solution density, N is Avogadro's number, and s is the sedimentation coefficient. It can also be obtained from the translational diffusion constant by eq 1.

Thus, experimentally determined values of the γ_i for solvated macromolecules can be calculated from the sedimentation coefficient, the rotary diffusion coefficients, and the product $M(1 - \bar{v}\rho)$. Fortunately, this product may be determined directly and accurately from sedimentation equilibrium measurements without requiring separate values of $\bar{v}$ and M.

Since

$$2\theta_e f_e^3 = 2kT(6\pi\eta r)^3/8\pi\eta r^3 = 54\pi^2\eta^2 kT \qquad (4)$$

introduction of eq 3 and 4 into eq 2b gives

$$\gamma_i = \frac{1}{54\pi^2 N^3 k}\frac{M^3(1 - \bar{v}\rho)^3}{s^3\eta^2 T}\sum_{j\neq i}\theta_j \qquad (5)$$

Because of concentration dependence of the experimental quantities, extrapolation to infinite dilution is necessary. If all data are converted into 25° in water, eq 5 becomes

$$\gamma_i = 2.612 \times 10^{-57}[M(1 - \bar{v}\rho)/s]^3\sum_{j\neq i}\theta_j \qquad (6)$$

The calculation of γ values from experimental data by eq 5 and 6 does not involve any assumptions regarding molecular shape or hydration. These experimentally obtained γ values may be compared with theoretical values computed for any hydrodynamic model. Here we consider only rigid ellipsoids in a Newtonian fluid.

In order to use the equations of Perrin for ellipsoids of revolution, we restrict ourselves to the axially symmetrical case ($a \neq b = c$). The justification and implications of discussing protein conformation in terms of hydrodynamically equivalent ellipsoids of revolution have been discussed by others (Scheraga and Mandelkern, 1953). We have the following relationships (Edsall, 1949, 1953) between the dielectric relaxation times, τ_{e_i}, and the rotary diffusion coefficients, θ_i.

For rotation of the symmetry axis around the transverse axes

$$2\theta_b = 2\theta_c = 1/\tau_{e_1} \qquad (7)$$

For rotation of one of the transverse axes around the other transverse axis and the symmetry axis

$$\theta_a + \theta_b = \theta_a + \theta_c = 1/\tau_{e_2} \qquad (8)$$

The rotary diffusion coefficient of the long axis of an ellipsoid of revolution can also be calculated from the electric birefringence or electric dichroism relaxation time, τ_n, according to the equation (O'Konski and Zimm 1950; Benoit, 1951).

$$\theta_b = 1/6\tau_n \qquad (9)$$

Theoretical values for γ_a and γ_b for ellipsoids of revolution may be calculated from the rotary and translational frictional ratios which have been evaluated for a wide range of axial ratios by means of Perrin's equations, and which are tabulated, *e.g.*, in Cohn and Edsall (1943).

These values of γ_a and γ_b for prolate and oblate ellipsoids of revolution are recorded in Table I. They can provide a test of internal consistency in that the axial ratios obtained from the two parameters must be equal if the molecule behaves hydrodynamically like a rigid ellipsoid of revolution.

It seems likely that determination of the two parameters from experimental results provides one of our most useful methods of distinguishing between prolate and oblate ellipsoids of revolution. We observe that for oblate ellipsoids of revolution γ_a is approximately equal to γ_b for all axial ratios, while for prolate ellipsoids the values of γ_a and γ_b diverge with increasing axial ratio.

Experimental Section

The bovine serum albumin used in these studies was crystallized bovine plasma albumin (list 2267) obtained from Armour Pharmaceutical Co., Kankakee, Ill. Lipids were removed as suggested by Foster (1960a,c). A 10% solution was prepared by dissolving the protein in 0.12 N HCl. If necessary, the pH was adjusted to 2.6–2.9 and the solution was stored at 5°. A slight turbidity, due presumably to fat droplets, developed after a few hours. After 3 days the solution was filtered through a Millipore filter (no. HA, 45 μ) and then submitted to gel filtration.

Gel filtration on Sephadex was performed under the conditions described by Pedersen (1962). The column was 4 cm in diameter and 150 cm long. The bed volume was 1900 ml and the void volume was 492 ml. The void volume was determined with a dyed dextran of molecular weight around 500,000, Cibacron Blått D × 500, kindly supplied by Dr. Gelotte, Pharmacia, Uppsala, Sweden. The column was packed with Sephadex G-100, bead form (no. 2946), with particle size 40–120 μ. The buffer was 0.200 N in NaCl and 0.05 M in Tris, adjusted to pH 8.1.

Following gel filtration, the monomer fraction was

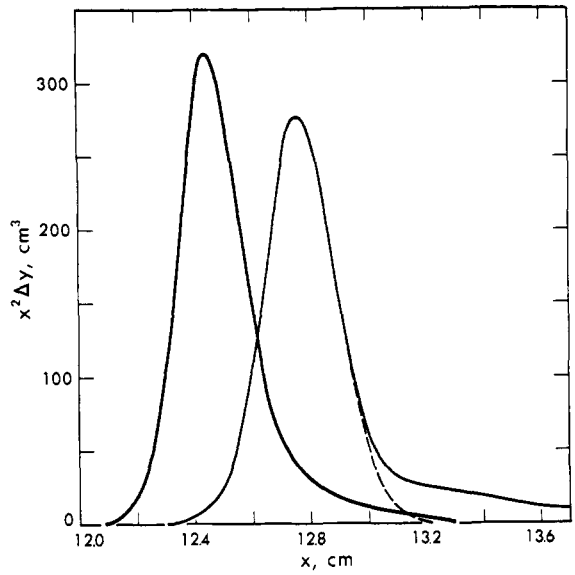

FIGURE 1: Analysis of Armour bovine serum albumin by sedimentation velocity. Schlieren diagrams corrected for radial dilution are plotted from pictures taken 88 and 120 min after the ultracentrifuge came up to a speed of 52,640 rpm. The protein concentration was 2 g/100 ml in a buffer 0.2 M in NaCl and 0.05 M in Tris (pH 8.15). The portion of the diagram considered due to monomer is indicated by the dashed line (expt 174.) Here x is the product of the magnification factor, F, and the radial distance, r, from the axis of rotation.

