Partial Specific Volumes and Interactions with Solvent Components of Proteins in Guanidine Hydrochloride†

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ABSTRACT: The partial specific volumes of twelve proteins were determined by density measurements. For these proteins in their native state, the determined values of partial specific volumes are generally in good agreement with the accepted literature values except in the case of bovine α-lactalbumin. The determined value of 0.704 is similar to that found for lysozyme. Preferential interaction parameters of 6 M guanidine hydrochloride with these proteins were also measured. For the twelve proteins studied, the preferential interaction with solvent components varies between 0 and 0.17 g of guanidine hydrochloride per g of protein. In no case is interaction preferential with water. The total binding of denaturant to protein is calculated and the correlation between the observed and expected number of denaturant molecules bound is found to be good, if peptide bonds and aromatic side chains are taken as the binding sites. The changes in volume upon transfer from dilute salt to 6 M guanidine hydrochloride of the proteins studied are calculated and compared with theoretical values reported in the literature.

Although an exact knowledge of the partial specific volume, $\bar{v}$, of a protein is essential for the determination of molecular weights from ultracentrifuge data and small-angle X-ray scattering, in the past this parameter was seldom measured. The molecular weights were calculated from values of $\bar{v}$ assumed, or calculated from amino acid composition. And yet the importance of accurate measurements of the partial specific volume was generally recognized, since a small error in that parameter is multiplied several fold in the calculation of the molecular weight, in particular when measurements are carried out in concentrated solutions of denaturant, such as 6 M Gdn·HCl,[1] a frequent practice in studies of subunit systems. Uncertainties in estimates of molecular weight can lead to wrong conclusions about the number of subunits in the native macromolecular assembly and to serious errors in the calculation of the thermodynamic parameters of associating systems. A classical example is the uncertainty which prevailed for several years about the exact number of polypeptide chains in rabbit muscle aldolase (Kawahara and Tanford, 1966; Schachman and Edelstein, 1966; Castellino and Barker, 1968; Reisler and Eisenberg, 1969; Meighen and Schachman, 1970).

Until recently, the methods available for the measurement of the partial specific volume either required frequently prohibitive amounts of material, as in conventional pycnometry, or involved long complicated procedures, as in the density gradient column technique (Linderstrøm-Lang and Lanz, 1935; Hvidt *et al.*, 1954; Reithel and Sakura, 1963). Recently, several new and elegant approaches to the measurement of $\bar{v}$, requiring small amounts of material, have been described. These include the H_2O–D_2O method of Edelstein and Schachman (1967), the magnetic float method of Ulrich *et al.* (1964), and the precision densimeter, based on the frequency of vibration of a "tuning fork," consisting of a sample-filled quartz tube, described by Stabinger *et al.* (1967). The commercial availability of the last two instruments and their ease of operation have made it possible to approach now in a systematic manner the measurement of partial specific volumes of proteins and other biological macromolecules in solvents of various compositions. Such a study has been initiated in our laboratory, and the purpose of this paper is to describe the results obtained with a number of proteins in their native state and denatured in 6 M Gdn·HCl.

Gdn·HCl is a well-characterized strong denaturant for proteins and has been frequently applied as the subunit dissociating agent in complex systems. The conformational and thermodynamic aspects of the denaturing action of Gdn·HCl have been the subject of extensive studies, and the present state of knowledge has been summarized recently (Tanford, 1968, 1970). One of the thermodynamic parameters which can be expected to reflect denaturation is the partial specific volume. Denaturation may be reflected in the value of $\bar{v}$ in two ways: first, if the volume of the protein changes on denaturation; second, if the protein undergoes interactions with solvent components. By proper combination of the partial specific volumes measured under various thermodynamic conditions, it is possible to obtain the values of the preferential solvent-binding parameter and of the change in volume involved during protein denaturation. In this paper, these quantities will be reported for a number of proteins.

Experimental Procedures

Materials

Extreme purity grade Gdn·HCl from Heico, Inc., was used without further purification, after filtration through a sintered-glass filter. The ultraviolet (uv) absorption of the solution was measured against deionized water with a Cary 14 spectrophotometer. Solutions of 6 M Gdn·HCl with less than 0.05 *A* at 240 nm and no absorbance from 260 to 400 nm were used. Iodoacetate and 2-mercaptoethanol were obtained from Eastman Kodak Co. All other chemicals were reagent grade and were used without further purification. Ribonuclease (type IIA, lot 107B-1290), bovine serum albumin (lot 56B-1290), and catalase (lot 125B-8500) were purchased from Sigma Chem-

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; PMG, buffer consists of 10^{-2} M sodium phosphate, 10^{-4} M GTP, and 5×10^{-3} M $MgCl_2$ at pH 7.0.

icals, Inc. Egg-white lysozyme (LYSF 647-8), chymotrypsinogen A (CG 763), carboxypeptidase A (COA 1 LC), and lima bean trypsin inhibitor (LBI 2 DA) were purchased from Worthington Biochemical Corp. α-Lactalbumin (lot W3730) was obtained from Schwarz/Mann. The samples of beef heart lactate dehydrogenase, α-chymotrypsin, and calf brain tubulin were the same as those used in other studies from this laboratory[2] (Fosmire and Timasheff, 1972; Lee *et al.*, 1973). β-Lactoglobulin A was prepared according to the method of Aschaffenburg and Drewry (1957).

Methods

Solvents Used in the Determination of Native Proteins. The partial specific volumes of the proteins in their native state were determined in the following solvents—RNase A, egg-white lysozyme, chymotrypsinogen A, α-chymotrypsin, and β-lactoglobulin A: 10^{-3} M HCl–0.1 M KCl (pH 3.0); calf brain microtubule protein (Tubulin): 0.1 M NaCl–PMG (pH 7.0); bovine serum albumin: 0.2 M NaCl (pH 7.0); carboxypeptidase A: 1.0 M NaCl–10^{-2} M potassium phosphate (pH 7.0); beef heart lactate dehydrogenase, α-lactalbumin, and catalase: 0.1 M NaCl–10^{-2} M potassium phosphate (pH 7.0); lima bean trypsin inhibitor: 10^{-2} M sodium phosphate (pH 7.0).

