Interpretation of Equilibrium Sedimentation Measurements of Proteins in Guanidine Hydrochloride Solutions. Partial Volumes, Density Increments, and the Molecular Weight of the Subunits of Rabbit Muscle Aldolase*

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ABSTRACT: The partial specific volume $\bar{v}_2^0$ at 25° of bovine serum albumin was found to decrease from 0.734 in 0.2 M NaCl to 0.728 in 6 M Gu·HCl–0.1 M β-mercaptoethanol; the apparent volume ϕ', defined by $(\partial\rho/\partial c_2)_\mu^0 = 1 - \phi'\rho^0$ (where $(\partial\rho/\partial c_2)_\mu^0$ is the density increment at constant chemical potential μ of diffusible solutes and ρ^0 is the density of the solvent), equals 0.721 in the latter solvent. For rabbit muscle aldolase in water we found $\bar{v}_2^0$ to be 0.737 and 0.739 at 20 and 25°, respectively. In 3, 4.5, and 6 M Gu·HCl at 25°, $\bar{v}_2^0$ was found to be practically unchanged 0.733, 0.732, and 0.733, respectively; ϕ' increased from 0.703 to 0.714, 0.725, and 0.730 in 3, 5, 6, and 7 M, respectively, of Gu·HCl, 0.1 M β-mercaptoethanol, at 25°. We show that the density increments in Gu·HCl solutions (in conjunction with published values of the equilibrium distribution in the ultracentrifuge) yield the correct molecular weight for bovine serum albumin and molecular weights corresponding to four subunits per native aldolase.

We discuss preferential interaction parameters $\xi_3^0 = (\partial w_3/\partial w_2)_\mu^0$ (subscript 3 refers to Gu·HCl and 2 to the protein component) and show that, in the case of aldolase, ξ_3^0 is positive, but decreases significantly with increasing Gu·HCl concentration. We also present kinetic data on denaturation of bovine serum albumin and aldolase in 6 M Gu·HCl, both in the absence and presence of variable concentration of β-mercaptoethanol, as followed by changes of solution viscosity with time. The sedimentation constant $s_{20,w}$ of aldolase in 0.15 M NaCl (pH 6) was found to be 7.43 in the limit of vanishing enzyme concentration (lowest concentration measured 0.02 mg/ml).

The correct establishment of the number of subunits of mammalian aldolase has, in recent years, been a much debated issue. As late as the middle of 1967 careful chemical evidence (Chan *et al.*, 1967; Sine and Hass, 1967) strongly supported three noncovalently bonded subunits per active enzyme. This stand was modified very soon thereafter (Morse *et al.*, 1967; *cf.* also Sia and Horecker, 1968) in favor of four subunits, now also supported by the extensive evidence of Penhoet *et al.* (1967).

For some years now Tanford and his collaborators have stressed the value of the physicochemical study of protein subunits in concentrated Gu·HCl solutions. On the basis of equilibrium sedimentation measurements Kawahara and Tanford (1966a) evaluated a molecular weight of 158,000 (in 0.15 M NaCl, pH 6) for the native enzyme and values ranging from 36,500 to 43,000 for the subunits (in 6 M Gu·HCl, 0.1 M β-mercaptoethanol). The spread in the latter values is due to the fact that the conventional plots, of Kawahara and Tanford, of ln c *vs.* r^2 (where c is the concentration of protein in grams per milliliter, and r is the distance from the center of rotation) curved away from the r^2 axis, and furthermore values for partial volumes, $\bar{v}$, were estimated only and not experimentally determined. In this, as well as in all the other sedimentation studies in Gu·HCl solutions, considerable effort was spent on the sedimentation measurements themselves, whereas values for partial volumes were usually assumed on the basis of diverse considerations, although it is well known that at high values of ρ^0 (the solvent density) calculation of the molecular weight is extremely sensitive to the value of the partial volume taken. In spite of the uncertainty in subunit molecular weight, Kawahara and Tanford (1966a) made a strong stand for four subunits per active enzyme. This was contested by Schachman and Edelstein (1966) who deduced three subunits from equilibrium sedimentation measurements in 3, 5, and 7 M Gu·HCl (they calculated a subunit molecular weight of 49,500 to 50,000 from their measurements). Lapanje and Tanford (1967) determined a molecular weight of 41,900 by osmotic pressure measurements in 6 M Gu·HCl, and Castellino and Barker (1968) found 42,400 by the same method in a similar solvent; they estimated 39,600–42,200 by equilibrium sedimentation, depending upon the value of the partial volume used.

The evidence for four subunits per native aldolase now appears convincing. We shall show in the following that proper interpretation of the equilibrium sedimentation data in terms of the thermodynamic theory of multicomponent systems (*cf.* Casassa and Eisenberg, 1964) leaves no doubt with respect to the correctness of these conclusions.