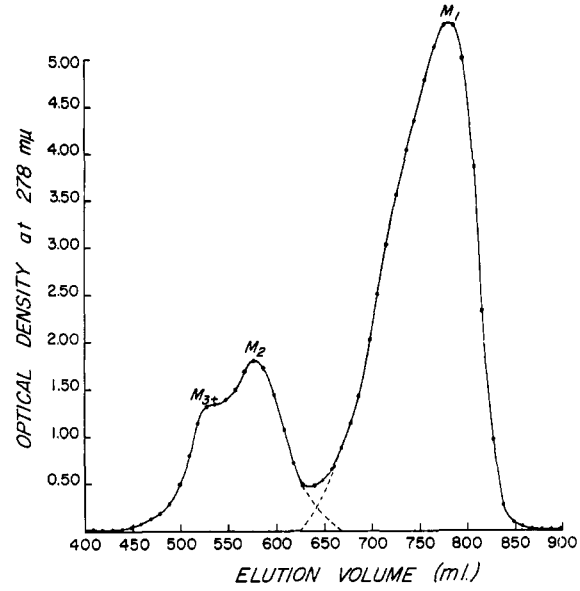

FIGURE 2: Gel filtration of Armour bovine serum albumin on Sephadex G-100. The column with a void volume of 494 ml was equilibrated with Tris-NaCl buffer (pH 8.1), ionic strength 0.250. The monomer and fractions containing higher oligomers are indicated by M_1, M_2, and M_3, etc. Tubes representing elution volumes of 700–820 ml were pooled as the monomer fraction, while volumes of 500–610 ml were pooled as the oligomer fraction.

pooled, concentrated by ultrafiltration (van Hofsten and Falkbring, 1960), dialyzed overnight against distilled water, and then deionized on a mixed-bed deionization column of the type referred to as "Column C" by Dintzis (1952). The isoionic solutions of pH 5.15 at 1% protein concentration freshly prepared as described here were used in all measurements reported here and in a preceding paper (Moser *et al.*, 1966). Where necessary, buffer composition was changed by dialysis, but for fear of irreversible association, lyophilization was not attempted.

The ultracentrifugation experiments were performed with a Spinco Model E ultracentrifuge. The characteristics of the instrument and treatment of data have been detailed elsewhere (Squire *et al.*, 1963).

Since concentration dependence and polydispersity were negligible, molecular weights were calculated from the slope of the log c *vs.* x^2 plot by means of the equation

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega\rho^2}\frac{\mathrm{d}\ln c}{\mathrm{d}r^2} \qquad (10)$$

where ω is the angular velocity; c is the protein concentration; r is the radial distance; and $x = Fr$, where F is the radial magnification factor. The value 0.734 (Dayhoff *et al.*, 1952) was used as the partial specific volume of bovine serum albumin. Sedimentation coefficients were converted into the usual standard state, $s_{20,w}$, by means of the equation

$$s_{20,w} = s_{obsd}\frac{\eta}{\eta_{20,w}}\frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho')} \qquad (11)$$

where η is the viscosity of the buffer at the temperature of the experiment, and ρ' in the expression $1 - \bar{v}\rho'$ is the density of the solution at the temperature of the experiment.

Results

Analysis of Armour Bovine Serum Albumin by Sedimentation Velocity and Gel Filtration. Because of the pronounced effect that bovine serum albumin oligomers (dimers and higher association products), frequently present in commercial preparations of bovine serum albumin would have on the dielectric dispersion results, it was essential that an estimation of the amount of oligomers be made. Sedimentation velocity appeared to be the method of choice. A 2% solution of Armour bovine serum albumin was examined by sedimentation velocity. The schlieren diagrams taken after 88 and 120 min at 52,640 rpm and corrected for radial dilution are given in Figure 1. The fraction of monomer was calculated from the ratio of the integral $\int x^2\Delta y dx$ over the portion of the peak assigned to monomer (at 120 min), divided by the same integral over the entire sample peak (at 88 min). Here Δy refers to the vertical distance between the schlieren images of solution and solvent. The fraction of monomer was found to be 0.895.

This result demonstrated that the amount of oligomer in this preparation was sufficient to seriously complicate the interpretation of dielectric dispersion experiments (Moser *et al.*, 1966), and removal of the polymerized material by gel filtration (Pedersen, 1962) appeared mandatory. The results of a typical gel filtration experiment are presented in Figure 2. In this experiment

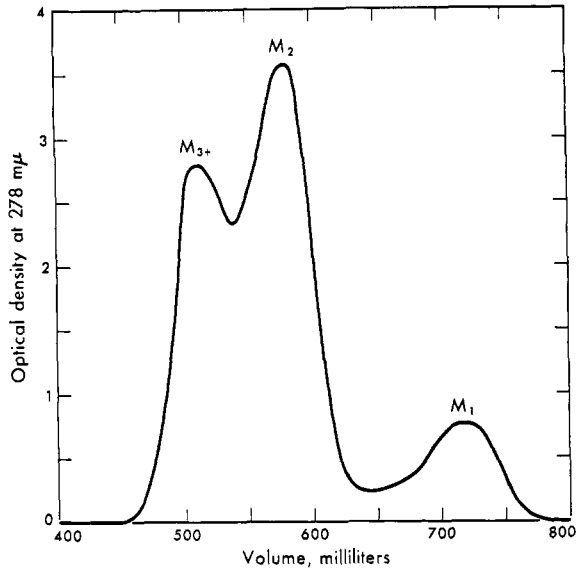

FIGURE 3: Gel filtration of pooled oligomer fractions from several experiments on Sephadex G-100. Pooled fractions obtained from several experiments as shown in Figure 2, and stored in the frozen state, were rerun on the same column under conditions essentially as described in Figure 2. Volumes of 570–660 ml were pooled as the dimer fraction.

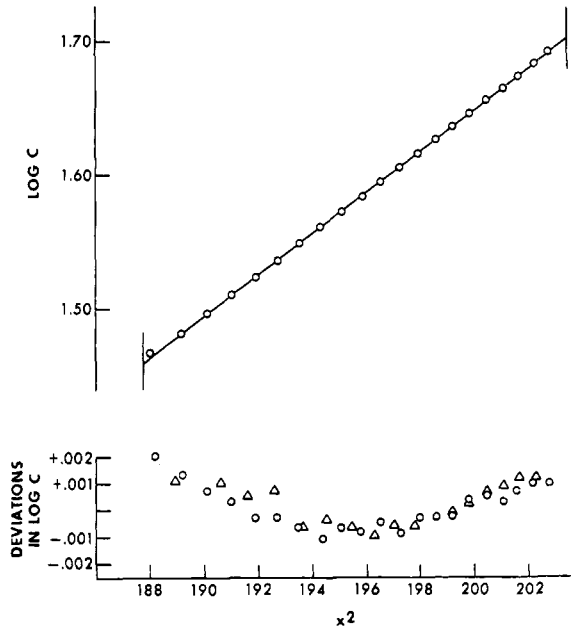

FIGURE 4: Plot of the logarithm of concentration (in fringes) *vs.* x^2 at sedimentation equilibrium of bovine serum albumin monomer in acetate buffer (pH 4.55), ionic strength 0.11 (expt 190, Table II). At the bottom of the figure are recorded the fringe deviations calculated for two of the experiments, 190 and 191.

a 2.0-g sample of bovine serum albumin, defatted as described above, was fractionated in a column that had been equilibrated with the Tris buffer (pH 8.15). After application of the protein, 20 ml at pH 2.9, gel filtration was continued with the Tris buffer. Comparison of the area of the elution diagram corresponding to the monomer with the total area indicated that the weight fraction of the monomer was only 79%.

