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Light-scattering Studies of the Interaction between Bovine Serum Albumin and Guanidine Hydrochloride in Aqueous Solutions[1)]

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The denaturation of bovine serum albumin (BSA) by guanidine hydrochloride (GuHCl) was studied by means of the light-scattering method. The Zimm plots were curved so that apparently imaginary values of the radius of gyration were obtained, but the modified Zimm plots, drawn by applying the correction factor for the optical anisotropy of segments, gave reasonable values of the weight-average molecular weight, the radius of gyration, and the second virial coefficient. The correction factor was calculated by using the experimental values of the scattering ratio at 90° of the scattering angle. The analysis of the intrinsic viscosity showed that the BSA molecules were elongated. According to these results, it has been concluded that the BSA molecules are streched by the denaturation the more as the GuHCl concentration is higher, and that the denatured BSA molecules, at first, form dimers by the side-by-side aggregation of two molecules, but that the intermolecular bindings, too, are broken with the further addition of GuHCl. The length reached at 5M GuHCl was about 1/5 of the fully stretched length. This suggests the possibility that the denatured BSA molecules can keep their local natural conformations.

It has been well established that proteins form complexes with various kinds of molecules and ions. The interaction between bovine serum albumin and sulfamines has also been studied by many investigators, including one of the present authors.[2)] As a continuation of these studies, the present authors intend to discuss the denaturation of bovine serum albumin (BSA) by guanidine hydrochloride (GuHCl) on the basis of a physico-chemical interpretation of the results from light-scattering measurements.

Bovine serum albumin is one of the well-studied proteins; its molecular weight is said to be about 70000, and its isoelectric is 6.4. Guanidine hydrochloride is a typical denaturating agent extensively used for various proteins. The mechanism of the denaturation is considered to involve the breaking-down of the secondary intramolecular bonds, such as ionic bonds, hydrogen bonds and hydrophobic bonds, as a result of the selective combination of GuHCl on the proteins, the conformation of the latter being changed according to the complex formation. The mechanism of the denaturation, however, has not yet been made very clear, particularly in relatively low concentrations of the denaturating agent or in the earlier stages of the denaturation process.

In the present investigation, the denaturation of BSA by GuHCl in aqueous solutions has been studied, including the low concentration range of the latter. The experimental method mainly used was light-scattering measurements, which were undertaken with particular attention paid to the following two points: (1) the variation in the refractive index increment, $(\partial n/\partial c)$, due to the complex formation, and (2) the deformation of the so-called Zimm plots due to the optical anisotropy of segments of the macromolecules.

Experimental

Materials. The guanidine hydrochloride used was a guaranteed reagent supplied by Nakarai Chemicals, Ltd. A solution of 6M was prepared gravimetrically, and from it solutions of various concentrations were prepared volumetrically.

The bovine serum albumin used was Fraction V from Armour Co. Ltd. (No. G5717, D. 1029), the same as that used in the previous paper.[3)] The concentration of BSA was determined by measuring the optical density at 278 mμ by means of a Shimadzu photoelectric spectrometer QR-50 with quartz cells 1 cm thick.

Water was distilled, refined by ion-exchange resin, and distilled again with an all-glass still.

Scattered Light Intensity. The method of the light-scattering measurements was the same as in the previous paper.[3)] A Shimadzu electrophotometric light-scattering photometer, PG-21, was used at 436 mμ and at 25°C. The

1) Presented at the 90th Annual Meeting of the Pharmaceutical Society of Japan held in Fukuoka in April, 1970.

2) M. Nakagaki *et al.*, *Yakugaku Zasshi*, **83**, 586 (1963); **84**, 516 (1964); **86**, 447 (1966); **87**, 817, 980, 1089 (1967).

3) M. Nakagaki and Y. Sano, This Bulletin, **45**, 1077 (1972).

reduced scattering intensity for unpolarized incident light, $R_u(\theta)$, was obtained and examined by the Zimm plot method, where θ is the scattering angle. The solutions and the solvent for the light-scattering measurements were made optically clear by ultrafiltration with Membrane Filter No. 15 from Sartorius Co., Ltd. It has been ascertained that the concentration of BSA does not change upon the ultrafiltration.

Scattering Ratio. The measurements were made by using the light-scattering photometer described above. The photometer was, for this experiment, equipped with a polarizer but not with an analyzer. The direction of the vibration of the electric vector of the light which passed through the polarizer was determined by using a reflecting glass plate set perpendicular to the plane of observation, for the intensity of the reflected light is greatest when the light vibrates perpendicularly to the plane of observation. The scattering ratio, $S(\theta)$, defined by the following equation:

$$S(\theta)=J_{//}(\theta)/J_{\perp}(\theta) \tag{1}$$

was determined, rotating the polarizer by 90°, where $J_{//}(\theta)$ and $J_{\perp}(\theta)$ are the Rayleigh ratios for incident beams vibrating, respectively, parallel to and perpendicular to the plane of observation, the latter being the plane containing the incident and scattered beams.

