Examination of the Dissociation of Multichain Proteins in Guanidine Hydrochloride by Membrane Osmometry*

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ABSTRACT: A number of proteins have been examined in dilute aqueous buffers and in concentrated guanidine hydrochloride. Sedimentation coefficients, intrinsic viscosities, and second virial coefficients of these proteins in 6.0 M guanidine hydrochloride–0.5 M mercaptoethanol suggest that all of the proteins are random coils and molecular weight measurements demonstrate that those with subunits are fully dissociated. Number-average molecular weights, determined by membrane osmometry for the native and guanidine hydrochloride dissociated proteins, are: bovine serum albumin, 68,320 ± 600 and 67,790 ± 1100; ovalbumin, 44,620 ± 300 and 46,530 ± 600; horse liver alcohol dehydrogenase (EC 1.1.1.2), 86,000 ± 1750 and 40,790 ± 300; rabbit muscle enolase (EC 4.2.1.11), 82,550 ± 800 and 36,500 ± 200; beef heart lactate dehydrogenase (EC 1.1.1.27), 136,290 ± 1400 and 36,180 ± 800; bovine methemoglobin, 63,720 ± 1100 and 15,840 ± 800; and rabbit muscle aldolase (EC 4.2.16), 156,500 ± 1000 and 42,400 ± 300. These results indicate that the proteins have one, one,two, two, four, four, and four subunits, respectively. For the native proteins weight-average molecular weights obtained by sedimentation equilibrium centrifugation agree well with those obtained by osmometry. The use of sedimentation equilibrium to determine the weight-average molecular weights of the guanidine hydrochloride dissociated proteins is complicated by the necessity of assuming a value of the partial specific volume, $\bar{v}$, for the polypeptide chains in a dissociating medium. However, molecular weights were obtained by this method which were consistent with the results from the membrane osmometry.

The subunit structures of proteins are of interest because of their possible role in biological control and as systems for the study of protein–protein interactions. It has been shown (Tanford *et al.*, 1966, 1967; Nozaki and Tanford, 1967; Lapanje and Tanford, 1967) that when proteins are dissolved in GuHCl–MSH (0.1),[1] dissociation into the component polypeptide chains occurs. The chains appear devoid of all noncovalent structure and exist as random coils.

The number of subunits in a protein has usually been obtained by determining its molecular weight by sedimentation equilibrium in the ultracentrifuge before and after dissociation into component polypeptide chains. The molecular weight calculated from centrifugation studies is dependent upon the value of the partial specific volume, $\bar{v}$, of the protein in the solvent used. For native proteins in dilute salt solutions the value of $\bar{v}$ is usually known or can be measured with sufficient accuracy to allow the calculation of useful weight-average molecular weights, M_w. However, few values of $\bar{v}$ have been measured in concentrated GuHCl and there are conflicting reports on the effect of high concentrations of GuHCl on $\bar{v}$. Since 6 M GuHCl has a high density, small inaccuracies in $\bar{v}$ produce a relatively large error in the calculated molecular weight. For example, in the case of aldolase sedimentation equilibrium centrifugation in GuHCl–MSH (0.1) has led to values of M_w for the subunits of approximately 40,000 (Kawahara and Tanford, 1966) and 50,000 (Schachman and Edelstein, 1966) which, when compared with native molecular weights of 160,000 and 150,000, indicate four or three polypeptide chains per molecule of native enzyme. The difference in subunit molecular weight in the studies cited lies in the values used for $\bar{v}$ (0.747 and 0.775 cc/g) rather than in the experimental results. The value of $\bar{v}$ in dissociating media depends upon the effect of unfolding on the value of $\bar{v}$ and on preferential interactions between the protein and components of the solvent system. Although methods are available to determine the direction and extent of these interactions (Schachman and Edelstein, 1966; Hade and Tanford, 1967; Noelken and Timasheff, 1967) there is still no clear-cut agreement as to their value for those proteins which have been examined by the various procedures.

Because of our interest in aldolase and the apparent difficulty in determining its molecular weight and the number of subunits in the molecule, we have attempted to determine these values using membrane osmometry. This method does not require knowledge of $\bar{v}$, only the experimentally determinable protein concentrations and osmotic pressures of the solutions are required. To provide an adequate comparison, we have examined six

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[1] GuHCl–MSH (0.1) refers to 6.0 M guanidine hydrochloride–0.1 M mercaptoethanol. GuHCl–MSH (0.5) refers to 6.0 M guanidine hydrochloride–0.5 M mercaptoethanol. All other abbreviations are as listed in *Biochemistry 5*, 1445 (1966).

other proteins in the native state and in GuHCl–MSH (0.5) solution using sedimentation equilibrium centrifugation, membrane osmometry, and viscosity determinations. In addition, we have attempted to determine the limitations of the membrane osmometry method using a commercially available instrument.

Materials and Methods

Proteins. Ovalbumin and bovine methemoglobin were obtained as lyophilized samples from Dr. H. B. Bull.

Bovine serum albumin and horse liver alcohol dehydrogenase (specific activity 2.2 units/mg) were purchased in an essentially salt-free, lyophilized form from Worthington Biochemicals and used without further purification.

Rabbit muscle aldolase (specific activity 10 μmoles of FDP/min per mg), rabbit muscle enolase (specific activity 27 units/mg), and beef heart lactate dehydrogenase (specific activity 360 units/mg) were purchased as ammonium sulfate suspensions from Boehringer-Mannheim Corp. For experiments using GuHCl–MSH the proteins were exhaustively dialyzed against water and lyophilized.

