Volume Change by Density. Ribonuclease in 0–8 M Guanidinium Chloride†

T. H. Crouch‡ and D. W. Kupke*

ABSTRACT: Volume changes (ΔV) accompanying interactions of proteins with other substances are conveniently determined in quantity with the rapid density techniques now available. It is shown that by constructing only two density-composition tables over the concentration range of interest of a protein-perturbing substance, the volume changes of interest and the partial specific volumes ($\bar{v}$) for each component in a three-component system can be generated as a smooth function of perturbant concentration. The ΔV of mixing, the ΔV in transporting protein from one concentration of perturbant to any other, and the ΔV in transporting the perturbant from solvent to protein solution can be calculated. The experimental system, water–isoionic ribonuclease–guanidinium chloride, was studied by this approach with three different rapid densimeters (two magnetic designs and the vibrating tube type). ΔV values for transporting ribonuclease from water to guanidinium chloride solutions between 0 and 8 M were positive and exhibited an apparent transition region between about 2 and 4 M guanidinium chloride at 20 °C. ΔV values for transporting the guanidinium chloride from water to water + ribonuclease were also positive but generally smaller; a marked reduction in these volume increases commenced at a substantially lower concentration of guanidinium chloride (<0.1 M) than that required to initiate the unfolding of the ribonuclease, suggesting that some of this perturbant interacts with protein prior to the unfolding process. Differences in the values of $\bar{v}$ of water and of guanidinium chloride in the presence and in the absence of ribonuclease were too small for the most part to assign values. At very low concentrations of guanidinium chloride, however, significantly higher values of $\bar{v}$ of guanidinium chloride were found with ribonuclease present than when absent. It is proposed that the substantial increase in volume (~+10 mL/mol of guanidinium chloride) attending the transport of this salt from water to water + ribonuclease in the mole/mole range [guanidinium chloride:ribonuclease] represents a spontaneous preference for the protein phase by analogy to that for the transfer of hydrocarbon from water to a nonpolar phase.

The density property has been utilized rarely for the determination of the total volume change, ΔV, of reactions despite the growing availability of rapid and sufficiently accurate density instruments. Volume change by density enlarges the experimental opportunities over that of volume difference techniques because the density method has become more versatile, rapid, and convenient and it requires less material per measurement. Additionally, the partial volume changes of the individual components can be collected concurrently.

In protein chemistry the volume change of interest at constant temperature and pressure is often the change observed by mixing a solution containing the protein with a solution containing a perturbant, such as a denaturant. By determining ΔV also on suitable control solutions it is possible to extract the volume change resulting only from the transport of the protein from one solvent medium to that of the perturbing medium (Katz and Ferris, 1966). Such differences in volume have been ascribed to changes in conformation of the protein. This volume change per gram of the protein is equivalent to the difference in the apparent specific volume, ϕ, of the protein component. The manner in which ϕ changes as a function of perturbant concentration requires that a set of measurements be made at a substantial number of different perturbant concentrations, a task which heretofore has been impractical. With rapid density techniques it becomes feasible to obtain ϕ as a smooth function of perturbant concentration from which values of ΔV accompanying the transport of a protein from a chosen concentration of perturbant to that at any other concentration can be calculated. From the same set of measurements, one may also calculate (1) the volume change for transporting the perturbant itself from water (or other solvent medium) to the protein–solvent solutions, (2) the total volume change of mixing any pair of compositions over the range encompassed, and (3) the differences in the partial specific volumes of perturbant and of solvent at each composition when protein is added to the system. This is accomplished by constructing two density-composition tables containing a suitable number of data points for the composition range of interest to yield a useful equation of each via curve-fitting procedures. The one curve is simply that of the two-component system of water (or suitable solvent medium), labeled component 1, and of the perturbant, labeled component 3, which is added in increasing amounts; such a density-composition table for this pair of components may be already available at the desired temperature. The second curve is that for the three-component system in which the macromolecule (component 2) coexists with component 1 at a fixed mass ratio as the amount of perturbant is varied. For the purpose of illustration, the three-component system of water, isoionic ribonuclease A (RNase), and guanidinium chloride (GdmCl)[1,2] was studied. It is shown that

† From the Department of Biochemistry, School of Medicine, The University of Virginia, Charlottesville, Virginia 22901. *Received February 22, 1977.* This work was supported by Grant No. BMS-75-01599 from the U.S. National Science Foundation.

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[1] Many authors refer to this substance as "guanidine·HCl". The compound behaves as a strong salt and we prefer the nomenclature "guanidinium chloride" which reflects this property. The latter designation is consistent with the usage commonly applied to other amine salts, e.g., ammonium chloride.

[2] Abbreviations used are: RNase, ribonuclease A; GdmCl, guanidinium chloride; NMR, nuclear magnetic resonance.

sufficient sensitivity is available under routine conditions for observing variations in ΔV (10^{-3} mL/g, or better) as a function of perturbant concentration. Upon smoothing and projecting these variations, the observed trends with composition may be related to other kinds of data and may also provide some unique insights into the processes (Kauzmann, 1959). For example, these trends may show whether a change in ΔV for transporting the macromolecule is accompanied by a concurrent change in ΔV for transporting the perturbant. With the system described here, the volume change upon transporting the perturbant, GdmCl, to the protein solution showed a change in character at concentrations below that where the volume change on transporting the protein to GdmCl solutions indicated a conformational transition. In addition, these experiments suggest that density may be found useful as a method for titrating small amounts of solute in the presence of a macromolecular species.

Experimental Section

Theory. The volume, V, of a solution at constant temperature and pressure may be defined in terms of the partial specific volumes, $\bar{v}$, and the number of grams, g, of each of the N number of components comprising the milieu. Thus:

$$V = \sum_{i=1}^{N} \bar{v}_i g_i \tag{1}$$

where the units of $\bar{v}$ are chosen for the present purpose in milliliters per gram (mL/g). Hence, a change in the value of $\bar{v}$ of a component may be demonstrable even where the change in total volume, ΔV, is very small or immeasurable upon mixing ingredients together.