Refractive Index Increment. The measurements were made by means of a Shimadzu photoelectric differential refractometer, DR-3 type (Brice type) at 436 mμ and at 25°C, according to the method described in the previous paper.[3]

Two kinds of refractive index increment, ϕ_c and ϕ_μ, are defined as follows:

$$\phi_c=(\partial n/\partial c_2)_{T,P,c_3} \tag{2a}$$

$$\phi_\mu=(\partial n/\partial c_2)_{T,P,\mu_3} \tag{2b}$$

where n is the refractive index of the solution, T is the temperature, P is the pressure, and c_2 and c_3 are the concentrations in g/m*l* of BSA and GuHCl respectively. The ϕ_c quantity is measured by diluting a mixed solution of BSA and GuHCl with a GuHCl solution whose concentration is the same as in the mixed solution. The ϕ_μ quantity, on the other hand, is measured by diluting it with a GuHCl solution which has been in membrane equilibrium with the mixed solution.

In the latter case, the membrane equilibrium was achieved by using a size-18/32 cellotube (diameter, 1.5 cm) of the Visking Co. It was swollen in pure water, soaked in a 0.05M EDTA solution for 30 min treated with hot water of 90°C for 30 min, washed sufficiently with pure water, and then used for the membrane equilibrium. The cellotube containing a mixed solution of BSA (about 0.5%) and GuHCl was shaken for 3 days at 25°C in an "outer" solution which contained GuHCl of the same concentration as the "inner" solution, the volume of the former being twice the latter. The concentration of GuHCl was determined by two methods: (1) by the measurement of the refractive index of the solution by means of the differential refractometer mentioned above, and (2) by the Mohr method of titrating the chloride ion with an $AgNO_3$ solution. The results of these two methods agreed well with each other. The concentration of BSA was determined spectrophotometrically, and it was ascertained that no BSA was present in the outer solution after the equilibrium.

Intrinsic Viscosity. The viscosity of the BSA solution containing GuHCl was measured by means of an Ostwald Viscometer in a thermostat at 25°C. It took about 120 sec for pure water to flow down. The intrinsic viscosity, $[\eta]$, of BSA was calculated according to the Huggins equation:

$$\eta_{sp}/(100c_2)=[\eta]+k[\eta]^2\cdot(100c_2) \tag{3}$$

where η_{sp} is the specific viscosity of the solution calculated by considering the GuHCl solution as the solvent. Here, c_2 is the concentration of BSA in g/m*l*, and k is the Huggins constant.

Density. A picnometer (about 35 m*l*) was used a 25°C. The methods of the measurements and of the analysis of the results were the same as those described in the previous paper.[3]

Results

Viscosity and Density. The viscosity, η, of the BSA solution containing GuHCl has been measured at various BSA concentrations, c_2(g/m*l*), by diluting the original solution with the solvent that contains GuHCl at the same concentration, [GuHCl] (M), as in the original solution. The relation between the reduced viscosity, $10^{-2}\cdot\eta_{sp}/c_2$, and the concentration of BSA, $100c_2$, is shown in Fig. 1 for various GuHCl

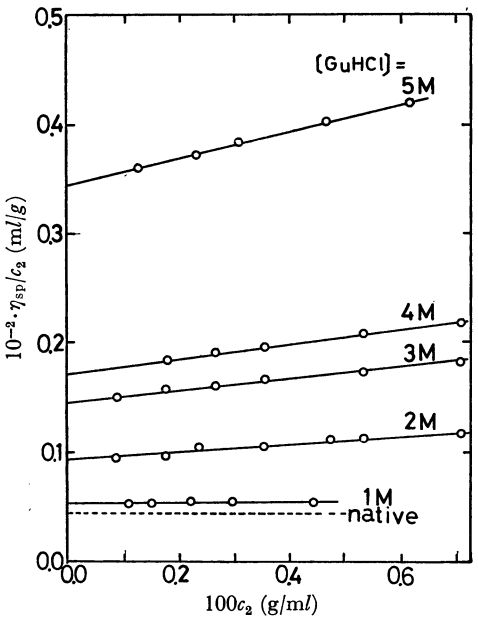

Fig. 1. Relations between the reduced viscosity and concentration of BSA for various GuHCl concentration.

concentrations. A linear relationship was seen for each case, with a good reproducibility. The dotted line in Fig. 1 is for native BSA, that is, for [GuHCl]=0. The intrinsic viscosity, $[\eta]$, the slope of the straight lines, $k[\eta]^2$, and the Huggins constant, k, obtained from these data according to Eq. (3) are shown in Table 1. The intrinsic viscosity of the denatured BSA increases

Table 1. Intrinsic viscosity and specific volume of denatured BSA

[GuHCl] (M)	$[\eta]$ (100 ml/g)	$k[\eta]^2$ (100 ml/g)^2	k	$\bar{v}_2$ (ml/g)
0	0.045	0	0	0.7265
1	0.054	0	0	—
2	0.093	0.033	3.4	—
3	0.145	0.054	2.1	0.7265
4	0.172	0.066	2.2	—
5	0.345	0.120	1.0	0.7265

with the GuHCl concentration, particularly in the range between 4M and 5M.

The partial specific volume of BSA, $\bar{v}_2$, in GuHCl solutions did not vary with the concentration of the latter; the average value was 0.7265 ml/g, as is shown in Table 1. This is in accord with the fact that the partial specific volume of BSA does not change upon the addition of urea, as has been reported in the literature.[4]

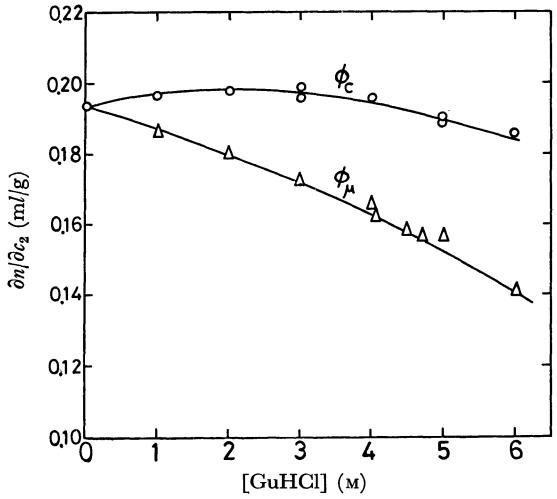

Fig. 2. Refractive index increments, ϕ_c(○) and ϕ_μ(△), of BSA in GuHCl solutions.