Preparation of Protein Solutions in 6 M Gdn·HCl. The apparent partial specific volumes of the proteins in 6 M Gdn·HCl (pH 7.1) were measured under conditions at which the chemical potential and the molality of solvent components were, in turn, kept identical in the reference solvent and in the protein solution. For measurements at constant molality of Gdn·HCl, the protein was exhaustively dialyzed against deionized distilled water; it was then freeze-dried and further dried under vacuum at 40° in the presence of phosphorus pentoxide for 24–48 hr. If the dried protein was soluble in 6 M Gdn·HCl, it was weighed into dry, tared test tubes, in aliquots covering a range of 5–20 mg of protein. To each tube 1.0 ml of solvent was added and the tubes were sealed quickly with Parafilm. Bovine serum albumin, RNase, α-chymotrypsin, β-lactoglobulin A, lima bean trypsin inhibitor, lysozyme, chymotrypsinogen A, and α-lactalbumin belonged to this group of proteins. If the dried protein was not soluble, as was true of tubulin, lactate dehydrogenase, catalase, and carboxypeptidase A, then, prior to freeze-drying, it was reduced with 2-mercaptoethanol in 6 M Gdn·HCl and then S-carboxymethylated with iodoacetate (Crestfield *et al.*, 1963). The reduced and S-carboxymethylated protein was then dialyzed against deionized distilled water, and dried, as described above. The protein solutions were then prepared in the same manner as above. Protein solutions for $\bar{v}$ measurements at constant chemical potential conditions were prepared by dialysis against 6 M Gdn·HCl for 4–5 days at 25°.

Protein Concentration Determination. The concentrations of native proteins were determined by measuring the absorbance of an aliquot which had been gravimetrically diluted with diffusate. For those proteins whose extinction coefficients were not known, the concentrations were determined by dry weight measurements. The values of the absorbances used were: ribonuclease A, 7.38 dl/(g cm) at 278 nm (Scott and Scheraga, 1963); lysozyme, 26.35 dl/(g cm) at 281.5 nm (Sophianopoulos *et al.*, 1962); chymotrypsinogen A, 19.7 dl/(g cm) at 282 nm (Jackson and Brandts, 1970); α-chymotrypsin, 20.3 dl/(g cm) at 280 nm (Aune and Timasheff, 1971); bovine serum albumin, 6.58 dl/(g cm) at 278 nm (Noelken and Timasheff, 1967); β-lactoglobulin, 9.6 dl/(g cm) at 278 nm (Townend *et al.*, 1960); carboxypeptidase A, 18.8 dl/(g cm) at 278 nm (Barzetzi *et al.*, 1963). The extinction coefficients of these protein solutions in 6 M Gdn·HCl were determined by uv spectroscopy. Aliquots of native protein stock solutions, 10–20 mg/ml in concentration, were diluted volumetrically to identical extents with the low salt buffers and with 6 M Gdn·HCl (pH 7.1) solvent. The uv spectra of these dilute solutions were recorded with a Cary 14 spectrophotometer 3–5 hr later. Knowledge of the extinction coefficients of the proteins in their native states and of the absorbance ratios, $A_{\text{native}}/A_{\text{denatured}}$, resulted in the extinction coefficients of these proteins in 6 M Gdn·HCl. The values of the extinction coefficients obtained are: ribonuclease A, 6.8 dl/(g cm) at 276 nm; lysozyme, 25.7 dl/(g cm) at 280 nm; chymotrypsinogen A, 17.3 dl/(g cm) at 280 nm; α-chymotrypsin, 18.7 dl/(g cm) at 280 nm; β-lactoglobulin A, 9.6 dl/(g cm) at 276 nm; lima bean trypsin inhibitor, 2.9 dl/(g cm) at 280 nm; α-lactalbumin, 9.4 dl/(g cm) at 276 nm.

The extinction coefficients in 6 M Gdn·HCl of the other proteins, as well as the reduced and S-carboxymethylated ones, were determined from dry weight measurements. These proteins were dialyzed exhaustively against deionized distilled water, freeze-dried, and further dried at 40° over phosphorus pentoxide in a vacuum oven for 24–48 hr. The dried protein was weighed into a predried, tared volumetric flask, 6 M Gdn·HCl solution was added gravimetrically, and the spectrum of the resulting protein solution was recorded. A combination of the protein concentration and absorbance yielded the extinction coefficient of the protein in 6 M Gdn·HCl. The values of extinction coefficient thus obtained are: beef heart lactate dehydrogenase, 11.0 dl/(g cm) at 276 nm; tubulin, 11.5 dl/(g cm) at 274 nm; catalase, 14.0 dl/(g cm) at 274 nm; carboxypeptidase A, 18.0 dl/(g cm) at 278 nm.

In the cases when the concentration was determined by uv absorbance, the absorption spectrum from 240 to 400 nm was recorded on a Cary 14 spectrophotometer. The light-scattering contribution to the absorbance was corrected for by the method of Leach and Scheraga (1960). In this procedure, extreme care must be exercised in the extrapolation of the log (absorbance) *vs.* log (wavelength) plots. If the contribution of light scattering was so great that the point at 310 nm fell on the extrapolated line from 380 to 320 nm, that particular sample was omitted, since this was regarded as giving too great an uncertainty in the concentration determination.

Density Measurements. The densities of the solvents and the protein solutions, a series of concentrations for each protein, were measured with a Precision Density Meter DMA-02 (Anton Paar, Gratz). The principle of the measurement is the variation of the natural frequency of a hollow oscillator when filled with liquids of different density and, thus, mass. Introduction of each liquid of different density changes the natural frequency or the reciprocal of frequency, the period, of the oscillator. In actual measurements, the time lapse, T, during a pre-set number of periods is measured by a crystal controlled timer.

The density of an unknown liquid is measured by reference to a known standard. The difference between the densities of two samples is given by

$$\rho_1 - \rho_2 = (1/A)(T_1^2 - T_2^2) \quad (1)$$

The constant A is an instrument constant and is obtained from calibration measurements with samples of known density. In the present studies, the standard solutions used for calibration

[2] M. J. Gorbunoff, G. J. Fosmire, and S. N. Timasheff (1974), manuscript in preparation.

were Spectroquality grade acetone, methanol, and absolute ethanol, as well as sucrose solutions (International Critical Tables, 1928). The instrument constant, A, did not change within a testing period of 6 months. All measurements were made at 20° with the cell compartment maintained at this temperature (±0.02°) with a refrigerated and heated circulating bath from Forma Scientific.

