Light Scattering Studies on the Thermal Denaturation of Bovine Serum Albumin[1)]

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Thermal denaturation of bovine serum albumin (BSA) was studied by means of light scattering and viscosity measurements. The degree of denaturation ($w_D{}^\circ$) was measured by using HABA, an anionic dye. The values of intrinsic viscosity, weight-average molecular weight and radius of gyration showed no appreciable changes when $w_D{}^\circ$ was within 85 percent, but increased remarkably when $w_D{}^\circ$ exceeded 85 percent. Thus, it has been concluded that the thermal denaturation proceeds in two steps, *i.e.*, intramolecular denaturation in the earlier stage and intermolecular association in the later stage. Analysis of the light-scattering data showed that the aggregated molecule was in a rod-like shape, whose length increased almost linearly with the weight fraction of the aggregated molecule w_n, the latter being obtained by analysis of the refractive index increment. On the other hand, the diameter did not change with the degree of denaturation, and its value was about 140 Å, which is almost equal to the largest dimension of the native BSA molecule. It has been concluded that the BSA molecules form aggregates by side-by-side association, whose length is about 2910 Å and diameter about 140 Å.

The thermal denaturation of proteins has been studied by many investigators with the use of the solubility method,[2)] Tiselius electrophoresis,[3)] ultracentrifugation,[3)] optical density difference,[4)] and acrylamide gel electrophoresis.[5)] From the results of these investigations, it was concluded that the polar groups of the inner parts of BSA are exposed to the surface of the molecule and the mechanism of the thermal denaturation of BSA differs at the initial stage from that at the later stage, some fractions of BSA aggregating while some remain undenatured. However, such points as the percentage of the aggregates in the solution and its variation with heating time and the dimension of the aggregated molecules, have not been clarified. With particular attention to these points, we have studied the mechanism of thermal denaturation of BSA by means of light scattering and viscosity measurements.

Experimental

Materials. The bovine serum albumin (here after called BSA) used was made by Armour Co., Ltd., (Fraction V, No. G5715, D. 1092), as in the previous paper.[6)] A solution containing about 0.25 percent BSA in 0.05M phosphate buffer (pH 7.2) was heated at the desired temperature between 25 and 90°C for the desired length of time in test tube fitted with a lid. It was dipped, into cold water in order to stop thermal denaturation.[2-5)] Under these conditions, turbidity did not increase very much. Phosphate buffer solution (pH regions 6.2—7.4) is said to be more suitable than acetate buffer solution, because the BSA in it shows less turbidity. The concentration of BSA was determined by measuring the optical density at 278 mμ. Water used was distilled, refined by ion-exchange resin, and distilled again with an all-glass still.[6)]

Degree of Denaturation. It is well-known[7)] that an anionic dye, 2-(4′-hydroxyphenylazo) benzoic acid (abbreviated HABA) is specifically bound to the native BSA and causes a remarkable optical change giving a new absorption band at 482 mμ at pH=7.2. The degree of denaturation was therefore determined with HABA by using the difference of optical density at 482 mμ from a blank solution of the same dye concentration as a reference, with a Shimadzu photoelectric photometer QR-50 with quartz cells 1 cm thick. A stock solution of 10^{-3}M HABA, stable for at least 10 months, was diluted to 5×10^{-4}M with a phosphate buffer. The dilute solution was stable for at least two weeks. Protein and dye reacted immediately and the colour of the complex was stable for several hours. Concentration of HABA used was 5×10^{-4}M and that of BSA about 0.1 percent or below. In these concentration regions, Beer's rule was satisfied. The degree of denaturation, $w_D{}^\circ$, was obtained experimentally by the relation

$$w_D{}^\circ = 1 - \Delta E/\Delta E_N \tag{1}$$

where ΔE is the optical density difference between the heat-denatured solution and the reference solution and ΔE_N is that of the native BSA.

Intrinsic Viscosity. Viscosity of BSA solution denatured at various temperatures and at various times was measured by the Ostwald type viscometer, the flow time of the clean water being about 120 sec at 25°C. The viscometer was cleaned immediately before use by chromate mixture, rinsed with distilled water many times to remove all traces of the acid, and then dried with acetone which had been distilled three times. Measurements were carried out in a thermostat kept at 25°C. The intrinsic viscosity, $[\eta]$ (ml/g), was determined according to the Huggins equation

$$\eta_{sp}/c = [\eta] + k[\eta]^2c \tag{2}$$

where η_{sp} is the specific viscosity of the solution, c the concentration of the BSA in g/ml, and k the Huggins constant.