Guanidine hydrochloride was purchased from Eastman Organic Chemicals. A total of 500 g was dissolved in 1.2 l. of boiling absolute ethanol and decolorized with charcoal. The ethanol solution was allowed to cool and the crystals were collected after 1 day. The guanidine hydrochloride was then recrystallized from absolute ethanol.

Sedimentation equilibrium studies were performed as described by Yphantis (1964) using a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics and a temperature control unit. An AN-D rotor was used at speeds above 20,000 rpm and an AN-J rotor at lower speeds. The Rayleigh patterns were photographed on Kodak spectroscopic plates, emulsion type II-G, with a Kodak type 77-A filter, placed directly over the light source. The cell used was a simple double-sectored cell equipped with sapphire windows. All experiments were performed at 20°.

With the exception of native bovine methemoglobin, native proteins were dissolved in the solvent to be used and dialyzed against the same solvent. The dialysate was used as the reference solvent. Fluorocarbon FC-43 was used as a base fluid to give a transparent cell bottom. Column heights of 3 mm were used and the rotor speed was chosen so that at equilibrium the meniscus concentration would be essentially zero and the effective reduced molecular weight, σ, would be approximately 5 cm^{-2} (Yphantis, 1964). Equilibrium times were approximated from $t_{eq} \backsim 2.3(b - a)/\omega^2 sr$ (Svedberg and Pedersen, 1940), where $b - a$ is the column height, ω is the angular velocity of the rotor, s is the observed sedimentation coefficient, and r is the distance from the center of rotation to the cell bottom. However, all runs were allowed to proceed until there was no further increase in fringe displacement with time. Following each run, a water blank was run without disassembling the cell to correct for minor effects of cell window distortions.

Interference patterns were measured with a Nikon two-dimensional comparator. The plate was carefully aligned in the comparator x coordinate and the displacement of any five fringes was measured along the y coordinate at intervals of the x coordinate. The results of the selected five fringes were averaged. The blank was evaluated in a similar fashion to obtain the results of an average fringe. The natural logarithms of the differences between the blank-corrected fringe displacements and the base line, ln D, were plotted against the position in the cell, x^2. When the meniscus concentration is zero, the weight-average molecular weight, M_w, was calculated as described by Yphantis (1964) (eq 1), where R is the

$$M_w = \frac{2RT}{\omega^2(1 - \bar{v}\rho)} \frac{d \ln D}{dx^2} \quad (1)$$

gas constant expressed as 8.314×10^7 ergs deg^{-1} $mole^{-1}$, ω is the angular velocity of the rotor, ρ is the solvent density, and $\bar{v}$ is the partial specific volume of the protein. If the ln D *vs.* x^2 plot is linear, then the molecular weight calculated by eq 1 is the only molecular weight of the original sample. If the plot is curved, an averaging technique must be used to obtain the average molecular weight of the original sample, *i.e.*, before redistribution has occurred.

Sedimentation velocities were measured in a Spinco Model E analytical ultracentrifuge, using schlieren optics. For all proteins except bovine methemoglobin, a Kodak no. 16 filter was placed directly over the light source and Kodak metallographic plates used for photography. In the case of bovine methemoglobin, a Kodak no. 25 filter and Kodak type 1-N spectroscopic plates were used. A single-sector cell with quartz windows was used for all native proteins and a double-sectored synthetic boundary cell with quartz windows was used for proteins in GuHCl–MSH (0.5). Sedimentation velocity experiments were run at 25°. Sedimentation coefficients are corrected to the density and viscosity of water at 20°.

Viscosities were determined at 25.0 ± 0.2°. The intrinsic viscosities of native and GuHCl–MSH (0.5)-dissociated rabbit muscle enolase, bovine serum albumin, and ovalbumin were performed with a Cole-Parmer falling-ball viscosimeter. The time of descent in water for a ball with a density of 2.53 g/cc was 120 sec. All other viscosities were measured using an Ostwald capillary viscosimeter with an outflow time of approximately 100 sec for water. Intrinsic viscosities of proteins were obtained by extrapolating the reduced viscosity to zero protein concentration (Huggins, 1942).

Osmometry. Osmotic pressures were obtained using a Hewlett-Packard high-speed membrane osmometer. The molecular weights of the native protein were determined at 25 ± 0.2°. However, in GuHCl the osmometer equilibrated very slowly and bubbles frequently formed in the membrane and capillary necessitating discontinuation of the experiment. The sluggishness was eliminated by addition of 0.001% detergent (Sparkleen) to the buffer and bubble formation was reduced by lowering the temperature to 5 ± 0.2°.

Deacetylated acetylcellulose membranes having molecular weight retention values of 20,000 (Hewlett-Packard Co., type B-19) were used. Studies on dissociated methemoglobin were performed using membranes with molecular weight retention ∽ 10,000 (Arro Co., type B-20). Prior equilibration of the membranes is critical. For experiments with native proteins, the membranes were placed for 24 hr in the buffer solution which was to be used, warmed to ∼10° above the temperature of the osmometer, and degassed for 5 min at water-aspirator pressure. The membranes were then installed in the instrument. For studies in GuHCl–MSH the membranes were first placed in distilled water for 24 hr, then successively in 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 M GuHCl solutions for 5 hr each. The membranes were then equilibrated in the GuHCl–MSH solution which was to be used in the osmometer for 24 hr. The quality of a given membrane preparation was determined by both the bubble rise time in the capillary (time required for the bubble to rise from the trap to the light source) and the value of the base line, P_0. A normal bubble rise time is 10–25 min, the longer times are required in GuHCl solutions and the shorter times in dilute buffers. The absolute value of the base line is a very important criterion of the adequacy of the setup. The value of P_0 in the experiments reported here was between 16.85 and 17.80 cm. Values much above 17.80 cm indicate leakage somewhere in the system and values much below 16.85 indicate trapped air bubbles on the solvent side of the membrane. In either event, continuation of the experiment was found to lead to erroneous results and the run was discontinued.