The general procedures used for density measurements were as follows. The instrument was warmed up and stabilized using deionized distilled water as standard. It was deemed ready only when at least three consecutive samples of water gave readings within $\pm 2 \times 10^{-5}$ sec at a preset count of 1×10^4. Once the instrument was stabilized, the cell was washed with five aliquots of 5 ml each of deionized distilled water, followed by three aliquots of 2 ml each of absolute ethanol. The cell was then air-dried with the pump on the instrument, a dry cell being indicated by a constant reading on the instrument. Then, the density of at least three solvent samples was measured, and any reading which varied by more than $\pm 5 \times 10^{-5}$ sec was discarded. For each individual sample the precision was $\pm 2 \times 10^{-5}$ sec. Each experiment consisted of measurements on four to five protein solutions within the concentration range of 3–20 mg/ml. After the measurement for each protein sample, the cell was washed and dried as described above. It is of particular importance that the readings for air before and after each sample should be similar. A deviation of $>20 \times 10^{-5}$ sec indicates the presence of impurities in the cell, and the washing and drying procedures must be repeated. Upon completion of the experiments on the protein samples, the instrument constant was rechecked, using deionized distilled water as standard.

Preparation of Solutions. Since all solutions must be free of large particles, the solvent was clarified by filtering through a sintered-glass filter before dissolving protein or using it for dialysis. Protein solutions for measurement at constant chemical potential were clarified, if necesssary, by filtering through Millipore filters (type LS, pore size 5.0 μm) before transferring into dialysis bags, and dialyzed against clean solvent. This permitted one to avoid excessive handling and possible evaporation after the solutions had reached constant chemical potential. Protein solutions prepared for measurements at constant molality were not filtrated. Large particles, if present in these solutions, were allowed to settle down before transferring to the density meter.

Care was taken to avoid evaporation, especially of the Gdn·HCl solutions, during dialysis and transfer of protein solutions from the dialysis system to the density meter. Generally the dialysis system was well sealed with Parafilm until the samples were ready for measurements. Just before measurement, the protein solutions in their dialysis bags were retrieved individually from the dialysis system with a stainless steel tricep. A sterile disposable 1-ml syringe with needle was used to transfer the solution from the dialysis bag to the density meter. The needle facilitated the transfer by minimizing the exposure of the solution to air. Following the direct transfer of the solution from the bag to the syringe, the needle was replaced by a female Luer adapter, which permitted easy injection of the solution into the cell. In all experiments involving Gdn·HCl the concentration of Gdn·HCl was nominal, its exact concentration being determined from the density of the final solvent in the diffusate.

The experimental results obtained from each measurement were the densities of the solvent and of the protein solution at a given concentration. The apparent partial specific volume, ϕ, was calculated with the following equation (Schachman, 1957; Kielly and Harrington, 1960; Cassasa and Eisenberg, 1961, 1964)

$$\phi = (1/\rho_0)\{1 - [(\rho - \rho_0)/c]\} \quad (2)$$

where c is the concentration of protein in grams per milliliter and ρ and ρ_0 are the densities of the solution and solvent, respectively, in grams per milliliter. The calculated values of ϕ were then plotted as a function of protein concentration, and the extrapolated value was taken as the partial specific volume, $\bar{v}_2^0$.

In density measurements, when the molal concentrations of diffusible components are kept identical in the solvent and solution, an apparent partial specific volume, ϕ_{m_3}, is obtained (Cassasa and Eisenberg, 1961, 1964); its extrapolated value to infinite dilution of protein gives the partial specific volume of the protein, $\phi_{2,m_3}^* = [\partial V/\partial g_2]_{T,m_3}$, in the particular medium. On the other hand, when it is the chemical potential of the added diffusible component (*e.g.*, Gdn·HCl) which is kept constant between solution and reference solvent (operationally this is accomplished by dialysis), the resulting apparent partial specific volume is ϕ_{μ_3} (Cassasa and Eisenberg, 1961, 1964); its extrapolated value to infinite dilution of the protein in chemical equilibrium with solvent is $\phi_{2,\mu_3}' = [\partial V/\partial g_2]_{T,\mu_3}$. Here V is the total volume of the solution, g_i is the concentration of component i in grams per gram of principal solvent, water, and μ_i is the chemical potential of component i. Following the notation of Scatchard (1946) and Stockmayer (1950), components 1, 2, and 3 are, respectively, water, protein, and added diffusible material.

From density measurements carried out both at constant chemical potential and constant composition of solvent components, it becomes possible to determine the extent of preferential interaction $[\partial g_3/\partial g_2]_{T,\mu_1,\mu_3} = \xi_3$ of the solvent components with the macromolecule, since (Cohen and Eisenberg, 1968)

$$[\partial g_3/\partial g_2]_{T,\mu_1,\mu_3} = \{[\partial \rho/\partial g_2]_{T,\mu_1,\mu_3} - [\partial \rho/\partial g_2]_{T,p,m_3}\}/[\partial \rho/\partial g_3]_{T,p,m_2} \quad (3)$$

From the definition of the partial specific volume, at infinite dilution, we may write

$$(1 - \phi_2'\rho_0)^0 = (1 - \phi_2^*\rho_0)^0 + \xi_3(1 - \bar{v}_3\rho_0) \quad (4)$$

The superscript 0 indicates infinite dilution of the macromolecular species. In practice, it is frequently found that the apparent partial specific volumes are independent of protein concentration, making it possible to equate ϕ with ϕ_2^* at the given conditions.

Density measurements can also be used to calculate the change in volume, ΔV, involved in transferring a native protein to a denaturing environment, since

$$\Delta V = M_2[\phi_2^* - \bar{v}_2] \quad (5)$$

where M_2 is the molecular weight of the protein.

When measured in this way, ΔV contains not only the change in the volume of the protein itself upon unfolding during denaturation, but also contributions from all other volume changes which occur in the system, such as differences in electrostriction in the two media, differences between the changes of volume of solvent components when they interact with protein, and ionization of buried groups.

TABLE I: Partial Specific Volumes and Preferential Interaction Parameters of Proteins.[a]

