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Temperature Dependence of the Second Virial Coefficient of Bovine Serum Albumin Solutions by the Archibald Ultracentrifugation Method*

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The generalized theory of the Archibald ultracentrifugation method has been extended to describe water-protein-salt and water-protein systems. In the former system a slight difference was found between the Archibald and the light scattering functions.

Using this method a preliminary study on the thermodynamic properties of isoionic bovine serum albumin solutions has been made. Particularly the temperature dependence of the second virial coefficient has been elucidated. It was found that the second virial coefficient almost vanishes in salt-free solutions at 10°C and that it becomes increasingly negative with increasing temperatures. The results are compared with light scattering data by Timasheff *et al.* taking into consideration the fluctuating charge theory of Kirkwood and Schumaker.

INTRODUCTION

The effects of electrostatic forces on intermolecular interactions between protein molecules in solution are represented in terms of the second virial coefficient A_2 in the osmotic pressure equation $\pi/cRT=1/M+A_2c+\cdots$ or in the light scattering equation $Hc/\Delta\tau=1/M+2A_2c\cdots$. A number of studies have been carried out on the effects of net charges on protein molecules and of ionic strength of the solution on the second virial coefficient. The thermodynamic behavior of protein molecules in solution with addition of simple electrolytes can be described well by the Gibbs-Donnan theory[1] and even better by the Scatchard theory[2]. These theories are concerned mainly with electrostatic interactions between protein molecules and those between protein molecules and small ions in the presence of simple electrolytes. Kirkwood and his associates[3~7] have studied the thermodynamic properties of isoionic proten solutions and found that the isoionic albumins in salt-free or in very low salt solutions have negative A_2. This tendency was interpreted by these authors as a result of attractive forces between protein molecules due to charge fluctuations on protein molecules.

Generally speaking, one may describe the change in magnitude of A_2 in terms of the pair potential of average force between two molecules. If the force is repulsive, A_2 should be positive. On the other hand, if the force is

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attractive in a certain range of distance between two molecules, there must be a certain temperature range where A_2 is negative. Furthermore studies of the temperature dependence of A_2 would provide valuable informations about the nature of intermolecular interactions. For example, one can expect to evaluate separately the enthalpy and entropy contributions to A_2 from a study of its temperature dependence. Schulz and his associates carried out studies in this area, however, they used almost entirely neutral polymers[8]. Although the effects of ionic strength and pH of protein solutions upon A_2 have been investigated, few reports concerning its temperature dependence were published. These thoughts motivated us to initiate the present study.

The present paper mainly concerns the following two points : To establish a workable technique for the determination of molecular weights and A_2 in protein solutions and to apply this technique for the study of isoionic solutions of bovine serum albumin (BSA). For the first purpose the Archibald ultracentrifugation technique[9] seems to be suitable. For many years the light scattering technique has successfully been applied in physicochemical studies of proteins, particularly in the determination of molecular weights and in studies of thermodynamic interactions[10-12]. In practice, this techique is less advantageous than the Archibald method, because it usually requires fairly large amounts of materials and a great care in purifying solutions. However, the Archibald method has neither of these requirements. Since Fujita *et al.*[15] have found that the Archibald method and the light scattering method provide similar information (Part I of this series), we decided to apply the Archibald method for the study of BSA solutions.

The Archibald method has also received wide application in the study of proteins[16][17]. However, its use has been confined practically to the determination of molecular weights, probably because no appropriate theory for the analysis of data was available. Therefore, we have reexamined, on the basis of the generalized theory[15], the applicability of the Archibald method to protein solutions with or without addition of simple electrolytes. This theory will be discussed in detail in the next section. By adopting the Archibald technique, we have studied isoionic solutions of BSA. Preliminary results of this study, in particular, the temperature dependence of A_2 of isoionic BSA in salt-free solutions will be reported in subsequent sections.

THEORETICAL

We consider a multicomponent system consisting of component 0 (principal solvent) and q species of solutes, which is subjected to centrifugation at constant temperature T and constant angular velocity of rotation ω. From the theory of isothermal sedimentation in terms of the thermodynamics of irreversible processes[18] one can write a general expression for the flow of each component, J_i. The application of the boundary condition in a centrifuge cell *i. e.*, the flow of any component vanishing at either end of the cell, leads to a set of q simulteneous equations with respect to $(\partial m_j/\partial r)$ $(j=1, 2, \cdots, q)$ at either

end of the cell

$$M_i(1-\bar{v}_i\rho)\omega^2 r=\sum_{j=1}^{q}\mu_{ij}\frac{\partial m_j}{\partial r} \qquad (1)$$