The oligomer fractions from several gel filtration experiments, stored in the frozen state, were pooled, concentrated by ultrafiltration, and rerun on Sephadex G-10 under the same conditions as above. The elution diagram from this experiment is given in Figure 3. The tubes corresponding to elution volumes 570–600 ml were pooled, concentrated, and used in the experiments characterizing the dimer fraction (Moser *et al.*, 1966).

Characterization of the Monomer Fraction by Sedimentation Equilibrium. The monomer fraction obtained from Sephadex gel filtration was studied in a series of three sedimentation equilibrium experiments. The purpose of these experiments was twofold: to apply a highly sensitive criterion of mass homogeneity, and to determine the molecular weight of the monomer. A high degree of mass homogeneity was shown by the fact that in all experiments the plot of the logarithm of concentration *vs.* the square of radial distance displayed only minor deviations from linearity (Figure 4). Details of the experimental conditions and the calculated values of the molecular weight from each of the experiments are given in Table II. Since the data in Table II show no concentration dependence in the molecular weight, the best estimate of the molecular weight of the bovine serum albumin monomer is the mean value 66,700 ± 400, where the uncertainty is given as the mean-square error.

On the lower part of Figure 4, we have plotted the fringe deviations from two of the experiments on a greatly enlarged scale. With the exception of one point, very close to the meniscus, and therefore somewhat unreliable, we note that most of the deviations are 0.001 fringe or less, but they do display a trend of curvature. Since the optics of the ultracentrifuge are focused at the customary midcell position, it is possible that these small deviations are a result of Wiener skewing. If the curvature is attributed to heterogeneity, the presence of 1% dimer or small molecules, or both, would account for the observed deviations.

Sedimentation Velocity Analysis of the Monomer and Dimer Fractions in Various Media. The schlieren diagrams from the sedimentation velocity experiment of the pure monomer fraction in Tris buffer (pH 8.15) provided additional evidence that the sample was monodisperse. Microcomparator readings of one of the photographs, corrected for radial dilution, are plotted in Fig-

TABLE II: Sedimentation Equilibrium of Bovine Serum Albumin Monomer.[a]

Expt	c_0 (g/100 ml)	Rpm	Mol Wt
190	0.917	6166	66,200
191	0.562	7447	67,200
192	0.342	9341	66,700
Mean			66,700 ± 400[b]

[a] pH 4.55 (acetate), μ = 0.110. [b] Standard deviation.

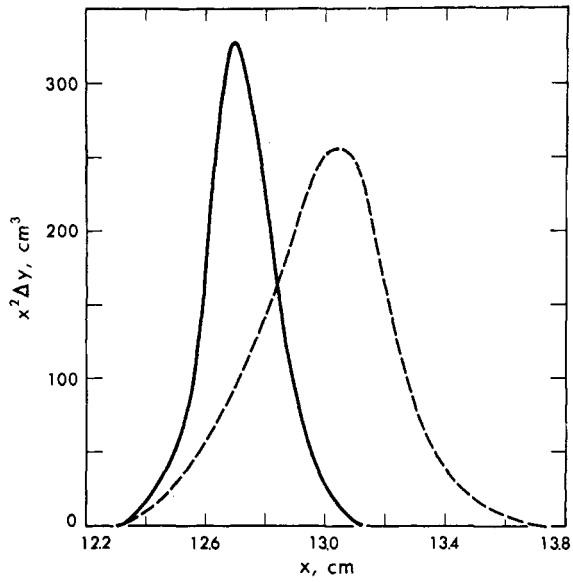

FIGURE 5: Analysis of bovine serum albumin monomer fraction by sedimentation velocity. Solid line: expt 178, protein concentration 1.95 g/100 ml in Tris buffer (pH 8.1), ionic strength 0.225. Dashed line: expt 179, deionized, protein concentration 2.17 g/100 ml. Both photographs were taken 112 min after the ultracentrifuge came up to speed at 52,640 rpm. The x axis of the plot from expt 178 was adjusted in order to superimpose the meniscus positions in the two experiments.

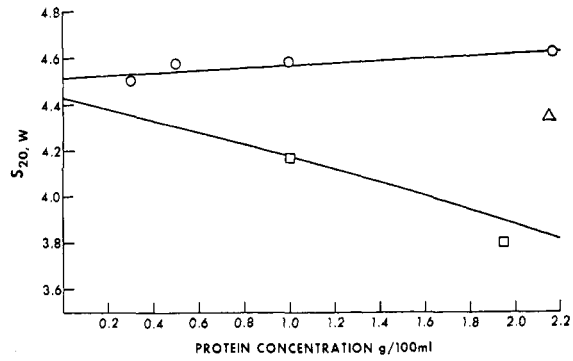

FIGURE 6: The concentration dependence of the sedimentation coefficient of deionized bovine serum albumin (○) is compared with the regression line obtained by Baldwin (1957) and converted into 20° (solid line) in acetate buffer (pH 4.6) and an ionic strength of 0.11. Also included are values of the sedimentation coefficient in a deionized solution to which 1 mole of NaCl/mole of protein has been added (△), and data obtained at pH 8.15 in Tris buffer (□).

ure 5. The sedimentation behavior of the same fraction in the deionized state was remarkably different, however. The sedimentation rate was substantially higher and boundary spreading was much greater. These two effects are also illustrated in Figure 5, where the microcomparator readings at equal sedimentation times are shown. The slight skew observed in the solid curve was clearly visible in the first photograph taken immediately after boundary formation in the synthetic boundary cell. Since this skew diminished with time, it was attributed to faulty boundary formation rather than heterogeneity.

The concentration dependence of the converted sedimentation coefficient, $s_{20,w}$, of the deionized protein, calculated from the second moment, is given in Figure 6 (circles). Also included in the same figure is the regression line calculated by Baldwin (1957) from his measurements of s *vs.* c in acetate buffer (pH 4.6) and ionic strength 0.11. While we have not made a thorough study of the s *vs.* c dependence in Tris buffer, we note that our two observations, also recorded in Figure 6, are consistent with extrapolation to the same value obtained with our data from deionized solutions.

The effect of a trace of electrolyte (1 mole of NaCl/mole of protein) on the sedimentation coefficient at a protein concentration of 2% is shown in Figure 6, as well as in Table III. The presence of this trace of electrolyte was sufficient to reduce the sedimentation coefficient nearly half-way to the value it would have had at ionic strength 0.11.

The schlieren diagram obtained from sedimentation velocity of the dimer fraction in acetate buffer is given in Figure 7. The peak is very nearly symmetrical, although a slight skew in the peak shape suggests the presence of higher association products, probably trimer present in an amount barely detectable. The converted sedimentation coefficient and conditions of the experiment are included in Table III.

