Dilatometric Study of the Interactions of Bovine Serum Albumin with Urea*

Sam Katz† and T. G. Ferris

ABSTRACT: This dilatometric study of the interaction of bovine serum albumin with urea relates the resultant volume changes to the conformational changes produced by the denaturation process. The volume changes were determined with a modified Teflon-sheathed Linderstrøm-Lang–Lanz [Linderstrøm-Lang, K., and Lanz, H. (1938), *Compt. Rend. Trav Lab. Carlsberg 24*, 1] type dilatometer. The experimental precision of ≤ 0.03 μl necessitated temperature control within $\pm 0.001°$. The following parameters were investigated: hydration state and protein and urea concentrations. The observed volume changes were converted by appropriate thermodynamic arguments to $(\bar{v}_{23} - \bar{v}_2)$, the difference between the partial molar volume of the protein in the denaturant and that in water. Albumin denatured in 4.0 M urea gave a positive value for $(\bar{v}_{23} - \bar{v}_2)$; however, with increased urea concentration this quantity decreased to negative values at urea concentration ≥ 7 M. Kinetic and thermodynamic data support the hypothesis that this process involves a series of sequential and/or parallel reactions to produce several species of denatured albumins as a function of urea concentration. Parallel experiments performed with quantitative planar acrylamide electrophoresis support this thesis.

Protein denaturation is initiated by a sequence of conformational changes which produce associated volume changes. The literature pertinent to the latter phenomenon is limited primarily to citations based on density measurements. A few reports indicate no demonstrable difference between the partial molar volumes of proteins in urea and that found in water (Reithel and Sakura, 1963). But the predominant view is that chemical denaturation causes a contraction of several hundred ml/100,000 g of protein (Charlwood, 1957; Kauzman, 1959). However, partial molar volumes evaluated from density measurements represent small differences between large numbers and are prone to error both on experimental and theoretical grounds (Krausz and Kauzman, 1965). Dilatometry was used by Christensen (1952) and by Kauzman (Simpson and Kauzman, 1953) to study the effect of urea on β-lactoglobin and OA,[1] respectively; volume decrements of the order of several hundred milliliters per mole were reported (see Discussion).

The lack of detailed data prompted this dilatometric study of the kinetics and thermodynamics of the volume changes produced by reaction of BSA with urea. A thermodynamic analysis serves to relate the observed volume changes to $(\bar{v}_{23} - \bar{v}_2)$, the difference in the partial molar volume of the protein in the denaturant and that found in water.[2] The unusual dependence of this parameter on urea concentration is explicable in terms of the existence of different populations and species of BSA(d) as a function of the conditions employed (Ferris and Katz, 1966).

Experimental Section

Apparatus. Modified Linderstrøm-Lang–Lanz dilatometers consisting of graduated 10.0-μl capillaries and bifurcated reaction vessels were fabricated (Linderstrøm-Lang and Lanz, 1938). The capillaries' calibration was validated by mercury displacement and by the excellent reproducibility of data. Teflon sheaths, permanently bonded to the capillary's 10/30 male standard taper joint with epoxy resins, eliminated the need for lubricants and the tendency to bind irreversibly upon prolonged use. The 35-ml reaction vessel, simulating an inverted Y, was fitted with a matched 12/30 female standard taper joint.

Recalibrated 1-ml volumetric pipets reproducible to ± 0.001 ml with water were used. The pipets were

* From the Naval Medical Research Institute, Bethesda, Maryland 20014, and Biochemistry Department, West Virginia University Medical Center, Morgantown, West Virginia 26506. *Received April 29, 1966.* This paper was presented in part at the 10th Annual Meeting, Biophysical Society, Boston, Mass., Feb 1966. Research was supported in part by a West Virginia University School of Medicine Institutional research grant and by a West Virginia University Senate research grant.

† Present address: Biochemistry Department, West Virginia University Medical Center.

[1] The abbreviations used were: BSA(h), bovine serum albumin, hydrated; BSA(c) bovine serum albumin, crystalline; BSA(d), bovine serum albumin, denatured; OA, ovalbumin; Hb, hemoglobin; HbCO, carbomonoxyhemoglobin.

[2] The conventional representation for quantities such as $\bar{v}_{12f}$ is $(\partial V/\partial n_1)_{T,P,n_2}$ and $\bar{v}_{12i}$ is $(\partial V/\partial n_1)_{T,P,n_2'}$, where the prime and absence of prime indicate the initial and final states, respectively (MacDougal, 1946). In view of the variety of systems referred to in this report, it was considered advantageous to use our notation to indicate specifically the presence or absence of given solutes in the system and hopefully minimize confusion by this device.

etched at the tip to evaluate errors attributable to surface tension, viscosity, and drainage. Standard solutions and methodology were used to reduce systematic errors. A 1-ml recalibrated microburet was used as a secondary standard. To introduce BSA(c), funnels designed to facilitate rapid weighing and transfer of protein were required in order to achieve the desired precision.

Materials. BSA, lot no. 70011 (Armour Pharmaceutical Co., Kankakee, Ill.), was used almost exclusively. BSA concentrations were determined by drying at 110 ± 5° for 36–48 hr and checked as required spectrophotometrically, $A_{279}^{1\%}$ 6.65. Urea (Mallinckrodt Co.) was recrystallized from 70%, v/v, ethanol at 50°, air dried, heated at 50° for 18 hr, and dried *in vacuo* for 24 hr. Heptane (Matheson Coleman and Bell), boiling range 98–99°, was purified with concentrated sulfuric acid, 10% $KMnO_4$ in 10% KOH, 10% $KMnO_4$ in 10% sulfuric acid, and distilled water, and redistilled. This was equilibrated against water prior to use.