$$(i=1,\ 2,\ \cdots,\ q\ ;\ r=r_1 \text{ and } r_2)$$

$$\mu_{ij}=(\partial\mu_i/m_j)_{T,\,P,\,m_{k(k\neq j)}} \qquad (1a)$$

$$\mu_i=\mu_i^{\circ}+RT\ln m_i+\mu_i^{(e)}. \qquad (1b)$$

The symbols used are : M_i and $\bar{v}_i$ are the molecular weight and the partial specific volume of component i, respectively ; ρ is the local density of the solution ; r, r_1 and r_2 are radial distances from the center of rotation to any arbitrary position, to the meniscus and to the bottom of the solution column, respectively ; m_j is the concentration of component j at the position r and time t expressed in any appropriate unit [for example, molarity (mole/l), partial density or c-scale (g/l), *etc.*] ; μ_i is the chemical potential per mole of component i and μ_i^0 and $\mu_i^{(e)}$ are the reference and the excess chemical potential, respectively.

First let us consider a salt-free solution of an isoionic protein. There are protein ions with zero average net charge, and very small amount of hydrogen and hydroxyl ions in the solution. If the contribution of these hydrogen and hydroxyl ions is neligible, the solution can be regarded practically as a two component system (water=component 0 and protein=component 1). Therefore the simulteneous equations (1) are reduced to a single equation and from this one can readily obtain the following equation at any given time :

$$(M_1)^*_{app}(t)=RT\phi_1 M_1^*(1/\mu_{11})/\phi_1 m_1 \qquad (2)$$

$$\phi_1=(\partial\tilde{n}/\partial m_1)_{T,\,P} \qquad (2a)$$

$$M_1^*=M_1(1-\bar{v}_1\rho), \qquad (2b)$$

where $\tilde{n}$ is the excess refractive index of the solution over the solvent, and ϕ_1 is the differential refractive index increment on the m-scale of the component 1. Here the quantity $(M_1)^*_{app}(t)$ is defined by experimentally measurable quantities as

$$(M_1)^*_{app}(t)=\frac{RT}{\omega^2 r}\frac{1}{\tilde{n}}\frac{\partial\tilde{n}}{\partial r} \qquad \text{at } r=r_1 \text{ and } r_2. \qquad (3)$$

(For practical procedures to determine quantities $\tilde{n}$ and $(\partial\tilde{n}/\partial r)$ at the meniscus or at the botton from a sedimentation pattern, see our previous papers or any other relevant articles[15~17)19)]). At the limit where $(M_1)^*_{app}(t)$ is extrapolated back to zero time, the values determined from both ends of the solution column should converge to the same value (provided the pressure effect is negligible). This will be termed the apparent reduced molecular weight, $(M_1)_{app}^*$. This should, of course, be a function of the initial concentration of the solute, *i. e.*, the concentration of the solute before centrifugation. By adopting the partial density c_1(g/l) as a concentration scale, one obtains the following equation from equations (1a)-(2b)

$$\frac{1}{(M_1)_{app}}=\frac{(1-\bar{v}_1\rho)}{(M_1)_{app}^*}=\frac{1}{M_1}(1+c_1\beta'_{11}) \tag{4}$$

$$\beta_{11}'=\frac{1}{RT}\left(\frac{\partial\mu_1^{(e)}}{\partial c_1}\right). \tag{4a}$$

Here the quantity $(M_1)_{app}$ has a dimension of molecular weight and is termed the apparent molecular weight. Equation (4) indicates that the solute molecular weight, M_1, may be determined from the intercept of the $1/(M_1)_{app}$ *versus* c_1 plot, while from the slope of the plot, β_{11}' may be evaluated*). It should be noted that the plot of $1/(M_1)_{app}$ *versus* c_1 should yield the same information as the plot of the light scattering function $H(c_1/\Delta\tau)$ *versus* c_1[6,14] where H is the well-known light scattering factor and $\Delta\tau$ is the excess turbidity over that of the solvent (water).