	$\bar{v}_2^0$ (native)[b]	$\phi_{2,m_3}^* = \phi_{m_3,c\to 0}$[b]	$\phi_{2,\mu_3}' = \phi_{\mu_3,c\to 0}$[b]	ξ_3 (from eq 4)[c]	$\{\partial m_3/\partial m_2\}_{\mu_1,\mu_3}$[d]	M_2
RNase A[e]	0.696 ± 0.001 (0.695)[f]	0.694 ± 0.001	0.694 ± 0.001	0.0 ± 0.01 (0.0)[g]	0	13,700
Lysozyme[e]	0.702 ± 0.001 (0.703)[h]	0.704 ± 0.002	0.694 ± 0.001	0.09 ± 0.02 (0.089)[g]	14 ± 3	14,300
Tubulin[i]	0.736 ± 0.001	0.736 ± 0.002	0.725 ± 0.002	0.10 ± 0.02 (0.10)[j]	55 ± 11	54,000
Chymotrypsinogen A	0.733 ± 0.001 (0.734)[k]	0.729 ± 0.001	0.712 ± 0.002	0.15 ± 0.02	41 ± 5	25,700
α-Chymotrypsin	0.738 ± 0.001 (0.736)[l]	0.732 ± 0.001	0.713 ± 0.002	0.17 ± 0.03	44 ± 7	25,000
Bovine serum albumin[e]	0.735 ± 0.002 (0.734)[m]	0.724 ± 0.001	0.717 ± 0.001	0.06 ± 0.01 (0.064)[g]	44 ± 7	68,000
Carboxypeptidase A[i]	0.748 ± 0.001	0.741 ± 0.002	0.735 ± 0.001	0.05 ± 0.01	20 ± 4	34,600
Lactate dehydrogenase (BH)[e,i]	0.741 ± 0.001	0.739 ± 0.001	0.736 ± 0.002	0.03 ± 0.01 (0.04)[n]	10 ± 3	36,000
Catalase[e,i]	0.730 ± 0.001	0.726 ± 0.001	0.725 ± 0.002	0.01 ± 0.01	6 ± 6	60,000
β-Lactoglobulin[e]	0.750 ± 0.002 (0.751)[o]	0.728 ± 0.002	0.719 ± 0.001	0.08 ± 0.02 (0.090)[g]	16 ± 4	18,400
Lima bean trypsin inhibitor	0.699 ± 0.001	0.699 ± 0.001	0.698 ± 0.003	0.01 ± 0.04	1 ± 4	9,000
α-Lactalbumin	0.704 ± 0.001 (0.729)[p]	0.701 ± 0.001	0.698 ± 0.002	0.03 ± 0.02	5 ± 3	14,300

[a] The values in parentheses are taken from the literature. [b] Values given in ml/g. [c] Values given in g/g. [d] Values given in mole/mole. [e] Proteins which show concentration dependence in ϕ_{2,m_3}^* measurements. [f] Ulrich *et al.* (1964). [g] Hade and Tanford (1967). [h] Sophianopoulos *et al.* (1962). [i] Proteins which are reduced and S-carboxymethylated for $\bar{v}$ measurements in 6 M Gdn·HCl. [j] Lee *et al.* (1973). [k] Skerjanc *et al.* (1970). [l] Schwert and Kaufman (1951). [m] Reisler and Eisenberg (1969). [n] Appella and Markert (1961). [o] Pedersen (1936). [p] Gordon and Ziegler (1955).

Results and Discussion

Partial Specific Volumes. Figure 1 presents results of density measurements and the calculation of the partial specific volumes of β-lactoglobulin A and tubulin. The apparent partial specific volume is plotted as a function of protein concentration, giving ϕ_2^* as the intercept. Partial specific volumes could be measured with a precision of 0.001–0.002. The factors which contribute to the uncertainty in $\bar{v}$ measurements are temperature fluctuation in the cell compartment and the precisions of the density and protein concentration determinations. A temperature change of 0.01° should result in a 2×10^{-6} g/ml change in density, which in turn corresponds to an uncertainty of ±0.0003 in the $\bar{v}$ measurement. A 3% uncertainty in protein concentration determinations introduces a 1% uncertainty in the $\bar{v}$ value. In density measurements, the time lapse, T, can be measured with a precision of $\pm 2 \times 10^{-5}$ sec. This corresponds to a precision of $\pm 4 \times 10^{-6}$ g/ml in density measurements, which represents an uncertainty of ±0.0006 in the $\bar{v}$ values. Thus, the uncertainty in the determination of $\bar{v}$ under the present experimental conditions could be not greater than ±0.003.

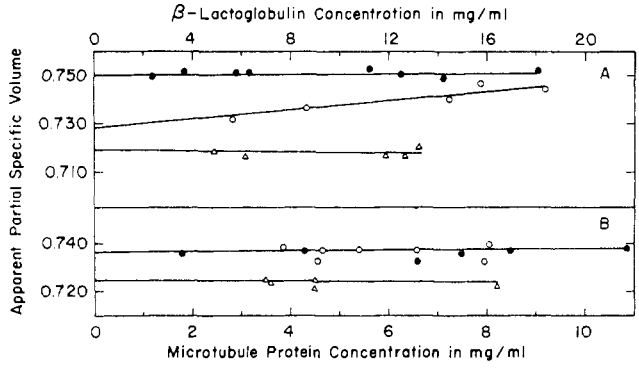

FIGURE 1: Relationships between apparent partial specific volumes and protein concentrations. (A) β-Lactoglobulin A in 0.1 M KCl–10^{-3} M HCl (pH 3.0) (●), in 6 M Gdn·HCl at constant molality (○), and in 6 M Gdn·HCl at constant chemical potential (△). (B) Calf brain microtubule protein in PMG–0.1 M NaCl (●), in 6 M Gdn·HCl at constant molality (○) and in 6 M Gdn·HCl at constant chemical potential (△). (On figures, Gdn·HCl is noted as GuHCl.)

The partial specific volumes of twelve proteins were determined in this manner in their native and denatured states. In all cases, there was little or no protein concentration dependence of the apparent partial specific volume in the native states and in Gdn·HCl at constant chemical potential. For some proteins, however, a definite concentration dependence of the apparent partial specific volumes was observed in measurements in Gdn·HCl at constant molality. This is exemplified in Figure 1, which presents typical plots of apparent partial specific volumes *vs.* protein concentration in the native state and in 6 M Gdn·HCl at constant chemical potential and constant molality for two proteins. In the case of β-lactoglobulin (Figure 1A), a definite concentration dependence is evident at constant molality of Gdn·HCl. No such dependence was observed at any conditions with calf brain microtubule protein. These examples clearly demonstrate that in accurate measurements of partial specific volumes of proteins, a concentration series is required to ascertain whether such a dependence exists. In the case that it does, an extrapolation is necessary to obtain the true value of the partial specific volume, and it is unwise to assume *a priori* an absence of concentration dependence.[3]

The results of partial specific volume measurements for the twleve proteins are summarized in Table I. The values of the partial specific volumes found for native ribonuclease A, lysozyme, chymotrypsinogen A, α-chymotrypsin, bovine serum

[3] The partial specific volume is related to the apparent quantities by $\bar{v}_- = \phi + g(d\phi/dg)$, where g is grams of macromolecules added to the solvent. For systems encountered in biophysical studies, the concentrations are often low enough that the concentration dependence of the apparent partial specific volume is undetectable. It is, however, the limiting value $\bar{v}_2^0 = \phi^0$ at $g = 0$ which is the quantity applied in analysis (Cassasa and Eisenberg, 1964).