Discussion

Our gel filtration data are in good agreement with those previously published by Pedersen (1962). The lack of symmetry of the monomer peak (Figure 2), as well as the appearance of appreciable amounts of monomer on rerunning the oligomer fraction, were both observed in the earlier study (Pedersen, 1962).

Analysis of the defatted bovine serum albumin by gel filtration indicated that 21% of the protein was in the form of association products, while the results of the sedimentation velocity experiments on the same material indicated only 10% oligomer. More recently, the molecular weight distribution of the same unfractionated sample was determined (Squire and Benson, 1967) by computer analysis of the data at sedimentation equilibrium. By this method it was found that the sample contained 85% monomer. The underestimate of the amount of oligomer by the sedimentation velocity method is due, at least in part, to the Johnston–Ogston effect (Schachman, 1959, pp 116–128). It is also possible that some of the highly associated bovine serum albumin may have sedimented away from the boundary.

The value for the molecular weight of the bovine serum albumin monomer, 66,700, calculated from the sedimentation equilibrium measurements reported here, is in good agreement with the value 66,500 calculated by Baldwin (1957) and the value 67,000 reported by Loeb and Scheraga (1956). Both these values from the literature were calculated from the results of sedimentation velocity and diffusion experiments in which the data were corrected to eliminate the effects of traces of association products. This value is also in good agreement with the value 66,836, which we calculated for the isoionic protein from the amino acid analysis of Spahr and

TABLE III: Sedimentation Velocity of Bovine Serum Albumin Monomer and Dimer.

Expt	Fraction	Buffer[a]	Protein Concn (g/100 ml)	$s_{20,w}$ (S)
174	Armour	pH 8.15	2.0	3.658
178	Monomer	pH 8.15	1.95	3.800
179	Monomer	Deionized	2.17	4.621[b]
180	Monomer	Deionized + NaCl[c]	2.17	4.344
182	Monomer	pH 8.15	1.0	4.169
181	Monomer	Deionized	1.0	4.583[b]
183	Monomer	Deionized	0.50	4.574[b]
184	Monomer	Deionized	0.25	4.501[b]
196	Dimer	pH 4.55	0.450	6.71

[a] Compositions of the buffers are given in the text. [b] Sedimentation coefficients so designated are calculated from the second moment, others from the maximum ordinate. [c] One mole of NaCl per mole of protein was added.

Edsall (1964). For other comparisons with data in the literature, see Phelps and Putnam (1960).

Early recognition (Svedberg and Pedersen, 1940) of the important role of charge effects in sedimentation of proteins has led to the almost universal practice of adding supporting electrolyte to the solvent; consequently, there have been few studies of sedimentation velocity of proteins at very low ionic strength. Pedersen (1958), in his study of charge and specific ion effects on the sedimentation coefficients of bovine serum albumin at low ionic strength, reported sedimentation coefficients for the isoionic protein of 3.75 S at 1% protein concentration and 4.0 S at 0.5%, while our values are somewhat higher (Figure 6).

The positive slope of the *s vs. c* curve in deionized solution as compared with the negative slope in buffered, high ionic strength solution (Figure 6) suggests that forces of attraction are substantially greater in the deionized state. The fact that within the limits of our experimental error[1] the limiting value of *s* at infinite dilution is independent of ionic strength suggests that the size and shape of bovine serum albumin are independent of ionic strength. The effects of ionic strength are in agreement with the results of other physicochemical measurements that have been made on bovine serum albumin solutions. For example, Timasheff *et al.* (1957) found that the concentration dependence of the reciprocal turbidity, as determined by light-scattering measurements, was negative at low salt concentrations and became positive with increasing concentration of NaCl. Krause and O'Konski (1959) observed that the specific Kerr constant of deionized bovine serum albumin increased with concentration, while untreated bovine serum albumin (crystalline bovine serum albumin dissolved in distilled water) showed a decrease in the specific Kerr constant with concentration. In addition, Tanford and Buzzell (1956) have studied the viscosity of solutions of bovine serum albumin over a wide range of pH and ionic strength. At the isoionic pH, they found that the slope and intercept of η_{sp}/c *vs.* c were both dependent upon ionic strength. They attributed the somewhat higher intrinsic viscosity of the deionized protein to an electroviscous effect rather than to a change in conformation. Their additional observation that the intrinsic viscosity is essentially constant from pH 4.3 to 7.3 suggests that our measurements at pH 5.15 are well within the pH region of structural stability.

Careful studies by Chen (1967) indicate that the amount of free fatty acids in Armour bovine serum albumin is less than 1 mole/mole of protein. Thus the effect of their removal on the partial specific volume or the molecular weight would be negligible. Chen (1967) has also reported that the optical rotatory dispersion and the sedimentation coefficient, as well as the characteristic pH of the N–F transition discussed by Foster (1960b), are unchanged by lipid removal at low pH. Thus the available evidence suggests that removal of the final traces of lipid does not result in a conformational change and that the structural transformations induced at low pH are reversed when the protein is returned to its isoelectric state. The conformational changes observed by Reynolds *et al.* (1967) on binding detergent anions appear to occur only at high binding ratios.

We suggest that the concentration dependence observed in all these experiments may be interpreted in terms of proton fluctuation forces discussed in the preceding paper (Moser *et al.*, 1966). In the deionized state the predominant interaction is an attractive force between fluctuating monopoles. When buffer salts or NaCl is added, anion binding (Scatchard *et al.*, 1950) results in a mean net negative charge, and the Coulombic repulsions become dominant. The interpretations of their own data by Timasheff *et al.* (1957) and Tanford and Buzzell (1956) are similar.

Molecular Dimensions of the Bovine Serum Albumin Monomer. The question arises as to whether it is reasonable to interpret the hydrodynamic data on bovine

[1] Because of the difficulties mentioned above in obtaining precise values for the sedimentation coefficient of deionized proteins, it seems likely that the discrepancies between the extrapolated values of *s* (in Figure 6) are not significant.

TABLE IV: Axial Ratio of Hydrated Bovine Serum Albumin.