In case of a protein solution in the presence of a simple electrolyte (water = component 0, protein = component 1, and neutral salt = component 2), one has to give a somewhat different definition of $(M_1)_{app}^*$. In practice, it is preferable to carry out an Archibald experiment by using a double-sector cell with the complete system (water-protein-salt) in one sector and the reference system (water-salt) in the other. Then the observed sedimentation pattern (for example, by a schlieren optics) would give

$$\begin{aligned}\Delta\frac{\partial\tilde{n}}{\partial r}&=\left(\frac{\partial\tilde{n}}{\partial r}\right)-\left(\frac{\partial\tilde{n}}{\partial r}\right)^R\\&=\phi_1(\partial m_1/\partial r)+\phi_2(\partial m_2/\partial r)-\phi_2(\partial m_2/\partial r)^R,\end{aligned} \tag{5a}$$

where $(\partial\tilde{n}/\partial r)^R$ and $(\partial m_2/\partial r)^R$ denote the values in the reference side of the cell. Here we have assumed that ϕ_2 has the same value in both the complete and the reference systems. And the application offithe well-known procedure of graphical integration of a schlieren pattern [15~17,19] would give

$$\begin{aligned}(\tilde{n}-\tilde{n}^R)_{r=r_1}&=(\tilde{n}-\tilde{n}^R)_{t=0}-\frac{1}{r_1^2}\int_{r_1}^{r_p}r^2\left(\Delta\frac{\partial\tilde{n}}{\partial r}\right)dr\\&=\phi_1m_1+\phi_2m_2-\phi_2m_2^R\end{aligned} \tag{5b}$$

(for the value at the bottom, replace r_1 by r_2), where r_p denotes an arbitrary position in the region where $\Delta(\partial\tilde{n}/\partial r)=0$, *i. e.*, the plateau region. Apparently it is convenient to define $(M_1)^*_{app}(t)$ by using these values**) as

$$(M_1)^*_{app}(t)=\frac{RT}{\omega^2r}\frac{(\partial\tilde{n}/\partial r)-(\partial\tilde{n}/\partial r)^R}{\tilde{n}-\tilde{n}^R}. \tag{6}$$

The thermodynamic expression for $(M_1)^*_{app}(t)$ may be obtained by the

*) If the protein sample contains dimer, trimer *etc.* formed by intermolecular couplings such as -S-S- linkage, which is often found to be the case, the protein component may be regarded as multicomponent in the sense that it is homologous but has molecular weight heterogeneity. Even in such a case, however, the partial specific volume and the specific refractive index increment may be assumed to be equal for all monomer, dimer, *etc.*. The procedure mentioned above, then, provides the weight average molecular weight of the protein component and the interaction parameter equivalent to that of the light scattering method[15].

similar procedure used in obtaining equation (2). The problem is how to define the flow equations in this system. Fujita[19] has shown that in the case of water and two electrolytes with three different ionic species, the phenomenological flow equations are perfectly the same as the corresponding flow equations for a ternary nonelectrolyte system, provided the definition of electrolyte components is properly given. Therefore the problem seems to be largely a matter of the choice of definition for the components.

Here we consider a case in which the neutral salt consists of one species of univalent cations and one species of univalent anions such as NaCl, and there are only two kinds of diffusible ions. We designate : water (principal solvent) as component 0 ; m_1 moles of protein as component 1, each mole of which has the mean charge of $\bar{Z}_1$ and associates υ_{1c} and υ_{1a} moles of diffusible cations and anions, respectively : m_2 moles of a 1−1 salt as component 2. We introduced the definition according to Scatchard [2)10)] which involves the net addition of only one mole of ion per each mole of protein component with the preservation of electroneutrality. Therefore $\upsilon_{1c}=-\upsilon_{1a}=-\bar{Z}_1/2$ and the amounts of total diffusible cations m_c and anions m_a should be $m_c=m_2-m_1(\bar{Z}_1/2)$: $m_a=m_2+m_1(\bar{Z}_1/2)$, respectively. Now we temporarily adopt the molarity (mole/l of solution) as the concentration scale.

By using the above definition, the chemical potential for each compoent can be written as follows[10] :

$$\mu_1=\mu_1^0+RT\ln m_1+RT\frac{\bar{Z}_1}{2}\ln\frac{m_2+(\bar{Z}_1/2)m_1}{m_2-(\bar{Z}_1/2)m_1}+\mu_1^{(e)} \tag{7a}$$

$$\mu_2=\mu_2^0+RT\ln[m_2^2-(m_1\bar{Z}_1/2)^2]+\mu_2^{(e)}. \tag{7b}$$

For the reference system, *i. e.*, the complete system less component 1, the chemical potential is

$$\mu_2^R=\mu_2^{0R}+2RT\ln m_2+\mu_2^{(e)R}. \tag{7c}$$

Now assuming that equation (1) is valid for each component as defined above, and taking the limit of zero time, we obtain the expression for $(M_1)_{app}^*$ from equations (1), (5a, b) and (b)

$$(M_1)_{app}^*=\lim_{t\to 0}(M_1)_{app}^*(t)$$
$$=\frac{RT}{m_1}\frac{(M_1^*+M_2^*)(1+\alpha\Gamma)}{(\mu_{11}-\mu_{22}\Gamma^2)}+M_2^*\left(\frac{1}{\mu_{22}}-\frac{1}{\mu_{22}^R}\right) \tag{8}$$