albumin, and β-lactoglobulin in the native state are identical with literature values within experimental uncertainty. The case of carboxypeptidase A is of particular interest. Since this enzyme does not dissolve in low ionic strength solvent, buffered 1.0 M NaCl had to be used as solvent. Bethune (1965) has carried out studies on the self-association of this enzyme in 1.0 M NaCl using an assumed partial specific volume of 0.75. At such a high concentration of salt, a significant contribution from preferential interaction with solvent components could be reasonably expected. Our measured value, 0.748, removes from Bethune's studies any uncertainty stemming from this parameter. The single major discrepancy from literature was found with α-lactalbumin. Our reproducibly obtained value of 0.704 is considerably below that of 0.729 previously reported. The lower value, 0.704, however, is almost identical with that of lysozyme, 0.702. Such an identity could be expected from the homology found between the amino acid sequences of these proteins (Brew *et al.*, 1967) and their overall structural similarity in solution indicated by small-angle X-ray scattering examination (Pessen *et al.*, 1971), and may be regarded as further support of the conformational similarity of these two proteins.

The values of the partial specific volumes in 6 M Gdn·HCl deserve particular attention. In the majority of cases, transfer from the native state into 6 M Gdn·HCl, without dialysis, results in either a small decrease in partial specific volume or no change at all. This indicates little or no overall volume change on transfer of the protein from native state in dilute buffer to an unfolded state in 6 M Gdn·HCl. Notable exceptions are β-lactoglobulin and bovine serum albumin, in which a considerable decrease in partial specific volume occurs. Other significant decreases are observed in carboxypeptidase A and α-chymotrypsin. In sedimentation equilibrium measurements of molecular weights of protein subunits in 6 M Gdn·HCl, the required value of the partial specific volume is that obtained after dialysis against the medium, namely, ϕ_2' extrapolated to zero protein concentration. In the past, this parameter has been rarely measured, the practice being to decrease the value of partial specific volume of the native protein by 0.004–0.010 (Sakura and Reithel, 1972), following the few observations reported in the literature (Kielly and Harrington, 1960). Comparison of columns 2 and 4 of Table I suggests, however, that such a practice is not devoid of hazards. While in half of the proteins studied here, this approximation is quite valid, there are several exceptions, in particular β-lactoglobulin ($\bar{v}_{2,\text{native}}^0 - \phi_{2,\mu_3}' = 0.031$), bovine serum albumin ($\bar{v}_{2,\text{native}}^0 - \phi_{2,\mu_3}' = 0.018$), α-chymotrypsin ($\bar{v}_{2,\text{native}}^0 - \phi_{2,\mu_3}' = 0.025$), and chymotrypsinogen A ($\bar{v}_{2,\text{native}}^0 - \phi_{2,\mu_3}' = 0.021$). An error of such magnitude in the partial specific volume may result in a very serious error in the calculated molecular weight when this value is combined with a sedimentation equilibrium experiment in 6 M Gdn·HCl. For example, using $\rho_0 = 1.1418$ g/ml in 6 M Gdn·HCl (Reisler and Eisenberg, 1969) results in values of the buoyancy term ($1 - \bar{v}_2^0\rho_0$) of 0.1437 and 0.1791 if $\bar{v}_2^0$ is taken as 0.750 and 0.719, in turn, *i.e.*, the values of $\bar{v}_2^0$ and ϕ_2' for β-lactoglobulin. We see that introduction of the native protein value of $\bar{v}_2^0$ into the molecular weight equation would result, in this case, in an error of 25%. For a dissociable system, this would introduce an uncertainty into the number of subunits above three. Comparison of columns 2, 3, and 4 of Table I shows that the difference between the partial specific volumes of the native protein and of the unfolded protein after dialysis may reflect a combination of two factors: (1) a volume change on denaturation, (2) preferential interaction with solvent components. While volume change is the major factor in the cases of β-lactoglobulin and bovine serum albumin, interactions with solvent may be the major contribution for α-chymotrypsin and chymotrypsinogen A.

The partial specific volumes in 6 M Gdn·HCl reported here are in general in reasonable agreement with available literature values. One notable exception is found in β-lactoglobulin, for which Reithel and Sakura (1963) reported a slight increase in the partial specific volume upon transfer of the native protein to 6 M Gdn·HCl and dialysis against this solvent. Later, these same authors (Sakura and Reithel, 1972) discussed the discrepancy between their results and the difference expected from the solvent interaction studies of Hade and Tanford (1967). They have suggested the possibility that hydrocarbon binding to protein in the density gradient column which they had used may interfere with $\bar{v}$ measurements of some proteins. This explanation for the erroneous value of the β-lactoglobulin partial specific volume in 6 M Gdn·HCl is quite plausible, since this protein is known to bind hydrocarbons (Wishnia, 1962; Wetlaufer and Lovrien, 1964; Davis and Dubos, 1947) to such an extent that, with it, the usual fluorocarbon layer cannot be used to determine the bottom of an ultracentrifuge cell (Adams and Lewis, 1968).

Protein–Solvent Interactions. The magnitudes of the preferential interaction parameter in 6 M Gdn·HCl calculated with eq 3 from the partial specific volumes are listed in columns 5 and 6 of Table I. In these calculations the values of $\bar{v}_3$ and ρ_0 used were 0.763 ml/g (Reisler and Eisenberg, 1969) and 1.1427 g/ml. The results are found to be in good agreement with those reported by Hade and Tanford (1967) using the isopiestic method, as well as with other reports in the literature (Lee *et al.*, 1973; Appella and Markert, 1961). This agreement indicates the ability of the present method to measure this parameter with a degree of accuracy approaching that of vapor pressure equilibrium. Particular advantages of the present method seem to reside in the requirement of smaller amounts of protein (0.7 ml at 3–18 mg/ml) and the rapidity and ease of the measurements themselves.

While measurements of the preferential interactions of solvent components with proteins are one of the means available in the study of mechanisms of protein denaturation (Tanford, 1968, 1970; Timasheff, 1970), they also may be used to estimate correct value of $\bar{v}_2$ to be used in molecular weight calculations (Timasheff and Inoue, 1968). This approach has been employed in a recent study on the molecular weight of lactate dehydrogenase (beef heart) (Fosmire and Timasheff, 1972) in which, using a ξ_3 value of 0.04 g of Gdn·HCl/g of lactate dehydrogenase in 5 M Gdn·HCl (Appella and Markert, 1961), it was estimated that ϕ_2' in 6 M Gdn·HCl should be equal to 0.736, *i.e.*, the presently determined value. In this case, the use of ϕ_2^* instead of ϕ_2' would have led to an error of only 3% in molecular weight.