Refractive Index Increment. The refractive index of the solution, n, was found to be linear to the BSA concentration, c_2 (g/ml). Two kinds of refractive index increment, ϕ_c and ϕ_μ, as defined by Eqs. (2a) and (2b) respectively, were obtained at various GuHCl concentrations, [GuHCl] (M). The results are shown in Fig. 2. The concentration of GuHCl in the outer solution at the membrane equilibrium was the same as that in the inner solution, at least within the limits of accuracy of the chemical analysis, since the concentration used here was relatively high. In spite of this, the values of these two refractive index increaments, ϕ_c and ϕ_μ, were definitely different from each other, as is shown in Fig. 2. This means that GuHCl is bound to BSA, as will be quantitatively discussed later on.

Light Scattering. The reduced scattering intensity for unpolarized incident light, $R_u(\theta)$, was measured as a function of the scattering angle, θ, at various BSA concentrations, c_2(g/ml), using GuHCl solutions of the respective concentrations. [GuHCl] (M), as the solvents. According to the usual Zimm-plot method, the apparent values of the weight-average molecular weight, M*, the radius of gyration, R_g*, and the second virial coefficient, A_2, are expected to be obtained on the basis of the following well-known equation:

$$\frac{K'\phi_\mu^2 c_2}{R_u(\theta)}=\frac{1}{M^*}\left(1+\frac{16\pi^2}{3\lambda^2}R_g^{*2}\sin^2\frac{\theta}{2}\right)+2A_2c_2 \quad (4)$$

where:

$$K'=2\pi^2 n_1^2/N_0\lambda_0^4 \quad (5)$$

4) S. Katz and T. G. Ferris, *Biochemistry*, **5**, 3246 (1966).

It has been established[5] that ϕ_μ, instead of ϕ_c, must be used in Eq. (4) for multi-solute systems.

In the case of [GuHCl]=0, it has been reported[3] that the Zimm plot of native BSA in a 0.01M sodium chloride solution (pH=5.40) is in accord with Eq. (4), that the value of the molecular weight (M^* in Eq. (4)) is 71900, which is in good agreement with the value reported in the literature,[6] and that the value of the second virial coefficient, A_2, is nearly equal to zero. On the other hand, the Zimm plot of the BSA denatured by the addition of GuHCl could not completely be analyzed according to Eq. (4).

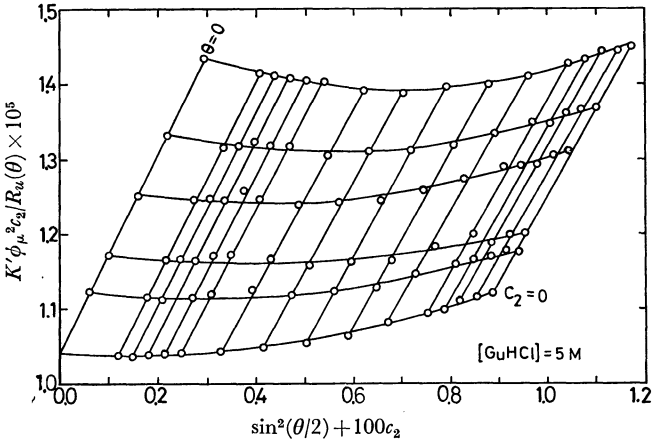

Fig. 3. Zimm plot of BSA at [GuHCl]=5M.

Table 2. Results of light scattering measurements

GuHCl (M)	$M^*\times10^{-4}$	$A_2\times10^4$ (mol·ml/g^2)	$M\times10^{-4}$	M/M_0	R_g (Å)
0	7.09	0	6.84	1.00	45
1	9.34	1.23	8.97	1.26	58
2	13.70	1.20	13.23	1.85	77
3	13.70	0.83	13.33	1.88	92
4	12.05	3.00	11.61	1.64	113
5	9.61	6.75	9.24	1.30	147

It can be seen from Fig. 3, which shows the case of [GuHCl]=5M as an example, that the plots obtained by the extrapolation to zero of θ are linear against c_2, and that the intersection and the inclination of the straight line give the apparent molecular weight, M^*, and the second virial coefficient, A_2, according to Eq. (4). The values of M^* and A_2 thus obtained are given in Table 2. However, the plots obtained by the extrapolation to zero of c_2 are not linear against $\sin^2(\theta/2)$, so Eq. (4) cannot be used to obtain the apparent value of R_g*. The initial slope (the slope at the limit of $\theta\to0$) cannot be used, either, because the slope is negative in the case of Fig. 3 and because R_g* cannot take an imaginary value. This anomalous behavior may be supposed to be due to the optical anisotropy of segments. The correction factor for this can be calculated, according to the theory presented previously,[7] by using the

5) J. G. Kirkwood and R. J. Goldberg, *J. Chem. Phys.*, **18**, 54 (1950); W. H. Stockmayer, *J. Chem. Phys.*, **18**, 58 (1950).