**) If one can measure the concentration distribution of protein component independently from that of salt by a special technique, for example, by use of a UV-absorption optics, $(M)_{app}^*(t)$ may be defined as

$$(M_1)_{app}^*(t)=(RT/\omega^2 r)(1/\tilde{n}_1)(\partial\tilde{n}_1/\partial r)$$
$$r=r_1 \text{ and } r_2,$$

where $\tilde{n}_1$ is the local concentration of protein component in a unit appropriate to the method employed. (It is not necessarily a refractive index increment but can be an optical density unit.) In this case, the Archibald function, $1/(M_1)_{app}$, contains only the factor $1+[(1-\bar{v}_2\rho)/(1-\bar{v}_1\rho)]I'_0$ in the denominator, while the numerator is completely the same to that of equation (9).

$$\Gamma = -\mu_{12}/\mu_{22} = (\partial m_2/\partial m_1)_{\mu_2} \tag{8a}$$

$$\alpha = \phi_2/\phi_1. \tag{8b}$$

It is conceivable that the second term at the right hand side of equation (8) may be neglected, especially in solutions with very low protein concentrations. By neglecting this term and by replacing the molarity by the partial density (number of grams of solute in one liter of solution), $c_1 = M_1 m_1$ and $c_2 = M_2 m_2$, equations (7a)～(7c) and (8)～(8b) yield

$$\frac{1}{(M_1)_{app}} = \frac{(1-\bar{v}_1\rho)}{(M_1)^*_{app}} = \frac{\dfrac{1}{M_1} + c_1\left[\dfrac{M_2\bar{Z}_1^2}{2M_1^2c_2\varepsilon} + \dfrac{\beta'_{11}}{M_1} - \left(\dfrac{2}{M_2c_2\varepsilon} + \dfrac{\beta'_{22}}{M_2}\right)(\Gamma')^2\right]}{\left[1 + \dfrac{(1-\bar{v}_2\rho)}{(1-\bar{v}_1\rho)}\Gamma'\right](1+\alpha'\Gamma')} \tag{9}$$

$$\Gamma' = \Gamma(M_2/M_1)_i^{\dagger};\ \alpha' = \alpha(M_1/M_2) \tag{9a, b}$$

$$\varepsilon = 1 - (\bar{Z}_1 M_2 c_1/2M_1c_2)^2 \tag{9c}$$

$$\beta'_{ij} = \frac{1}{RT}\left(\frac{\partial \mu_i{}^{(e)}}{\partial c_j}\right) \quad (i, j = 1 \text{ and } 2). \tag{9d}$$

After determining $(M_1)_{app}$ as a function of c_1 with fixed value of c_2, the plot of $1/(M_1)_{app}$ *versus* c_1 yields the slope factor and from the intercept of the plot $c_1 = 0$ it yields

$$(M_1)_{app,\,0} = M_1\left[1 + \left(\frac{1-\bar{v}_2\rho}{1-\bar{v}_1\rho}\right)_{c_1=0}\Gamma'_0\right](1+\alpha'_0\Gamma'_0) \tag{10}$$

where the suffix 0 indicates that the corresponding quantites are those obtained at the limit of $c_1 = 0$. This equation shows that the extrapolation of $(M_1)_{app}$ to $c_1 = 0$ does not yield a true value of molecular weight, M_1, unless other factors have been evaluated from independent measurements[9]. The correction terms, however, are often found to be negligible in many protein systems.

At this point it is interesting to note that the light scattering function and the Archibald function for a water-proteinsalt system are dissimilar. According to Timasheff and Coleman[14], the former was given as

$$Hc/\Delta\tau = \frac{1}{(1+\alpha'\Gamma')^2}\left\{\frac{1}{M} + c_1\left[\frac{M_2\bar{Z}_1^2}{2M_1^2c_2\varepsilon} + \frac{\beta'_{11}}{M_1} - \left(\frac{2}{M_2c_2\varepsilon} + \frac{\beta'_{22}}{M_2}\right)(\Gamma')^2\right]\right\}. \tag{11}$$

By comparing equations (9) and (11), it is readily seen that only the factor in the denominators of the two equation differs. At the limit of $c_1 = 0$ with a fixed value of c_2, the light scattering function gives

$$(\Delta\tau/Hc_1)_{c_1=0} = M_1(1+\alpha'_0\Gamma'_0)^2. \tag{12}$$

Therefore the values from the intercepts of the Archibald and the light scattering functions at $c_1 = 0$ are different by a factor of

$$\{1 + [(1-\bar{v}_2\rho)/(1-\bar{v}_1\rho)]_{c_1=0}\Gamma'_0\}/(1+\alpha'_0\Gamma'_0). \tag{13}$$

This relation, in turn, might be useful in evaluating the factor Γ'_0 from the light scattering and the Archibald data of the same water-protein-salt system,

if there is any appreciable difference between them.