It is usually possible to obtain any $\bar{v}_i$ at finite concentrations by density from the slope:

$$\left(\frac{\partial \rho}{\partial c_i}\right)_m = \left(\frac{1 - \bar{v}_i \rho}{1 - \bar{v}_i c_i}\right) \tag{2}$$

where ρ is the density of the solution in grams per milliliter, subscript m refers to constant composition (i.e., molality) of all components except for the ith one, and c is its concentration in grams per milliliter. With this choice of concentration units, eq 1 reduces to $\Sigma_i \bar{v}_i c_i = 1$ whereby $\bar{v}$ of only $N - 1$ components need be determined. Weight per weight definitions of the concentration are readily interconverted; for example, the weight fraction, $W_i = g_i/\Sigma_i g_i$, is related to c_i by the density such that $c_i = \rho W_i$.

Unlike with most crystalloidal substances, the values of $\bar{v}$ of macromolecules change so little with concentration that the density is usually observed to be a linear function of c over a fairly broad range in c. For such solutes it is useful to consider eq 2 as $c_i \rightarrow 0$, which equation when rearranged reduces to:

$$\bar{v}_2^0 = \frac{1}{\rho^0}\left[1 - \left(\frac{\partial \rho}{\partial c_i}\right)_m^0\right] \tag{3}$$

where superscript zero refers to conditions at vanishing macromolecule concentration and subscript 2 refers to a macromolecular component. Over the concentration range where $\bar{v}_2 = \bar{v}_2^0$, the quantity $\bar{v}_2^0$ is equivalent experimentally to ϕ_2, the apparent specific volume, which is the observed change in volume upon adding a unit mass of the component to a solvent medium. Thus:

$$V = \phi_2 g_2 + \sum_{j \neq 2} \bar{v}_j^0 g_j \tag{4}$$

where the second term on the right equals V^0, the volume of the solvent in the absence of component 2. Differentiation with respect to g_2 and holding the second term constant, in accord with the definition of ϕ, shows that $\phi_2 = \bar{v}_2^0 = \bar{v}_2$ at finite concentrations so long as ϕ_2 does not vary with g_2. The general equation for obtaining any ϕ_i (except for water or the major solvent component) becomes:

$$\phi_i = \frac{1}{\rho^0}\left[1 - \left(\frac{\rho - \rho^0}{c_i}\right)_m\right]_{i \neq 1} \tag{5}$$

where subscript 1 refers to the major solvent component. Apparent specific volumes have the great practical advantage in that the values of $\bar{v}$ for all other components need not be determined each time a mass of the component being varied is added. Thus, the values of $\bar{v}_j^0$ (eq 4) of all other components remain constant in expressions utilizing ϕ_2 as the mass of a protein component is varied. Density-composition tables from which to calculate $\bar{v}_j^0$ exist in the literature for a variety of two-component solvent systems commonly used for the proteins, or, if such tables are unavailable, they now can be obtained conveniently with very high accuracy via rapid density techniques.

The change in volume, ΔV, upon transporting a given mass of component $i \neq 1$ from one medium to another of a different composition is seen from eq 4 to be simply the difference in the value of ϕ_i in the two media. This result arises by arbitrarily holding constant the volume V^0 containing all components other than i in the two media in accordance with the definition of ϕ. The volume change per gram of i in transporting it from medium A to medium B, if $d\phi_i/dg_i = 0$ as is commonly observed with the proteins, is:

$$\frac{\Delta V}{g_i} = \phi_{i(B)} - \phi_{i(A)} = \Delta\phi_i \tag{6}$$

(For a general review of these volume principles cf. Kupke (1973).)

Materials and Dry Weights. The guanidinium chloride (GdmCl) was either the Ultra Pure grade from Schwarz/Mann or the less purified product from British Drug House, Ltd. (Poole, England). These preparations were twice crystallized in the cold from absolute methanol after treating the saturated solutions of GdmCl in methanol (40 °C) with well-washed activated carbon and filtering. The harvested crystals were pulverized and then dried in vacuo at 40 °C. The degree of purity fell within the titration and absorbance guidelines outlined by Nozaki (1972). Pertinent to our purpose, the various samples exhibited the same density per unit of weight fraction in water to within ~5 parts in 10^6. Most, but not all, of the Ultra Pure samples were indistinguishable from the further purified product, and these were used occasionally after simply pulverizing and drying. The final material was maintained under vacuum at room temperature until just before use.

The bovine pancreatic ribonuclease A (RNase) was either the type XII-A from Sigma Chemical Co. or the chromatographic grade from P-L Biochemicals, Inc. Water solutions of these preparations were deionized through a well-washed mixed-bed ion-exchange column (Amberlite M1 under a layer of 0.1 vol of MB-3), dialyzed against cold water, and filtered through 0.22 μm Millipore filters. No systematic differences in the specific volume, specific absorbance, or pH were then observed between these two sources. The pH of these solutions ranged from 9.6 to 9.8. The dry weights of concentrated stock solutions in water were determined in quadruplicate on each preparation in such a manner that differences in the weight fraction between extreme values in a set were less than 1 part