Procedure. Heptane, freshly prepared urea, and protein solutions were degassed prior to use. Into one arm of the reaction vessel 1.000_0 ml of aqueous solution was pipetted and into the other 8.00 ml of urea. Heptane was layered carefully over these solutions and the capillary affixed. The assembly was submerged in a 200-l. water thermostat maintained at 30.0°, regulated to ±0.001° (Katz, 1963). After equilibration, capillary adjustment, and thermal equilibration, mixing was performed cautiously to avoid breaking the manometric column due to thermal effects. Readings were recorded at minute intervals until steady-state values were reached. This required 10–15 min, but readings were continued at less frequent intervals for 1–4 days.

The requirements for mixing BSA(c) with urea solution were more demanding. Stringent precautions were essential to minimize water pickup, a major source of error. To degas the protein, sufficient heptane was added to cover the BSA and the system was evacuated. The remainder of the procedure was conventional. The solution time for BSA(c) in water was 10–45 min; for urea solution 0.25–2 hr was required, depending on the weight of protein.

Thermodynamic Analysis

The mixing processes considered are as follows.

$$\mathrm{P}\ (n_2,\ 1.000_0\ \mathrm{ml}) + \mathrm{H_2O}\ (n_1,\ 8.00\ \mathrm{ml}) \longrightarrow \mathrm{P}\ (n_2,\ 9.00\ \mathrm{ml}) \quad \text{(I)}$$

$$\mathrm{H_2O}\ (n_1,\ 1.000_0\ \mathrm{ml}) + \mathrm{urea}\ (n_3,\ 8.00\ \mathrm{ml}) \longrightarrow \mathrm{urea}\ (n_3,\ 9.00\ \mathrm{ml}) \quad \text{(II)}$$

$$\mathrm{P}\ (n_2,\ 1.000_0\ \mathrm{ml}) + \mathrm{urea}\ (n_3,\ 8.00\ \mathrm{ml}) \longrightarrow \mathrm{P\cdot urea\cdot H_2O}\ (9.00\ \mathrm{ml}) \quad \text{(III)}$$

$$\mathrm{P}\ (n_2,\ c) + \mathrm{H_2O}\ (n_1,\ 8.00\ \mathrm{ml}) \longrightarrow \mathrm{P}\ (n_2,\ 8.00\ \mathrm{ml}) \quad \text{(IV)}$$

$$\mathrm{P}\ (n_2,\ c) + \mathrm{urea}\ (n_3,\ 8.00\ \mathrm{ml}) \longrightarrow \mathrm{P\cdot urea\cdot H_2O}\ (8.00\ \mathrm{ml}) \quad \text{(V)}$$

The symbol n_i represents the number of moles of the following components; water by 1 or I, protein by 2, urea by 3; P is the symbol for protein while the numerical terms indicate the volume in milliliters prior to mixing.

The thermodynamic expression for the volume change produced by mixing an aqueous solution of protein with water, ΔV, process I, is

$$\Delta V = n_1(\bar{v}_{12f} - \bar{v}_{12i}) + n_1(\bar{v}_{12f} - \bar{v}_1^0) + n_2(\bar{v}_{2f} - \bar{v}_{2i}) \quad (1)$$

The symbol $\bar{v}_{i2}$ represents the partial molar volume of substance 1, water, in solution with a given concentration of substance 2, the protein; the superscript 0 indicates the pure substance; while the letters i and f represent the initial and final states of the system.[2]

The corresponding expression for process II, the volume change produced by mixing 1.000_0 ml of water with n_3 moles of urea contained in 8.00 ml of solution, can be represented by the following general thermodynamic equation

$$\Delta V_1 = n_1(\bar{v}_{13f} - \bar{v}_1^0) + n_1(\bar{v}_{13f} - \bar{v}_{13i}) + n_3(\bar{v}_{3f} - \bar{v}_{3i}) \quad (2)$$

The statement for the volume change, ΔV, produced by adding n_2 moles of protein in water to a solution of urea, process III, is as follows.

$$\Delta V = n_1(\bar{v}_{123} - \bar{v}_{12}) + n_1(\bar{v}_{123} - \bar{v}_{13}) + n_3(\bar{v}_{32} - \bar{v}_3) + n_2(\bar{v}_{23} - \bar{v}_2) \quad (3)$$

The following derivation is based on the premise that the mixing of BSA(h) with either water or urea solutions satisfies the criteria for an "athermal" system, namely, that the volume and enthalpy changes produced by mixing these components are negligibly small or zero (Hildebrand and Scott, 1950). Evidence based on experiment will prove that the alteration of the partial molar volumes of these entities due to the introduction of BSA(h) is a second-order effect compared to that produced by dilution of urea with water in these systems. If we accept these propositions, the first three terms on the right side of eq 3 may be replaced with the equivalent expression for the dilution of urea with water, $\Delta V_{(v-\phi)}$, to give

$$\Delta V = \Delta V_{(v-\phi)} + n_2(\bar{v}_{23} - \bar{v}_2) \quad (4)$$

where the term $\Delta V_{(v-\phi)}$ represents the volume change when $(v - \phi)$ ml of water are added to 8.00 ml of urea solution. For this system, the quantity $(v - \phi)$ represents the difference between the volume of protein solution introduced, v or 1.000 ml, minus the protein's displacement volume, ϕ, *i.e.*, the product of the protein weight, g_2, times its partial specific volume, ρ_2, or $\rho_2 g_2$. It should be noted that the value for $\Delta V_{(v-\phi)}$ can be derived from graphical analysis of experimental values or else from an equation derived from quasi-