The thermodynamic treatment of multicomponent systems takes into account the fact that in such a system preferential interactions of the macromolecular solute component with the solvent or with low molecular weight solutes (present as buffer) makes the use of equations pertaining to simple two component systems inapplicable. The equations for equilibrium sedimentation of compressible multicomponent systems

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can be solved exactly for many situations of practical interest, and may be cast in particularly simple form if certain derivatives of solution density at constant chemical potential (at dialysis equilibrium) of diffusible solutes are considered. Whenever two component theory is used where multicomponent theory is applicable, a conceptual error is made. The practical consequences of this error may usually not be gauged *a priori*. With respect to the study of equilibrium sedimentation of nucleic acids the problem has been recently examined (Cohen and Eisenberg, 1968). With respect to protein solutions consequences from improper use of two component theory in situations requiring multicomponent theory can only be evaluated *a posteriori* from experiments with protein molecules of known molecular weight. Thus Edelstein and Schachman (1967) have shown that correct partial specific volumes and molecular weights of proteins may be obtained by a differential sedimentation equilibrium technique in multicomponent water–protein–salt and heavy water–protein–salt solutions. We have, on the other hand, seen above that the neglect of multicomponent theory in concentrated Gu·HCl solutions, may lead to incorrect molecular weights of proteins; in the present instance the uncertainty in the determination of the molecular weight of the aldolase subunits was sufficient to make a decision between three to four subunits per native enzyme extremely precarious.

In the present study we determined partial volumes and density increments of rabbit muscle aldolase and bovine serum albumin in concentrated Gu·HCl solutions, as well as some other properties of these solutions. We discuss preferential interaction parameters, experimentally determined for some protein systems in 6 M Gu·HCl by Kirby Hade and Tanford (1967) and by Noelken and Timasheff (1967), in relation to the density measurements. Finally, on the basis of the density increments determined by us, and equilibrium sedimentation results reported in the literature, we calculate the molecular weights of bovine serum albumin and aldolase subunits, at various Gu·HCl concentrations. We conclude that the size of the subunits determined by equilibrium sedimentation in concentrated Gu·HCl solutions corresponds to four subunits per native aldolase molecule. We hope the result of the present analysis will be useful toward the unambiguous evaluation of controversial molecular weights by equilibrium sedimentation of other proteins and protein subunits as well.

Materials and Methods

Rabbit muscle aldolase was purchased from C. F. Boehringer und Soehne GmbH (Mannheim, Germany) as crystalline suspension in ammonium sulfate solution. Bovine serum albumin was a crystalline and lyophilized product from Sigma Chemical Corp.

The Gu·HCl used in this study was prepared from guanidine carbonate (Analar BDH) and purified as described by Nozaki and Tanford (1967). Before use it was dried *in vacuo* at 60° over phosphorus pentoxide for 24 hr. Other reagents were the best available commercial products. Dialysis bags (Visking) were thoroughly rinsed and boiled with double distilled water until no absorption at 276 mμ could be observed in the rinsings.

The dialyses were conducted in a cylinder in a continuous slow rotation about the long axis. Efficient equilibration is essential. It was promoted by a large air bubble (inside the bag) which continuously mixed the dialyzed solution (Cohen and Eisenberg, 1968).

Concentrated stock solutions of desalted aldolase and bovine serum albumin were obtained by dissolving the crystalline proteins in conductivity grade distilled water followed by an exhaustive dialysis against several changes of water, in the cold (~4°). When the equilibrating solvent was 0.2 M NaCl, the dialysis was allowed to take place at room temperature for the last few hours. Prior to use the concentrated stock solutions were filtered through 0.8-μ Millipore filters.

Solutions of proteins for density increment measurements at constant chemical potential μ of Gu·HCl were obtained as follows. The crystalline proteins were dissolved in 5 ml (β-mercaptoethanol content as specified in each case) of Gu·HCl solution and filtered through 0.8-μ Millipore filters into dialysis bags. Each experimental run consisted of four protein samples, in the concentration range of 4–11 mg/ml, which were dialyzed with two changes against Gu·HCl solution at room temperature, for 24–30 hr. The pH of protein solutions in Gu·HCl was found to be around 6.

Solutions for partial specific volume determinations were prepared by weighing dried samples of Gu·HCl into tightly closing plastic bottles and adding in the next step appropriate (weighed) amounts of desalted concentrated stock solution of aldolase (of known concentration). Reference solutions were obtained by adding water, in place of protein solution, to the Gu·HCl samples. The amount of water added was so adjusted as to provide a calibration curve for densities of Gu·HCl solutions against molality. Density differences, $\Delta\rho$, were calculated by comparing the densities of protein containing solutions with the densities of reference solutions of identical molality. A similar procedure has been used in the study of the partial specific volumes of DNA in concentrated salt solutions (Cohen and Eisenberg, 1968).

Concentration Determinations. Concentrations of protein solutions in both water and 0.2 M NaCl were determined by use of the extinction coefficients 0.67 for $E^{0.1\%}_{1\text{ cm}}$ of bovine serum albumin at 279 mμ and 0.91 for $E^{0.1\%}_{1\text{ cm}}$ of aldolase at 280 mμ.