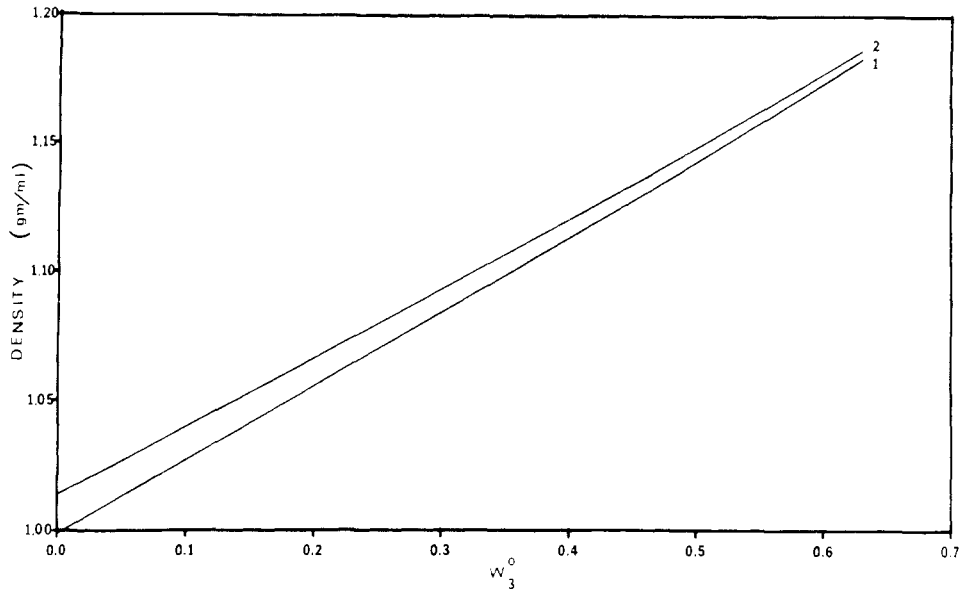

FIGURE 1: Density vs. weight fraction, W_3^0, of guanidinium chloride with respect to water at 20 °C: curve 1, water–guanidinium chloride; curve 2, water–isoionic ribonuclease A–guanidinium chloride. (Curves drawn to fit the data via eq 7 and the coefficients of Table I.)

in 500 (usually these differences approached 1 part in 10^3). For the drying procedure, 4 small glass vessels with caps (~400 μL each) containing about 250 μL of solution (7 to 12 mg of protein) and 2 similar vessels of water, all of known weight (to within ±10 μg), were evaporated at ~45 °C in a vacuum oven while flushing with dry nitrogen gas until nearly dry. The vessels were then heated at 105 °C in vacuo for 3 to 4 days. The dry weights, corrected to vacuum conditions, were obtained after extrapolating the series of observed weights as a function of time when the vacuum seal was broken after cooling the heated vessels (Hunter, 1966). In this way the variable pickup of moisture by the samples while on the analytical balance was accounted for. The vessels themselves increased in weight ~10 μg or less during the weighings, whereas the vessels containing protein increased 50–90 μg. The relative humidity of the weighing room was maintained at a level of between 50 and 60%. The heatings and weighings were repeated at least twice more; the last two weights per sample varied by 10 μg or less and, sometimes, this final value agreed with that after the first heating. The value of $\bar{v}$ in water at 20 °C, based on these dry weights for the 6 preparations used here, was 0.7033 ± 0.0015 mL/g. The specific absorbance in water at 20 °C was 0.726 ± 0.002 mL/mg at 277.5 nm for a solution pathlength of 1 cm.

Density Determinations. Samples for the density determinations were prepared gravimetrically in 1- to 2-g amounts using a Mettler M-5 analytical balance. Volumes of deionized RNase solutions or of deionized water were added to the dried GdmCl in a manner to minimize evaporation error. For samples which were very dilute in GdmCl, weighed portions of water or water–RNase were mixed with weights of an accurately known concentration of GdmCl in water. Densities involving RNase solutions were determined within several hours because small but significant increases in the density were observed after several days if the weight fraction of GdmCl was high ($W_3 > 0.2$); these increases in density could not be unambiguously ascribed to evaporation. Since the objective was to maintain a constant molality of protein throughout the density series as the molality of GdmCl (g_3/g_2) was varied, the appropriate volumes of a protein stock solution were added, via gas-tight syringes, to the dry GdmCl. The value of g_3/g_1, after correction for the difference in air buoyancies of the weighed material, was calculated via:

$$g_3/g_1 = g_3/m_p(1 - W_2^0)$$

where W_2^0 is the known weight fraction of RNase in the stock solution containing no GdmCl and m_p is the buoyancy-corrected mass of this solution added to the corrected mass, g_3, of the GdmCl.

Densities were determined on triplicate samples at 20 °C with one of two different magnetic suspension densimeters (Senter, 1969; Almeida and Crouch, 1971)[3] or by the electronic vibrating-tube method (Mettler/Paar, Model DMA 02C). On occasions, samples of the same solution were compared in the three instruments concurrently. These densimeters gave comparable results for the purpose at hand, although the magnetic method was about four times more rapid and required about one-third as much material. In general, the precisions with replicate samples of a given solution were within 2 to 4 parts in 10^6 in the density. The overall precision with separately prepared solutions of GdmCl with a given stock solution of RNase ranged from 5 to 10 parts in 10^6. For the calibrations, water solutions of vacuum-dried sucrose (U.S. National Bureau of Standards, Lot 17) were used in conjunction with the density-composition tables at 20 °C (Plato, 1900). Water solutions of heat-dried Suprapur grade cesium chloride (E. Merck, Darmstadt, Germany) were also prepared and used as secondary calibration standards for convenience in the higher density range where sucrose solutions become viscous.

Results and Discussion

Figure 1 shows the densities at 20 °C of the water–GdmCl mixtures (curve 1) and those of water–RNase–GdmCl (curve 2) as a function of W_3^0, the weight fraction of GdmCl in water (exclusive of the protein in curve 2). Thus, for curve 1, $W_3^0 = g_3/(g_1 + g_3) = W_3$ at any composition, whereas in curve 2, $W_3^0 > W_3$ owing to the presence of a constant amount of protein per gram of water. By this choice of units for the concentration

[3] Details for the theory and practice of the magnetic method are described by Kupke and Beams (1972).

TABLE I: Coefficients of Eq 7 for Curves of Figure 1.