Examination of column 5 of Table I reveals a particularly significant feature. For the twelve proteins studied, the preferential interaction with solvent components varies between zero (RNase A) and 0.17 g of Gdn·HCl/g of protein (α-chymotrypsin). In no case is interaction preferential with water. Since the solvent is identical in all cases, this variation in ξ_3 must be related to the properties of the protein molecules themselves.

Tanford (1968, 1970) has shown that in 6 M Gdn·HCl most proteins are devoid of specific structural features. Since the polypeptide backbone chains are equally exposed to solvent under such circumstances, the variations in the preferential interaction parameter must reflect some variations in amino acid composition. It is known that the different amino acid side chains differ from each other in their affinity for water and

TABLE II: Protein–Solvent Interactions in 6 M Gdn·HCl.

Protein	$(\partial g_3/\partial g_2)_{T,p,\mu_3}$ (g/g)	A_1 (g/g)	A_3 g/g	A_3 mol/mol	$A_{3,\text{calcd}}$ (mol/mol)
Lactate dehydrogenase	0.03	0.455	0.488	184	182
Tubulin	0.10	0.487	0.581	331	302
α-Lactalbumin	0.03	0.522	0.556	84	81
Lima bean trypsin inhibitor	0.01	0.498	0.511	48	50
β-Lactoglobulin A	0.08	0.514	0.598	115	102
Carboxypeptidase A	0.05	0.361	0.414	149	204
Bovine serum albumin	0.06	0.445	0.508	362	359
Chymotrypsinogen	0.15	0.390	0.543	145	143
Lysozyme	0.09	0.360	0.450	67	75
α-Chymotrypsin	0.17	0.360	0.533	139	136
Ribonuclease	0.0	0.503	0.507	72	86
Catalase	0.01	0.465	0.478	300	313
Aldolase	0.089[a]	0.384	0.476	199	222

[a] Hade and Tanford (1967).

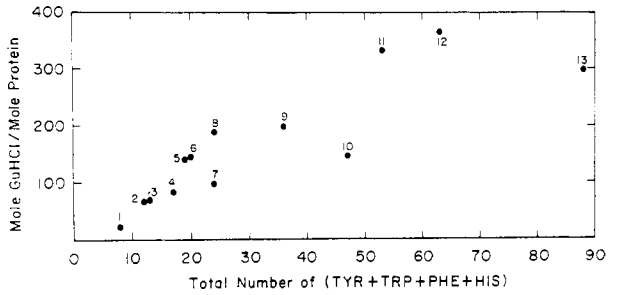

FIGURE 2: Relationship between the number of moles of Gdn·HCl bound to a mole of protein, A_3, and the total number of aromatic amino acids. The proteins are: (1) lima bean trypsin inhibitor, (2) lysozyme, (3) ribonuclease A, (4) α-lactalbumin, (5) α-chymotrypsin, (6) chymotrypsinogen, (7) β-lactoglobulin, (8) lactate dehydrogenase, (9) aldolase, (10) carboxypeptidase A, (11) tubulin, (12) bovine serum albumin, and (13) catalase. The value for aldolase is taken from Reisler and Eisenberg (1969).

Gdn·HCl (Nozaki and Tanford, 1970). An attempt was made, therefore, to correlate the preferential interaction parameter with various characteristics of the amino acid compositions of these proteins. An attempt to correlate the preferential interaction parameter with the average hydrophobicity,[4] $H\phi_{av}$, of the proteins showed the absence of such correlation.

Preferential interaction is an expression of the difference between the interactions of each solvent component with the protein. Since these may vary independently for different proteins, a better way to compare the different proteins would be through the actual amount of Gdn·HCl bound to each protein. This can be calculated from the preferential interaction parameter (Inoue and Timasheff, 1972), if the absolute degree of hydration is known, since

$$(\partial g_3/\partial g_2)_{T,p,\mu_3} = A_3 - g_3 A_1 \qquad (6)$$

where A_3 is absolute solvation, *i.e.*, the actual amount of denaturant bound to the protein, A_1 is absolute hydration, and g_3 is the solvent composition, expressed as grams of component 3 per gram of water. In practice, this calculation is complicated by our poor knowledge of protein hydration. A range of values for A_1 of proteins can be found in the literature (Kuntz, 1971; Bull and Breese, 1968). These are frequently a function of the technique used (Kuntz, 1971; Bull and Breese, 1968; Tanford, 1961; Timasheff, 1963). For the purpose of our presentation, and in order to maintain internal consistency, A_1 was calculated for each protein from the hydration of its constituent amino acids according to the method of Kuntz (1971), since there are no experimental values of A_1 for a number of proteins used in this study. It is true that the Kuntz procedure might overestimate the value of hydration for native proteins. In the presence of denaturant, however, the proteins are unfolded, with a majority of residues in contact with solvent, and the assumption that the values of hydration for these proteins are equivalent to the summation of those of their amino acid constituents assumes greater validity. Introduction of these values of A_1 into eq 6 gave A_3 for each protein. The results are summarized in Table II. The value of g_3 in these calculations was 1.007 g of Gdn·HCl/g of H_2O.

The interaction of Gdn·HCl with proteins may occur at a variety of sites. The favorable free energy of transfer of hydrophobic side chains and, in particular, of aromatic residues from aqueous medium to 6 M Gdn·HCl suggests these as very likely sites of interaction (Tanford, 1970; Nozaki and Tanford, 1970). On the other hand, the hydrogen-bonding ability of the guanidinium group should favor its interaction with peptide bonds. Indeed, Robinson and Jencks (1965) have postulated that a Gdn·HCl molecule could hydrogen bond to two peptide bonds forming a cyclic structure.

With these possibilities in mind, attempts were made to correlate A_3 with various compositional variables of the proteins. Figure 2 shows the relationship between the amount of Gdn·HCl bound per mole of protein and the total number of aromatic amino acids in each protein. A fair correlation seems to exist, with the exception of catalase and carboxypeptidase A. Plots of A_3 *vs.* the number of (Tyr + Trp + Phe + His + Ala + Leu + Ile + Val + Met) or (Tyr + Trp + Phe) or Tyr resulted in increasingly poor correlation. It might seem, therefore, that while aromatic and possibly hydrophobic side chains are binding sites for Gdn·HCl, they cannot account for

[4] Hydrophobicity has been defined by Bigelow (1967) as the average free energy of transfer of the amino acid side chains of a protein from an aqueous environment to a nonpolar environment, based on the values given by Tanford for ethyl alcohol (Tanford, 1962).