Data	Method of Analysis	a/b
τ_{ϵ_1}, τ_{ϵ_2}, s, $M(1 - \bar{v}\rho)$	γ function	3.0[a]
τ_n, s, $M(1 - \bar{v}\rho)$	γ function	3.6[a]
τ_n, $[\eta]$, M	δ function	6.2[a]
τ_{ϵ_1}, $[\eta]$, M	δ function	4.4[a]
s, $[\eta]$, M	β function	See text[b]
$(\tau_{\epsilon_1} = 3\tau_n)$, τ_{ϵ_2}	Perrin equations	3.0[c]
s, $[\eta]$, M	At^3	See text[d]
X-Ray scattering		2.5[e]
		3.9[d]
K_s and $[\eta]$		2.4[f]
Electron microscopy		3.5[g]

[a] This work. [b] Scheraga and Mandelkern (1953). [c] Moser *et al.* (1966). [d] Champagne (1957). [e] Luzzati *et al.* (1961). [f] Creeth and Knight (1965). [g] Chatterjee and Chatterjee (1965).

serum albumin in terms of the model of a rigid ellipsoid of revolution. The evidence for the rigidity of isoionic bovine serum albumin has already been summarized by Moser *et al.* (1966). The good agreement among rotary diffusion coefficients by dielectric dispersion, fluorescence depolarization, and electric birefringence suggests that the macromolecule is rigid at the pH employed in this study. It is well known (Foster, 1960b) that at low pH the macromolecule expands and becomes flexible (Harrington *et al.*, 1956; Riddiford and Jennings, 1966a,b), and recent studies by Weber and Young (1964a,b) show that in this state, peptic hydrolysis yields a few large fragments. This has led to considerations of a molecular model consisting of a rigid linear aggregate of spheres in contact under isoionic conditions, and in an expanded state at low pH, and this model is in reasonable accord with much of the physical–chemical data (Bloomfield, 1966). The translational frictional coefficient of the close-packed model was shown to be equal to that of a prolate ellipsoid of revolution of equal axial ratio and very nearly the same length. This suggests that it is meaningful to use the prolate ellipsoid of revolution to interpret the hydrodynamic properties of bovine serum albumin, and that one may expect to estimate dimensions in reasonable agreement with those of a linear rigid array of subunits, in spite of the differences in shape. Some calculations by Bloomfield *et al.* (1967) on variously shaped aggregates of spheres, and experiments by Broersma (1960), indicate that holes in structures have a remarkably small effect on hydrodynamic properties.

In a preceding paper (Moser *et al.*, 1966) we have reported the dielectric dispersion relaxation times, τ_{ϵ_1} and τ_{ϵ_2}, which gave the best least-squares fit between a theoretical Debye curve for two relaxation times and the experimental results at various concentrations. From these two relaxation times extrapolated to zero concentration, $\tau_{\epsilon_1} = 0.22$ μsec and $\tau_{\epsilon_2} = 0.074$ μsec, and Baldwin's (1957) value for the sedimentation coefficient at infinite dilution, $s_{25,w} = 5.01 \times 10^{-13}$ sec, and the value $M(1 - \bar{v}\rho) = 17{,}890$ from this research, we have calculated the two γ functions from eq 6–8, $\gamma_a = 0.54$ and $\gamma_b = 1.61$.[2] From Table I, we see that these values correspond to axial ratios of 3.5 and 5.1, respectively.

An analysis was also made of the dispersion curve extrapolated to zero concentration, utilizing a computer program that found the best values of two relaxation times, with the constraint introduced that the calculated values of γ_a and γ_b correspond to the same axial ratio. The mean-square deviation of the normalized dielectric increments was minimized with the values $\tau_{\epsilon_1} = 0.202$ μsec, $\tau_{\epsilon_2} = 0.098$ μsec, and an axial ratio $p = a/b = 3.0$ (Table IV). The root-mean-square deviation for 20 points on the normalized dispersion curve was 0.0120. The root-mean-square deviation from known sources of error was estimated to be 0.02. If instead of the value $s_{25,w} = 5.01$ S, obtained by Baldwin, we use the value 5.11 S suggested by our data on deionized solutions, the calculated value of the axial ratio is increased only by 0.4.

For reasons discussed in the preceding paper (Moser *et al.*, 1966), we consider the best value of θ_b to be the one obtained from the electric birefringence data extrapolated to infinite dilution, $\theta_b = 2.18 \times 10^6$ sec^{-1}. Introduction of this value into eq 6 gives a value $\gamma_a = 0.52$, which by interpolation (Table I) corresponds to an axial ratio of 3.6, in close agreement with the results of the above calculations. An oblate shape for bovine serum albumin is excluded by the γ-function analysis since $\gamma_a = 0.52$ is well below the theoretical value for any oblate ellipsoid of revolution. This is in agreement with earlier studies (Oncley, 1943; Krause and O'Konski, 1959; Moser *et al.*, 1966).

The harmonic mean relaxation time, $\bar{\tau}_h$, was calculated from the two dielectric dispersion relaxation times (obtained with the consistency restraint) with

$$\frac{1}{\bar{\tau}_h} = \left(\frac{1}{\tau_{\epsilon_1}} + \frac{2}{\tau_{\epsilon_2}}\right)\frac{1}{3} = 8.45 \times 10^6 \text{ sec}^{-1} \qquad (12)$$

or $\bar{\tau}_h = 0.118$ μsec. This value is in good agreement with the value 0.124 μsec found by Harrington *et al.* (1956), but appreciably lower than the value 0.155 μsec of Steiner (1953), both obtained from fluorescence polarization studies.

The δ function of Scheraga and Mandelkern (1953)

$$\delta = \frac{600}{Nk}\left(\frac{\eta_0\theta}{T}\right)[\eta]M \qquad (13)$$

provides a relationship for calculating the axial ratio of

[2] This involved the assumptions that the dielectric increments are due to permanent dipole orientation, and that the macromolecule approximates a rigid ellipsoid of revolution, assumptions which were justified earlier for this protein (Moser *et al.*, 1966). It should be kept in mind that in other systems or under other conditions, these assumptions may not be adequate. For example, at the pK of the amino or carboxyl groups, the phenomenon of proton fluctuation may become significant.

the solvated macromolecule from the anhydrous molecular weight, M, the intrinsic viscosity, $[\eta]$, and the rotary diffusion coefficient, θ_b. Careful measurements of the intrinsic viscosity of highly purified bovine serum albumin have been made. Tanford and Buzzell (1956) and Reynolds *et al.* (1967) report values of 0.037 dl g^{-1}. Yang and Foster (1954) report 0.038, and Loeb and Scheraga (1956) report 0.041. We use the most recent value, 0.037. The δ function calculated from the electric birefringence relaxation time, τ_n, is 1.16, corresponding to an axial ratio of 6.2, while the δ function calculated from the long dielectric dispersion relaxation time is 4.4. In both calculations, the molecular weight was taken as 66,700, and the values for the axial ratios were obtained by interpolation in Table II of Scheraga and Mandelkern (1953). The values for θ_b from electric birefringence, 2.18×10^6 sec^{-1}, and from dielectric dispersion, 2.48×10^6 sec^{-1}, were taken from Table II of Moser *et al.* (1966).

Creeth and Knight (1965) have applied the "rule of Wales and Van Holde" to proteins, and from an extensive tabulation of published data have concluded that the ratio of K_s, the coefficient of the concentration dependence of the reciprocal sedimentation coefficient, to $[\eta]$ is 1.6 for compact spherical macromolecules. For elongated particles they deduce an empirical relationship between the axial ratio and the ratio of $K_s/[\eta]$ of the form

$$\log p = 1.56(1.7 + K_s/[\eta]); \quad 0.2 < K_s/[\eta] < 1.7 \quad (14)$$

Taking the value $K_s = 5.4$ from their compilation and $[\eta] = 3.7$ ml/g, we calculate an axial ratio of 2.4. This value has also been included in Table IV.