Coeff of W_3	Curve 1 (H_2O-GdmCl)	Curve 2 (H_2O-RNase-GdmCl)
a_0	$0.998\ 265\ 3 \pm 3.15 \times 10^{-6}$	$1.013\ 362\ 7 \pm 1.02 \times 10^{-5}$
a_1	$0.296\ 306\ 4 \pm 5.10 \times 10^{-4}$	$0.288\ 422\ 9 \pm 5.77 \times 10^{-4}$
a_2	$-0.129\ 821\ 5 \pm 1.50 \times 10^{-2}$	$-0.142\ 976\ 4 \pm 8.89 \times 10^{-3}$
a_3	$0.725\ 210\ 8 \pm 1.77 \times 10^{-1}$	$0.668\ 828\ 0 \pm 5.45 \times 10^{-2}$
a_4	$-2.593\ 957 \pm 1.05$	$-1.598\ 501\ 1 \pm 1.57 \times 10^{-1}$
a_5	$6.683\ 422 \pm 3.42$	$1.997\ 452\ 8 \pm 2.13 \times 10^{-1}$
a_6	$-11.132\ 93 \pm 6.21$	$-0.984\ 268\ 5 \pm 1.10 \times 10^{-1}$
a_7	$10.599\ 57 \pm 5.87$	
a_8	$-4.328\ 860 \pm 2.26$	

variable, the density difference between the two curves at any W_3^0 is viewed at the same mass ratio of GdmCl to water. The data in curve 2 are from measurements using six different stock solutions of RNase in water which varied in weight fraction, W_2^0 [$=g_2/(g_1 + g_2)$], from 0.048 to 0.062 on the basis of dry-weight analyses. The concentrations of these solutions were not adjusted to a common value to avoid evaporation and other error attending such meticulous manipulations. Hence, the points for curve 2 represent the data after normalizing all density values to those for a standard stock solution of weight fraction, $W_2^{0(s)} = 0.05$ before any GdmCl was added. This reduction is justified because the values of ϕ_2 were found to be independent of RNase concentration within the error of the method at various levels of GdmCl over the range encompassed. The raw data for this normalization were treated as outlined in Appendix A. The data points (57 for curve 1 and 117 for curve 2, as normalized) are omitted from Figure 1; the smooth curves are computer-drawn representations based on polynomial regression analyses. Both curves fit the form:

$$\rho = \sum_{i=0}^{N} a_i W_3^i \qquad (7)$$

where the coefficients, a_i, of W_3^i are as listed in Table I. The values of N (8 for curve 1 and 6 for curve 2) were chosen such that the variance was at the first minimum with respect to N. The standard error was $\pm 1.22 \times 10^{-5}$ g/mL for the water-GdmCl series and $\pm 2.64 \times 10^{-5}$ g/mL for the series containing RNase.

With the densities known for any composition of a series, such as the 2 series in Figure 1, the total volume change, ΔV_{mix}, of mixing together assigned masses of any two solutions in a series can be calculated. Details for this procedure are outlined in Appendix B. If ρ is linear with c_2 (curve 2), the method can be applied to any concentration of protein desired; hence, ΔV_{mix} can be calculated for a solution from curve 1 mixed with a solution from curve 2. These volume changes may be useful in constructing other experiments, but they are not dealt with further here.

To illustrate the method of obtaining the volume change upon transporting protein from one concentration of perturbant to another, we may focus on two pairs of points on the two curves of Figure 1. Each pair of points is taken at a specified value of W_3^0. We choose the first pair where no GdmCl is present ($W_3^0 = 0$) and the second pair at $W_3^0 = 0.50$ (~6 M GdmCl). In principle, an amount (50 mg) of RNase is being transported from 1 g of water to another gram of water containing 1 g of GdmCl. Since the total weight fraction, W_2, of RNase can be calculated for the two points in the upper curve (Appendix A), the values of ϕ_2 are given by eq 5 after multiplying W_2 by the density in each case to obtain the respective values of c_2. Hence, the difference, $\Delta\phi_2$, gives immediately the change in volume for carrying 1 g of RNase from water to 6 M GdmCl via eq 6; no other information is required. Obviously, this exercise may be repeated in order to obtain the volume change in transporting the protein component between any of the GdmCl concentrations encompassed in Figure 1. For this purpose, it is convenient to calculate the values of ϕ_2 at suitable intervals over the total range of W_3^0. Figure 2 shows ϕ_2 as a function of W_3^0 (and the molarity of GdmCl in the protein solution). The points represent experimental values, while the smooth curve represents the values of ϕ_2 calculated from the two smoothed curves in Figure 1. The standard error of the data points with respect to the generated curve is ± 0.0008 mL/g.

It is apparent that the specific volume of RNase is generally greater in GdmCl at all concentrations than in water, even after maximal denaturation of the protein in this salt (>4 M). This result may seem surprising in view of the standard explanation that the exposure to water of hydrophobic residues on the protein should cause a contraction in the specific volume. It should be recalled, however, that such volume changes depend on the differences in the various interactions of all components and cannot be predicted on the basis of a single kind of interaction between only the protein and the water. We have observed this increase in ϕ_2 for RNase at denaturing concentrations of GdmCl with a variety of RNase preparations over a period of several years. On the other hand, the addition of β-mercaptoethanol (0.005 to 0.1 M) gave rise to a precipitous drop in the values of ϕ_2 (>−0.013 mL/g) at GdmCl concentrations between about 3 and 6 M. Presumably, the random coil form interacts much more intimately with solvent than do the conformations with intact disulfide bridges.

The concentration range from the crest to the center of the shallow well in Figure 2 (~1.9 to 4 M GdmCl) corresponds with the region defined by Hantgan et al. (1974) and others for the transition to a denatured conformation. (Note: a much deeper well was observed with some preparations which had stood several days after mixing; these values are not included in Figure 2.)