Light Scattering Measurements. The method of measurements was the same as that given in a previous paper.[8)] A

[1)] This paper was presented at the 24 th Annual Meeting of the Chemical Society of Japan, Gifu, November, 1971.

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[4)] M. F. Kerekes, *Naturwissenschaften*, **17**, 537 (1963).

[5)] A. Aoki, K. Hiramatsu, K. Kimura, S. Kaneshima, Y. Nakamura, and K. Sano, *Bull. Inst. Chem. Res.*, *Kyoto Univ.*, **47**, 274 (1969).

[6)] Nakagaki and Y. Sano, This Bulletin, **45**, 2100 (1972).

[7)] D. R. Rustein, E. F. Ingenito, W. E. Reynolds, and J. M. Burke, *J. Clin. Invest.*, **33**, 211 (1954); H. T. Wrenn and T. V. Feichtmeir, *Amer. J. Clin. Pathol.*, **26**, 960 (1956).

[8)] M. Nakagaki and Y. Sano, This Bulletin, **45**, 1011 (1972).

Shimadzu electrophotometric light scattering photometer PG-21 was used at 436 mμ and at 25°C. The reduced scattering intensity for unpolarized incident light was obtained and examined by the Zimm plot method. The solutions and solvent were made optically clean by ultrafiltration with Membrane Filter No. 15 from Sartorious Co. Ltd. It has been ascertained that the concentration of the thermally denatured BSA does not change by ultrafiltration.

Refractive Index Increment. Measurements were carried out with a Shimadzu photoelectric differential refractometer DR-3 (Brice type) at 436 mμ and at 25°C.

Density. A picnometer of about 35 ml was used at 25°C.

Results

Degree of Denaturation. The weight fraction of denatured BSA, w_{D}°, was obtained by using HABA for the solutions heated at various temperatures for various lengths of time. The results are shown in Figs. 1 and 2. We see from Fig. 1 that the value of w_{D}° increases with time and seems to reach a final value determined by the temperature. When the temperature was below 53°C, the w_{D}° value reaches, at most 25 percent, while at or above 80°C it reaches 90 percent in about 20 min. At 56—60°C, it increases gradually with time.

The w_{D}° values obtained at various temperatures at the constant heating time of 100 min are plotted in Fig. 2. The value was less than 10 percent below 50°C but increased remarkably in the temperature range 50—60°C. Thus, w_{D}° reached about 80 or 85 percent when the BSA solution was heated at 60°C for 100 min.

Intrinsic Viscosity. Two samples obtained by heating (A) at various temperatures for 100 min, and (B) at 60°C for various lengths of time, were studied, the values of the intrinsic viscosity $[\eta]$ being obtained for the BSA of various degrees of denaturation, w_{D}°. The results are shown in Fig. 3, where open circles denote the BSA heated at various temperatures for 100 min (case A), and closed circles that heated at 60°C for various lengths of time (case B). Both plots lie on the same curve when plotted against w_{D}^{0}. This suggests that the state of BSA molecules and therefore the mechanism of the thermal denaturation in heated solutions does not depend on the method of heating but only on the degree of denaturation w_{D}°.

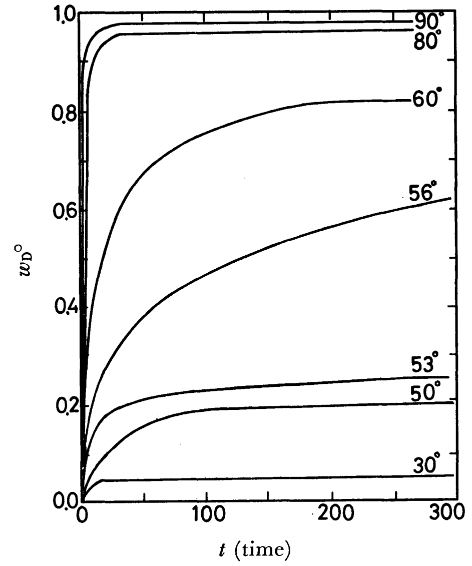

Fig. 1. Degree of denaturation, w_{D}°, obtained at various heating temperatures and heating times, with HABA (at 482 mμ) in 0.05M phosphate buffer at pH 7.2.