Finally, we have included in Table IV the value for P found by Chatterjee and Chatterjee (1965) from electron microscopy.

An examination of Table IV leads to the conclusion that all the methods of analysis for macromolecular shape listed are in reasonable accord with a prolate ellipsoid of axial ratio 3.5 with the exception of the δ, the β, and the Λt^3 functions. Yang (1961) has pointed out that four groups have calculated essentially equal values of the Scheraga–Mandelkern β function for bovine serum albumin, 2.05×10^6, which is less than the minimum theoretical value, 2.12×10^6, the value for a sphere. The value of β for a prolate ellipsoid of revolution of axial ratio 3.5 is 2.18×10^6 (Scheraga, 1961), which illustrates the insensitivity of β to the axial ratio, in contrast with γ_a and γ_z (Table I). Thus, relatively small experimental errors can cause large changes in p values estimated from β. Harrington *et al.* (1956) demonstrated failures of β to give the shapes of viruses, and Schachman (1959) has recommended caution in the use of β. The experimental errors for bovine serum albumin measurements, however, seem to be smaller than required to explain the discrepancy between p, computed from β and the closely related Λt^3 function (Champagne, 1957), and the more reliable values. The discrepancy may be due to the use, in the derivation of β, of the relation attributed to Lansing and Kraemer (1936), $M_h(1 - \bar{v}_h\rho) = M(1 - \bar{v}\rho)$, where the M_h and $\bar{v}_h$ refer to the hydrated protein and

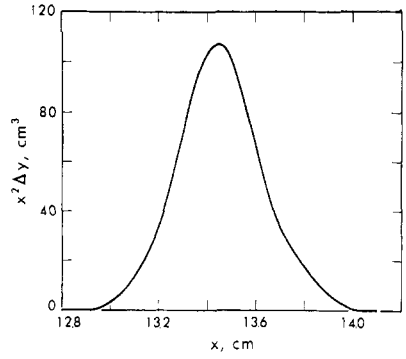

FIGURE 7: Analysis of bovine serum albumin dimer fraction by sedimentation velocity. Conditions of the experiment are as described in Figure 1, except that the protein concentration was 0.45 g/100 ml. The photograph from which this figure was drawn was taken 104 min after the ultracentrifuge came up to speed.

M and v refer to the anhydrous material. This equation appears rigorously applicable in sedimentation equilibrium theory, that is, when considering thermodynamic properties, but may not be sufficiently accurate when used for hydrodynamic behavior. Tanford and Buzzell (1956) suggested that serum albumin cannot be represented as an ellipsoid of revolution, but the problems of combining hydrodynamic and thermodynamic data to derive hydration values recently have been outlined (Scheraga, 1961), and we suggest that further developments in this area are needed to understand the failures with the β function. No adequate explanation could be found for the large p obtained from the δ function. Part of these discrepancies may be resolved if hydrodynamic equations can be developed for generalized ellipsoids and other rigid shapes, and if β would be computed for more realistic models, in the manner recently illustrated by Bloomfield *et al.* (1967).

Our measurements of rotary diffusion and sedimentation coefficients provide enough information to separate the effects of shape and hydration. With the most reliable axial ratio, $p = 3.5$, obtained through the γ functions, the hydrodynamically effective volume, V_e, was calculated in different ways. Table V shows three equations, taken from Yang's (1961) review, together with the results. In the first equation, the intrinsic viscosity, $[\eta] = 0.037$ dl g^{-1}, was taken from Reynolds *et al.* (1967), and the Simha viscosity factor $\nu = 4.17$, corresponding to an axial ratio of 3.5, was taken from Cohn and Edsall (1943, p 519). The value for f_e used in eq 2 of the table was calculated from the relation $f/f_e = 1.147$, corresponding to an axial ratio of 3.5 (Table I), and $f = 5.929 \times 10^{-8}$ g sec^{-1}, from the value $s_{25,w} = 5.01 \times 10^{-13}$ sec. The value ζ_e used in the third equation is calculated from the relation $\zeta_b/\zeta_e = 2.870$, corresponding to an axial ratio of 3.5 (Table I), with $\zeta_b = kT/\theta_b$, where θ_b is in one case the rotational diffusion coefficient obtained from dielectric dispersion, 2.475×10^6 sec^{-1}, and in the other, from birefringence relaxation, 2.18×10^6 sec^{-1}. The fractional hydration values in the final column, V_{H_2O}/V_p, are calculated from the relation $(V_e - V_p)/V_p$, where $V_p = M\bar{v}/N$. It can be seen that all calculations lead to values of 0.39 with a standard devia-

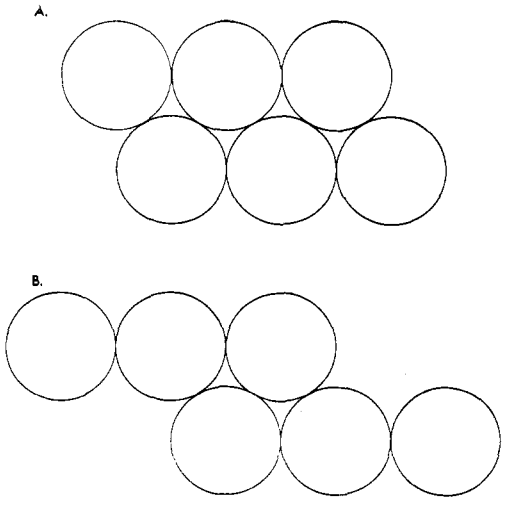

FIGURE 8: Models used in estimating the conformation of the bovine serum albumin dimer.

tion of 0.14. The mean value for the effective hydrodynamic volume, V_e, of the four recorded in Table V from hydrodynamic measurements is 11.2×10^{-20} cm^3. While hydration values are of interest, it should be cautioned that their physical significance should not be taken too literally because of the simplified model we are required to use. With this value and an axial ratio of 3.5, we calculate the dimensions 2a = 138 Å and 2b = 30 Å for hydrated bovine serum albumin.

Conformation of the Dimer. The presence of traces of monomer and higher polymers in our dimer preparation complicated the interpretation of the dielectric dispersion results (Moser *et al.*, 1966). It seems unlikely, however, that their presence has introduced appreciable error into the sedimentation coefficient of the dimer. The sedimentation coefficient of the dimer, s_d, was found to be $s_{20,w}$ = 6.71 S at a concentration of 0.450 g/100 ml. The sedimentation coefficient of the monomer, s, at the same concentration from Baldwin's (1957) study is 4.41 S. The ratio of the sedimentation coefficients may be simply related to the shape-dependent part of the frictional ratios provided that the partial specific volume is unchanged by dimerization and that the change in degree of hydration may be neglected.