EXPERIMENTAL

Preparation of Isoionic BSA Solutions

A crystalline BSA sample from Behring Werk A. G., Marburg/Lahn (Lot No. 17) was obtained through the courtesy of Professor G. V. Schulz (Institut für physikalische Chemie der Universität Mainz). The procedure for purification employed by Timasheff *et al.*[6] was followed in this experiment.

800 mg of the BSA sample were dissolved at 4°C into 20 ml of distilled water. Distilled water was prepared by distilling commercial pure water with a small amount of $KMnO_4$ in an all Pyrex glass still. The purity of the water was tested by specific conductivity measurements. The specific conductance of distilled water used was less than 1.44×10^{-6} ohm^{-1} cm^{-1}.

The BSA solution was first filtrated through a sintered glass filter of ultrafine porosity and then the filtrate was dialyzed against several changs of distilled water for four days at 4°C. The pH of the solution at the end of dialysis was 5.2. The solution was then passed through an ion-exchange column which contained 40 ml of a 2 : 1 mixture of anionic (Amberlite I. R. 120) and cationic (Amberlite I. R. A. 400) resins. The resins had been converted to the hydrogen and the hydroxyl form, respectively, by the methods described by Timasheff *et al.*[6]. After passing through the column, the concentration of the recovered protein solution was about 2.5 % by weight and its specific conductance and pH were found to be 3.54×10^{-6} ohm^{-1} cm^{-1} and 4.7, respectively. The solution seemed to be salt-free.

The resulting solution was stored at 4°C for later use in the Archibald experiments. At each experiment the stock solution was diluted with distilled water to a desired concentration.

All salt solutions (M/10 NaCl solutions) were prepared by diluting a 1 M NaCl solution obtained with reagent grade chemical. A calculated amount of this solution was added to the protein solutions to bring the final salt concentration to M/10.

Determination of Protein Concentration

All concentrations were determined by means of ultraviolet absorption at 280 mμ in a Beckman model DU spectrophotometer. The value for the absorption coefficient $[E]^{1\%}_{1cm}=6.5$ was used as the standard for spectrophotometric determination of protein concentrations. In preliminary experiments, the concentration was also measured by the micro-Kjeldahl technique using a factor of 6.25 for conversion of nitrogen content to protein concentration.

Determination of Sedimentation Coefficient

For the characterization of the BSA sample, sedimentation velocity experiments were carried out useing a Spinco model E ultracentrifuge at the Research Institute of Sionogi & Co. The measurements were performed at 20°C with rotational velocity 59,780 r.p.m. for salt-free solutions. The sedimentation coef-

ficient obtained after extrapolation to zero protein concentration was $S_0=4.00$ (in Svedberg units). This value agrees quite well with that obtained by Schlamowitz *et al.*[20]. The authors wish to thank for the cooperation of the Sionogi Research Institute.

Archibald Ultracentrifugation Experiments

For all the Archibald ultracentrifugation experiments, a Phywe air-driven ultracentrifuge was used. The fluctuation of the rotational speed was less than 1 % of the average value, and the temperature of the rotor was kept within± 0.5°C around the desired value during each centrifugation run. The details of the instrument and experimental procedure have been reported in our previous papers[15)21)]. Therefore it will not be recounted here. A small amount of Dow Corning No. 555 silicone oil was used as a bottom liquid only for salt solutions.

For the partial specific volume of BSA at different temperatures, values published by Charlwood[22)] and by Cox and Schumaker[23)] were employed. These values are listed in Table 1 together with the value of the buoyancy factor $(1-\bar{v}_1\rho)$.

Table 1. Values of the partial specific volume, $\bar{v}_1$, and the buoyancy factor, $(1-\bar{v}_1\rho)$, for the BSA solutions studied.

Temp. (°C)	$\bar{v}_1$ (ml/g)	$(1-\bar{v}_1\rho)$
10	0.725	0.2752
15	0.729	0.2717
22	0.734	0.2676
25*	0.735	0.2641
30	0.738	0.2652

*) Values for M/10 NaCl solution of isoionic BSA.