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

7) M. Nakagaki, This Bulletin, **34**, 834 (1961).

value of the scattering ratio at $\theta=90°$.

Scattering Ratio. If the segments of a macromolecule are completely isotropic and are sufficiently small compared to the wavelength of light, the light scattered at $\theta=90°$ must be completely polarized and vibrating perpendicularly to the plane of observation, even when unpolarized light is used as the incident light. If the incident light is linearly polarized, the intensity of the scattered light at $\theta=90°$ must be zero when the incident light is vibrating parallel to the plane of observation, as long as the segments are isotropic and sufficiently small.

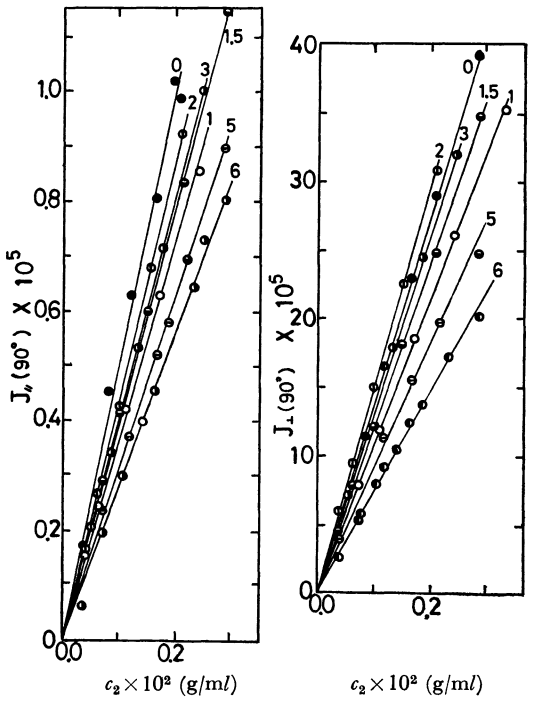

Fig. 4. The proportionality of the Rayleigh ratios, $J_\perp$ (90) and $J_{//}(90)$, to BSA concentration, at various GuHCl concentrations.

On the other hand, if the segments are optically anisotropic, the scattered light is depolarized even at $\theta=90°$, or the scattered light has some intensity even when the linearly-polarized incident light is vibrating parallel to the plane of observation. The Rayleigh ratio, that is, the scattered light intensity per unit of solid angle from a unit of the volume of the solution per unit of the intensity of the incident light, was measured at $\theta=90°$ for the incident light vibrating perpendicular to and parallel to the plane of observation; the results, $J_\perp$ (90°) and $J_{//}$ (90°) respectively, are shown in Fig. 4 as functions of the BSA concentration, c_2 (g/ml). The parameter values in Fig. 4 show the GuHCl concentration, [GuHCl] (M). In each case, the scattered light intensity is proportional to the BSA concentration.

The values of the scattering ratio at $\theta=90°$, Q, defined by the following equation:

$$Q=S(90°)=J_{//}(90°)/J_\perp(90°) \tag{6}$$

are given in Table 3 for various GuHCl concentrations. The scattering ratio is of the same numerical value as the depolarization ratio for unpolarized incident light, but the latter has an experimental disadvantage because perfectly unpolarized incident light is rather difficult to obtain. The scattering ratio is, therefore, used in the present paper. As is shown in Table 3, the scattering ratio is slightly dependent on the GuHCl concentration, although it is nearly independent of the BSA concentration.

Table 3. Scattering ratio and anisotropy of BSA segments

[GuHCl] (M)	$Q\times10^2$	p_a	m
0	3.70	1.65	1.199
1	3.40	1.62	1.199
2	2.96	1.57	1.198
3	3.08	1.59	1.194
4	3.15	1.59	1.194
5	3.33	1.62	1.182

Discussion

Variation in the Refractive Index Increment due to the Complex Formation. In a ternary system, water (1)-protein (2)-low-molecular-weight substance (3), the analysis of the refractive index increment and, therefore, of the light-scattering data is simple if one can treat the aqueous solution of the third component as a solvent (1+3) and the ternary system as a solution of the protein (2) in the solvent (1+3). According to this conventional method, the refractive index increment, ϕ_c, defined by Eq. (2a) is measured by diluting the ternary system with a "solvent" (1+3) whose concentration is c_3 (g/ml).

If, however, a special interaction such as complex formation exists between the protein (2) and the third component (3), the "free" concentration or the chemical potential of the third component decreases with the complex formation. This results in a change in the composition of the solvent (1+3). In order to obtain the refractive index increment, ϕ_μ, defined by Eq. (2b), the solvent composition must be kept constant by diluting the ternary system with a solution which is in a membrane equilibrium with the ternary system.