Starting with the general equation relating the sedimentation coefficients to the frictional coefficient, eq 3, it is clear that the ratio of sedimentation coefficients for dimer and monomer is given by

$$s_d/s_m = 2f_m/f_d \tag{15}$$

The frictional ratio of both species can be split into shape- and hydration-dependent parts (Oncley, 1941)

$$\frac{f}{f_0} = \frac{f}{f_e}\frac{f_e}{f_0} \tag{16}$$

so that

$$\frac{s_d}{s_m} = 2\,\frac{(f/f_e)_m(f_e/f_0)_m(f_0)_m}{(f/f_e)_d(f_e/f_0)_d(f_0)_d} \tag{17}$$

If dimer and monomer are assumed to have the same partial specific volume and hydration, it is evident that

$$(f_e/f_0)_m = (f_e/f_0)_d \tag{18}$$

Furthermore, the ratio of the frictional coefficients of the equivalent anhydrous spheres also reduces to a simple expression

$$\frac{(f_0)_m}{(f_0)_d} = \frac{1}{2^{1/3}} \tag{19}$$

Thus, introducing eq 18 and 19 into eq 17 gives

$$\frac{s_d}{s_m} = 1.59\frac{(f/f_e)_m}{(f/f_e)_d} \tag{20}$$

where $1.59 = 2^{2/3}$. Since the observed ratio of sedimentation coefficients of dimer to monomer is s_d/s_m = 6.71/4.41 = 1.52, it is apparent that f/f_e of the dimer is somewhat greater than that of the monomer, which means a larger a/b for the dimer. Introducing the observed ratio of sedimentation coefficients, 1.52, into eq 20 together with the value 1.147 from Table I for the frictional ratio of the monomer of axial ratio 3.5, we calculate f/f_e of the dimer equal to 1.20. This corresponds to an ellipsoid of axial ratio 4.25. Thus, the simplest model for the dimer formed by prolate ellipsoids of axial ratio 3.5 is a displaced side-by-side dimer having the hydrodynamic properties of an ellipsoid of revolution of axial ratio 4.25. This would correspond to a displacement of about 0.6 the length of the monomer molecule.

The above calculation involves crude approximations. It may be interesting to compare the above conclusion with one from a somewhat more sophisticated treatment due originally to Kirkwood (1954) and recently extended by Bloomfield *et al.* (1967). In making these calculations, we represent the monomer as a linear ar-

TABLE V: Effective Hydrodynamic Volume and Hydration of Bovine Serum Albumin Axial Ratio 3.5.

Equations[a]	V_e (cm^3)	Hydration (V_{H_2O}/V_p)
(1) $V_e = \frac{100M[\eta]}{N\nu}$	9.80×10^{-20}	0.21
(2) $V_e = \frac{4\pi}{3}\left(\frac{f_e}{6\pi\eta_0}\right)^3$	12.10×10^{-20}	0.49
(3) $V_e = \frac{\zeta_e}{6\eta_0}$	10.80×10^{-20} [b]	0.33
	12.26×10^{-20} [c]	0.51
(4) X-Ray scattering (pH 5.1)	13.0×10^{-20}	

[a] Equations 1–3 are taken from Yang's review (1961, p 339). The X-ray-scattering data are those of Luzzati *et al.* (1961). [b] With θ_b obtained from dielectric dispersion (Moser *et al.*, 1966). [c] With θ_b obtained from birefringence decay (Moser *et al.*, 1966).

ray of three spheres of radius, r, and consider two alternative models for the dimer as indicated in Figure 8A,-B. The frictional ratio f_d/f_m of dimer and monomer was calculated for each of the models by means of the equation

$$f = \frac{6\pi\eta Nr}{1 + \frac{r}{N}\sum_{l=1}^{N}\sum_{s=1}^{N}{}'\langle R_{ls}^{-1}\rangle}$$

taken from Bloomfield *et al.* (1967). Here N is the number of spheres in the total aggregate. Thus $N = 3$ for the monomer and 6 for the dimer, and r need not be specified since it disappears on forming the ratio, f_d/f_m, of the frictional coefficients. The double sum refers to the sum of all the reciprocal distances, R_{ls}, between the quasi-subunits. In this way, we obtain a frictional ratio for model A, to monomer, $f_A/f_m = 1.215$, which on substitution into eq 15 leads to a ratio of sedimentation efficients, $s_A/s_m = 1.646$. With model B, we calculate $f_B/f_m = 1.312$ and $s_B/s_m = 1.524$, a value which agrees very well with the observed ratio 1.521. This model for the dimer resembles closely the one suggested by Bloomfield (1966).

Conclusion

In the studies reported in this and an earlier publication, Moser *et al.* (1966), we have outlined present methods of estimating size and shape of proteins from a combination of electric and hydrodynamic measurements using bovine serum albumin as an example. We have tested the assumptions that are implicit in this approach and they appear valid in this system. Thus, the present study further tends to reinstate dielectric dispersion as a method of studying the size and shape of proteins, especially when used in conjunction with electric birefringence and sedimentation measurements.

Acknowledgment

The authors gratefully acknowledge the interest of Professor C. H. Li, Director of the Hormone Research Laboratory, University of California.