The ΔV for transporting 1 g of the denatured protein from 4 M GdmCl to higher concentrations of the salt increases rather remarkably (~+0.01 mL/g_2 or ~+137 mL/mol of RNase from 4 to 8 M GdmCl). A similar effect was noted also by Skerjanc et al. (1970) for chymotrypsinogen in high concentrations of urea. If the RNase is maximally denatured above 4 M GdmCl, the increase in volume upon transporting this protein from 4 M to higher concentrations of GdmCl might be explained by the loss of electrostricted water to the bulk solvent phase. RNase, however, could not be salted out in saturated GdmCl at 20 °C ($W_3^0 = 0.675$). Copious precipi-

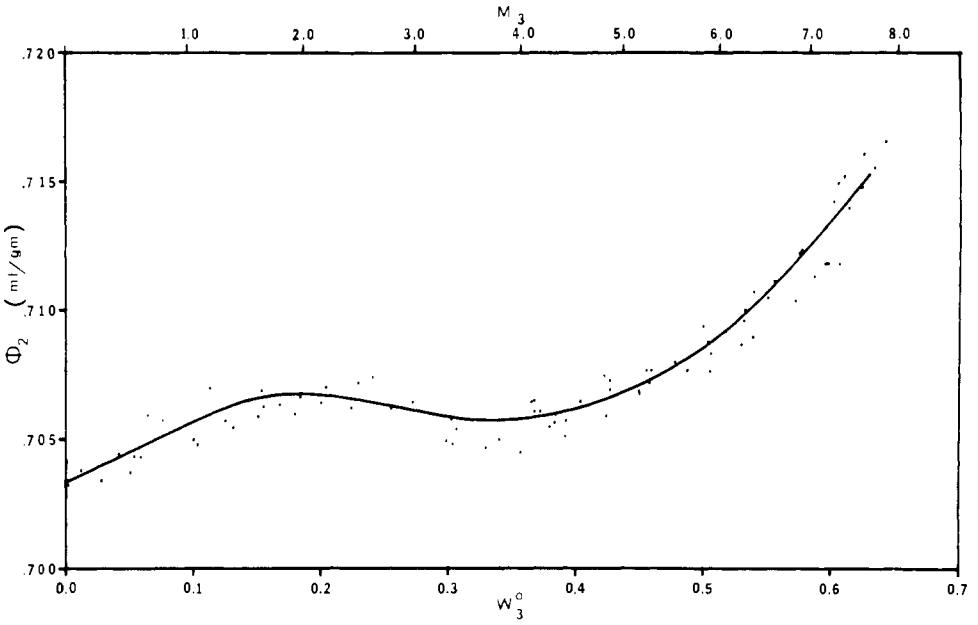

FIGURE 2: Apparent specific volume, ϕ_2, of isoionic ribonuclease A vs. weight fraction, W_3^0, of guanidinium chloride with respect to water, and vs. the molarity, M_3, of guanidinium chloride in the ribonuclease solutions (20 °C). The points represent the values of ϕ_2 calculated from the experimental values of the densities (~30 data points clustering at $W_3^0 \rightarrow 0$ are omitted). The solid curve represents values of ϕ_2 calculated from the smoothed curves of Figure 1.

tation ensued upon reducing the temperature, but the supernate contained nearly all of the RNase. (Indeed, it appeared that the presence of 5% RNase reduced the solubility of GdmCl slightly, relative to the same value of g_3/g_1 in water alone.) Since the values of $\bar{v}$ and ϕ of a component are often composition dependent, the partial volumes of the nonprotein components should be taken into account (see below).

We assume that the values of ϕ_2 in Figure 2 are equivalent to $\bar{v}_2$ at each concentration of GdmCl since no dependence of ϕ_2 on c_2 was apparent experimentally at selected values of W_3. Combining the values of $\bar{v}_2$ and $\bar{v}_3$ for curve 2 of Figure 1 into the relation $\Sigma_i \bar{v}_i c_i = 1$, the values of $\bar{v}_1$ are obtained by difference. Similarly, the values of $\bar{v}_1$ for curve 1 of Figure 1 (the protein-free series) are obtained after calculating $\bar{v}_3$ in that system. The values of $\bar{v}_3$ in each curve were calculated with eq 2 after determining the slopes via computer from the smoothed curves of Figure 1 in which the density values obtained by eq 7 with the coefficients of Table I were used to convert the weight fractions to c_3.[4] Curves showing the variation of $\bar{v}_1$ in the presence and absence of RNase, respectively, as a function of GdmCl concentration are depicted in Figure 3a, and those for the variation of $\bar{v}_3$ in the same two media are shown in Figure 3b. The differences between values of $\bar{v}_1$ and between values of $\bar{v}_3$ at a common value of W_3^0 resulting from the presence of RNase are seen to be too small to warrant confidence in them without further repetitive experiments. In other systems such differences may be more dramatic. Nonetheless, the trends suggest that $\bar{v}_1$ decreases slightly and that $\bar{v}_3$ increases slightly with W_3^0 after RNase is presumably denatured maximally in this system beyond 4 M GdmCl. The apparent divergence between values of $\bar{v}_3$ as $W_3^0 \rightarrow 0$, however, is dealt with presently because, if true, a substantial difference in the interactions of GdmCl in water and in water + RNase is indicated at very low concentrations of this salt. In passing, the polynomial fittings to the curves of Figure 1 become insensitive as the concentration of GdmCl approaches zero; therefore, the curves of Figure 3b lead to an inaccurate representation of the values of $\bar{v}_3$ in the two solvents as $W_3^0 \rightarrow 0$.

The volume change on transporting the guanidine salt from water to water + RNase is expressed as a function of W_3^0 in Figure 4. This volume change per gram of GdmCl is given by the difference in the apparent specific volumes, $\phi_{3(r)} - \phi_3 = \Delta\phi_3$, of GdmCl in the two solvents at any given mass ratio of GdmCl to water (where r refers to the RNase solutions). The value of ϕ_3 in each solvent (water and water + RNase) at a particular value of W_3^0 was calculated according to eq 5 via the densities from eq 7 and Table I and by applying the density of pure water (0.998234 g/mL at 20 °C) and that of the GdmCl-free protein solution (1.013363 g/mL at 20 °C) for the appropriate values of ρ^0. Unfortunately, these volume differences at the higher values of W_3^0 approach the limits of the precision exercised in our experiments. The trend in $\Delta\phi_3$ with W_3^0, however, is essentially accurate, and the values of $\Delta\phi_3$ are of the same degree of reliability as the two smoothed curves which were generated from the experimental values of the two density-composition tables.