Spectroscopic determination of protein concentration in presence of Gu·HCl was based on a careful study of the perturbation of the ultraviolet spectra of protein solutions at various Gu·HCl concentrations. The absorption maximum of the two proteins in Gu·HCl shifted to λ 276 mμ with a concurrent decrease in absorbance A. Solutions of proteins in Gu·HCl for spectroscopic studies were prepared by diluting stock solutions of desalted protein with Gu·HCl and were read against the proper blank. Readings were stable and reproducible over a wide range of protein concentrations over many days; β-mercaptoethanol, if present, did not interfere with spectroscopic measurements, provided its evaporation was prevented, and proper blanks were used.

In conclusion, bovine serum albumin concentrations (in milligrams per milliliter) in the presence of 6 M Gu·HCl were obtained from the readings in this solvent at 276 mμ by multiplying the observed A values by 1.075/0.67. The observed A values of aldolase at 276 mμ in presence of 3, 5, 6, and 7 M Gu·HCl were multiplied by 1.115/0.91, 1.110/0.91, 1.107/0.91, and 1.105/0.91, respectively, to yield the enzyme concentration.

Density measurements were made with the digital precision

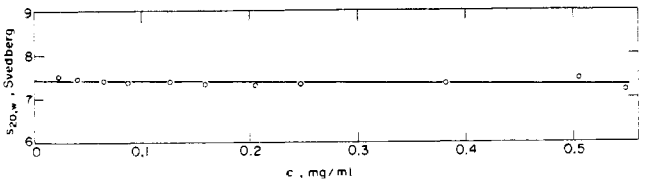

FIGURE 1: Sedimentation coefficients $s_{20,w}$ of rabbit muscle aldolase in 0.15 M NaCl (pH 6) *vs.* protein concentration.

densitometer DMA-02 produced by Anton Paar K. G. (Graz, Austria) according to the design of Stabinger *et al.* (1967). Experimental precision, checked with specially prepared salt samples, was limited to 2×10^{-6} g/ml in density due to the thermostat performance (temperature constancy around 20 and 25° was $\pm 1\text{–}2 \times 10^{-2}$°) and small fluctuations in room temperature. Every run of experiments (four protein samples at different concentrations) was preceded and followed by a check of the calibration constant of the instrument. In the absence of protein, the density difference between Gu·HCl solution inside and outside the dialysis bags was found to be negligible, and no correction for membrane asymmetry was required.

Viscosity measurements were made in Ostwald–Cannon–Fenske-type capillary viscometers with a flow time for water of about 250 sec. The temperature was maintained at 25 ± 0.02°. Protein solutions for viscosity measurements were prepared by diluting the desalted stock solutions of proteins with Gu·HCl solutions. Viscosity values were followed as a function of time.

Sedimentation Measurements. Sedimentation constants of aldolase in 0.15 M NaCl solution (pH 6.0) ware measured in 12- and 30-mm double-sector cells with a Spinco Model E ultracentrifuge equipped with ultraviolet optics, photoelectric scanner, multiplex, and temperature control unit. Sedimentation velocity scans at low aldolase concentrations were taken at 235 and 280 mμ at 20° and at a speed of 40,000 rpm. The reported values of the sedimentation constants were corrected to water at 20°.

Basic Equations. The basic equations used in this work have been derived previously (*cf.* Casassa and Eisenberg, 1961, 1964). The differential expression for the concentration distribution of a macromolecular electroneutral component 2 in a compressible three component system (component 1 is the principal solvent, component 3 is a low molecular weight salt), at constant temperature, at equilibrium in a centrifugal field potential $\omega^2 r^2/2$, at vanishing concentration of component 2, at distance r from the center of rotation of the centrifuge rotor, is given by

$$\mathrm{d} \ln c_2/\mathrm{d}r^2 = (\omega^2/2RT) M_2 (\partial\rho/\partial c_2)_\mu{}^0 \qquad (1)$$

where c_2 is the concentration of component 2 (in grams per milliliter), ω is the angular velocity, ρ is the density of the system, $\mathrm{d}r^2 = 2r\mathrm{d}r$; the subscript μ indicates constancy of chemical potentials of components diffusible through a semipermeable membrane and the superscript zero refers to vanishing polymer concentrations. Temperature is constant throughout this work. The molecular weight M_2 is unambiguously defined in terms of the weight of material included in the definition of c_2. We have specialized above to a rather simple system and more general cases are easily analyzed.

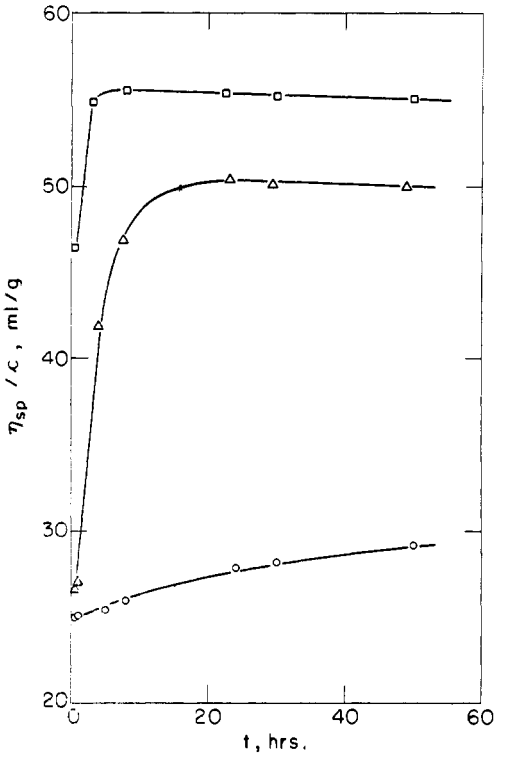

FIGURE 2: Viscosity of bovine serum albumin solutions in 6 M Gu·HCl, at 25°, as a function of time; (○) no β-mercaptoethanol; (△) 0.01 M β-mercpatoethanol; (□), 0.1 M β-mercaptoethanol.