The osmometer was always operated in the reverse mode. With the bubble at the bottom of the capillary, the solvent reservoir was run to the top of the elevator. The selector control was switched to the *DET* position and the bubble rise was observed in the capillary. When the amperage rose above an arbitrarily fixed reference value, the selector switch was turned to the *RUN* position and the control switch to reverse. In this mode of operation the optical system of the osmometer responds to movements of the lower meniscus of the bubble rather than the top meniscus. The reverse mode of operation offers an advantage over the normal mode of operation. With small bubbles, rapid pressure changes can cause the bubble to completely overshoot the control position. When this occurs in the normal mode of operation, the servo system drives the bubble to the membrane clamp from which it cannot be recovered. In the reverse mode, the servo system drives the bubble to the bubble trap from which it is easily recovered.

When the osmometer was properly set up, the base-line value can be reproduced with a precision of at least ±0.08 cm. Following the establishment of a base line, the instrument was charged with a series of protein solutions in order of increasing concentration. The base-line value, P_0, subtracted from the equilibrium value, P, gives the osmotic pressure of a particular protein concentration. The base line was repeated after every third protein sample to determine whether the membrane was changing during the experiment. No significant alteration in the value of P_0 was observed.

The number-average molecular weight of a protein was determined by the equation

$$M_n = \frac{RT}{\left(\frac{\pi}{c}\right)_{c=0}} \tag{2}$$

The gas constant, R, is expressed in terms of the particular buffer used and has a value of $84.7/\rho$ cc cm mole^{-1} deg^{-1}, where ρ is the density of the buffer at the temperature of the experiment. The value of $(\pi/c)_{c=0}$ was obtained using a linear least-squares analysis of the data with π/c as the dependent variable and c in grams per liter as the independent variable. Densities of the solvents were obtained using Gay-Lussac specific gravity bottles in conjunction with a circulating water bath maintained at constant temperature.

A general equation relating osmotic pressure to molecular weight is

$$\pi/c = RT(1/M + BC + Cc^2 \ldots .) \tag{3}$$

Values for the second virial coefficients, B, were calculated from the slope of the plots of π/c *vs.* C by linear least-squares analysis of the data using eq 4.

$$B = \frac{\Delta(\pi/c)}{\Delta c}\frac{1}{RT} \tag{4}$$

Preparation of Solutions. For sedimentation equilibrium studies the protein was dissolved in the buffer to be used to a final concentration of 0.05–0.10 mg/ml and dialyzed against several changes of the same buffer. The last dialysate served as the reference. The conditions used for native proteins were: (a) bovine serum albumin, 0.1 M potassium phosphate–0.1 M potassium chloride (pH 6.0); (b) ovalbumin, 0.1 M potassium phosphate–0.1 M potassium chloride (pH 6.0); (c) rabbit muscle enolase, 0.1 M Tris-HCl–0.05 M magnesium chloride (pH 6.8); (d) horse liver alcohol dehydrogenase, 0.1 M potassium phosphate–0.1 M potassium chloride (pH 7.0); under these conditions no zinc is lost from the protein (Vallee and Hoch, 1957); (e) beef heart lactate dehydrogenase, 0.1 M potassium phosphate–0.1 M potassium chloride (pH 6.8); (f) bovine methemoglobin, 0.05 M potassium phosphate (pH 7.5) (no dialysis); and (g) rabbit muscle aldolase, 0.1 M Tris-HCl–0.1 M mercaptoethanol (pH 6.8), 0.15 M glycylglycine–0.1 M mercaptoethanol (pH 7.5), 0.1 M sodium acetate–0.1 M mercaptoethanol (pH 5.5), 0.1 M potassium phosphate (pH 7.0), and 0.1 M potassium phosphate–0.1 M sodium chloride–0.01 M mercaptoethanol (pH 6.5).

For sedimentation equilibrium studies in dissociating solvents alcohol dehydrogenase was dissolved in 6.0 M GuHCl, all other proteins were dissolved in 6.0 M GuHCl–0.5 M MSH (pH 6), except for bovine methemoglobin which was run at pH 7.5. The solutions were made 0.01 M in EDTA for enolase and methemoglobin.