The curve in Figure 4 shows that the protein in 1 g of water titrates the guanidinium salt such that the volume increases more at any given value of W_3^0 than when the same amount of this salt is added to 1 g of water alone. These volume differences are greater at the denaturant concentrations which are below the range (~2–4 M) where the unfolding transition takes place. Evidence from NMR spectroscopy shows that various identifiable groups on RNase undergo changes in chemical shift at pretransition concentrations of this salt, as well as at concentrations in the transition region (Benz and Roberts, 1975). For example, the interactions observed by these authors at 0.5–0.7 M GdmCl correspond to the wrinkle in the curve of Figure 4.

The almost constant residual volume difference (~+0.0003

[4] It is often simpler in practice to eliminate c_3 for the calculation of $\bar{v}_3$ by rewriting eq 2 as follows:

$$\bar{v}_3 = \frac{\rho - (1 - W_3)(\partial\rho/\partial W_3)_{m,T,P}}{\rho^2}$$

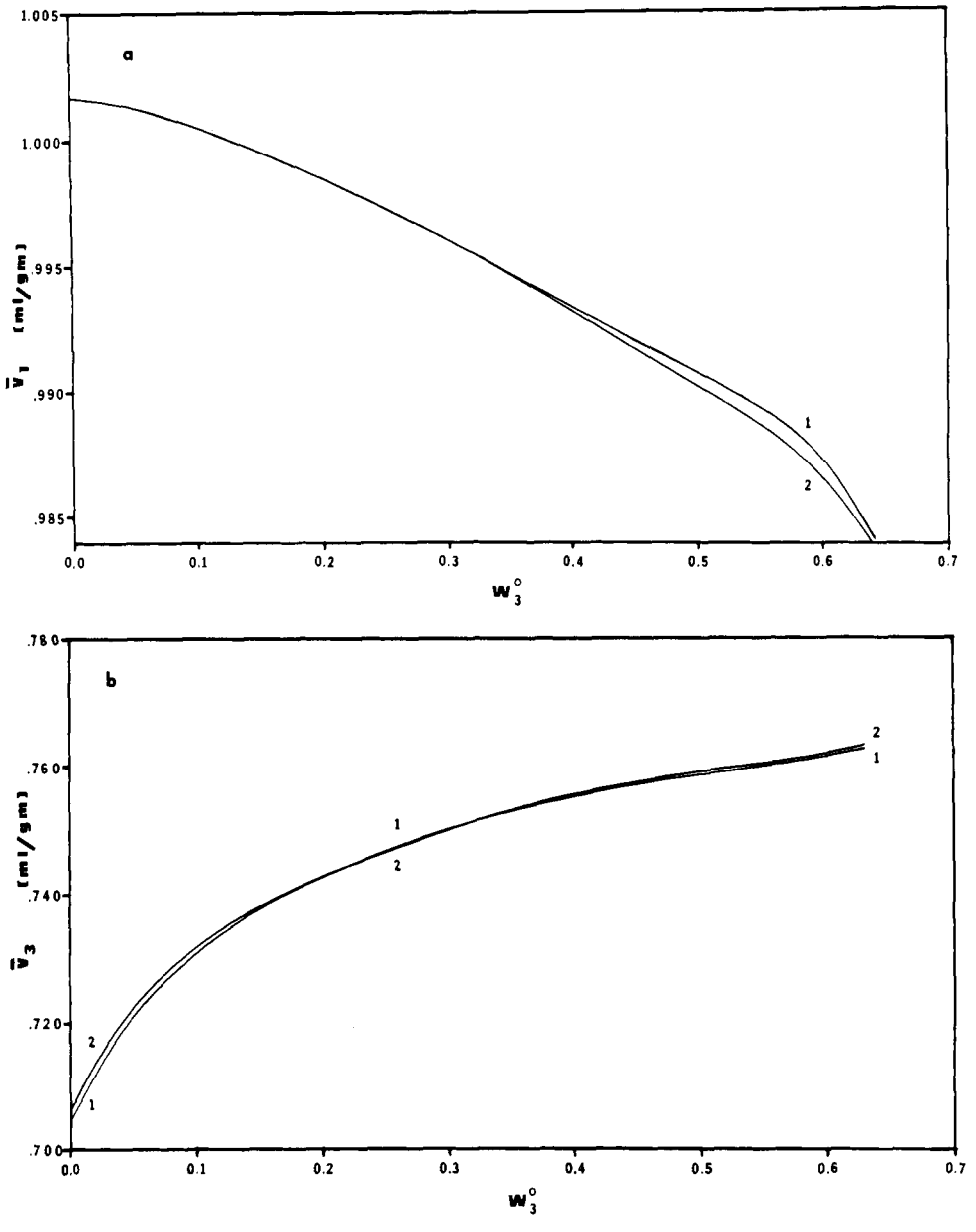

FIGURE 3: (a) Partial specific volume, $\bar{v}_1$, of water vs. weight fraction, W_3^0, of guanidinium chloride with respect to water (20 °C): curve 1, water–guanidinium chloride; curve 2, water–isoionic ribonuclease A–guanidinium chloride. (b) Partial specific volume, $\bar{v}_3$, of guanidinium chloride vs. weight fraction, W_3^0, of guanidinium chloride with respect to water (20 °C): curve 1, water–guanidinium chloride; curve 2, water–isoionic ribonuclease A–guanidinium chloride. The curves in both a and b represent the values of $\bar{v}_i$ calculated from the smoothed curves of Figure 1.