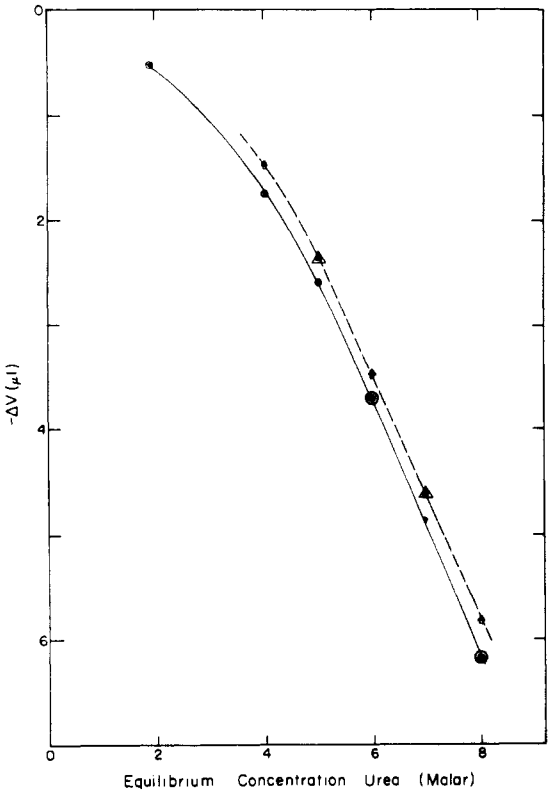

FIGURE 1: The volume changes produced by transferring 1.000 ml of aqueous solution to 8.00 ml of urea solution. Top curve: 9% BSA added to urea, process III; the symbols ▲△ represent duplicate experiments. Bottom curve: water added to urea, process II; the symbols O● represent duplicate experiments. Temperature was 30.0°.

thermodynamic arguments.[3] Thus eq 4 is a statement that the observed volume change, ΔV, represents the sum of the volume changes due to the dilution of urea with water and the contribution due to the change of the protein's partial molar volume from that in a denaturant to that found in water (native state).

The corresponding thermodynamic expressions for transferring crystalline protein to water and to urea solutions are given by eq 5 and 6, respectively.

$$\Delta V_{12} = n_1(\bar{v}_{12} - \bar{v}_1^0) + n_2(\bar{v}_2 - \bar{v}_2^0) \qquad (5)$$

and

$$\Delta V_{123} = n_1(\bar{v}_{132} - \bar{v}_{13}) + n_2(\bar{v}_{23} - \bar{v}_2^0) + n_3(\bar{v}_{32} - \bar{v}_3) \qquad (6)$$

These expressions are self-explanatory. The physical state of these systems after mixing are identical with that of processes I and III after mixing, yet the volume changes and the thermodynamic equations differ sufficiently to permit a critical assessment of the various premises invoked (see Discussion).

Calculation of Potential Error

Since the values for $(\bar{v}_{23} - \bar{v}_2)$ derived from eq 4 represent a small difference between large numbers, the assessment of potential error is essential. The error estimates were made using the theory of propagation of errors (Wilson, 1952).

Errors of 0.03 μl in ΔV and in $\Delta V_{(v-\phi)}$ introduce errors of 13 and 22 ml/mole for the term $(\bar{v}_{23} - \bar{v}_2)$, respectively, while errors of 0.003 in ρ_2 and of 3000 in the selection of M, molecular weight of protein, engender an uncertainty of 1–2 ml/mole. A fluctuation of 0.001° introduces a shift of 0.05 μl in the dilatometric reading. These considerations define the degree of experimental precision required. Each point depicted graphically is the mean of an experiment performed in triplicate, the acceptable range being ≤ 0.03 μl. Any value cited represented the mean of three to six such values with the spread ≤ 0.04 μl.

Results

Mixing of Water with BSA in Water. The addition of 8 ml of water to 1 ml of BSA solution where the initial concentration ranged from 4 to 12% showed no demonstrable volume change, *i.e.*, $\Delta V = 0.0 \pm 0.03$ μl. By referring to eq 1 and by noting that primary variable is the weight of the protein, n_2, it is apparent that in order for ΔV to be zero each of the bracketed terms must be equal to zero. These data demonstrate that, over a wide range of BSA(h) concentrations, 0.5–12%, that the partial molar volume of water in the presence of BSA(h), $\bar{v}_{12}$, and the partial molar volume of BSA(h) in water, $\bar{v}_2$, are concentration independent. An even more striking observation is that the value for $\bar{v}_{12}$, the partial molar volume of water in the presence of BSA(h), is identical with $\bar{v}_1^0$, the partial molar volume of pure water. Evidence that the enthalpy changes in these dilution processes were negligible was demonstrated by the absence of thermal effects upon mixing. Further corroborative evidence that the heat of mixing was a small quantity was derived from enthalpies of dilution calculated from adsorption isotherms of water with BSA and hemoglobin (Dole and McLaren, 1947; Eley and Leslie, 1964). The absence of thermal and volume effects fulfills the criteria for "athermal systems"; this is indicative of an insignificant degree of interaction between these molecular species (Hildebrand and Scott, 1950).