Several theoretical papers discussing the relation between the ϕ_c and ϕ_μ are found in the literature.[5,8] In most cases reported so far, the difference between ϕ_c and ϕ_μ is small enough, particularly when the concentration of the third component is smaller than about 0.5M. If, however, the c_3 concentration is higher than this, or if the third component has a particularly strong affinity to the protein, the difference between these two quantities becomes important. In this case, the following equation is used at a constant temperature and pressure:

$$\left(\frac{\partial n}{\partial c_2}\right)_{\mu_3}=\left(\frac{\partial n}{\partial c_2}\right)_{c_3}+\left(\frac{\partial n}{\partial c_3}\right)_{c_2}\cdot\left(\frac{\partial c_3}{\partial c_2}\right)_{\mu_3} \tag{7}$$

Here, the quantity $(\partial n/\partial c_3)_{c_2}$ is the refractive index increment of the third component at a constant protein

8) R. H. Ewart, C. P. Roe, P. Debye, and J. R. McCartney, *J. Chem. Phys.*, **14**, 687 (1946); H. C. Brinkman and J. J. Hermans, *ibid.*, **17**, 574 (1949); J. Ooi, *J. Polymer Sci.*, **28**, 459 (1958); B. E. Read, *Trans. Faraday Soc.*, **56**, 382 (1960).

concentration, c_2. Since the value does not change very much, the value in water, ϕ_3, may be used for it as long as the protein concentration is not high. The quantity;

$$\delta' = \left(\frac{\partial c_3}{\partial c_2}\right)_{\mu_3} \tag{8}$$

is the amount of the third component bound to the protein (g/g), if c_2 and c_3 are expressed in g/m*l*. Therefore, Eq. (7) may be rewritten as follows:

$$\phi_\mu = \phi_c + \phi_3 \cdot \delta' \tag{9}$$

The apparent binding ratio, δ', can thus be calculated from the data shown in Fig. 2 and the experimental value for GuHCl: $\phi_3 = 0.1938$ m*l*/g. The value of δ' is negative, however, as is shown in Table 4, because ϕ_μ is smaller than ϕ_c. The negative value of the apparent binding ratio, δ', is due to the effect of the volume excluded by BSA molecules. The actual value of the binding ratio, δ (g/g), is obtained by means of:

$$\delta = \delta' + c_3\bar{v}_2 \tag{10}$$

Here, $\bar{v}_2$ is the specific volume of BSA, 0.7265 (m*l*/g), and is independent of the GuHCl concentration, c_3, as is shown in Table 1.

The molar binding ratio of GuHCl per amino-acid residue of BSA, δ_m (mol/mol), may be calculated on the basis of the average molecular weight per amino-acid residue, $\bar{M}_a = 112.8$, which is obtained by means of the data on amino-acid composition of BSA reported in the literature.[9] As may be seen in Table 4, the value of δ_m increases with the GuHCl concentration, but it is less than 1 even at concentrations as high as 6M GuHCl.

TABLE 4. BINDING RATIOS OF GuHCl TO BSA

[GuHCl] (M)	δ' (g/g)	δ (g/g)	δ_m (mol/mol)
1	−0.0465	0.1159	0.1369
2	−0.0929	0.2318	0.2738
3	−0.1280	0.3363	0.3972
4	−0.1662	0.4439	0.5243
5	−0.1863	0.5334	0.6299
6	−0.2338	0.6503	0.7679

Optical Anisotropy of BSA Segments and of Benzene. It has already been discussed theoretically in a previous paper[7] that the angular variation in the scattered light intensity from macromolecules depends on the optical anisotropy of the segments, and that the degree of the anisotropy could be estimated by means of measurements using polarized incident light.

In the case of isotropic segments which can be treated as Rayleigh spheres, the Rayleigh ratio for unpolarized incident light, $J_u(\theta)$, is proportional to the $(1+\cos^2\theta)$ factor. According to this fact, the reduced scattering intensity, $R_u(\theta)$, used in Eq. (4) may be defined by the following equation:

$$R_u(\theta) = J_u(\theta)/(1+\cos^2\theta) \tag{11}$$

If the segments are Rayleigh scatterers, the value of $R_u(\theta)$ is independent of the scattering angle, θ, except the anglar dependency due to the interference factor which contains the term of the radius of gyration.

In the case of anisotropic segments, however, $R_u(\theta)$ is dependent on the scattering angle, θ. If the segments are considered to be Rayleigh-Gans scatterers, as in the previous paper,[7] the Rayleigh ratios are given as follows, since $R_u(90°) = J_u(90°)$:

$$\frac{J_{/\!/}(\theta)}{R_u(90°)} = \frac{2Q}{1+Q} + 2\left(\frac{1-Q}{1+Q}\right)\cos^2\theta \tag{12a}$$

$$\frac{J_\perp(\theta)}{R_u(90°)} = \frac{2}{1+Q} \tag{12b}$$

$$\frac{J_u(\theta)}{R_u(90°)} = 1 + \left(\frac{1-Q}{1+Q}\right)\cos^2\theta \tag{12c}$$

Before going further, these relations were examined experimentally by using benzene, a standard liquid commonly used to determine the cell constants of the light-scattering apparatus. The values of $R_u(90°)$ and Q for benzene are known from the literature[10] to be $R_u(90°) = 4.85 \times 10^{-5}$ and $Q = 0.41$, the latter being equal to the depolarization ratio, ρ_u. Eqs. (12a—c) for benzene are, therefore:

$$\left.\begin{aligned} J_{/\!/}(\theta) \times 10^5 &= 2.82 + 4.06\cos^2\theta \\ J_\perp(\theta) \times 10^5 &= 6.89 \\ J_u(\theta) \times 10^5 &= 4.85 + 2.03\cos^2\theta \end{aligned}\right\} \tag{13}$$

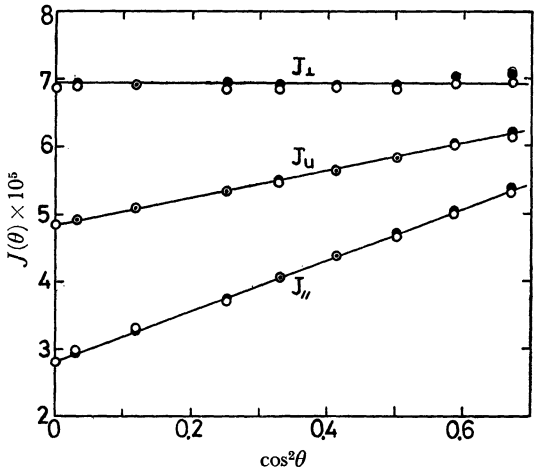

Fig. 5. The relation between the Rayleigh ratios, $J(\theta)$ and $\cos^2\theta$, for benzene. (○) $\theta<90°$, (●) $\theta>90°$.