mL/g_3) at all concentrations above 4 M, where the unfolding is presumed to be complete, may not reflect any further interactions of this salt with the protein (the slight peak at W_3^0 = 0.65 may result from the insensitivity of this smoothing procedure at the ends of the two curves in Figure 1). The upward curves of ϕ_3 vs. W_3^0 both in the presence and absence of RNase were essentially parallel in this range (>4 M). Hence, the constant difference in the volume increment after adding a given amount of GdmCl to 1 g of water and after adding the same amount to 1 g of water + RNase at all $W_3^0 > 0.35$ may be viewed in terms of the availability of water. If 1 g of the protein effectively removes 0.50 g of water at all $W_3^0 > 0.35$, the effect is to raise the value of ϕ_3 by the constant amount shown in Figure 4, provided that the protein–denaturant interaction is negligible in this range. Thus, the amount of water removed by the protein when W_2^0 = 0.500 (i.e., g_2/g_1 = 0.0526_3) is 0.50 g_1/g_2 times 0.0526_3 g_2/g_1, or 0.0263 g of water per g of the total water present. Hence, 0.9727 g of water is available for solvating the GdmCl. The reduced amount of available water would raise the effective concentration at, for example, W_3^0 = 0.4000 to 0.4064. The value of ϕ_3 at the latter concentration in the absence of RNase is the same as that found at W_3^0 = 0.4000 in the presence of the RNase. This calculation neglects the effect on ϕ_3 by the comparatively small proportion of GdmCl which may be "bound" to the protein (or more precisely, the amount of GdmCl which is not of the bulk-solvent phase). In these calculations, however, we assume that the additional GdmCl above W_3^0 = 0.35 (>4 M) does not contribute to the "bound" amount. [We estimate the "bound" amount as $0.53g_3/g_2$ (or about 4% of the total GdmCl in the preceding example) by applying the value for the unavailable water, $0.50g_1/g_2$, in combination with the preferential interaction parameter; some values for the latter were determined in this concentration range by the density-equilibrium dialysis

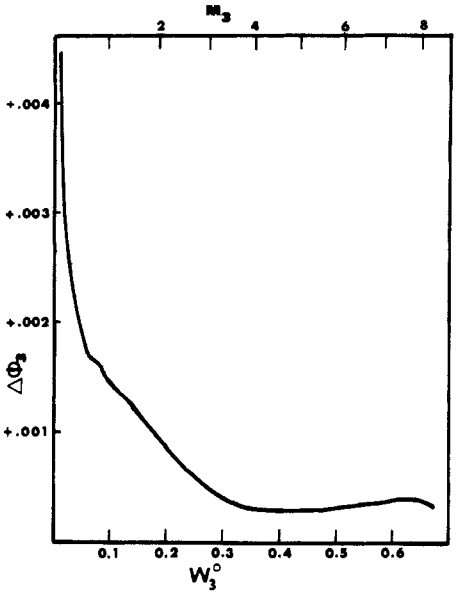

FIGURE 4: The difference in the apparent specific volume, $\Delta\phi_3$ (= $\Delta V/g_3$), of guanidinium chloride vs. the weight fraction, W_3^0, of guanidinium chloride with respect to water upon transporting the salt from water to water + ribonuclease (20 °C). The curve represents the difference in the values of ϕ_3 calculated from the smoothed curves of Figure 1 at a common value of W_3^0. M_3 is the molarity of guanidinium chloride in the ribonuclease solutions for comparing this data with those from other kinds of studies.

method (Kupke and Beams, 1972; Kupke, 1973). This "bound" amount corresponds to about 76 mol of GdmCl per mol of isoionic RNase in the posttransition region, in approximate agreement with conclusions drawn from other kinds of studies (cited in Benz and Roberts, 1975).]

The upward trend in Figure 4 as $W_3^0 \rightarrow 0$, however, suggests that the presence of RNase increases the values of ϕ_3 (and $\bar{v}_3$) at very low concentrations of GdmCl (which is not evident from the differences in $\bar{v}_3$ of Figure 3b). This trend was confirmed by the results of a test with very low concentrations of GdmCl. The values of $\bar{v}_3^0$ were estimated from the limiting slope as $c_3 \rightarrow 0$ at five different concentrations of RNase (c_2 = 0 to 0.048 g/mL). Although true values of $\bar{v}_3^0$ may not have been achieved in these cases, it was clear that the partial specific volume of GdmCl increased significantly with RNase concentration as $c_3 \rightarrow 0$. Over the range in c_2 which was encompassed in this special study, $\bar{v}_3^0$ (apparent) increased linearly from 0.692 to 0.719 mL/g_3, or $d\bar{v}_3^0/dc_2 \simeq +0.56$ mL^2 g_3^{-1} g_2^{-1}. In these experiments, the densities were determined in triplicate, or more, on 10 to 40 different concentrations of the 5 series ranging from ~0.01 to 0.2% GdmCl. The values of $\bar{v}_3^0$ (apparent) were obtained via eq 3 from a linear least-squares fit to the data in each series. The assignment of linearity was arbitrary, but over this short concentration range the substantial differences in the values illustrate sufficiently the increase in $\bar{v}_3^0$ with c_2. A confirmatory aspect is provided by the observation that the individual values of ϕ_3 *increased* as $c_3 \rightarrow 0$ in the four series containing RNase, but *decreased* as $c_3 \rightarrow 0$ in the series containing no RNase (the latter trend is commonly observed for many salts and other small solutes in water). Indeed, individual values of ϕ_3 at a level of ~1 mol of GdmCl per mol of RNase (though subject to greater error in this range) were approximately 0.8 mL/g or even somewhat higher. [An apparent break occurred in plotting ϕ_3 vs. W_3^0 at ~3 mol of GdmCl per mol of RNase; cf. Benz and Roberts (1975).] Thus, a relatively large positive volume change (on the order of +10 mL/mol of GdmCl) attends the transport of GdmCl from water to water + RNase at about equimolar concentrations of GdmCl:RNase. This increase in volume is analogous to that observed when hydrocarbons are transported from water to a nonpolar phase, and suggests that GdmCl spontaneously interacts with the protein phase—the particular interaction decreasing with increasing concentrations of GdmCl. Whether other salts exhibit such increases in specific volume in the presence of about equimolar concentrations of protein is not known. Nonetheless, the titration behavior by a density approach with selected solutes, such as coenzymes, allosteric effectors, etc., may be found useful.