For a monodisperse macromolecular component 2 we can easily derive $M_2(\partial\rho/\partial c_2)_\mu{}^0$ from the slope d ln $c_2/\mathrm{d}r^2$; knowledge of $(\partial\rho/\partial c_2)_\mu{}^0$ or, at low concentrations c_2, the reduced density increment $\Delta\rho/c_2$ at dialysis equilibrium, permits evaluation of M_2. Nothing further need be known.

Preferential interactions are reflected in the values of $(\partial\rho/\partial c_2)_\mu{}^0$; in the three component system under consideration preferential interactions of component 2 with the low molecular weight components may be due to binding of component 1 (hydration), binding of component 3 (Gu·HCl), or Donnan effects due to electrostatic charges residing on the macromolecular. To a good approximation

$$(\partial\rho/\partial c_2)_\mu{}^0 = (1 - \bar{v}_2{}^0\rho^0) + \xi_3{}^0 (1 - \bar{v}_3\rho^0) \qquad (2)$$

$$= (1 + \xi_3{}^0) - \rho^0 (\bar{v}_2{}^0 + \xi_3{}^0\bar{v}_3) \qquad (2a)$$

where $\xi_3{}^0 = (\partial w_3/\partial w_2)_\mu{}^0$ is the weight of component 3 in grams to be added to the solution, per gram of component 2 added, in order to maintain constancy of μ_1 and μ_3; $\bar{v}_2{}^0$ and $\bar{v}_3$ are the properly defined partial specific volumes $(\partial V/\partial w_2)_{P,w_3}{}^0$ and $(\partial V/\partial w_3)_{P,w_2=0}$, respectively, the partial derivatives of the volume with respect to the weight molalities w_2 and w_3 are to be taken at constant pressure, temperature and composition of the solvent. What is usually called the partial specific volume of a protein in concentrated Gu·HCl solutions is an apparent quantity ϕ', defined operationally (*cf.* Casassa and Eisenberg, 1964) in analogy to an equation applicable

TABLE I: Densities and Derived Quantities for Bovine Serum Albumin in 0.2 M NaCl and in 6 M Gu·HCl–0.1 M β-Mercaptoethanol at 25°.[a]

Solvent (M)	ρ^0	$(\partial\rho/\partial c_2)_\mu{}^0$	σ	ϕ'	$\bar{v}_2{}^0$
NaCl (0.2)	1.0053	0.2643	0.0013	0.732	0.734
Gu·HCl (6)	1.1415	0.1759	0.0015	0.722	0.729
Gu·HCl (6)	1.1407	0.1785	0.0010	0.720	0.727

[a] Each set of data consists of four experimental points in the concentration range 4.6–11.2 mg/ml. No trend with concentration was observed. $\partial\rho/\partial c_2$ is the average of four $\Delta\rho/c_2$ determinations within each set; σ is the standard deviation.

to two component systems only

$$(\partial\rho/\partial c_2)_\mu{}^0 = 1 - \phi'\rho^0 \quad (3)$$

The quantity ϕ' is not simply related to a partial volume, and its variation with concentration and solvent density, ρ^0, is not easily predictable. The partial specific volume, $\bar{v}_2{}^0$, at low protein concentrations, is given by

$$(\partial\rho/\partial c_2)_{P,w_3}{}^0 = 1 - \bar{v}_2\rho^0 \quad (4)$$

Results

Viscosity and Sedimentation. Sedimentation and viscosity measurements were undertaken mainly to characterize enzyme solutions and to determine experimental conditions suitable for the density work. We have determined (Figure 1) the sedimentation coefficients of aldolase at concentrations appreciably lower than reported in the literature (*cf.* Kawahara and Tanford, 1966a), in 0.15 M NaCl (pH 6). We find $s^0_{20,w} = 7.43$ S in the limit of zero enzyme concentration, this value decreases to 7.12 at 1 mg/ml. These data are slightly lower than literature reports, but do not seem to support dissociation, under these conditions, of the native enzyme (mol wt $\simeq$ 158,000) to smaller fragments.