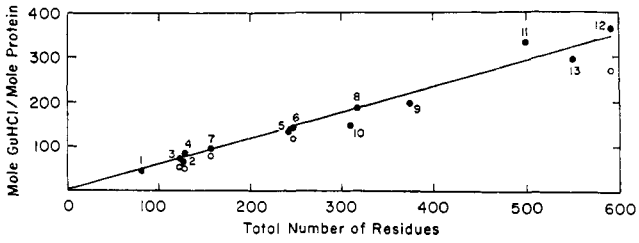

FIGURE 3: Relationship between the number of moles of Gdn·HCl bound to a mole of protein, A_3, and the total number of amino acid residues in each protein or subunit of an associated protein. The proteins and their respective numbers are the same as Figure 2. Data obtained with A_1, values computed by the method of Kuntz (1971) (●) and Bull and Breese (1968) (○).

the total extent of interaction. Figure 3 shows A_3 as a function of the total number of amino acid residues in each protein or in a subunit of an associated system. A good linear relationship with a slope of 0.58 is obtained. For comparison, values of A_3 calculated for proteins whose A_1 had been determined experimentally (Bull and Breese, 1968) are also included in Figure 3. Although these are somewhat lower than those obtained using the Kuntz procedure to determine hydration, a linear correlation still prevails.

Assuming that the Robinson and Jencks (1965) postulate of two peptide bonds interacting with a single Gdn·HCl molecule is valid and that aromatic amino acid side chains are also good candidates for binding sites (Tanford, 1970; Nozaki and Tanford, 1970), the expected number of binding sites for each protein was calculated. This is given as the summation of [(total number of peptide bonds/2) + total aromatic amino acids]. The results are tabulated in column 6 of Table II. Figure 4 is a plot of the number of Gdn·HCl molecules bound, A_3, *vs.* the expected number of binding sites. An excellent correlation is obtained, and the least-squares line drawn through the points has a slope of 1.004. It may be concluded, therefore, that peptide bonds and aromatic side chains are the best Gdn·HCl binding sites. This is in agreement with the conclusion of Robinson and Jencks (1965) that the interaction between Gdn·HCl and peptides is neither strictly hydrophobic nor nonhydrophobic, but both. This conclusion is further supported by attempts to correlate A_3 with hydrophobicity, as defined by Bigelow (1967), and with the average free energy of transfer, ΔF_t, of the proteins from aqueous medium into 6 M Gdn·HCl, calculated from the solubility data of Nozaki and Tanford (1970). In neither case was there any correlation found.

Although the binding of Gdn·HCl to proteins might be accompanied by a change in the extent of hydration, this effect cannot be large. Neurath and Bull reported already in 1936 that there was no noticeable differences in hydration between native and denatured proteins, while Kuntz (1971) could find no change in hydration when bovine serum albumin was denatured by urea.

While the degree of Gdn·HCl interaction with proteins is not related to the average free energy of transfer of the amino acid side chains from water to the denaturing medium, it was interesting to see whether interaction with Gdn·HCl and protein unfolding are mutually related for a single protein, as has been found for protein denaturation by 2-chloroethanol (Timasheff, 1970; Inoue and Timasheff, 1972). For this, the preferential interaction of lysozyme with solvent components in Gdn·HCl solutions between 1 and 6 M was determined and, from it, A_3 was calculated. In Figure 5 the variation in number of moles of Gdn·HCl bound per mole of lysozyme as a function of solvent composition is compared with the change in optical rotation reported by Tanford and coworkers (1966). Two features are evident: first, in general the conformational change parallels the absolute degree of interaction of the denaturant with lysozyme; second, binding of Gdn·HCl to lysozyme occurs already below 3 M, *i.e.*, in the region in which no conformational change is observed. At higher solvent concentrations, both quantities level off, or increase only very slowly. The fact that GuHCl binding precedes unfolding, a situation identical with protein denaturation by 2-chloroethanol (Timasheff, 1970), suggests that, in the case of this denaturant, as well, denaturation is mediated by intimate contact between the denaturant and portions of the protein molecule. The resulting interactions between denaturant molecules and the protein weaken the forces which stabilize the globular conformation. This permits an initial loosening of the structure, which, upon binding of further denaturant molecules, leads to the eventual destruction of the native structure. While in the case of 2-chloroethanol the prevalent protein–denaturant interactions appear to involve hydrophobic residues (Timasheff, 1970), Gdn·HCl could function through interactions with a variety of groups, principally peptide bonds and aromatic side chains.

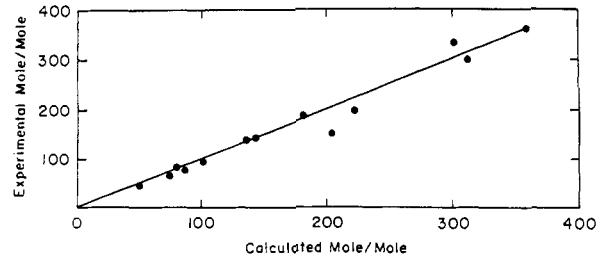

FIGURE 4: Relationship between experimental A_3 and calculated A_3.

Volume Changes. Using the data of Table I and eq 5, the changes in volume upon transfer from dilute salt to 6 M

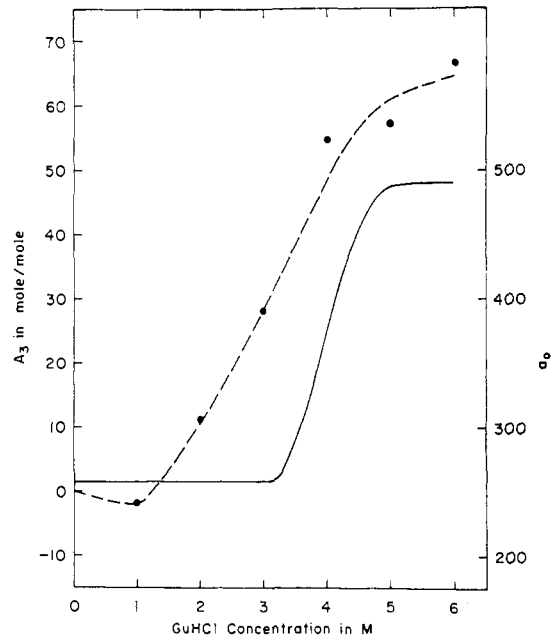

FIGURE 5: Variation with solvent composition of Gdn·HCl binding to lysozyme and of optical rotation measurements in Gdn·HCl, which are taken from Tanford *et al.* (1966). Filled circles and dashed line: binding of Gdn·HCl to the protein, A_3, at pH 5.5, 25°; solid line: optical rotation measurements at pH 5.5, 30°.