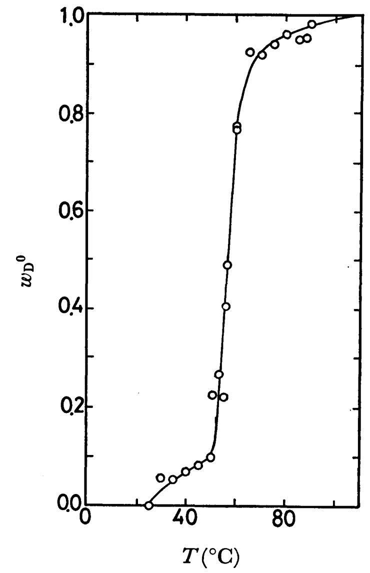

Fig. 2. Degree of denaturation, w_{D}°, obtained at various heating temperatures for 100 min.

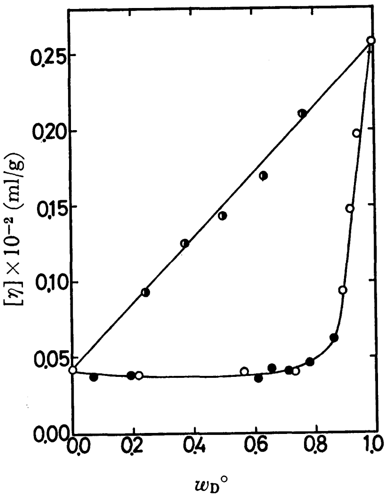

Fig. 3. Intrinsic viscosities, $10^{-2}[\eta]$, (ml/g) obtained by heating (A) at various temperatures for 100 min. (open circles) and (B) at 60°C for various times (closed circles) and (C) for the mixed solution of the native BSA and the denatured BSA, the latter having been heated at 90°C for 100 min. (half-open circles).

Half-open circles in Fig. 3 show the values of intrinsic viscosity obtained for the mixture of the solutions of untreated native BSA and of the almost fully denatured BSA, the latter being obtained by heating at 90°C for 100 min. It is seen that the additivity of the intrinsic viscosity against w_{D}° holds for the mixed solution, and that the value of $[\eta]$ of the mixture is entirely different from that of the partially denatured solutions. It is thus concluded that the actual solution is not a simple mixture of fully denatured BSA and native BSA.

The curve $[\eta]$ *vs.* w_D° is bent at about $w_D^\circ=0.85$. When w_D° was below 85 percent, the intrinsic viscosity showed no appreciable change but it increased remarkably when w_D° exceeded 85 percent. The data indicate that the mechanism of thermal denaturation differs for below and above 85 percent of w_D°. In the initial stage of heating, intramolecular denaturation which does not affect the value of $[\eta]$ would mainly occur, while in the later stage, intermolecular aggregation which gives rise to an increase of $[\eta]$ would mainly occur. Light scattering measurements were carried out in order to clarify the results.

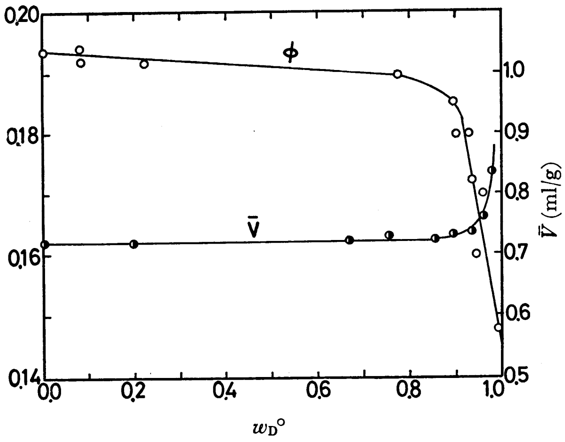

Fig. 4. Refractive index increment, ϕ, (ml/g) and the partial specific volume, $\bar{V}$, (ml/g) at various degree of denaturation.

Refractive Index Increment. The refractive index of the thermally denatured solution n was found to be linear with respect to BSA concentration c (g/ml). As the inclination of the straight lines, the value of the refractive index increment ϕ (ml/g) was determined for each thermally denatured solution and the result is given in Fig. 4. The quantity ϕ is the apparent refractive increment defined as follows.

$$\phi = (\mathrm{d}n/\mathrm{d}c)$$

We see that ϕ does not change appreciably for w_D° values less than 85 percent but decreases remarkably at values exceeding 85 percent. This is in line with the variation of intrinsic viscosity.