We aimed to use the smallest possible concentrations of β-mercaptoethanol consistent with the requirement of complete unfolding of the polypeptide chains in Gu·HCl solutions and prevention of oxidative reaggregation by the formation of sulfur bridges. We followed the change with time of reduced specific viscosity η_{sp}/c, in the case of bovine serum albumin, as well as aldolase. In Figure 2 we show that, in the case of bovine serum albumin high concentration of β-mercaptoethanol (0.1 M), in 6 M Gu·HCl is essential for complete unfolding of the molecule. In the absence of β-mercaptoethanol, η_{sp}/c increases slowly with time; in the presence of β-mercaptoethanol the increase of η_{sp}/c is more rapid, and at 0.1 M of the reagent the viscosity maximum is reached rather quickly, no further increase results at higher β-mercaptoethanol concentration. We find at $c = 4.8$ mg/ml, $\eta_{sp}/c = 55.6$ ml/g, and calculate 56.0 at this concentration from the values of Tanford *et al.* (1967) (they report an intrinsic viscosity $[\eta] = 52.2$ and a Huggins coefficient $k = 0.29$).

In the case of aldolase (Figure 3) a different type of behavior

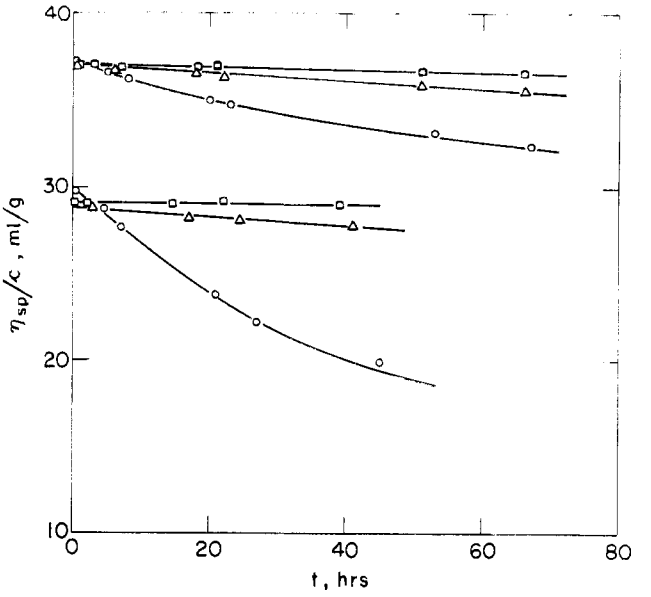

FIGURE 3: Viscosity of rabbit muscle aldolase solutions in 6 M (top curves) and 3 M (lower set of curves) Gu·HCl. at 25°, as a function of time; symbols as in Figure 2.

is observed. In the absence of β-mercaptoethanol the viscosity corresponding to the unfolded chains is almost immediately reached, thereafter η_{sp}/c decreases slowly with time. In the presence of 0.01 and 0.1 M β-mercaptoethanol the decrease of η_{sp}/c with time becomes inconsequential; on the time scale of the density measurements 0.01 M β-mercaptoethanol was found to be adequate. At 6 M Gu·HCl we find at $c = 3.25$ mg/ml, $\eta_{sp}/c = 37.0$ ml/g and calculate 36.7 from the data of Tanford *et al.* (1967) (they find $[\eta] = 35.3$ and $k = 0.35$). In 3 M Gu·HCl η_{sp}/c is somewhat lower (29.1 ml/g at 3.00 mg/ml) which is an indication that 6 M Gu·HCl is a better solvent than 3 M Gu·HCl for this enzyme; already at 3 M Gu·HCl, chain unfolding appears to be essentially complete (Castellino and Barker, 1968).

Densities and Derived Quantities. BOVINE SERUM ALBUMIN. We have determined densities at dialysis equilibrium of solutions of bovine serum albumin in 0.2 M NaCl and in 6 M Gu·HCl, 0.1 M β-mercaptoethanol, aqueous solutions. In this and all further experiments the Gu·HCl concentration is nominal, and the exact concentration is determined by the density of the final solvent in the dialysis. The results of the experiments with bovine serum albumin are summarized in Table I. From the densities the density "increments" $(\partial\rho/\partial c_2)_\mu{}^0$ in column 3 have been calculated. In column 5 we report the quantity ϕ' calculated by the use of eq 3 and in column 6 we list the partial specific volumes $\bar{v}_2{}^0$, calculated by eq 2.

For the calculation of $\bar{v}_2{}^0$ from $(\partial\rho/\partial c_2)_\mu{}^0$ and ρ^0 by eq 2 we require a knowledge of $\xi_3{}^0$ and $\bar{v}_3$; for bovine serum albumin in water we follow the calculation of Casassa and Eisenberg (1961) and find a small increase from 0.732 to 0.734, the value commonly accepted for $\bar{v}_2{}^0$ of bovine serum albumin in water and dilute salt solutions.

The interaction parameters in 6 M Gu·HCl have been measured for bovine serum albumin by Noelken and Timasheff (1967) by refractive index determinations and light-

TABLE II: Densities and Derived Quantities for Rabbit Muscle Aldolase in Water and in Gu·HCl–0.01 M β-Mercaptoethanol, 25° (Except Where Otherwise Indicated).[a]