Osmometry and intrinsic viscosity experiments were conducted using the same solvents as used for sedi-

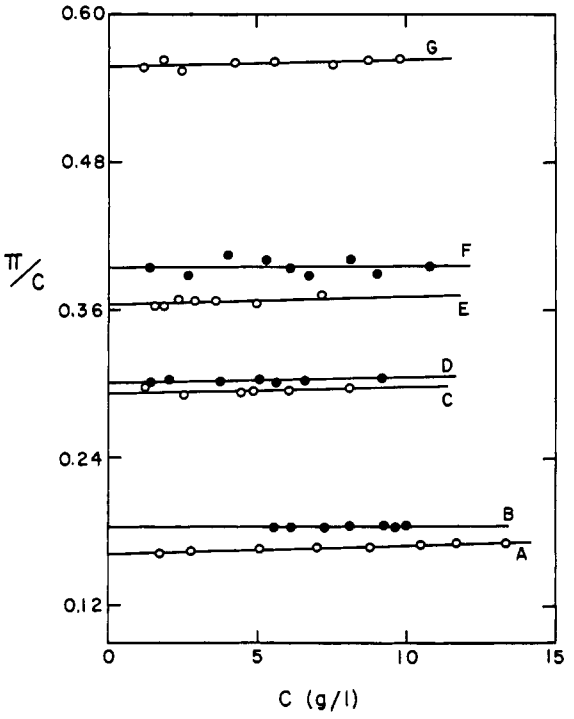

FIGURE 1: Plots of π/c *vs.* c for native proteins. (A) Aldolase, (B) lactate dehydrogenase, (C) enolase, (D) alcohol dehydrogenase, (E) serum albumin, (F) methemoglobin, and (G) ovalbumin.

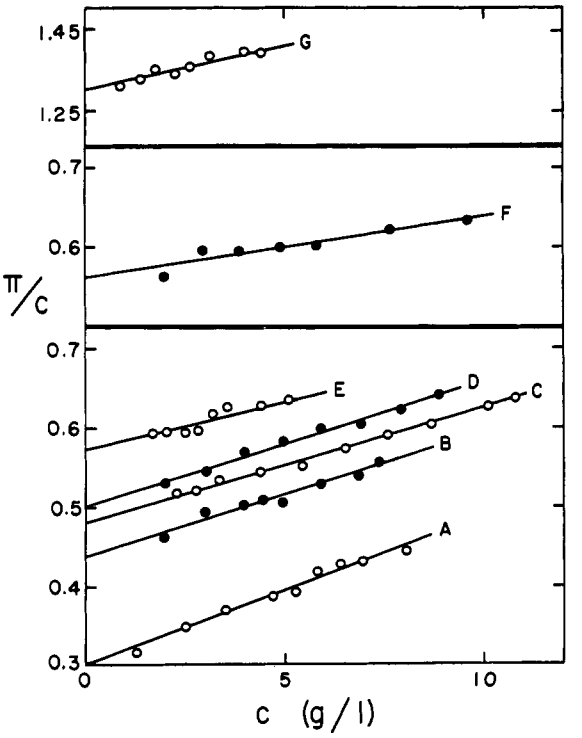

FIGURE 2: Plots of π/c *vs.* c for proteins in GuHCl–MSH (0.5). (A) Serum albumin, (B) ovalbumin, (C) aldolase, (D) alcohol dehydrogenase, (E) lactate dehydrogenase, (F) enolase, and (G) methemoglobin.

mentation equilibrium. For native ovalbumin, serum albumin, and alcohol dehydrogenase, the lyophilized protein was dissolved in buffer and exhaustively dialyzed against the same buffer. For native aldolase, enolase, and lactate dehydrogenase, the solutions were freed of ammonium sulfate by centrifugation and dialysis against the selected buffer. In all cases the final dialysate was used to equilibrate the osmometer. Native methemoglobin was weighed and dissolved in buffer. Buffer concentrations and methemoglobin weights were adjusted for the amount of H_2O in the lyophilized sample.

Guanidine hydrochloride dissociated protein solutions used for osmometry and intrinsic viscosity studies were prepared by slowly adding a carefully weighed sample of the lyophilized protein to the same GuHCl–MSH solution as used for the sedimentation equilibrium experiments and adjusting to the desired final volume with the same solvent. The amount of water in each lyophilized protein was determined by weighing a sample of the protein before and after drying in a vacuum oven at 107° for 48 hr. Corrections were made for the effect of this added water on the concentration of GuHCl–MSH and protein.

Sedimentation velocity experiments were performed on protein solutions which had been used in the osmometer.

For osmometry and viscometry on native proteins concentrations were determined by measuring the optical density of the protein solution and preparing a series of dilutions. The following extinction coefficients $E_{1\text{ cm}}^{0.1\%}$ at 280 mμ were used: serum albumin, 0.680; ovalbumin, 0.735; alcohol dehydrogenase, 0.455; enolase, 0.90; lactate dehydrogenase, 1.492; and aldolase, 0.938.

Partial Specific Volumes. For native proteins the following $\bar{v}$ at 20° were used: serum albumin, 0.729 (Dayhoff *et al.*, 1952); ovalbumin, 0.744 (Dayhoff *et al.*, 1952); enolase, 0.747 (Holt and Wold, 1961); alcohol dehydrogenase, 0.75 (Ehrenberg and Dalziel, 1958); lactate dehydrogenase, 0.740 (Appella and Markert, 1963); and aldolase, 0.742 (Taylor and Lowry, 1956). A value of 0.75 was assumed for bovine methemoglobin analogous to that for other hemoglobins (Kirshner and Tanford, 1964). When a value of $\bar{v}$ had not been determined at 20°, an increase of 0.001 cc/g per deg was assumed (Taylor and Lowry, 1956). However, minor variation in this parameter does not significantly effect the results. On the other hand, variations in the value of $\bar{v}$ of proteins in GuHCl–MSH have a great effect on the value of the molecular weight because of the higher density of the solvent. Precise literature data on the effect of GuHCl–MSH on $\bar{v}$ is lacking. Kielley and Harrington (1960) found a change of 0.01 cc/g in the $\bar{v}$ of myosin in GuHCl. However, little change of $\bar{v}$ GuHCl for a number of proteins has been reported (Reithel and Sakura, 1963). Hade and Tanford (1967) determined that when a number of proteins, including serum albumin and aldolase, are dissolved in 6.0 M GuHCl, preferential binding of GuHCl to the polypeptide chains occurs. This has the effect of lowering the $\bar{v}$ from the native value. Preferential binding of GuHCl to serum albumin was also shown by Noelken

TABLE I: The Molecular Weights of Native and Dissociated Proteins and the Number of Subunits as Determined by Osmometry.