Light Scattering. The reduced scattering intensity for unpolarized incident light, R_θ, was measured as a function of the scattering angle θ at various BSA concentration c (g/ml), in 0.05M phosphate buffer as a solvent. According to the Zimm plot method, apparent values of the weight-averaged molecular weight M, the radius of gyration R_g and the second virial coefficient A_2 are obtained on the basis of the following equation:

$$\frac{K'\phi^2 c}{R_\theta} = \frac{1}{M}\left(1+\frac{16\pi^2 n_0^2}{3\lambda_0^2}R_g^2\sin^2\frac{\theta}{2}\right) + 2A_2 c \qquad \text{(3a)}$$

where

$$K' = 2\pi^2 n_0^2/N_0\lambda_0^4 \qquad \text{(3b)}$$

Here, N_0 is Avogadro's number, λ_0 is the wavelength in a vacuum, the value used in this experiment being 4360 Å.

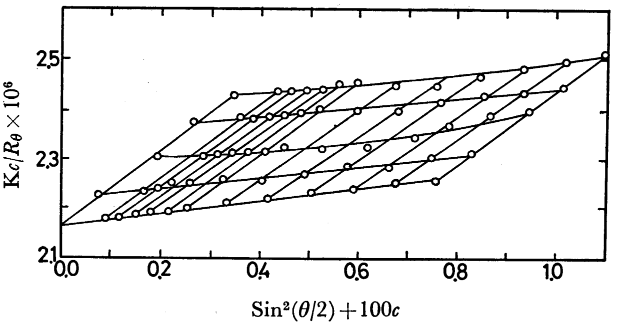

Fig. 5. Zimm plot for the thermally-denatured BSA at 60°C for 100 min.

It has been reported[8] that the Zimm plot of native BSA in 0.01M sodium chloride solution (pH=5.40) is narrow because the value of the second virial coefficient A_2 is nearly equal to zero, and that the value of the molecular weight M is 71900, which is in good agreement with the value reported.[9] The Zimm plot of the BSA thermally denatured by heating at 60°C for 100 min is shown in Fig. 5 as an example. The plots obtained by the extrapolation to zero of θ are linear against c. From the straight line, the apparent molecular weight M and the second virial coefficient A_2 can be calculated according to Eq. (3). The curves of the reduced scattering intensity against $\sin^2(\theta/2)$ at finite concentration are slightly concave upwards, but the curve obtained by the extrapolation to zero of c is almost linear against $\sin^2(\theta/2)$. The apparent radius of gyration R_g can therefore be obtained from the inclination of the straight line. The values of M, R_g, and A_2 thus obtained are given in Tables 1 and 2, which give the results obtained by the methods of heat treatment A and B, respectively, and Fig. 6 which gives the results of M and R_g plotted as a function of the degree of denaturation w_D°. We see that the increase of M is relatively small when w_D° exceeds this value. Similarly, the value of R_g is nearly constant when w_D°

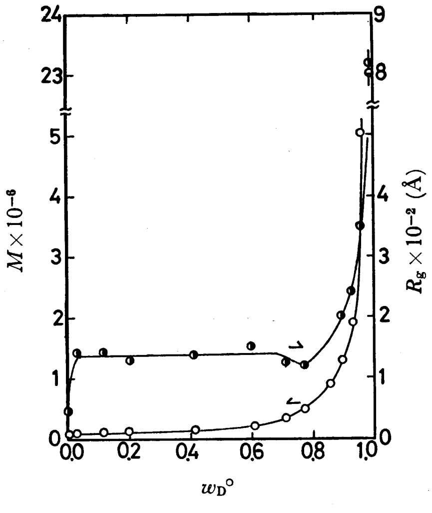

Fig. 6. Apparent molecular weight, M, and apparent radius of gyration, R_g, obtained at various degree of denaturation.

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[9] J. T. Edsall, H. Edelhoch, R. Lontie, and P. R. Morrison, *J. Amer. Chem. Soc.*, **72**, 464 (1950).

Table 1. Results of light scattering, intrinsic viscosity and specific volume of denatured BSA heated at various temperatures for 100 min

T°C	Native	55°C	60°C	65°C	70°C	80°C	90°C
$M\times10^{-6}$	0.0719	—	0.4628	1.263	1.890	5.025	23.04
$A_2\times10^5$ (mole ml/g^2)	0	—	3.08	1.93	1.50	0.638	0.175
R_g(Å)	45	—	116	201	242	354	816
$[\eta]\times10^{-2}$ (ml/g)	0.0405	0.0395	0.0475	0.0940	0.1468	0.1974	0.2582
ϕ (ml/g)	0.1935	—	0.1896	0.1850	0.1799	0.1702	0.1475
$\bar{V}$(ml/g)	0.7165	0.7190	0.7200	0.7338	0.7353	0.7577	0.8362
$w_{\rm D}^\circ$	0	0.400	0.770	0.895	0.930	0.960	0.990