References

Baldwin, R. L. (1957), *Biochem. J. 65*, 503.
Benoit, H. (1951), *Ann. Phys. 6*, 561.
Bloomfield, V. (1966), *Biochemistry 5*, 684.
Bloomfield, V., Dalton, W. O., and Van Holde, K. E. (1967), *Biopolymers 5*, 135.
Broersma, S. (1960), *J. Chim. Phys. 32*, 1626.
Champagne, M. (1957), *J. Chim. Phys. 54*, 378.
Chatterjee, A., and Chatterjee, S. N. (1965), *J. Mol. Biol. 11*, 432.
Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Peptides and Amino Acids, New York, N. Y., Reinhold.
Creeth, J. M., and Knight, C. K. (1965), *Biochim. Biophys. Acta 102*, 549.
Dayhoff, M. O., Perlmann, G. E., and MacInnes, D. A. (1952), *J. Am. Chem. Soc. 74*, 2515.
Dintzis, H. M. (1952), Ph.D. Dissertation, Harvard University, Cambridge, Mass.
Edsall, J. T. (1949), *Fortschr. Chem. Forsch. 1*, 119.
Edsall, J. T. (1953), *Proteins 1*, 549.
Foster, J. F. (1960a), *J. Am. Chem. Soc. 82*, 3741.
Foster, J. F. (1960b), *in* The Plasma Proteins, Vol. 1, Putnam, Frank W., Ed., New York, N. Y., Academic, Chapter 6.
Foster, J. F. (1960c), *J. Am. Chem. Soc. 81*, 865.
Harrington, W. F., Johnson, P., and Ottewill, R. H. (1956), *Biochem. J. 62*, 569.
Kirkwood, J. G. (1954), *J. Polymer Sci. 12*, 1.
Krause, S., and O'Konski, C. T. (1959), *J. Am. Chem. Soc. 81*, 5082.
Lansing, W. D., and Kraemer, E. O. (1936), *J. Am. Chem. Soc. 58*, 1471.
Loeb, G. I., and Scheraga, H. A. (1956), *J. Phys. Chem. 60*, 1633.
Luzzati, V., Witz, J., and Nicolaieff, A. (1961), *J. Mol. Biol. 3*, 379.
Moser, P., Squire, P. G., and O'Konski, C. T. (1966), *J. Phys. Chem. 70*, 744.
O'Konski, C. T., and Zimm, B. H. (1950), *Science 111*, 113.
Oncley, J. L. (1941), *Ann. N. Y. Acad. Sci. 41*, 121.
Oncley, J. L. (1943), *in* Proteins, Amino Acids and Peptides, Cohn, E. J., and Edsall, J. T., Ed., New York, N. Y., Reinhold, Chapter 22.
Pedersen, K. O. (1958), *J. Phys. Chem. 62*, 1282.
Pedersen, K. O. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 157.
Phelps, R. A., and Putnam, F. W. (1960), *in* The Plasma Proteins, Putnam, F. W., Ed., New York, N. Y., Academic, Chapter 5.
Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6*, 973.
Riddiford, C. L., and Jennings, B. R. (1966a), *J. Am. Chem. Soc. 88*, 4359.
Riddiford, C. L., and Jennings, B. R. (1966b), *Biochim. Biophys. Acta 126*, 171.
Scatchard, G., Scheinberg, I. H., and Armstrong, S. H., Jr. (1950), *J. Am. Chem. Soc. 72*, 535.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, p 242.
Scheraga, H. A. (1961), Protein Structure, New York, N. Y., Academic.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Spahr, P. F., and Edsall, J. T. (1964), *J. Biol. Chem. 239*, 850.
Squire, P. G., and Benson, S. (1967), *in* Protides of the Biological Fluids, Peeters, H., Ed., New York, N. Y., Elsevier, p 441.
Squire, P. G., Starman, B., and Li, C. H. (1963), *J. Biol. Chem. 238*, 1389.
Steiner, R. F. (1953), *Arch. Biochem. Biophys. 46*, 291.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University.
Tanford, C., and Buzzell, J. G. (1956), *J. Phys. Chem. 60*, 225.

Timasheff, S. N., Dintzis, H. M., Kirkwood, J. G., and Coleman, B. D. (1957), *J. Am. Chem. Soc. 79*, 782.
van Hofsten, B., and Falkbring, S. O. (1960), *Anal. Biochem. 1*, 463.
Weber, G., and Young, L. B. (1964a), *J. Biol. Chem. 239*, 1415.
Weber, G., and Young, L. B. (1964b), *J. Biol. Chem. 239*, 1425.
Yang, J. T. (1961), *Advan. Protein Chem. 16*, 323.
Yang, J. T., and Foster, J. F. (1954), *J. Am. Chem. Soc. 76*, 1588.

Steroid–Protein Interactions. XVIII. Isolation and Observations on the Polymeric Nature of the Corticosteroid-Binding Globulin of the Rat*

Gerald J. Chader† and Ulrich Westphal

ABSTRACT: Chromatographic techniques in combination with gel filtration resulted in the isolation of a corticosteroid-binding globulin from pooled rat serum. The isolated corticosteroid-binding globulin–corticosterone complex was homogeneous by sedimentation velocity ($s^0_{20,w}$ = 3.56 S), paper electrophoresis, and immunoelectrophoresis (α_1-globulin). A molecular weight of 61,000 ± 1100 was obtained by the approach to sedimentation equilibrium method whereas the corticosterone content indicated a molecular weight of approximately 53,000 for the active steroid-binding species. A carbohydrate content of 27.8% was found. Distinct differences in certain amino acid residues such as half-cystine may account for differences in steroid-binding and polymeric properties between rat corticosteroid-binding globulin and corticosteroid-binding globulin from the human and rabbit. The number of high-affinity binding sites for corticosterone in the pure rat corticosteroid-binding globulin was determined to be $n = 1$. The association constants of the corticosterone complex at 4 and 37° were $k = 5.1 \times 10^8$ and 2.8×10^7 M^{-1}, respectively. Thermodynamic calculations gave a negative enthalpy change and a negative entropy change for the interaction. Rat corticosteroid-binding globulin was found to be polymeric in nature. Removal of steroid from the isolated steroid–protein complex ("stripping") resulted in at least four polymeric peaks discernible during ultracentrifugation; a concomitant loss in steroid-binding affinity was observed. Recombination with 1 mole of corticosterone/mole of corticosteroid-binding globulin restored most of the corticosteroid-binding globulin activity and also reversed the polymerization resulting in one homogeneous sedimentation peak of the $s_{20,w}$ value of the original monomeric complex. Polyacrylamide disc electrophoresis of "stripped" rat corticosteroid-binding globulin showed a polymeric pattern similar to that observed in the ultracentrifuge. Considerably less polymeric banding of corticosteroid-binding globulin occurred before stripping substantiating the conclusion that dissociation of the complex and removal of corticosteroid from the protein led to polymerism. The reactions are an example of the regulatory control by a steroid hormone of the quaternary structure of a steroid-binding protein.

Soon after the discovery of the corticosteroid-binding globulin (Daughaday, 1956; Bush, 1957) or transcortin (Slaunwhite and Sandberg, 1959) in human plasma, it was observed that the blood of the rat also contains macromolecules which bind glucocorticoid hormones with high affinity (Daughaday, 1958; Slaunwhite and Sandberg, 1959). This finding was of particular significance to the biochemist since it opened the way for experimental study of the factors involved in this highly specific association between steroid and protein (Westphal, 1961).

Corticosteroid-binding activity in rat serum was found to be dependent upon the corticosterone level (Westphal *et al.*, 1963); the binding ability is associated with an α_1-globulin (Westphal and DeVenuto, 1966). The corticosteroid-binding globulin level is influenced

* From the Biochemistry Department, University of Louisville, School of Medicine, Reynolds Building, Louisville, Kentucky 40208. *Received May 27, 1968.* The present work was supported by Grants AM-04040 and AM-06369 from the National Institute of Arthritis and Metabolic Diseases, by Research Career Award GM-K6-14,138 from the Division of General Medical Sciences of the U. S. Public Health Service, and by Contract DA-49-193-MD-2263 from the U. S. Army Medical Research and Development Command. Some of the present results have been reported (Chader and Westphal, 1968). For the preceding paper of this series, see Ganguly and Westphal (1968).

† Present address: Department of Biological Chemistry, Harvard Medical School, Boston, Mass. 02115.