RESULTS AND DISCUSSION

Fig. 1 shows the plots of $(M_1)_{app}(t)$ against time for BSA in M/10 NaCl solutions measured at 25°C. It is seen that in each case $(M_1)_{app}(t)$ obtained

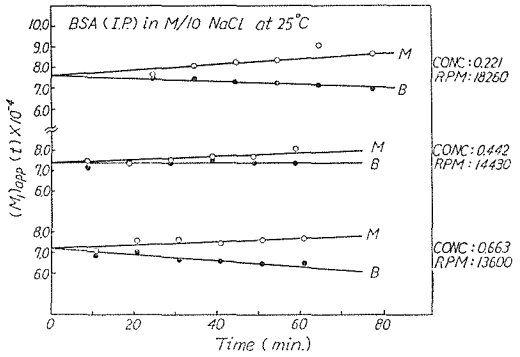

Fig. 1. Plots of $(M_1)_{app}(t)$ *versus* time t for BSA in M/10 NaCl solution meassured at 25°C. M and B indicate data from the meniscus and the bottom, respectively.

from the meniscus increases whereas that obtained from the bottom decreases with time.

Fig. 2 shows the similar plot for salt-free solutions at three different tem-

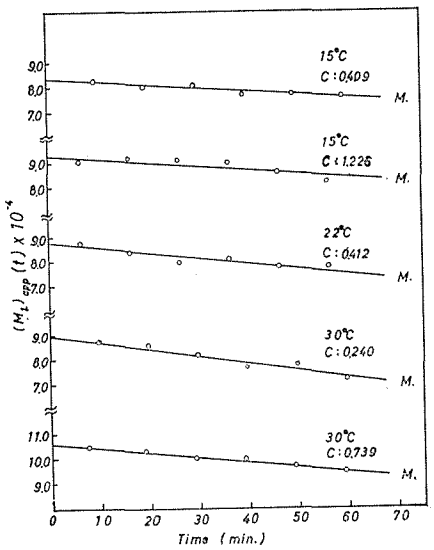

Fig. 2. Plots of $(M_1)_{app}(t)$ *versus* time t for isoionic BSA in salt-free solutions at various initial concentrations and temperatures as indicated. M indicates data from the meniscus.

peratures. In this case, addition of silicone oil as botton liquid resulted in the appearance of cloudy precipitates in the solution and gave blurred schlieren pictures. Therefore silicone oil was not added to the solutions and the data from only the meniscus were used for evaluation of $(M_1)_{app}(t)$. In any one of these cases, $(M_1)_{app}(t)$ from the meniscus is found decreasing with time.

In previous papers[15)17)21)] and also in Part III of this series by Toyoshima and Fujita[24)] the qualitative nature of the dependence of $(M^1)_{app}(t)$ on time was discussed in detail. It was deduced that two major effects, *i. e.*, the nonideality and the polydispersity effect are responsible for the time dependence of $(M_1)_{app}(t)$. In the case of a nonideal monodisperse system it was predicted that $(M_1)_{app}(t)$ from the meniscus should increase or decrease with time if the nonideality parameter is positive or negative, respectively. The opposite should be the case for $(M_1)_{app}(t)$ from the bottom[15)17)21)]. As is shown in Figure 3, the parameter is positive for BSA in M/10 solutions and negative for salt-free isoionic BSA solutions. The time dependence of $(M_1)_{app}(t)$ found in these experiments is in accord with the qualitative prediction given in previous papers.

Fig. 3 shows $1/(M_1)_{app}$ as a function of initial protein concentration for salt-free solutions of isoionic BSA at four different temperatures ranging from 10° at 30°C and M/10 NaCl solutions at 25°C. The dotted lines in the figure are the light scattering data by Timasheff *et al.*[6)]. This curve shows a high upward curvature in the range of low protein concentrations (less than 0.2g/dl) for

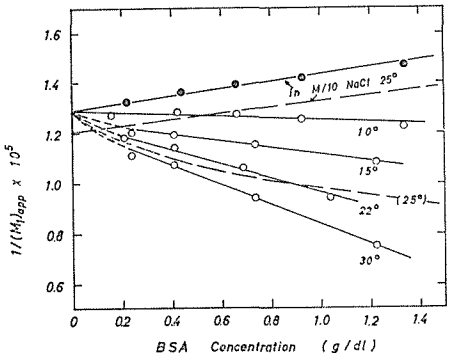

Fig. 3. Plots of $1/(M_1)_{app}$ *versus* BSA concentration, c_1. Filled circles are data for M/10 NaCl solution at 25°C : open circles are data for salt-free solutions at various temperatures as indicated : dotted lines are the corresponding light scattering data obtained by Timasheff *et al.*[6].