The experimental values obtained for benzene purified by the method described in the previous paper[3] are shown in Fig. 5, with open circles for $\theta<90°$ and closed circles for $\theta>90°$, together with the straight lines drawn according to Eq. (13). The agreements between the experiment and the theory are satisfactory.

Since Eqs. (12a—c) have been justified experimentally, the treatment may be extended to explain the anomalous behaviour of the Zimm plots of BSA, an example of which has been shown in Fig. 3. For this purpose, the following equation, which can be derived easily from Eq. (12c), may be used:

$$P_u(\theta) = \frac{R_u(\theta)}{R_u(0)} = 1 + Q\frac{1-\cos^2\theta}{1+\cos^2\theta} \tag{14}$$

9) W. H. Stein and S. Moore, *J. Biol. Chem.*, **178**, 79 (1949).

10) C. L. Carr and B. H. Zimm, *J. Chem. Phys.*, **18**, 1616 (1950).

Before doing this, the Q-values of BSA given in Table 3 will be discussed further. The Q quantity is related to the excess-polarizability ratio, p_α, defined by:

$$p_\alpha = \alpha_a/\alpha_b \tag{15}$$

where α_a and α_b are the principal values of the excess polarizability (in excess of that of the solvent) of the segments parallel to and perpendicular to, respectively, the axis of the rotation of the spheroidal segments. The relation is given by the following equation:[11]

$$Q = \frac{(2/15)(p_\alpha-1)^2}{1+(2/3)(p_\alpha-1)+(1/15)(p_\alpha-1)^2} \tag{16}$$

It is obvious that $Q=0$ for optically-isotropic segments ($p_\alpha=1$). The p_α-values of BSA obtained by Eq. (16) are given in Table 3. It may be seen that the value of p_α changes only a little with the GuHCl concentration and shows its minimum point at about 2—3M of the latter. This is related to the side-by-side dimerization of BSA molecules, as will be discussed later on.

As for the magnitude of the p_α value, it has already been concluded in a previous paper[7] that the value should be within the following range, if the anisotropy is due to the anisometry, that is, the segments are considered to be non-spherical particles of an isotropic material. If the value of p_α is outside the range, the segments must have intrinsic anisotropy:

$$(m^2+1)/2 > p_\alpha > 1/m^2 \tag{17}$$

where m is the relative refractive index of the segments.

The value of m may be estimated according to the following equation:[7]

$$\phi_\mu = (m-1)n_1\bar{v}_2 \tag{18}$$

Here, $\bar{v}_2$ is the specific volume of BSA, as is given in Table 1, and n_1 is the refractive index of the solvent, that is, the GuHCl solution of the corresponding concentration. As is shown in Table 3, the value of m is about 1.2, which is the value usually obtained for various organic substances in water.

The range given by Eq. (17) for this m-value is:

$$1.21 > p_\alpha > 0.70 \tag{19}$$

The p_α value of BSA shown in Table 3 is definitely outside this range. The optical anisotropy of BSA may, therefore, be concluded to be intrinsic.

Modified Zimm Plot of Denatured BSA. For the macromolecules of anisotropic segments, it has already been stated[7] that the light-scattering equation must be modified by replacing M^* of Eq. (4) with $M\cdot P_u(\theta)/F$, where $P_u(\theta)$ is the function already given by Eq. (14), and where the factor, F, is given by the following equation:[12]

$$F = 1-(7/6)Q \tag{20}$$

Equation (4) can, therefore, be rewritten as follows, since $P_u(0)=1$:

$$[P_u(\theta)/F]\cdot[K'\phi_\mu^2 c_2/R_u(\theta)] = \frac{1}{M}\left(1+\frac{16\pi^2}{3\lambda^2}R_g^2\sin^2\frac{\theta}{2}\right)+2\frac{A_2}{F}c_2 \tag{21}$$

11) The quantity, p_α, defined here is the reciprocal of that used in Ref. 7. Equation (16) may be derived readily from Eqs. (21) and (29) of the same paper.

12) The equation $F=1+Q/2$, derived from Eqs. (29) and (37) of Ref. 7, is in error. This point will be discussed in a separate paper.