Addition of Water to Urea Solutions. The dilution of urea solutions with water, process II, evokes an immediate large contraction due to the volumetric changes and the sizable endothermic reactions. Steady-state readings are reached 3–10 min after mixing depending on the urea concentration employed. The data are summarized by the lower curve in Figure 1 and in the second column in Table I. The parabolic

[3] This was checked by inserting the partial molar volumes for these constituents (Gucker *et al.*, 1938) and making the requisite calculation.

TABLE I: Volume Change as a Function of Urea Concentration.

Equil Urea Concn (M)	$-\Delta V_1$[a] (μl)	g_2[b] (g/ml)	$-\Delta V_{exp}$[c] (μl)	$(\bar{v}_{23} - \bar{v}_2)$[d] (ml/mole of BSA)
4.0_0	1.74 (±0.01)	0.0900	1.44 (±0.01)	135
5.0_1	2.58 (±0.02)	0.0884	2.35 (±0.02)	45
6.0_0	3.685 (±0.005)	0.0900	3.44 (±0.02)	0
7.0_0	4.87 (±0.03)	0.0900	4.61 (±0.03)	−45
8.0_0	6.18 (±0.02)	0.0903	5.79 (±0.02)	−12

[a] Addition of 1.00_0 ml of water to 8.00 ml of urea solutions; bracketed values indicate the range of data. [b] Weight of BSA. [c] Addition of 1.00_0 ml of BSA solution to similar urea solutions. [d] In these calculations the partial specific volume of BSA(h) was taken at 0.736 (Charlwood, 1957), mol wt 66,000 (Yphantis, 1964); temperature of experiments was 30.0°.

relationship between ΔV_1 and the equilibrium urea concentration can be converted to a linear form by plotting the absolute value of the square root of the volume change, $|\Delta V_1^{1/2}|$, against c_3, the equilibrium urea concentration. The rationale for this conversion follows from inspection of the equation relating the partial molar volume of urea with concentration (Gucker *et al.*, 1938). The values for $\Delta V_{(v-\phi)}$ were obtained from an equation derived from quasi-thermodynamic arguments or from a graphical analysis of data where 0.900, 1.000, and 1.100 ml of water were added to 8.00 ml of urea solutions encompassing concentrations from 4.00 to 8.00 M. The agreement between the two sets of data was within experimental error, ±0.03 μl.

Addition of BSA(h) to Urea Solutions. Since the values of $(\bar{v}_{23} - \bar{v}_2)$, the difference between the partial molar volume of BSA in urea and that value in water, are based on eq 4 it was necessary to design experiments to test the validity of this equation. A series of experiments were performed where the protein content was invariant and the amount of water was the variable and the converse situation where the primary variable was the protein. Exemplifying the first approach 90.7 mg of BSA in volumes of 1.00_0 and 1.10_0 ml, respectively, were mixed with 8.00 ml of 9.00 M urea. The volume changes were −5.71 and −6.30 μl, giving values for $(\bar{v}_{23} - \bar{v}_2)$ of −15 and 0 ml/mole, which are within experimental error. In another experimental set the BSA used ranged from 57.7 to 114.0 mg with the volume of water spanning 0.965–0.915 ml. The pipet used delivered 0.998 ± 0.001 ml of water at 24°. The volume changes ranged from −5.95 to −5.72 μl for these systems. The values for $(\bar{v}_{23} - \bar{v}_2)$ ranged from 14 to −30 ml/mole apparently indicative of a slight dependence on protein concentration or the operation of a systematic error. Proof of a deficiency in pipetting was established by visual inspection, by dry weights, and by weighing of the solutions delivered. A positive error in the volume delivered was found for protein at concentration ≤6% and negative errors at concentration ≥9%. The corrected values for $(\bar{v}_{23} - \bar{v}_2$ were −10 ± 20 ml/mole with no evidence for a protein concentration effect. This constitutes satisfactory agreement with the value of −12.3 ml/mole, standard deviation of 8, representing 14 independent measurements over a 6-month period.

These data confirm the premise that the primary determinant is the amount of water added to the urea, and that the protein's contribution is relatively minor. In the first experiment a 10% variation in water caused a 11.5% variation in ΔV, whereas a 240% variation in protein weight evoked only a 3.4% change in ΔV, with the volume changes mirroring the variation in water content.

The effect of varying the urea concentration is summarized in Figure 1 and Table I. The pH of BSA(h) was in the isoionic range 5.1 ± 0.1 (Katz and Ellinger, 1963); in urea the pH of BSA exhibited a urea concentration effect. In 4.0 M urea the pH was 6.4 ± 0.3 and this function increased monotonically with urea concentration to a value of 6.9 ± 0.3 in 8.0 M urea (Bull *et al.*, 1964).

The kinetics for the volumetric changes in process III were similar to that of process II. The volume readings for systems ≥7 M urea were invariant after mixing, whereas at lower urea concentrations there was a slight decrease in volume with time, ≤0.005 μl/hr. The data reported will be limited to the initial steady-state readings.