Solvent	ρ^0	$(\partial\rho/\partial c_2)_{P,m_3}{}^0$	$(\partial\rho/\partial c_2)_\mu{}^0$	σ	ϕ'	$\bar{v}_2{}^0$
1. H_2O, 20°	0.9982	0.2639		0.0005		0.737
2. H_2O, 25°	0.9970	0.2630		0.0005		0.739
3. 3 M Gu·HCl	1.0725		0.2458	0.0030	0.703	
4. 3 M Gu·HCl[b]	1.0735	0.2136		0.0021		0.733
5. 4.5 M Gu·HCl[b]	1.1076	0.1896		0.0020		0.732
6. 5 M Gu·HCl	1.1140		0.2051	0.0031	0.714	
7. 6 M Gu·HCl	1.1413		0.1732	0.0050	0.725	0.734
8. 6 M Gu·HCl[b]	1.1417	0.1661		0.0008		0.730
9. 6 M Gu·HCl[b]	1.1418	0.1622		0.0030		0.734
10. 7 M Gu·HCl	1.1634		0.1484	0.0040	0.732	
11. 7 M Gu·HCl	1.1637		0.1524	0.0020	0.738	

[a] *Cf.* footnote *a* to Table I. [b] No β-mercaptoethanol used in these experiments.

scattering measurements, and for this and other proteins by Kirby Hade and Tanford (1967) by isopiestic distillation. Partial volumes $\bar{v}_3$ of Gu·HCl can be derived from the density measurements of Kawahara and Tanford (1966b) and Kielley and Harrington (1960). Their density measurements are spaced over wide intervals; we determined densities of Gu·HCl solutions in the range 5.97–6.03 M and found $\bar{v}_3$ = 0.763 in this range.[1] In the last column of Table I, $\bar{v}_2{}^0$ has been calculated with the use of $\xi_3{}^0 = 0.064$ as given by Kirby Hade and Tanford (1967). If we take the average $\bar{v}_2{}^0 = 0.728$ of our two sets of experiments, then we find that $\bar{v}_2{}^0$ for bovine serum albumin decreases upon transfer from water to 6 M Gu·HCl by about 0.006 ml/g, which corresponds to about 400 ml/mole of protein; this is the expected decrease (Kauzmann, 1959; Tanford, 1968) but we would like to state that our experiments were not designed to determine this difference with any degree of accuracy. If we use the considerably higher value of $\xi_3{}^0 = 0.18$ as given by Noelken and Timasheff (1967) we find $\bar{v}_2{}^0 = 0.741$, a value apparently too high to be correct. The dilatometric measurements (in the absence of β-mercaptoethanol of Katz (1968) do not extend to high enough Gu·HCl concentration to settle this issue.

For the determination of molecular weights from equilibrium sedimentation in 6 M Gu·HCl we use eq 1; $(\partial\rho/\partial c_2)_\mu{}^0$ should correspond exactly to the density of the solution used in the sedimentation experiment. As such solutions cannot be exactly reproduced we assume that ϕ' does not change appreciably in a small range around 6 M Gu·HCl. We use $\phi' = 0.721$, (the average of the two values given in Table I) and calculate $(\partial\rho/\partial c_2)_\mu{}^0$ by use of the exact value of the density given (we cannot precisely evaluate molecular weights from experiments in which the density is not reported). From the slope d ln c_2/dr^2 and ρ^0 given by Castellino and Barker (1968) we calculate a molecular weight of 65,800 for the unfolded bovine serum albumin peptide chain.

Rabbit Muscle Aldolase. We have determined densities of aldolase solutions under various experimental conditions; the results are summarized in Table II and Figure 4. In row 1 and 2 of Table II we present densities of aldolase in water at two temperatures. From the densities, partial volumes, $\bar{v}_2{}^0$, were calculated by eq 4, applicable to two component systems ($m_3 = 0$). We see that in the range from 20 to 25° $\bar{v}_2{}^0$ increases by 0.002 ml/g. This moderate increase with temperature corresponds closely to the temperature dependence of $\bar{v}$ of some proteins reported by Hunter (1967) and is substantially less than reported by Taylor and Lowry (1956) for aldolase (*cf.* also Sia and Horecker (1968) for partial volumes of aldolase under various conditions of salt concentration and pH).

Measurements in Gu·HCl–0.01 M β-mercaptoethanol are reported in rows 3–11 of Table II. In rows 3, 6, 7, 10, and 11 we report $(\partial\rho/\partial c_2)_\mu{}^0$ at 3, 5, 6, and 7 M Gu·HCl; $(\partial\rho/\partial c_2)_\mu{}^0$ decreases almost linearly with increase in ρ^0 (Figure 4). From $(\partial\rho/\partial c_2)_\mu{}^0$ we have calculated, at each Gu·HCl concentration, the apparent quantity ϕ' by eq 3. It is seen that ϕ' increases somewhat with Gu·HCl concentration. In the last column (row 7) we have calculated $\bar{v}_2{}^0$ of aldolase in 6 M Gu·HCl by eq 2, with the use of $(\partial\rho/\partial c_2)_\mu{}^0$, $\xi_3{}^0 = 0.082$, as determined by Kirby Hade and Tanford (1967) by isopiestic distillation, and $\bar{v}_3 = 0.763$ (*cf.* previous section). Direct sets of determination of $\bar{v}_2{}^0$ from $(\partial\rho/\partial c_2)_{P,m_3}{}^0$ in 3, 4.5, and 6 M Gu·HCl (without addition of β-mercaptoethanol) are reported in rows 4, 5, 8, and 9 of Table II. The results in the last column of rows 8 and 9 are quite close to the result calculated with the help of $\xi_3{}^0$ in the last column of row 7. We see that the decrease of $\bar{v}_2{}^0$ from water (0.739) to 6 M Gu·HCl (average value 0.733) is about 0.006 ml/g (250 ml/mole), which is of the same order as found in the case of bovine serum albumin. It is seen (*cf.* last column, rows 4, 5, and 7–9) that, to within the accuracy of our experiments, $\bar{v}_2{}^0$ does not depend on Gu·HCl concentration in the range 3–6 M.