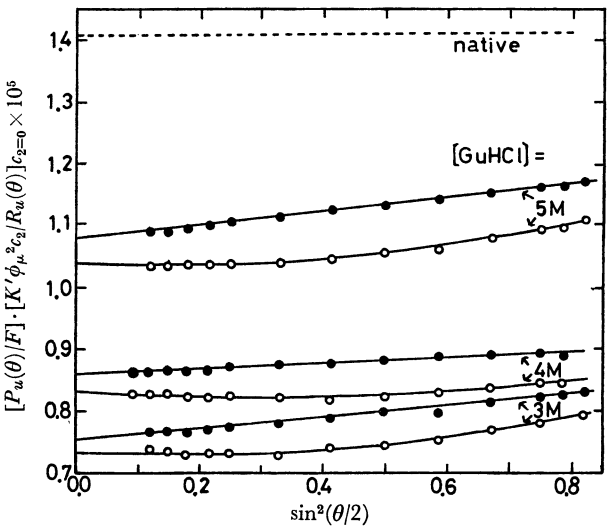

Fig. 6. Usual (open circles) and modified (closed circles) Zimm plots of BSA at $c_2\to 0$, for various GuHCl concentrations.

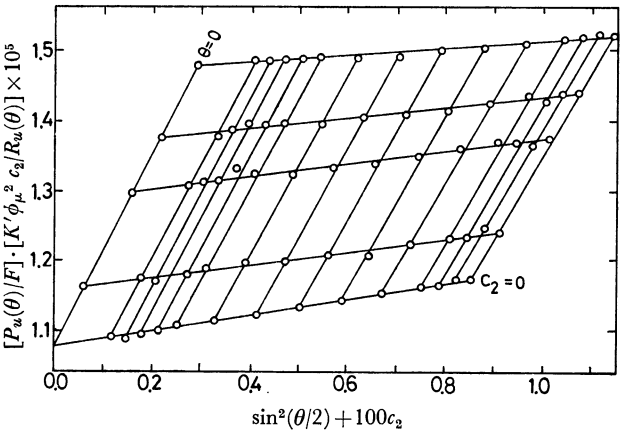

Fig. 7. Modified Zimm plot of BSA at [GuHCl]=5M.

The left side of Eq. (21) can be plotted on the ordinate to draw the modified Zimm plot, because $P_u(\theta)$ and F can be calculated by using the value of Q obtained experimentally, as is shown in Table 3.

It can be seen from Fig. 6 that the plots for $c_2\to 0$ are curved in the usual Zimm plots, as is shown by the open circles, but that the plots are on straight lines in the modified Zimm plots, as is shown by the closed circles. As an example, the modified Zimm plot of BSA in 5M GuHCl is given in Fig. 7; it is composed of straight lines only, in contradistinction to the usual Zimm plot of the same system shown in Fig. 3. It may, therefore, be concluded that the curved feature of the usual Zimm plot, and also the apparently imaginary value of the radius of gyration, R_g^*, are due to the optical anisotropy of BSA. The correct values of the molecular weight, M, and the radius of gyration, R_g, as obtained from the modified Zimm plot, are given in Table 2.

Molecular Weight of Denatured BSA. The weight-average molecular weight, M, of BSA, obtained from the modified Zimm plot, increases with the GuHCl concentration at first and then reaches a maximum point at about 3M GuHCl, as is shown in Table 2. The ratio (M/M_0) of the molecular weight to that of

native BSA is nearly equal to 2 at the maximum point. This means that dimers are formed by the intermolecular binding of two denatured BSA molecules. With a further increase in the GuHCl concentration, however, the intermolecular binding seems to be broken, because the molecular weight decreases to as low as $M/M_0=$ 1.30 at 5M GuHCl.

The following conclusions may, therefore, be drawn: (1) GuHCl, at first, breaks the intramolecular binding of BSA and changes the shape of the molecules, as will be discussed in the next section. (2) The broken bonds, which have been brought to the outer surface of the molecule by the change in the molecular shape, combine with those of another denatures BSA molecule to form a dimer. (3) These intermolecular bonds, too, are broken with a further increase in the GuHCl concentration.

Molecular Shape and Intrinsic Viscosity of Denatures BSA. As has been described above, the following three quantities, the intrinsic viscosity, $[\eta]$; the molecular volume, V_m (which is equal to $M\bar{v}_2/N_0$, where N_0 is the Avogadro number), and the radius of gyration, R_g, of BSA at various GuHCl concentrations have been determined. On the basis of these results, the shape and size of BSA molecules may be discussed if the molecular shape is approximated by a suitable model, as has already been shown in a previous paper[13] on chondroitin sulfate.

If the denatured BSA molecule is approximated by a random coil;[14]

$$[\eta]=6^{3/2}\Phi R_g^3/M \tag{22}$$

where $\Phi=2.87\times10^{21}$.

In the case of a cylindrical rod or a sheroid,

$$[\eta]=0.025\bar{v}_2\cdot\nu(p) \tag{23}$$

where $\nu(p)$ is the shape factor as a function of the axial ratio, p.

$$p=L/D=a/b \tag{24}$$

where L and D are the length and diameter respectively of the cylindrical rod, and where a and b are the radii of the sheroid in the direction parallel to and parpendicular to respectively, the axis of rotation. The shape factor is given by one of the following equations.[15]

$$\nu(p)=0.373+0.08p^2\left\{\frac{1}{3(\ln 2p-1.8)}+\frac{1}{(\ln 2p-0.8)}\right\} \tag{25a}$$

For a prolate sheroid ($p>1$),

$$\nu(p<15)=1+0.163(p-1)^{1.508} \tag{25b}$$

$$\nu(p>15)=0.64+0.08p^2\left\{\frac{1}{3(\ln 2p-1.5)}+\frac{1}{(\ln 2p-0.5)}\right\}$$

For a oblate spheroid ($p<1$),

$$\nu(p)=1+0.272\frac{1-p}{p}-0.251\left\{\frac{1-p}{1-0.075p}\right\} \tag{25c}$$

The value of $[\eta]$ may, therefore, be calculated if the value of the axial ratio, p, is known.