TABLE III: Change in Volume for Proteins upon Transferring from the Native to the Denatured State in 6 M Gdn·HCl.[a]

Protein	$-\Delta V$ (ml/mol)	$-\partial V = \Delta V$/Residue (ml/mol of Residue)
RNase	30 ± 30	0.24
Lysozyme	−30 ± 40 (54)[b]	−0.23
Tubulin	0 ± 160	0.00
Chymotrypsinogen A	100 ± 50 (10)[c,d]	0.40
α-Chymotrypsin	150 ± 50	0.62
Bovine serum albumin	750 ± 200 (12)[d,e]	1.27
α-Lactalbumin	40 ± 30	0.31
Lactate dehydrogenase (BH)	70 ± 70	0.22
Catalase	240 ± 100	0.44
β-Lactoglobulin	400 ± 60 (600)[f,g]	2.50
Lima bean trypsin inhibitor	0 ± 20	0.00

[a] The values in brackets are taken from literature. [b] Skerjanc and Lapanje (1972). [c] Skerjanc *et al.* (1970). [d] Values determined in 8 M urea. [e] Katz and Ferris (1966). [f] Values determined in 38% urea. [g] Christensen (1952).

Gdn·HCl of the proteins studied were calculated from the difference in apparent partial specific volume measured at constant molality of Gdn·HCl and under native conditions. The results are presented in Table III. The reported values of ΔV for transfer of the proteins from water to 6 M Gdn·HCl require that corrections be made to obtain $\bar{v}_2^0$ in water; it may be reasonably assumed, however, that $\bar{v}_2^0$ measured in dilute buffer is identical with or very close to that in water, any difference being smaller than experimental error. This assumption is not valid, however, in the case for carboxypeptidase A for which the native $\bar{v}_2^0$ was measured in 1 M salt. Hence, it is not included in Table III. As expected (Reisler and Eisenberg, 1969; Tanford, 1968; Kauzmann, 1959; Skerjanc and Lapanje, 1972; Christensen, 1952; Katz and Ferris, 1966) the magnitudes of ΔV are small for all proteins, the decrease in volume never exceeding a few hundred milliliters per mole. The accuracy and precision of ΔV measurements obtained by density determinations, however, do not permit an evaluation of the results more than on a qualitative basis. In general, the values agree reasonably well with those reported in the literature. It is encouraging to notice, for example, that for β-lactoglobulin the volume change is of similar magnitude as that reported by Christensen (1952), who, by using a dilatometric technique, found a decrease of 612 ml/mol when β-lactoglobulin was transferred from 26 to 38% urea.

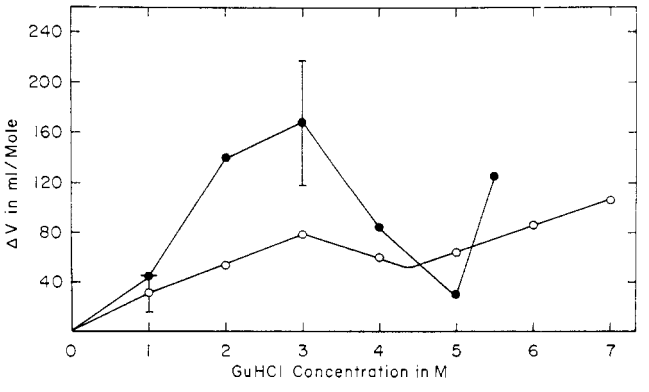

FIGURE 6: Change of the partial molar volume of lysozyme upon transfer from water to Gdn·HCl as a function of Gdn·HCl concentration at pH 5.5 and 25°. (●) Data obtained from density measurements and (○) data from dilatometry (Skerjanc and Lapanje, 1972). The bars indicate the largest and smallest deviations in these measurements.

As stated above, the volume changes which occur during protein denaturation are a summation of the effects of the conformational change, change in the solvation volume, and change in the electrostriction around the side chains of polar amino acid residues. The contribution of the conformational change can be resolved into at least two factors: first, to differences in the volume occupied by atoms packed in different manner in various conformations and, second, to the void volumes which exist in various conformations. In the case of subunit systems, a contribution to ΔV can also result from the separation of the subunits, the release of previously buried groups to contact with solvent and the annihilation of possible intersubunit void spaces. An example of the magnitude of ΔV that could be expected from conformational change alone is given by the results of Noguchi and Yang (Noguchi and Yang, 1963, 1971; Noguchi, 1966; Makino and Noguchi, 1971) who have determined the volume change involved in helix–coil and β-coil transitions. These investigators have estimated a $\Delta V_{\text{helix-coil}}$ of −0.6 to −1.0 ml per mol of residue and a $\Delta V_{\beta\text{-coil}}$ of −1.90 to −2.35 ml per mol of residue. In the last column of Table III, the volume changes actually observed in the various proteins are presented as milliliters of volume change per mole of residue. It is evident that the volume change per residue varies between essentially none (tubulin, lima bean trypsin inhibitor) to values too large to be accounted for in terms of the quantities obtained by Noguchi and Yang.

At least for four of the proteins studied (RNase A, lysozyme, chymotrypsinogen, and α-chymotrypsin) the uncertainty can be attributed to the fact that $\bar{v}_2$ of these proteins in the native and denatured states were measured at different pH values. Katz and Miller (1971) and Krausz and Kauzmann (1965) have demonstrated clearly that the ΔV obtained at different pH values cannot be interpreted directly unless the contribution of protonation to ΔV can be accounted for. However, the necessary information to calculate the magnitude of this contribution to the proteins studied here is not available at present.

The change in volume of lysozyme upon denaturation by Gdn·HCl is shown in Figure 6, as a function of Gdn·HCl concentration and compared with results of dilatometric experiments (Skerjanc and Lapanje (1972). There is, in general, a fair correlation between the two sets of results. The sinusoidal relation between ΔV and concentration of denaturant, seen in Figure 6, is a general phenomenon observed in the dilatometric studies of the denaturation of other proteins by urea or Gdn·HCl (Skerjanc *et al.*, 1970; Katz, 1968; Skerjanc and Lapanje, 1972; Christensen, 1952; Katz and Ferris, 1966). Skerjanc and Lapanje (1972) have attributed the decrease in ΔV observed at 3–4 M Gdn·HCl to the unfolding of the molecule, and the gradual increase in volume observed below and above 3–4 M Gdn·HCl to the binding of Gdn·HCl to the protein.

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