Table 2. Results of light scattering, intrinsic viscosity, and specific volume of denatured BSA heated at 60°C for various times

Time (min)	15	30	65	200
$M\times10^{-6}$	0.1296	0.1907	0.3397	0.8800
$A_2\times10^5$ (mol. ml/g^2)	0.169	0.288	0.203	2.02
R_g (Å)	177	153	153	163
$[\eta]\times10^{-2}$ (ml/g)	0.0398	0.0352	0.0407	0.0623
ϕ (ml/g)	0.1921	0.1902	0.1899	—
$\bar{V}$ (ml/g)	0.7180	0.7200	0.7200	—
$w^\circ_{\rm D}$	0.196	0.610	0.710	0.856

is below 85 percent, and increases remarkably when $w_{\rm D}^\circ$ exceeds this value.

Discussion

Mechanism of Denaturation. It is thought that the mechanism of denaturation changes at about 85 percent of the degree of denaturation. No appreciable change in $[\eta]$, ϕ, and R_g and relatively small increase of M, observed at the earlier stage of heating, would suggest intramolecular denaturation in the range where $w_{\rm D}^\circ$ is less than 85 percent. When $w_{\rm D}^\circ$ exceeds 85 percent, the values of M, R_g, and $[\eta]$ increased and ϕ decreased remarkably, from which the intermolecular aggregation of denatured molecules could be suggested. There will, therefore, be at least three components in the solutions, that is, native (N), intramolecularly denatured (D), and intermolecularly aggregated (D_n) components. The following reaction would occur between them:

$$n{\rm N} \longrightarrow n{\rm D} \longrightarrow {\rm D}_n \tag{4}$$

Component D is mainly formed in the stage where $w_{\rm D}^\circ$ is less than 85 percent. The value of $[\eta]$, R_g, ϕ, M, and $\bar{V}$ of this component are nearly the same as those of the native BSA, although the component differs from that of native BSA in conformation. Component D_n is formed by the intermolecular aggregation of the intramolecularly denatured BSA mainly in the stage where $w_{\rm D}^0$ exceeds 85 percent. Many papers have dealt with qualitative studies of aggregation of denatured proteins,[2–5)] but not much with quantitative studies.

Weight Fraction of Denatured Components. The following equation is derived for the refractive index increment of a solution containing z macromolecular components at constant temperature T, and at constant pressure P.

$$\left(\frac{\partial n}{\partial c}\right)_{T,P} = \sum_{i=1}^{i=z}\left(\frac{\partial n}{\partial c_i}\right)_{T,P,c_{j\neq i}} \cdot \left(\frac{\partial c_i}{\partial c}\right)_{T,P} \tag{5}$$

where

$$c = \sum_{i=1}^{i=z} c_i \quad \text{and} \quad w_i = c_i/c, \tag{6}$$

n being the refractive index of the solution and c the concentration for the total solutes g/ml, related to the concentration of i-th component according to Eq. (6) where w_i is the weight fraction of the i-th component. If the refractive index increment by the i-th component $(\partial n/\partial c_i)_{P,T,c_j}$ is expressed by ϕ_i, Eq. (5) can be written as follows.

$$\phi = \sum_{i=1}^{i=z} \phi_i w_i \tag{7}$$

where ϕ is the apparent refractive index increment obtained for the total solutes in the mixed solution. The following equations can be obtained for a mixed solution of three macromolecular solutes N, D, and D_n mentioned above:

$$1 - w_{\rm D}^\circ = w_{\rm N} \tag{8a}$$

$$w_{\rm D}^\circ = w_{\rm D} + w_n \tag{8b}$$

$$\phi = \phi_{\rm N} w_{\rm N} + \phi_{\rm D} w_{\rm D} + \phi_n w_n \tag{8c}$$

where the subscripts N, D, and n represent N, D, and D_n, and $w_{\rm D}^\circ$ is the degree of denaturation obtained experimentally by using HABA according to Eq. (2).

Since the value of ϕ in Fig. 4 showed no appreciable change when $w_{\rm D}^\circ$ was within 85 percent, it may be proper to assume that the value of the refractive increment of component D is nearly equal to that of native BSA, *viz.*, $\phi_{\rm N} \fallingdotseq \phi_{\rm D}$. Thus, according to Eq. (8):

$$w_n = (\phi - \phi_{\rm N})/(\phi_n - \phi_{\rm N}) \tag{9a}$$

$$w_{\rm D} = w_{\rm D}^\circ - w_n \tag{9b}$$

The results obtained are shown in Fig. 7 by using the values $\phi_n=0.1422$(ml/g) and $\phi_{\rm N}=0.1935$(ml/g). The curve of $w_{\rm D}$ has a maximum point as expected for the intermediate component of a successive reaction as Eq. (4). The maximum point is for $w_{\rm D}^\circ=85$ percent. On the other hand, w_n showed no appreciable increase when $w_{\rm D}^\circ$ is less than 85 percent. When $w_{\rm D}^\circ$ exceeds this value, $w_{\rm D}$ increased remarkably as a result of the intermolecular aggregation.