Molecular Weight of Aldolase Subunit. The molecular weight of the aldolase subunit from equilibrium sedimentation can now be calculated at various Gu·HCl concentrations with the help of the experimental slopes d ln c_2/dr^2, $(\partial\rho/\partial c_2)_\mu{}^0$

[1] In conjunction with the measurements with aldolase we also determined $v_3 = 0.757$ in the range 4.49–4.52 M and $\bar{v}_3 = 0.748$ in the range 3.00–3.07 M of Gu·HCl.

from Table II and eq 1. Considering that the Gu·HCl concentrations in Table II are nominal only, we calculate $(\partial\rho/\partial c_2)_\mu^0$, to be used in eq 1, from ϕ' and the appropriate density ρ^0, with the use of eq 3; alternatively $(\partial\rho/\partial c_2)_\mu^0$ values at given ρ^0 values may be derived from Figure 4.

The following results were obtained. Schachman and Edelstein (1966) report linear plots of ln c_2 *vs.* r^2; from the slopes (M(1 − $\phi'P$)) given by them, 10,070, 7820, and 5590 at 3, 5, and 7 M Gu·HCl (no β-mercaptoethanol used, no densities ρ^0 reported), we calculate mol wt 40,400, 39,500, and 37,300, respectively. From the corresponding linear plot of Castellino and Barker (1968) at 6 M Gu·HCl–0.5 M β-mercaptoethanol, $\rho^0 = 1.15$, we calculate mol wt 37,900.

These values lead rather closely to 4 subunits per native enzyme M = 156,000–160,000. The equilibrium sedimentation plot ln c_2 *vs.* r^2 of Kawahara and Tanford (1966a) is curved (indicating apparent polydispersity) and yields a lower molecular weight (34,300) for the weight average molecular weight with our value 0.725 for ϕ'. It is interesting to note that these authors were the first to deduce correctly four subunits per enzyme, although they used values of ϕ' (0.747–0.735 in 6 M Gu·HCl) appreciably larger than that now experimentally determined by us.

Discussion

Lack of suitable data for $(\partial\rho/\partial c_2)_\mu^0$, or partial volumes and interaction parameters, has sometimes led to an analysis of sedimentation equilibrium data in terms of the dependence of the slope d ln c_2/dr^2 upon solvent density ρ^0 (*cf.* Schachman and Edelstein, (1966).

It was observed that such a plot is often linear, at least over a limited range of densities ρ^0, and experimental data were interpreted in terms of the intercepts (obtained by long extrapolations) at $\rho^0 = 1$ (pure water) and d ln $c_2/dr^2 = 0$ (buoyancy conditions). If we rewrite eq 2 in terms of preferential interaction with respect to component 1, water

$$(\partial\rho/\partial c_2)_\mu^0 = (1 - \bar{v}_2^0\rho^0) + \xi_1^0(1 - \bar{v}_1\rho^0) \quad (5)$$

$$= (1 + \xi_1^0) - \rho^0(\bar{v}_2^0 + \xi_1^0\bar{v}_1) \quad (5a)$$

then we see that (by either eq 2 or 5) to be meaningful these procedures require that all $\bar{v}$'s and ξ's be independent of concentration, or ρ^0. We have previously shown that this is not true for DNA in NaCl and CsCl solutions (Cohen and Eisenberg, 1968). It is not true in the present case (aldolase in Gu·HCl) either, although the plot of $(\partial\rho/\partial c_2)_\mu^0$ is linear in ρ^0, with a negative slope (Figure 4). Differentiation of eq 2 yields

$$-\mathrm{d}\left(\frac{\partial\rho}{\partial c_2}\right)_\mu^0 \Big/ \mathrm{d}\rho^0 = (\bar{v}_2^0 + \xi_3^0\bar{v}_3) - \frac{\mathrm{d}\xi_3^0}{\mathrm{d}\rho^0} \times (1 - \rho^0\bar{v}_3) + \rho^0\left(\frac{\mathrm{d}\bar{v}_2^0}{\mathrm{d}\rho^0} + \xi_3^0\frac{\mathrm{d}\bar{v}_3}{\mathrm{d}\rho^0}\right) \quad (6)$$

We have determined $\bar{v}_2^0$ and $\bar{v}_3$ at three Gu·HCl concentrations. From eq 2 we can now calculate ξ_3^0 which, we find, decreases with increase in Gu·HCl concentration (Figure 5), we do not claim any accuracy in this calculation (which depends on the small difference between $(\partial\rho/\partial c_2)_\mu^0$ and $(1 - \bar{v}_2^0\rho^0)$) but it is interesting to note that the value determined by Kirby Hade and Tanford (1967) agrees with the value deduced here on the basis of an indirect evaluation, the extremely close agreement is most probably coincidental.