In contrast to process II, the data for process III when plotted as $|\Delta V^{1/2}|$ *vs.* c_3, the urea concentration, deviated from linearity when c_3 was ≤5 M urea. The values for $(\bar{v}_{23} - \bar{v}_2)$ exhibited a unique functional dependence on urea concentration. In 4.0 M urea this term was 135 ml/mole of BSA and then this quantity dropped sharply with increasing urea concentration to a value of −45 ml/mole of BSA in 7.0 M urea, an apparent point of inflection since it increased to −12 ml/mole of BSA in 8.0 M urea (Table I). The possibility of systematic errors is minimized by the use of identical pipets, a rigorously standardized technique, and a

standard BSA concentration to reduce drainage errors. Excluding the existence of a systematic error or a deficiency in eq 4 these data suggest that the properties and/or species of BSA(d) differed substantially as a function of the urea concentration (see Discussion for corroborative evidence).

Addition of BSA(c) to Aqueous Solutions. The volume decrements produced by the transport of BSA(c) to either water or to 8.0 M urea, processes IV and V, were a function of BSA weight and moisture content. These data are summarized in Table II. The spread of the data represents errors due to hygroscopic effects, material loss in transfer, in degassing, and incomplete solution. The values for the reduced volume changes, the volume change divided by the BSA weight, $\Delta V/w$, for a given batch of BSA showed no dependence on the BSA concentration. This was proved by experiments where the weight of BSA(c) varied from 65 to 150 mg and the diluent volume from 4 to 8 ml.

TABLE II: ΔV of Solution of BSA(c) in H_2O or in 8.00 M Urea.[a]

BSA Lot No.	HO_2 Content (%)	BSA(c)–H_2O[b] $-\Delta V/w$ (μl/g)	BSA(c)–Urea (8.00 M) $-\Delta V/w$ (μl/g)
A70011	0.5	26.0 (±1.0)	25.8 (±1.0)
A70011	0.4	25.8 (±0.8)	—
A70011	3–4	—	23.1 (±0.2)
A70011	5	21.95 (±1.0)	21.9 (±0.6)
W69312	7.5	12.9 (±1.1)	12.8 (±0.9)

[a] Weight of BSA ranged from 65 to 150 mg, volume of diluent from 4 to 8 ml; temperature was 30.0°. [b] Bracketed values indicate range of data.

The variation of $\Delta V/w$ with moisture content is indicated by the decrease of values for this quantity from -26 μl/g of BSA for BSA(c), with a moisture content of 0.45%, to -22 μl/g for BSA, 5% moisture. The value of -12.9 μl/g found for lot no. 69312 may be due to its high moisture content, 7.5%, or more probably is symptomatic of the changes attendant to its prolonged storage after opening. The value for the volume change for BSA(c), moisture content of 0.45%, when converted from a weight basis to volume basis, was -35 μl/ml of BSA. This differs markedly from the 7.5% decrement reported by Chick and Martin (1913). Since the density of the protein solutions, as determined by these investigators, agrees to within 1% of the accepted values (Charlwood, 1957), it appears that the discrepancy resides either with the protein preparation employed (salts present) and/or with their determination of the density of crystalline protein (possible air entrapment).

The volume changes for processes IV and V were found to be a function of the weight of protein introduced; the values for the reduced volumes for both of these systems proved to be concentration independent. More specifically, the addition of differing volumes of water to systems where the BSA(c) weight was constant gave volume changes which were independent of the volume of water and dependent only on the weight of protein. It is obvious (see eq 5) that the quantity $(\bar{v}_{12} - \bar{v}_1^0)$ is zero, a result in accord with the findings of process I. Other experiments where the volume of water was constant and the protein the variable showed a direct linear relationship between the values for ΔV_{12} with the weight of protein introduced. Therefore, the quantity, $\Delta V_{12}/w$, is a constant. By reference to eq 5 it is apparent that the partial molar volume of BSA(h), $\bar{v}_2$, is concentration independent in agreement with the conclusion derived from process I.

A similar experimental design was used for process V, the addition of BSA(c) to 8.00 M urea. Here the volume changes produced with 8.00 M urea were identical, within experimental error, to the preceding set where the solvent was water (see Table II). The slightly reduced values noted for the urea system can be attributed to about 1–5% of the protein which did not dissolve. The time required for the solution of the protein in this medium was appreciably longer than that required for water, *i.e.*, 15–120 min as contrasted to 5–15 min. Furthermore, while the aqueous systems were homogeneous by visual inspection some of the protein dissolved in 8.00 M urea was present in a transparent gelatinous state. However, the pattern for the solubility of the urea–BSA system simulated that of the water–BSA system since the observed volume change ΔV_{123} exhibited a dependence only on the weight of BSA(c) and was independent of the volume. The analysis of these data indicates that ΔV_{123}, the observed volume change, is a function only of the second term on the right side of eq 6. The cancelation of the first and third terms is indicative of the operation of the Gibbs–Duhem relationship here. Consequently, $\Delta V_{123}/n_2$ is equal to $(\bar{v}_{23} - \bar{v}_2^0)$, and in view of direct linear relationship of this function with the protein weight, then $\bar{v}_{23}$ is independent of the protein concentration in 8.00 M urea. This observation corroborates the similar conclusion derived from the mixing studies of BSA(h) with urea solutions, process III.