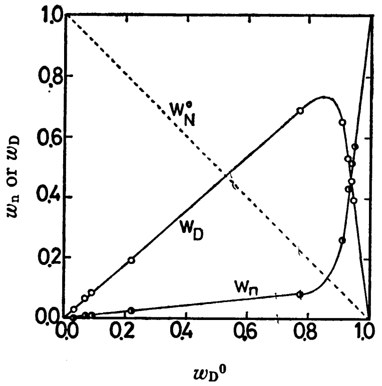

Fig. 7. Weight fraction of the monomeric, denatured BSA, W_D, and of the aggregate, W_n, at various degree of denaturation W_D°.

Physico-chemical Properties of Aggregated Molecules. For the light scattering of a solution containing z macromolecular components, the following equation derived by the theory of fluctuation of the chemical potential was used in the previous paper,[8)]

$$\frac{K'c}{R_\theta} = \frac{1}{\sum\limits_{i=1}^{i=z} M_i w_i \phi_i^2}\left\{1 + \frac{16\pi^2 n_0^2}{3\lambda_0}\left(\frac{\sum\limits_{i=1}^{i=z} M_i w_i \phi_i^2 R_{gi}^2}{\sum\limits_{i=1}^{i=z} M_i w_i \phi_i^2}\right)\sin^2\frac{\theta}{2}\right\} + 2c\frac{\sum\limits_{i=1}^{i=z}\sum\limits_{j=1}^{j=z} A_2^{(ij)} M_i M_j P_i(\theta) P_j(\theta) w_i w_j \phi_i \phi_j}{\left(\sum\limits_{i=1}^{i=z} M_i P_i(\theta) w_i \phi_i^2\right)^2} \tag{10}$$

where R_θ is the reduced scattering intensity of the solution containing z macromolecular components, $P_i(\theta)$ the interference factor of the i-th macromolecular component, $A_2^{(ij)}$ the second virial coefficient describing the interaction between the i-th and j-th components, w_i the weight fraction of the i-th component in the total solute, M_i the weight-average molecular weight of the i-th component, ϕ_i the refractive increment of the i-th component, n_0 is the refractive index of the solvent and λ_0 the wavelength in a vaccum (the value 4360 Å was used). From this equation the following equations can be obtained for a mixed solution of three macromolecular species, N, D, and D_n,

$$M\phi^2 = M_N w_N \phi_N^2 + N_D w_D \phi_D^2 + M_n w_n \phi_n^2 \tag{11a}$$

$$R_g^2 M\phi^2 = R_{gN}^2 \phi_N^2 M_N w_N + R_{gD}^2 \phi_D^2 M_D w_D + R_{gn}^2 \phi_n^2 M_n w_n \tag{11b}$$

where M, ϕ, and R_g are experimental values.

From Eq. (11a) and the relations $M_D \fallingdotseq M_N$, and $\phi_D \fallingdotseq \phi_N$, the value of M_n can be obtained:

$$M_n = \{M\phi^2 - (1-w_n) M_N \phi_N^2\}/(w_n \phi_n^2) \tag{12}$$

The results are given in Fig. 8 and Table 3. The degree of association n can be obtained as (M_n/M_N).

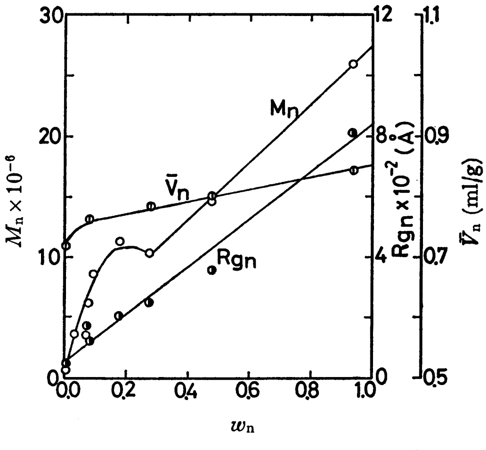

Fig. 8. Molecular weight, M_n, radius of gyration, R_{gn}, and partial specific volume, $\bar{V}_n$, of the aggregate at various degree of denaturation.