Protein[a]	Solvent Density (g/cm³)	$RT \times 10^{-4}$ (cm 1 mole^{-1})	π/c (cm l. g^{-1})	M_n	Subunits ±3%
Serum albumin	1.012	2.4941	0.365 ± 0.003	68,320 ± 600	1.0
Serum albumin + G	1.150	2.0475	0.302 ± 0.005	67,790 ± 1,000	
Ovalbumin	1.012	2.4941	0.559 ± 0.003	44,620 ± 300	1.0
Ovalbumin + G	1.150	2.0475	0.440 ± 0.006	46,530 ± 600	
Alcohol dehydrogenase	1.012	2.4941	0.290 ± 0.006	86,000 ± 1,750	2.1
Alcohol dehydrogenase + G	1.150	2.0475	0.502 ± 0.003	40,790 ± 300	
Enolase	1.009	2.5015	0.303 ± 0.003	82,550 ± 800	2.3
Enolase + G	1.150	2.0475	0.561 ± 0.003	36,500 ± 200	
Methemoglobin	1.008	2.5040	0.393 ± 0.007	63,720 ± 1,100	4.0
Methemoglobin + G	1.150	2.0475	1.293 ± 0.006	15,840 ± 800	
Lactate dehydrogenase	1.012	2.4941	0.183 ± 0.002	136,290 ± 1,400	3.8
Lactate dehydrogenase + G	1.150	2.0475	0.566 ± 0.013	36,180 ± 800	
Aldolase	1.008	2.5040	0.160 ± 0.001	156,500 ± 1,000	3.7
Aldolase + G	1.150	2.0475	0.483 ± 0.003	42,400 ± 300	

[a] Sources of proteins and buffer concentrations are described under Materials and Methods. G = proteins in GuHCl–MSH (0.5).

and Timasheff (1967). However, Schachman and Edelstein (1966) found that aldolase preferentially bound H_2O when dissolved in GuHCl solutions. This would increase $\bar{v}$ over the native value. We feel that the weight of the evidence, including this study, supports the finding that the $\bar{v}$ of proteins in GuHCl–MSH should be either unchanged or slightly decreased when compared with the value for the native protein. The molecular weights of all proteins in GuHCl–MSH (0.5) are reported as a range of values assuming a $\bar{v}$ which is unchanged and a $\bar{v}$ which is decreased by 0.01 cc/g (maximum decrease observed).

Results

Osmometry. Typical data from the Mechrolab osmometer are plotted in Figures 1 and 2. Values for $\pi/c_{c=0}$ were obtained by linear least-squares analysis of the data plotted. The points shown are averages of two experimental values obtained on duplicate samples during a single experiment. In no case did the values differ by more than 0.08 cm and an average difference was 0.03 cm. Values of M_n for the native and dissociated proteins, solvent densities, and number of subunits are given in Table I.

Sedimentation velocity photographs obtained on dissociated protein solutions used in the osmometer are shown in Figure 3. In all cases a single symmetrical peak is present. The values of sedimentation coefficients of native and dissociated proteins corrected to the density and viscosity of water at 20° ($s_{20,w}$) are given in Table III.

Sedimentation Equilibrium. Plots of ln D *vs.* x^2 for proteins in ordinary buffers and in GuHCl–MSH (0.5) are shown in Figures 4–7. Each point is the difference between the average of readings made on five fringes and the average of readings made on five fringes of the water blank at the same value of x. Each reading could be reproduced within ±7 μ and the values plotted have a maximum standard deviation of ±8 μ. Only readings having a displacement of more than 50 μ above the base line were used to compute molecular weights. Values for M_w in ordinary and dissociating media and the range of subunits are given in Table II.

Intrinsic viscosities, $[\eta]$, of all protein solutions are listed in Table III. Outflow times were reproduced within 0.3%.

Second virial coefficients, B, were obtained by linear least-squares analysis of the slopes of the lines in Figures 1 and 2 as described in Materials and Methods. The results for native and dissociated proteins are given in Table III.

Discussion

Kawahara and Tanford (1966) reported that sedimentation equilibrium centrifugation of rabbit muscle aldolase in GuHCl–MSH (0.1) indicated that the native molecule consisted of four subunits, two each of mol wt 47,000 and 32,000. Prior to that time, data from many laboratories had supported the view that the enzyme consisted of three very similar subunits. Since Kawahara and Tanford's report a number of observations have been made supporting the four-subunit nature of the enzyme. The conflicting literature reports are summarized in Table IV.