salt-free solutions. In the present study we neither confirmed nor disproved the appearance of this high curvature, because Archibald experiments could not be carried out with high precision in the concentration range less than about 0.2g/dl. The slope of $1/(M_1)_{app}$ *versus* c_1 plot for 10°C data seems to be almost zero so that the molecular weight, M_1, of BSA is evaluated to be 7.75×10^4 from the intercept of linear extrapolation with respect to c_1. This value coincides fairly well to that obtained by light scattering measuremnts by Edsall *et al.*[10] Other curves for the data at different temperatures were drawn by assuming this value as the molecular weight of BSA. The evaluated M_1 and the slope factor, $M_1\beta_{11}'$, which is equivalent to $2BM^2_2/1000$ defined by Edsall *et al.*[10], are listed in Table 2. The slope factor in salt-free solutions becomes

Table 2. Molecular weight, M_1, the interaction parameters, $M_1\beta'_{11}$ and the root mean square charge, $<Z_1^2>^{1/2}$, of isoionic BSA in salt-free solutions at various temperatures.

Temp. (°C)	M_1	$M_1\beta'_{11}$[b]		$<Z_1^2>^{1/2}$[c]
		c_1=5g/l	c_1=10g/l	
10	7.75×10^4	− 240	− 240	1.5
15		−1290	−1080	2.9
22		−2230	−1950	3.4
25[a]	8.09×10^4 8.25×10^4	−2430	−1760	3.56
30		−3060	−2700	3.9

[a] Light-scattering data by Timasheff *et al.*[6].

[b] $M_1\beta'_{11}=\frac{1}{RT}\left(\frac{\partial\mu_1}{\partial m_1}\right)^{c)}$, which is equivalent to $2BM^2_2/1000$ defined by Edsall *et al.*[10].

[c] The root mean square charge in protonic unit on a BSA molecule calculated by equation [10] and Fig. 4.

more and more negative as the temperatures rises. For M/10 NaCl solutions, the intercept is almost identical to that of salt-free solutions. This suggests that the correction factor in equation (10) is neglible. In contrast to the salt-

free case, the slope is positive and the slope factor is evaluated to be +870.

For a protein solution in the presence of neutral salt, the following factors contribute to the coefficient of c_1, as had already been pointed out by Timasheff and Coleman[14]. The first term of this coefficient in equation (9) is a consequence of the component definition used and is always positive. The third term indicates the contribution of the interaction between the protein and salt, which can be calculated from salt-binding data and thermodynamic on protein-free salt solutions. The β_{11}' term, which represents protein-protein interaction, involves contribution from several attractive and repulsive forces (in general, the former gives negative and the latter positive contribution). At high salt concentrations, BSA molecules acquire net negative charges due to chloride ion-binding. This results in a strong repulsive force between protein molecules and gives a positive contribution to the coefficient of c_1. The value of +870 found in the present experiment corresponds roughly to the case of $\bar{Z}_1$ value of about −10 〔c. f. Edsall *et al.*[10]〕. Also an appreciable difference can be seen between the light scattering plots of Timasheff *et al.* and that of the Archibald plot for M/10 NaCl solutions (c. f. Fig. 3). It is unlikely that this is due to the difference pointed out in equation (3), because the correction factor seems to be quite small (acording to Timasheff *et al.*, for example, the factor $\alpha_0\,\Gamma'_0$ is of the order of 3.5×10^{-3} for 0.15M NaCl solutions of BSA). Perhaps this is due to the differences in BSA samples, (Timasheff's sample might have contained different amounts of dimers, trimers, etc. than ours).

The experession for the parameter β_{11}' of isoionic protein, *i. e.*, protein with an average zero net charge but non-zero mean square charge, in salt-free solution, was given originally by Kirkwood and Schumaker[3] and later by Timasheff and Coleman [14]. The theory includes the attractive force due to the fluctuations in charge and charge configurations on a protein molecule. According to their theory, in a salt-free solution of isoionic protein, the term $c_1\beta_{11}'$ assumes the form of a power series in $c_1^{1/2}$ as :

$$c_1\beta_{11}' = \frac{c_1}{RT}\left(\frac{\partial\mu_1^{(c)}}{\partial c_1}\right) = -\frac{\pi^{1/2}N^{1/2}e^3<\bar{Z}_1^2>^{3/2}}{2(DkT)^{3/2}M_1^{1/2}}c_1^{1/2} + B^0c_1 + \cdots \qquad (14)$$