It is seen that the value of M_n increases remarkably with the increase of w_n, the increase being almost linear when w_n exceeds 0.3. The last column of Table 3 shows values obtained by the extrapolation of w_n to 1, according to the data shown in Fig. 8. We see that the weight-average molecular weight of the aggregate is 27.2×10^6 at the maximum, and the aggregate is composed of 378 molecules of denatured BSA.

The conformation of the intramolecularly denatured BSA, D, is expected to differ more or less from that of the native BSA. The result given in Fig. 6, however, shows that the radius of gyration remains constant in the initial stage of thermal denaturation where w_D° is less than 85 percent. The radius of gyration of the component D, R_{gD}, is therefore assumed to be nearly equal to that of native BSA, R_{gN}. The value of the radius of gyration of the aggregate, R_{gn}, can then be obtained according to the equation easily derived from Eq. (11b):

$$R_{gn}^2 = \{R_g^2 M\phi^2 - R_{gN}^2 M_N \phi_N^2 (1-w_n)\}/(w_n M_n \phi_n^2) \tag{13}$$

The results are shown in Fig. 8 and Table 3. We see that R_{gn} increases almost linearly with the increase of w_n. The value of the radius of gyration of the aggregate itself was about 841 Å at the maximum, as obtained by extrapolation of w_n to 1. This value is about 19 times larger than that of the native BSA.

For the partial specific volume of polymer mixtures, the additivity rule is usually assumed. In the present case of three solutes,

$$\bar{V} = \bar{V}_N w_N + \bar{V}_D w_D + \bar{V}_n w_n \tag{14}$$

The value of $\bar{V}$ remains almost constant in the region of w_D° less than 85 percent, where the intramolecular denaturation mainly takes place. The partial specific volume of the denatured, monomeric BSA, $\bar{V}_D$, is therefore assumed to be nearly equal to that of the

Table 3. Results of light scattering and specific volume of the aggregates

w_n	0.0791	0.1724	0.2759	0.4726	0.9331	1.000
$M_n \times 10^{-6}$	6.42	7.73	10.3	14.7	25.8	27.2
R_{gn} (Å)	126	206	249	355	816	841
n	89.3	108	143	205	359	378
$\bar{V}_n$ (ml/g)	0.7607	0.7710	0.7847	0.8037	0.8448	0.8990

native BSA, $\bar{V}_N$. If $\bar{V}_D \doteqdot \bar{V}_N$ in Eq. (14), the value of the partial volume of the aggregate $\bar{V}_n$, can be obtained. The results are shown in Table 3 and Fig. 8. We see from Fig. 8 that values of $\bar{V}_n$ increase almost linearly with increase of w_n and that the value of $\bar{V}_n$ is 0.8990 ml/g at the maximum association state where w_n is equal to 1.

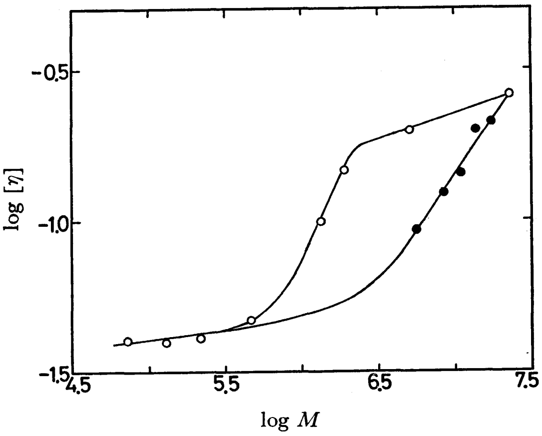

Fig. 9. Log-log plots of intrinsic viscosity against apparent molecular weight.

Dependence of $[\eta]$ *and* R_g *on* M. The log-log plots showing the dependency of the intrinsic viscosity on weight average molecular weight are given in Fig. 9. Open circles denote the experimental values obtained for variously denatured solutions and closed circles those obtained for the mixed solutions of the native BSA and completely denatured BSA. The latter was obtained heating at 90°C for 100 min, its molecular weight being 2.30×10^7. The exponent a in the Kuhn-Mark equation $[\eta]=KM^a$ was obtained from the slope of curves in Fig. 9. In the former, three values of the exponent a 0.054, 1.1, and 0.166 are obtained for lower, intermediate and higher M values, respectively. In the latter mixed solutions, the values of a are 0.055 and 0.75 for lower and higher M-values, respectively.