In view of these experimental findings, the following relationship can be derived.

$$\frac{\Delta V_{123} - \Delta V_{12}}{n_2} = (\bar{v}_{23} - \bar{v}_2) \qquad (7)$$

This equation provides another independent method for evaluating $(\bar{v}_{23} - \bar{v}_2)$. From the data in Table II a value of 0 ± 45 ml/mole of BSA in 8.0 M urea was

calculated for $(\bar{v}_{23} - \bar{v}_2)$.[4] This is in fair agreement with the corresponding value of −12 ml/mole determined previously, thus providing additional evidence attesting to the validity of eq 4.

Discussion

Since the role of eq 4 is such a key factor in this discussion, a reevaluation of the supporting evidence is warranted. The assumption made was that the volume changes produced by introducing BSA(h) to water and/or urea are second-order effects compared to that produced by the interaction of water with urea or that these systems meet the criteria for "athermal system" (Hildebrand and Scott, 1950). Data supporting this postulate are as follows: (i) the volume changes produced by the dilution of BSA(h) with water are zero; (ii) the partial molar volume of water in the presence of BSA(h), $\bar{v}_{12}$, is identical with pure water, $\bar{v}_1^0$; (iii) the enthalpy change for dilution of BSA(h) is negligible; (iv) the volume changes produced by adding BSA(h) to urea solutions are dependent primarily on the amount of water added and exhibit only a secondary dependence on the amount of protein introduced; (v) the volume changes resulting from the addition of BSA(c) to water and to 8.00 M urea were virtually identical; (vi) the difference between $\bar{v}_{23}$, at any urea concentration, and $\bar{v}_2$ is a small term relative to the value of the molar volume of BSA, 48.6 l.; and (vii) the agreement between the values of $(\bar{v}_{23} - \bar{v}_2)$ determined by two independent means, BSA(h) and BSA(c), support the validity of thermodynamic equations proposed.

The striking variation of $\bar{v}_{23}$ with urea concentration, Table I, has virtually no precedence in the literature. The change in the physical properties produced by the denaturation of BSA by urea as determined by difference spectra (Glazer *et al.*, 1963), optical rotation (Simpson and Kauzman, 1953), and viscosity measurements (Frensdorff *et al.*, 1953) exhibit a sigmoidal functional dependence on the denaturants' concentrations with the parameters being determined by the environmental conditions and also by the observational technique used. These curves differ materially from the sinusoidal relationship found here for $(\bar{v}_{23} - \bar{v}_2)$. The transitional changes observed dilatometrically and by optical rotation occurred immediately after exposure to urea and exhibited very little additional change with the course of time. However, the magnitude and kinetics for the viscosity changes differ from the above and exhibit a distinctive functional dependence on such variables as pH, temperature, urea, and protein concentration (Frensdorff *et al.*, 1953; McKenzie *et al.*, 1963). These data have been interpreted as indicating a transformation from the native globular conformation to a "randomly coiled polymer" for the unfolded and unaggregated molecule. Recently, a more revealing insight into the mechanism for this process was afforded by ultracentrifugal studies by Gutter and associates (1957) which revealed the presence of three components in 3–5 M urea, pH 4.5–5.3, but only one peak at lower and higher pH values.

Substantive evidence for the existence of a multiplicity of reaction routes and products for BSA–urea interaction was established by means of acrylamide gel electrophoresis (Ferris and Katz, 1966). At urea concentrations ≤ 3 M, no changes were observable; however, in 4 M urea there appeared a species of denatured albumin, S_j, consisting of eight to ten subfractions which were characterized by electrophoretic mobilities 5–25% of the parent BSA. With increasing urea concentrations the population of the S_j fractions decreased but this was accompanied by an increase in a second category of denatured albumins, I_k, consisting of six to ten subspecies with electrophoretic mobilities about 20–80% of BSA.

Several reaction mechanisms can be proposed to explain the distributions of populations and species of BSA(d) as a function of urea concentration. Preliminary to any discussion of mechanism, note should be made that most commercial BSA consists of 85–95% monomer, 5–10% dimer, and the remainder *x*-mer (Hartley *et al.*, 1962; Petersen and Foster, 1965), thus permitting the operation of a minimum of three parallel reaction networks. However, the most important set of reactions centers on the monomer in view of its high concentration and since the denaturation rate constants for the dimer and *x*-mer are considerably lower than that of the monomer. Furthermore, since the conversion of monomer to dimer at this pH is negligible, the possibility of competitive reactions from this source may be discounted (Kay and Edsall, 1956). One reaction mechanism can be based on the hypothesis that native BSA consists of a microheterogeneous population of proteins, therefore the spectra of denatured proteins is merely a reflection of this phenomenon (Petersen and Foster, 1965). However, the analysis of the kinetics of these systems does not lend support to the microheterogeneity concept, *per se*. The mechanism advocated here involves the conversion of the BSA monomer *via* an activated complex by a sequence of parallel and sequential reactions to any of several major species of BSA(d) depending on the conditions employed.

It may be concluded from electrophoretic, dilatometric, and ultracentrifugal data (Gutter *et al.*, 1957) that the species which predominate at 4 M urea has a molecular structure which differs considerably from the species existing at higher urea concentrations. Thus the partial molar volume determined at a given urea concentration represents the mean value of the population of the denatured protein(s) present in the system at the time of measurement.