$$B^0 = \frac{7\pi Na^3}{6M_1} + \frac{2\pi Ne^4<\bar{Z}_1^2>^2a}{M_1(DkT)^2} + 2B', \qquad (14a)$$

where $<\bar{Z}_1^2>$ is the mean square charge of a protein molecule in protonic units e, D is the dielectric constant of the medium, k is the Boltzmann constant, N is the Avogadro number and a is the Debye-Hückel parameter of the protein molecule. The first term of equation (14a) is the excluded volume obtained by assuming that the protein molecule is a sphere of radius a , while the third term $2B'$ represents the contribution of the effect of all other intermolecular forces (van der Waals forces, fixed multipole moments *etc.*). The equation indicates that a plot of $1/(M_1)_{app}$ *versus* $c_1^{1/2}$ for a salt-free isoionic protein solution should be linear at low protein concentrations and that the mean square charge $<\bar{Z}_1^2>$ can be evaluated from the slope.

In light scattering experiments[6,13,14] with salt-free isoionic BSA, bovine serum

mercaptalbumin, human serum mercaptalbumin and conalbumin, it was found that in agreement with the prediction of the fluctuating charge theory[3] the plot of $H(c_1/\Delta\tau)$ *versus* $c_1^{1/2}$ is close to linear. Although we could not carry out Archibald measurements at low enough concentrations to observe an upward curvature in the $1/(M_1)_{app}$ *versus* c_1 plot, it seemed to be worthwhile to test the linearity in the $1/(M_1)_{app}$ *versus* $c_1^{1/2}$ plot. Results are shown in Fig. 4.

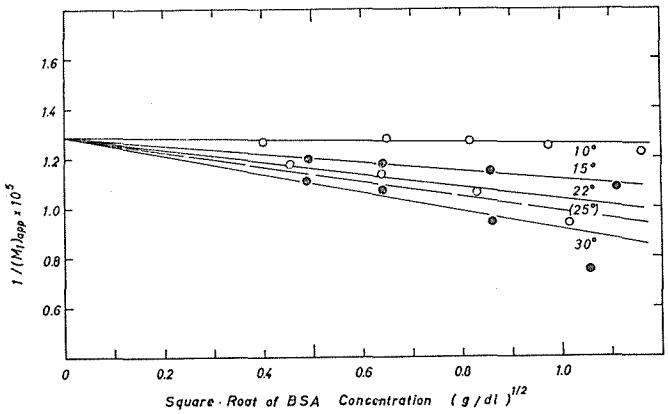

Fig. 4. Plots of $1/(M_1)_{app}$ *varsus* square root of BSA concentration, c_1. Data for salt-free solution in figure 3 are replotted : dotted line again shows the light scattering data by Timasheff *et al.* at 25°C[6].

The straight lines have been satisfactory from the intercept corresponding to the M_1 value of 7.75×10^4 to obtain the best fit for experimental points. Linearity is better in the plots at 10°C and 15°C and rather poor for 22° and 30°C data. At high concentrations deviation is considerable. However, values of $<\bar{Z}_1^2>$ were estimated from the slope of these lines using equation (14). The values are listed in the last column of Table 2. These results suggest that the mean square charge increases as the temperature rises if the fluctuating charge theory is correct. It cannot be established, however, if this deviation is due to an increase in the mean square charge with increasing temperature or some other factor.

Certain polar polymer systems with lower consolute temperatures[25] exist, *i. e.*, aqueous solutions of polyethylene glycol and of polypropylene glycol, whose second virial coefficient decreases with rising temperature. This has been interpreted as the result of negative mixing entropies due to orientation-dependent interactions between polar groups of polymer and solvent molecules. This might be the case in the present system.

On the other hand the apparent attractive forces between protein molecules could be caused by rapid, reversible association-dissociation reactions. Such reactions were found to be taking place in some enzyme systems such as insulin[26], trypsin[27] and α-chymotrypsin[28]. It is certainly true that in usual BSA preparations, dimers, trimers and oligomers contribute perhaps as much as 10% of total BSA. And these dimers and oligomers could actually be separated from the monomers by gel filtration technique[29]. However, some evidence ob-

tained to date by various authors suggest that reversible association-dissociation reactions are unlikely to occur[30~33]. Rather, association reactions in BSA system seem to be slow irreversible processes and the final polymer content in each BSA preparation is likely to depend on the particular procedure employed. More extensive studies are required to elucidate the nature of the temperature dependence of intermolecular interactions between isoionic BSA molecules.

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