Log-log plots of R_g *vs.* M for the results from thermally treated solutions (open circles) and for calculated values of the aggregated molecules themselves (closed circles) are shown in Fig. 10. The relations are linear and expressed by the empirical equation: $R_g=K'M^b$. The value of the constants, $K'=0.185\times 10^{-8}$ and $b=0.496$, were obtained for the solutions, and $K'=1.68\times 10^{-17}$ and $b=1.578$ for the aggregates.

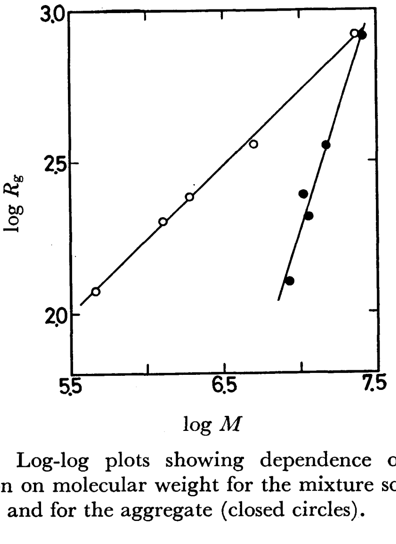

Fig. 10. Log-log plots showing dependence of radius of gyration on molecular weight for the mixture solutes (open-circles) and for the aggregate (closed circles).

The results indicate that native and intramolecularly denatured species are approximated with a compact, comparatively spherical shape because of the low a and b values, while the intermolecularly aggregated species is approximated with rigid, cylindrical rod molecules because of the relatively large a and b values.

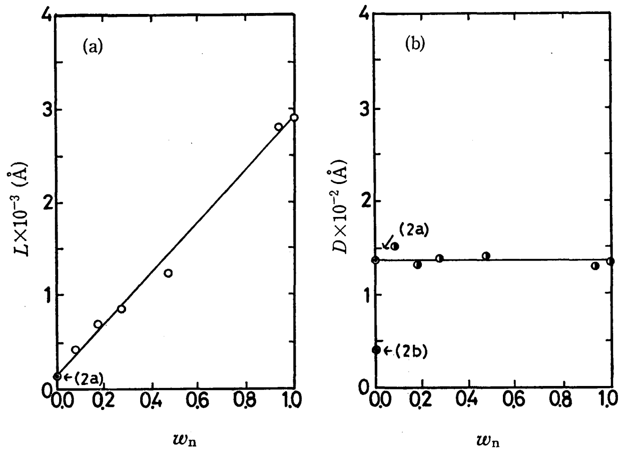

Fig. 11. Variation of molecular length L and diameter D by association.

TABLE 4. MOLECULAR DIMENSIONS OF THE AGGREGATES AS CYLINDRICAL RODS

w_n	L (Å)	D (Å)	p	L/n
0.0791	435	154	2.8	4.87
0.1724	715	133	5.4	6.65
0.2759	862	141	6.1	5.32
0.4726	1231	142	8.7	6.02
0.9331	2828	128	22.1	7.88
1.000	2913	133	21.9	7.70
		average 139		average 6.41

Size and Shape of Aggregated Molecules. If the aggregate is assumed to be rigid and the mass is distributed uniformly all over the volume of the aggregate, the size and shape of the aggregate can be calculated from the above data of M_n, $\bar{V}_n$, and R_{gn}. The dimensions and axial ratio of the aggregates as cylindrical rod molecules are given in Fig. 11 and Table 4, where L and D are the length and diameter, respectively, of the rod molecule, and p is the axial ratio $(=L/D)$. The diameters of the native BSA as a rigid prolate spheroid with major axis a and minor axis b are $2a=140$ Å and $2b=70$ Å as shown in Fig. 11 by double circles at $w_n=$zero. We see from Fig. 11a that the length L increases almost linearly with the increase of the weight fraction of the aggregate w_n. The value of the length of the aggregate obtained by extrapolation of w_n to zero agreed with the length of the native BSA: $2a=140$ Å. The length per one BSA in an aggregate

L/n (n is the degree of association) is about 6.41 Å, irrespective of the value of w_n (Table 4).

On the other hand, the diameter of the aggregated molecule D was almost constant irrespective of w_n (Fig. 11b). The average value of diameter D thus obtained was 140 Å, which is equal to the length of the native BSA, $2a$, instead of the diameter $2b$. This would indicate that the aggregate is formed by side-by-side association, so that the diameter of the aggregate is almost constant, but the length of the aggregate increases linearly with the degree of aggregation. At $w_n=1$, the length and diameter of the aggregate are 2910 and 140 Å, respectively, and one aggregate consists of 378 molecules.