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Appendix A

We wish to convert the observed density of the protein solution at each g_3/g_1 in a series, where only component 3 is varied, to a standard density, $\rho^{(s)}$, which reflects a designated value of g_2/g_1 (grams of protein per gram of water) at any value of g_3/g_1 (or W_3^0). Thus, different stock solutions may be utilized in preparing a density-composition table over a range in component 3 levels without the chore and additional error of attempting to match each stock solution to some common value of W_2^0. The densities of curve 2, Figure 1, were converted to the basis of $W_2^{0(s)} = 0.05$ (or $g_2^{(s)}/g_1 = 0.0526$). The conversion to a standard density assumes that ϕ_2 is constant in c_2 at each value of g_3/g_1, an assumption which should be tested by observing ρ vs. c_2 on new systems. Thus, at constant temperature and pressure, the standard density is given by eq 5, which in linear form is:

$$\rho^{(s)} = (1 - \phi_2\rho^0)c_2^{(s)} + \rho^0 \tag{8}$$

In practice, $c_2^{(s)}$ is given by $\rho^{(s)}W_2^{(s)}$ where $W_2^{(s)}$ at each g_3/g_1 is obtained from the assigned value of $W_2^{0(s)}$ by:

$$W_2^{(s)} = \frac{W_2^{0(s)}(1 - W_2^{0(s)})^{-1}}{1 + g_3/g_1 + W_2^{0(s)}(1 - W_2^{0(s)})^{-1}} = \frac{g_2^{(s)}/g_1}{1 + g_3/g_1 + g_2^{(s)}/g_1} \tag{9}$$

Substituting eq 9 into eq 8 and recalling the definition of $c_2^{(s)}$ gives eq 10.

$$\rho^{(s)} = \rho^0[1 - (1 - \phi_2\rho^0)W_2^{(s)}]^{-1} \tag{10}$$

Values of ϕ_2 need not be determined at each g_3/g_1 in order to obtain $\rho^{(s)}$ since eq 5 can be rewritten:

$$\phi_2 = \frac{1}{\rho^0}\left[1 - \frac{(1 - \rho^0/\rho)}{W_2}\right] \tag{11}$$

whereby eq 10 becomes:

$$\rho^{(s)} = \rho^0\left[1 - \left(1 - \frac{\rho^0}{\rho}\right) W_2^{(s)}/W_2\right]^{-1} \tag{12}$$

In this case ρ and W_2 are the experimentally determined values with the actual stock solutions employed; $W_2^{(s)}$ is given by eq 9 and ρ^0 is the value at g_3/g_1 containing no protein as obtained via eq 7 and the appropriate coefficients (Table I in our case).

In projecting the relationship between the standard density and the weight fraction of component 3 (as in Figure 1), the

standard weight fraction, $W_3^{(s)}$, must be used. The latter is related to the assigned value of $W_2^{0(s)}$ by:

$$W_3^{(s)} = \frac{g_3/g_1}{1 + g_3/g_1 + W_2^{0(s)}(1 - W_2^{0(s)})^{-1}} \quad (13)$$

where each g_3/g_1 is the experimental value and is independent of $W_2^{0(s)}$ (or $g_2^{(s)}/g_1$). In the case of Figure 1, curve 2, the standard density was plotted as a function of W_3^0 instead of $W_3^{(s)}$ for ease of comparing the data with those from the water–GdmCl series (curve 2). W_3^0, being independent of the presence of any component 2, is also given by eq 13 with $W_2^{0(s)}$ preset to zero.

Appendix B

The ΔV of mixing of any two solutions A and B can be determined from the masses, m_A and m_B, which are to be mixed and the densities before and after mixing. The total volume before mixing is the sum of the masses each divided by its density, i.e., $[m_A/\rho_A + m_B/\rho_B]$. Determination of the density, ρ_{AB}, after mixing yields the final volume, $[m_{AB}/\rho_{AB}]$, where AB denotes the mixture. The difference in volume is the ΔV of mixing given by eq 14.

$$\Delta V_{mix} = \frac{m_{AB}}{\rho_{AB}} - \left[\frac{m_A}{\rho_A} + \frac{m_B}{\rho_B}\right] \quad (14)$$

With a regression curve available for a known concentration of protein, W_2^0, or a standard concentration, $W_2^{0(s)}$, such as in curve 2 of Figure 1, ρ_A and ρ_B are obtained directly for the chosen solutions of weight fractions $W_{3,A}$ and $W_{3,B}$, respectively, via eq 7 and the appropriate coefficients. The value of ρ_{AB} for the mixture is found similarly after calculating $W_{3,AB}$ in terms of the designated masses, m_A and m_B, of solutions of weight fraction $W_{3,A}$ and $W_{3,B}$, respectively. Thus:

$$W_{3,AB} = \frac{W_{3,A}m_A + W_{3,B}m_B}{m_A + m_B} \quad (15)$$

Obviously, any number of solutions in any proportion along a regression curve may be used for evaluating the total volume change of mixing if the effect of a perturbant is essentially reversible. In addition, such ΔV can be evaluated for any concentration of protein if ρ is a linear function of c_2 by generating curves at assigned values of $W_2^{0(s)}$ as shown in Appendix A. For mixing a solution from curve 1 with a solution from curve 2 (Figure 1), no further curves need be generated for ΔV_{mix} if $(d\rho/dc_2)_m$ is constant at various W_3^0.

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