It was our purpose in this study to determine the number of subunits in rabbit muscle aldolase by os-

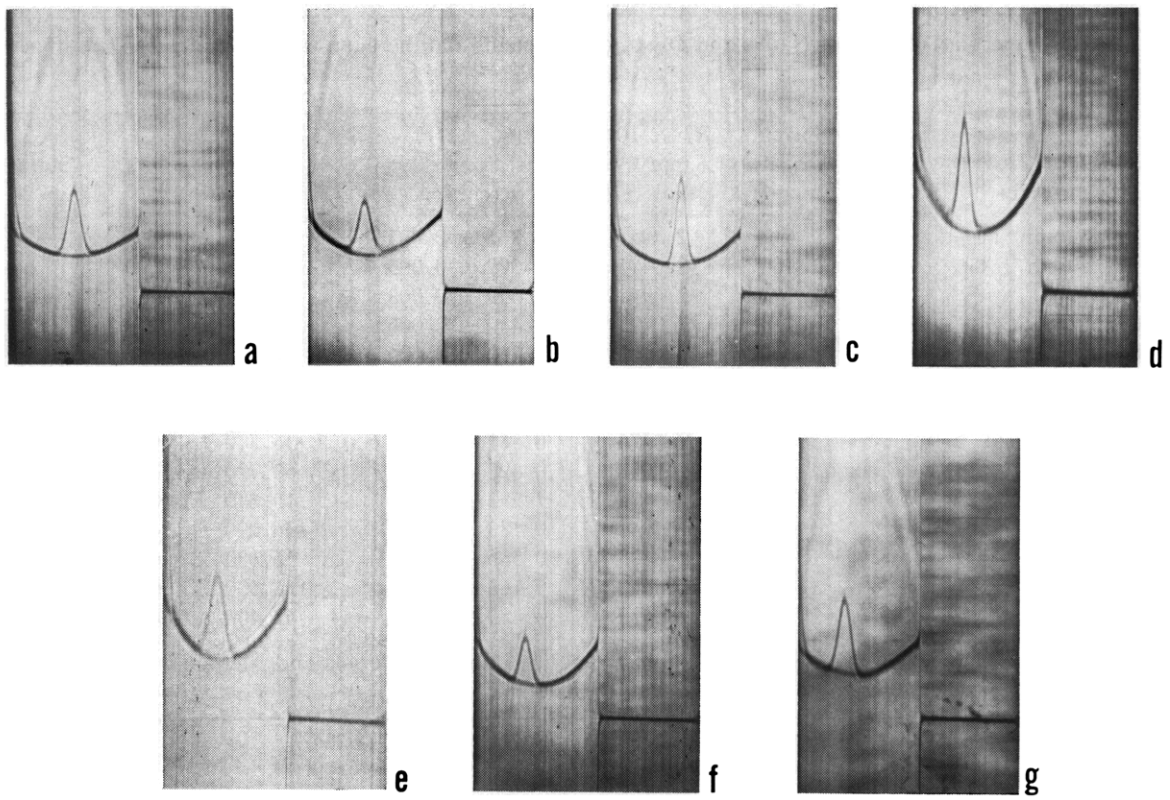

FIGURE 3: Sedimentation velocity photographs of proteins in GuHCl–MSH (0.5). In all cases a synthetic boundary cell was used. The times given represent the time after the centrifuge was taken to 59,780 rpm. (A) Serum albumin, 40 min; (B) ovalbumin, 16 min; (C) alcohol dehydrogenase, 16 min; (D) enolase, 16 min; (E) methemoglobin, 32 min; (F) lactate dehydrogenase, 16 min; and (G) aldolase, 16 min. The direction of sedimentation is right to left.

mometry since this method, unlike ultracentrifugation, does not require knowledge of $\bar{v}$ for the protein in dissociating media. Using membranes which retain only the macromolecule, and extrapolating all observations to zero protein concentration, the response of the instrument to a protein in a dissociating medium should increase over its response to the protein in a nondissociating medium by a factor equal to the number of subunits produced. To provide an adequate evaluation of the method several "standard" proteins have been examined. In addition, all protein solutions were examined by sedimentation equilibrium centrifugation

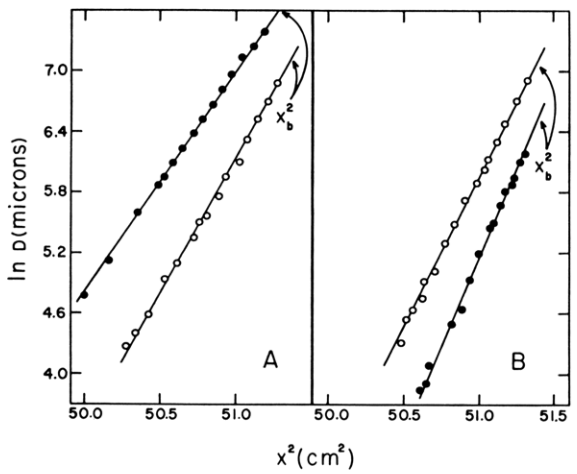

FIGURE 4: Plots of ln D *vs.* x^2. (A) Native ovalbumin (○), 33,450 rpm, and ovalbumin in GuHCl–MSH (●) (0.5), 39,460 rpm. (B) Native serum albumin (○), 27,690 rpm, and serum albumin in GuHCl–MSH (●) (0.5), 37,020 rpm. X_b^2 represents the cell bottom.