The volume increase observed for BSA upon its transfer from water to 4 M urea can be explained by several

[4] The value for $(\bar{v}_{23} - \bar{v}_2)$ calculated from the data in Table II is about 6 ml/mole. The slightly more positive values for the urea systems reflect a systematic error due to the incomplete dissolution of BSA(c), 1–5% undissolved in the urea solutions, resulting in an apparent positive value.

possible mechanisms. One hypothesis, based on light-scattering studies, postulates that there is an isotropic expansion of BSA due to the preferential imbibition of urea by BSA (Kay and Edsall, 1956). Another explanation is that in urea solutions there is a diminution of the protein's intramolecular hydrophobic and electrostatic bonding energies thereby causing a decrease of structural integrity (Nozaki and Tanford, 1963). Regardless of the mechanism, the expansion of BSA in dilute urea is consistent with the small rise in reduced viscosity found in 2–5 M urea and with the considerable reduction in mobility in acrylamide gel electrophoresis. The small change in viscosity is compatible with maintenance of the protein's globular structure and a consequent minor increase in frictional resistance. The drastic reduction in electrophoretic mobility indicates that "electrophoretic diameter" of this species of BAS(d) has increased to that of the gel pore thus resulting in a marked molecular sieving effect. The reduction in electrophoretic mobility is too large to be ascribed to the change in pK values for the protein's prototropic groups upon transfer from water to urea solutions since these values are not altered appreciably by this medium effect (Wu and Dimler, 1963; Bull *et al.*, 1964).

At higher urea concentrations the resultant decrease in helical structure becomes the dominant factor since a helix–coil transition causes a volume decrease of -0.5 to -1.0 ml/amino acid residue (Noguchi and Yang, 1953). Consequently BSA with a 50% helical content and consisting of 570 residues (Davies, 1965) could undergo a contraction of -140 to -280 ml/mole providing urea denaturation were to cause a complete loss of helical character. Callaghan and Martin (1962) on the basis of ORD studies state that there is 12% reduction of helical content for BSA upon exposure to 4 M urea with the major loss in helicity occurring at higher urea concentrations. Thus the volume decreases found for ($\bar{v}_{23} - \bar{v}_2$) parallels this helical–coil transition. Other factors which can contribute to this volume decrease are intrinsic in the rearrangement of a globular molecule to a more random structure thus exposing more surface with the consequent transfer of apolar and some polar residues from essentially a hydrophobic to a hydrophilic millieu (Perutz, 1965). The effects should resemble that produced by the transport of either hydrocarbons or polar molecules from a non-aqueous medium to water, a decrease of 10–20 ml/mole (Masterton, 1954; Cohn and Edsall, 1943; Linderstrøm-Lang and Jacobsen, 1941). The net volume change represents the summation of all of these factors but the dominant factor for this stage is the helix–coil transition.

The change in $\bar{v}_{23}$ with urea concentration has certain semblance to changes which occur to the activation volume, ΔV^*, upon thermal and/or pressure denaturation. The value for ΔV^* for heat denaturation of OA to 70° decreased from 24 to -38 ml/mole as the pressure increased from 0 to 5000 kg/cm^2 (Suzuki, 1960), while isobaric experiments, at 4500 kg/cm^2, gave values which were initially more negative than the preceding set; here the values for ΔV^* decreased from -92 to -38 ml/mole as the temperature increased from 10 to 70°. Similar results were obtained for HbCO. The author postulated the existence of different mechanisms and products as a function of the experimental conditions employed.

Evidence supporting this hypothesis, but also demonstrative of the difficulty at arriving at any generalization, is the wide variations in the values for the volume changes produced by thermal denaturation. The data appear to vary with the type of protein, experimental conditions, and also with the technique employed. Heymann (1936) denatured BSA, OA, and serum globulin at 80° and found volume increases of 200 ml/100,000 g of protein by dilatometry. However, more recent dilatometric studies of the denaturation of ribonuclease (RNAase) at 45°, pH 2.8, gave values of -240 ± 100 ml/mole (Holcomb and Van Holde, 1962). Yet Gill and Glogovsky (1965) using a similar system but employing pressures up to 1400 atm found volume change of -30 ± 10 ml/mole. The latter authors proposed that the thermal denaturation of RNAase proceeds *via* a "r step" sequence and the techniques employed measured different stages of the process. To reconcile the negative values for RNAase with Heymann's positive results for globular proteins one must assume either grossly different macromolecular organization or that the denaturation mechanism operational at 80°, at isoelectric pH, differs considerably from that at 45°, pH 2.8. Additional evidence lending support for the multiplicity theorem for protein denaturation are the time-dependent positive and negative volume changes observed when OA, BSA, and chymotrypsin were exposed to pH extremes (Rasper and Kauzman, 1962).

Christensen (1952) investigating the denaturation of β-lactoglobulin by urea reported a contraction of 600 ml/mole as determined dilatometrically. In contrast to our results a pronounced time dependence prevailed with only 43% of the reaction completed in 13 hr; 8 days was required for a steady-state value. Conceivably the changes can be due to the dissociation of β-lactoglobulin from a tetramer to a monomer (Timasheff and Townend, 1964) and the randomization of the secondary structure which is largely pleated sheet or β structure in contrast to the α-helical structure characteristic for BSA (Timasheff and Susi, 1966).

In view of the preceding there seems to be substantial basis for the hypothesis that the denaturation of BSA by urea proceeds *via* several reaction routes generating a minimum of two major species of BSA(d) each of which are composed of several subspecies. The mechanism(s) and distribution of species are dependent on the conditions employed (Poland and Scheraga, 1965; Ferris and Katz, 1966).