The molecular volume, V_m, is given by the equation:

$$V_m=\frac{\pi}{4}L^3/p^2=\frac{4}{3}\pi a^3/p^2 \tag{26}$$

The radius of gyration, R_g, is given as follows. For a cylindrical rod,

$$R_g^2=\frac{L^2}{12}(1+3/2p^2) \tag{27a}$$

For a sheroid,

$$R_g^2=\frac{a^2}{5}(1+2/p^2) \tag{27b}$$

According to these equations, the molecular dimensions, L and D or a and b, and the axial ratio, p, can be calculated.

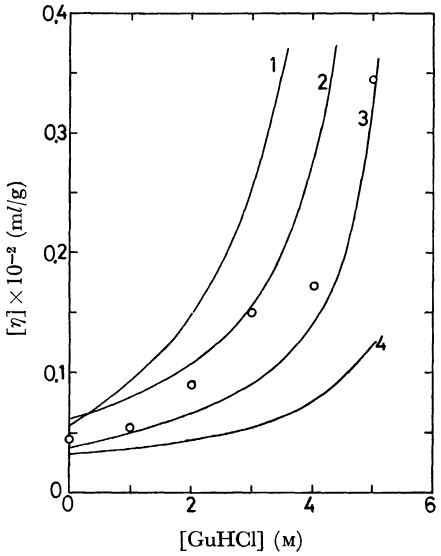

Fig. 8. Intrinsic viscosity of BSA in GuHCl solutions.
(1) Random coil, (2) cylindrical rod, (3) prolate spheroid and (4) oblate spheroid.

The value of the intrinsic viscosity, $[\eta]$, obtained experimentally and theoretically are compared in Fig. 8. The experimental values, already given in Table 1 and shown in Fig. 8 by open circles, are close to the theoretical curve of the prolate spheroid, except at a GuHCl concentration of about 3M, where the experimental plot is close to the theoretical curve of the cylindrical rod, probably because the dimer formation is the most complete at this GuHCl concentration. At any rate, the experimental plots are located between the theoretical curve of the prolate spheroid and that of the cylindrical rod. It may, therefore, be concluded that the denatured BSA molecules are shape in elongated.

TABLE 5. DIMENSIONS OF DENATURED BSA MOLECULES AS PROLATE SPHEROIDS

[GuHCl] (M)	a (Å)	b (Å)	p	b_1 (Å)	p_1
0	71.2	16.8	4.23	16.8	4.23
1	91.7	17.0	5.40	14.8	6.20
2	122.4	17.8	6.87	12.8	9.56
3	145.5	16.1	9.02	11.8	12.3
4	179.3	13.8	13.0	10.6	16.9
5	231.9	10.6	21.9	9.31	24.9

13) M. Nakagaki and K. Ikeda, This Bulletin, **41**, 553 (1968).
14) P. J. Flory, "The Principles of Polymer Chemistry," Cornell University Press (1953), p. 611.
15) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940); *J. Chem. Phys.*, **13**, 188 (1945); W. Kuhn and H. Kuhn, *Helv. Chim. Acta*, **28**, 97 (1945); H. A. Scheraga, *J. Chem. Phys.*, **23**, 1526 (1955).

The dimensions and the axial ratio of BSA molecules as prolate spheroids are shown in Table 5. These values were calculated on the basis of the radius of gyration determined by the light-scattering measurements and on the basis of the molecular volume determined by the density measurements.

It may be mentioned here that the axial ratio, p, increases from 4.23 to 21.9 with the GuHCl concentration, in contradistinction to the excess-polarizability ratio, p_α, which scarcely changes with the GuHCl concentration, as shown in Table 3. This is because p_α expresses the optical anisotropy of each segment, while p expresses the anisometry of the molecule as a whole, and the molecule is composed of many segments.

According to Table 5, the length, $2a$, of BSA molecules increases monotonously with the GuHCl concentration, but the diameter, $2b$, has a maximum point at about 2M GuHCl. This maximum may be supposed to be due to the maximum of the dimerization of BSA at about this GuHCl concentration, suggesting that the dimerization is realized by a side-by-side combination of two denatured BSA molecules. If this is so, then the radius, b_1, and the corresponding axial ratio, p_1, of one BSA molecule in the aggregate may be calculated by means of the following equations:

$$b_1 = b/\sqrt{M/M_0}$$
$$p_1 = a/b_1 \tag{28}$$

As is shown also in Table 5, the value of b_1 monotonously decreases, and that of p_1 monotonously increases, with the GuHCl concentration. It may, therefore, be concluded that the denaturating agent, GuHCl, breaks the intramolecular bonds of BSA to make the molecular shape the longer as the GuHCl concentration is higher, and that two molecules of denatured BSA aggregate side-by-side to form a dimer.

The largest length of a BSA molecule observed here is $2a=464$ Å at 5M GuHCl. This is about 1/5 of the length of a fully-stretched BSA molecule, that is estimated to be about 2200 Å for BSA containing 602 amino-acid residues.[9] This is in contrast to sodium chondroitin sulfate C whose length is between 1/2 ($J=\infty$) and 2/3 ($J=0.01$M) of the fully-stretched length,[13] where J is the ionic strength. The relatively small stretching of the BSA molecule by denaturation suggests that denatured BSA molecules keep their natural local conformation even in a GuHCl solution as concentrated as 5M.