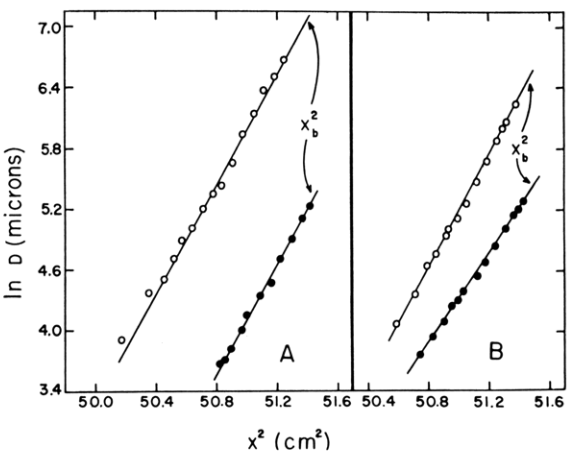

FIGURE 5: Plots of ln D *vs.* x^2. (A) Native enolase (○), 25,986 rpm, and enolase in GuHCl–MSH (●) (0.5), 44,770 rpm. (B) Native alcohol dehydrogenase (○), 25,992 rpm, and alcohol dehydrogenase in GuHCl (●), 42,040 rpm. X_b^2 represents the cell bottom.

TABLE II: The Molecular Weights of Native and Dissociated Proteins and the Number of Subunits as Determined by Sedimentation Equilibrium.

Protein[a]	Slope of ln D *vs.* x^2	$\bar{v}_{20°}$	M_w[b] ± 4%	Subunits
Serum albumin	3.108	0.729	68,750	1.1 ± 0.2
Serum albumin + G	3.487	0.729–0.719	69,480–64,480	0.9 ± 0.2–1.1 ± 0.2
Ovalbumin	2.653	0.744	43,040	0.9 ± 0.2–1.0 ± 0.2
Ovalbumin + G	2.314	0.744–0.734	45,220–42,060	
Alcohol dehydrogenase[c]	2.830	0.750	82,540	2.0 ± 0.2–2.2 ± 0.2
Alcohol dehydrogenase + G	2.256	0.750–0.740	40,950–37,670	
Enolase[c]	3.350	0.747	78,360	1.9 ± 0.2–2.0 ± 0.3
Enolase + G	2.787	0.747–0.737	43,360–40,130	
Methemoglobin	1.180	0.750	66,300	4.0 ± 0.3–4.3 ± 0.4
Methemoglobin + G	1.316	0.750–0.740	16,410–15,150	
Lactate dehydrogenase	3.330	0.740	141,440	3.7 ± 0.3–4.0 ± 0.3
Lactate dehydrogenase + G	2.305	0.740–0.730	37,850–35,750	
Aldolase[d]		0.742	156,100	3.7 ± 0.2–3.95 ± 0.3
Aldolase + G[e]		0.742–0.732	42,200–39,600	

[a] Proteins and solvents are identical with those used for osmometry. G = GuHCl–MSH (0.5). [b] Values of R and T are given in Materials and Methods. Rotor speeds are given in figure legends. [c] The temperature of this run was 25°. [d] The molecular weight represents an average of the three runs presented in Figure 7A. [e] The molecular weight represents an average of the two runs presented in Figure 7B.

to obtain values for M_w, by viscosity measurements to determine the extent of unfolding produced on dissociation, and by sedimentation velocity to establish the homogeneity of the dissociated samples.

Osmometry Studies. The Mechrolab high-speed membrane osmometer Model 503 was found to give values for molecular weights in good agreement with the literature values obtained by other methods. The values obtained can be reproduced with reasonable precision. For example, the molecular weights obtained for native aldolase in a series of determinations were 152,000–158,000. The method requires large amounts of protein since a series of 1.0-ml samples containing between 1 and 10–15 mg of protein per ml must be used to obtain an accurate extrapolated value of the osmotic pressure at a protein concentration of zero. The extrapolation also covers the range of protein concentrations used for sedimentation studies and for enzyme activity measurements so that the osmometer data cannot give information on dissociation occurring at low concentrations. The instrument requires some skill to assemble and approximately 1 day is required to determine whether it is functioning properly. The membranes must be carefully adapted to the medium to be used and initial temperature equilibration is slow. However, once a properly performing membrane is installed the osmotic

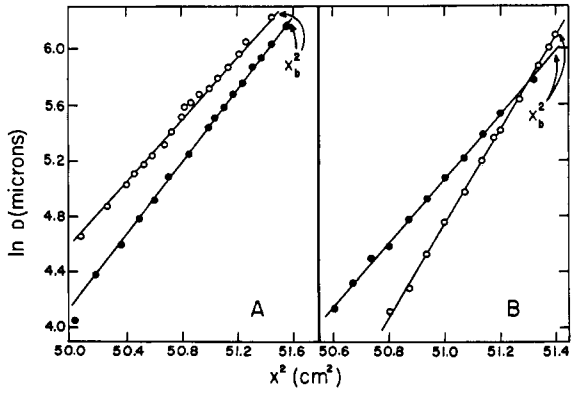

FIGURE 6: Plots of ln D *vs.* x^2. (A) Native methemoglobin (○), 18,000 rpm, and methemoglobin in GuHCl–MSH (●) (0.5), 50,740 rpm. (B) Native lactate dehydrogenase (○), 20,410 rpm, and lactate dehydrogenase in GuHCl–MSH (●) (0.5), 42,034 rpm. X_b^2 represents the cell bottom.