Acknowledgments

We wish to thank Professor Kauzman for reading and suggesting some changes in this manuscript and to acknowledge our indebtedness to Mr. R. High and

Mr. T. Kilduff of the Harry Diamond Laboratories, Washington, D. C.; to Mr. G. Pearse and Mr. Hugh Stupp, Machinist Shop, National Naval Medical Center; and to Mr. Hoye Walls, West Virginia University Medical Center Instrumentation Unit, for advice relevant to the design, fabrication, and bonding of the Teflon sleeves. The design and the construction of the basic electronic components for the thermostat was by Mr. M. Eicher and Mr. T. Connors, Electronic Labs, Naval Medical Research Institute. Additional circuitry was fabricated by Mr. L. McIntyre and Mr. James McCune, West Virginia University Medical Center Instrumentation Unit.

References

Bull, H. B., Breese, K., Ferguson, G. L., and Swenson, C. A. (1964), *Arch. Biochem. Biophys. 104*, 297.

Callaghan, P., and Martin, N. H. (1962), *Biochem. J. 83*, 144.

Charlwood, P. A. (1957), *J. Am. Chem. Soc. 79*, 776.

Chick, H., and Martin, C. J. (1913), *Biochem. J. 7*, 92.

Christensen, K. L. (1952), *Compt. Rend. Trav. Lab. Carlsberg 28*, 37.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, pp 156, 370.

Davies, D. R. (1965), *Progr. Biophys. Mol. Biol. 15*, 189.

Dole, M., and McLaren, A. D. (1947), *J. Am. Chem. Soc. 69*, 651.

Eley, D. D., and Leslie, R. B. (1964), *in* The Structure and Properties of Biomolecules and Biological System, Duchesne, J., Ed., New York, N. Y., Interscience, p 238.

Ferris, T. G., and Katz, S. (1966), *Nature 211*, 586.

Frensdorff, H. K., Watson, M. T., and Kauzman, W. (1953), *J. Am. Chem. Soc. 75*, 5157.

Gill, S. J., and Glogovsky, R. L. (1965), *J. Phys. Chem. 69*, 1515.

Glazer, A. M., McKenzie, H. A., and Wake, R. G. (1963), *Biochim. Biophys. Acta 69*, 240.

Gucker, F. T., Gage, F. W., and Moser, C. G. (1938), *J. Am. Chem. Soc. 60*, 2582.

Gutter, F. J., Peterson, E. A., and Sober, H. A. (1957), *Arch. Biochem. Biophys. 72*, 194.

Hartley, R. W., Jr., Peterson, E. A., and Sober, H. A. (1962), *Biochemistry 74*, 3087.

Heymann, E. (1936), *Biochem. J.* 30, 127.

Hildebrand, J. H., and Scott, R. R. (1950), The Solubility of Nonelectrolytes, 3rd ed, New York, N. Y., Reinhold, pp 107, 135.

Holcomb, D. M., and Van Holde, K. G. (1962), *J. Phys. Chem. 66*, 1999.

Katz, S. (1963), *J. Chem. Educ.*, 40, 266.

Katz, S., and Ellinger, F. (1963), *Biochemistry 2*, 406.

Kauzman, W. (1959), *Advan. Protein Chem. 14*, 1.

Kay, C. M., and Edsall, J. T. (1956), *Arch. Biochem. Biophys. 65*, 354.

Krausz, L. M., and Kauzman, W. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1234.

Linderstrøm-Lang, K., and Jacobsen, C. F. (1941), *Compt. Rend. Trav. Lab. Carlsberg 24*, 1.

Linderstrøm-Lang, K., and Lanz, H. (1938), *Compt. Rend. Trav. Lab. Carlsberg 21*, 315.

MacDougal, F. H. (1946), Thermodynamics and Chemistry, 3rd ed, New York, N. Y., Wiley, p 26.

Masterton, W. L. (1954), *J. Chem. Phys. 22*, 1830.

McKenzie, H. A., Smith, M. B., and Wake, R. G. (1963), *Biochim. Biophys. Acta 69*, 222.

Noguchi, H., and Yang, J. T. (1963), *Biopolymers 1*, 359.

Nozaki, Y., and Tanford, C. (1963), *J. Biol. Chem. 238*, 4074.

Perutz, M. F. (1965), *J. Mol. Biol. 13*, 1965.

Peterson, H. A., and Foster, J. F. (1965), *J. Biol. Chem. 240*, 2503.

Poland, D. C., and Scheraga, H. A. (1965), *Biopolymers 3*, 401.

Rasper, J., and Kauzman, W. (1962), *J. Am. Chem. Soc. 84*, 1771.

Reithel, F. J., and Sakura, J. D. (1963), *J. Phys. Chem. 67*, 2497.

Simpson, R. B., and Kauzman, W. (1953), *J. Am. Chem. Soc. 75*, 5139.

Suzuki, K. (1960), *Rev. Phys. Chem. Japan, 29*, 91.

Timasheff, S. N., and Susi, H. (1966), *J. Biol. Chem. 241*, 249.

Timasheff, S. N., and Townend, R. (1964), *Nature 203*, 517.

Wilson, E. B. (1952), Introduction to Scientific Research, New York, N. Y., McGraw-Hill, p 272.

Wu, Y. V., and Dimler, R. J. (1963), *Arch. Biochem. Biophys. 103*, 310.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.