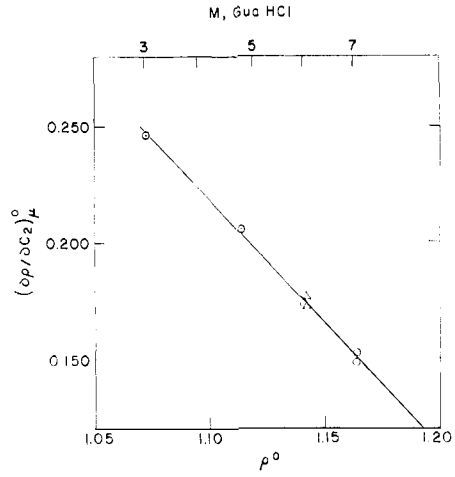

FIGURE 4: Density increments $(\partial\rho/\partial c_2)_\mu^0$ of rabbit muscle aldolase solutions, at 25° as a function of ρ^0; O, experimental points; Δ calculated from $(\partial\rho/\partial c_2)_{P,m_3}^0$ in Table II and eq 2 and 4, with ξ_3^0 of Kirby Hade and Tanford (1967).

The experimental slope $-\mathrm{d}(\partial\rho/\partial c_2)_\mu^0/\mathrm{d}\rho^0$ in Figure 4 equals 1.065; if we assume ξ_3^0, $\bar{v}_2^0$, and $\bar{v}_3$ to be constant, we calculate (from the first term on the right hand side of eq 6) 0.44 for ξ_3^0 at 6 M Gu·HCl (with $\bar{v}_2^0 = 0.733$ and $\bar{v}_3 = 0.763$). This is in complete disagreement with the experimental value $\xi_3^0 = 0.082$ of Kirby Hade and Tanford (1967), and of Figure 5. We must thus consider all terms on the right hand side of eq 6; the first term equals (with $\xi_3^0 = 0.082$) 0.733 + 0.062 = 0.795; for the second term we estimate, from Figure 5, $\mathrm{d}\xi_3^0/\mathrm{d}\rho^0 = -2.0$, and find $-2.0 \times 0.129 = -0.258$ for this term; the contribution of the last term is rather small, with $\mathrm{d}\bar{v}_2^0/\mathrm{d}\rho^0$ negligible and $\mathrm{d}v_3/\mathrm{d}\rho^0 \simeq 0.2$ we find $1.142 \times 0.082 \times 0.2 = 0.019$ for this term. The sum of the three terms

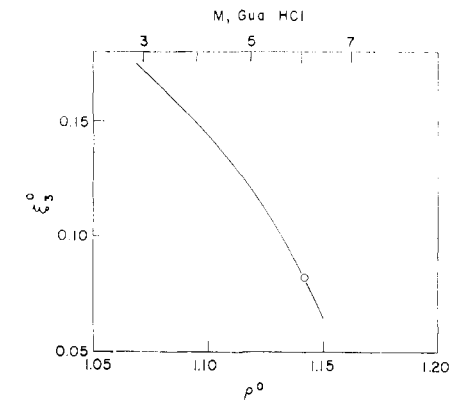

FIGURE 5: Preferential interaction parameter ξ_3^0, as a function of Gu.HCl concentration, for rabbit muscle aldolase, at 25°; full curve, calculated from eq 2, $(\partial\rho/\partial c_2)_\mu^0$ from Figure 4, $\bar{v}_2^0 = 0.733$ (at all Gu·HCl concentrations) and $\bar{v}_3$ interpolated from values as reported; O, from isopiestic distillation data of Kirby Hade and Tanford (1967).

equals 1.072 which is rather close to the experimental slope, 1.065. We have gone into this calculation in some detail in order to emphasize quite clearly that in spite of the linear behavior of $d(\partial\rho/\partial c_2)_\mu{}^0/d\rho^0$ with ρ^0 (or $d \ln c_2/dr^2$ with ρ^0, for that matter) incorrect conclusions may easily be reached.

It is important to point out that in spite of the fact that $(\partial\rho/\partial c_2)_\mu{}^0$ and partial volume determinations are not precise enough for quantitative evaluation of $\xi_3{}^0$ at various Gu·HCl concentrations, the decrease of $\xi_3{}^0$ with increasing Gu·HCl concentration (Figure 5) is a necessary conclusion from the value of the slope $d(\partial\rho/\partial c_2)_\mu{}^0/d\rho^0$ of Figure 4; $d\xi_3{}^0/d\rho^0$ must be significantly negative to raise the calculated slope (right-hand side of eq 6) to the experimental value. We prefer not to involve this formal thermodynamic presentation with customary, but necessarily tenuous molecular interpretations.

We finally conclude that the lack of precise knowledge of partial volumes and interaction parameters in protein Gu·HCl solutions may lead to erroneous results. On the other hand, we have shown that there are no difficulties, either conceptual or practical, to prevent proper analysis of equilibrium sedimentation, density, and derived data, in these multicomponent systems.

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