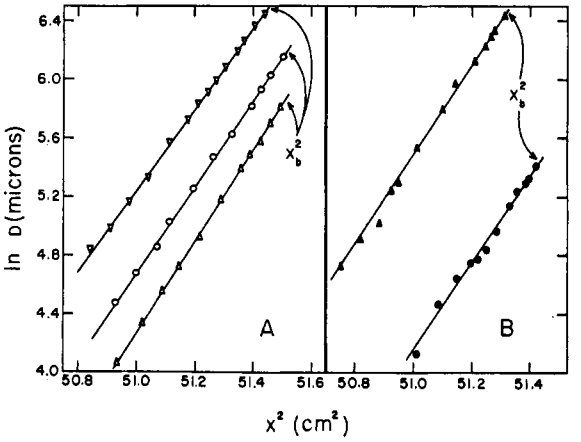

FIGURE 7: Plots of ln D *vs.* x^2. (A) Native aldolase (▽), pH 5.5, 17,000 rpm, native aldolase (○), pH 7.5, 17,980 rpm, and native aldolase (△), pH 6.5, 19,160 rpm. (B) Aldolase in GuHCl–MSH (▲) (0.5), 44,000 rpm, and aldolase in GuHCl–MSH (●) (0.5), 44,790 rpm. X_b^2 represents the cell bottom.

TABLE III: $s_{20,w}$, $[\eta]$, and B for Native and Dissociated Proteins.

Protein[a]	$s_{20,w} \pm 0.1$[b] sec^{-1} (S)	$[\eta] \pm 1\%$ (cc g^{-1}) Obsd	Calcd[c]	$B \times 10^5 \pm 7\%$ (cc mole g^{-2})
Serum albumin	4.41	3.6		2.9
Serum albumin + G	1.99	51.3	51.3	92.4
Ovalbumin	3.53	4.4		2.4
Ovalbumin + G	1.39	34.6	35.5	76.0
Alcohol dehydrogenase	5.39	3.6		4.5
Alcohol dehydrogenase + G	1.53	34.1	34.9	77.8
Enolase	5.59	3.7		1.0
Enolase + G	1.53	33.0	33.5	32.4
Methemoglobin	4.40	3.4		1.3
Methemoglobin + G	0.96	19.8	19.7	109.0
Lactate dehydrogenase	7.18	3.8		2.0
Lactate dehydrogenase + G	1.55	32.4	32.1	81.7
Aldolase	7.80	3.4		2.8
Aldolase + G	1.35	35.4	35.6	70.8

[a] Proteins and solvents are identical with Tables I and II. [b] Protein concentrations range from 4 to 8 mg per ml. [c] $[\eta] = 0.684n^{0.67}$, where n is the number of amino acid residues per chain (Tanford *et al.*, 1966).

pressure data are easily obtained. It is significantly easier to obtain satisfactory data at 5° than at 25°. Use of this method requires only that the concentration of the protein solutions be known with precision. The osmometer is insensitive to small differences in the concentration of diffusible ions and it is not necessary to prepare solutions of proteins for osmometry by exhaustive dialysis against the solvent to be used in the reference compartment.

Aldolase. The ratio of the molecular weights of the native and GuHCl–MSH dissociated protein obtained by osmometry (M_n) and by sedimentation equilibrium centrifugation (M_w) both indicate that the protein has at least four subunits. The absolute value for the molecular weights obtained by the two methods agree very closely and are also in good agreement with the values reported by Kawahara and Tanford (1966). Kawahara and Tanford observed curvature in the plots of ln D *vs.* x^2 for both the native and dissociated enzyme. They observed that at concentrations below 0.2 mg/ml, which corresponds to ~ln 5.4 on Figures 4–7, native aldolase dissociates into subunits of $M_w \backsim 80{,}000$. We have been unable to observe this effect. To explore the reasons for this difference in behavior we have examined aldolase by equilibrium sedimentation at pH values of 5.5, 6.5, and 7.5. Since the enzyme is known to dissociate at pH values below 3 it was felt that dilution and a slightly lowered pH might produce dissociation. However, no curvature of ln D *vs.* x^2 plots was observed (see Figure 7A). The recent observation of Woodfin (1967) that aldolase reacts with its substrate, fructose diphosphate (FDP), to form a derivative which is colored and which is dissociated led us to examine the effects of a small proportion of FDP on the sedimentation behavior of the native enzyme. Aldolase and FDP in a 1:1 ratio were mixed and kept at 4° for 1 week. A sedimentation equilibrium experiment produced a linear ln D *vs.* x^2 plot in which the molecular weight was 10% higher than that for the native enzyme. However, there was no indication of half-molecules at any point in the cell. When examined by sedimentation velocity the material appeared homogeneous with a single, symmetrical peak having $s_{20,w} = 7.8$ S. In GuHCl–MSH (0.1) Kawahara and Tanford also observed that the subunit population was heterogeneous. The molecular weights of the individual chains which best fit their data were 32,000 and 47,000 but, as they pointed out, the experimental error allows for considerable deviation from these values. The divergence of their plots of ln D *vs.* x^2 was observed at displacements of less than 100 μ. Although we have taken careful and repeated measurements in five separate experiments at low protein concentration we have not observed deviation from linearity in any of the plots of ln D *vs.* x^2 for the dissociated protein. When fringe displacements of less than 100 μ are measured the error increases significantly (Yphantis, 1964) and corrections for the water blank can be of the same order of magnitude as the displacement. We have found that small deviations from linearity can be observed in this region, but that they are largely due to errors in estimating displacement. Repeated readings of the same plate can give plots which deviate from linearity in opposite directions. Although variations in the quality of the plate and in the corrections for the water blank may allow accurate readings to be made of small fringe displacements, we conclude that the plots of ln D *vs.* x^2 for aldolase in GuHCl–MSH (0.5) are linear and that all of the subunits have essentially the same molecular weight. As shown in Figure 3g, the sedimentation pat-

TABLE IV: Literature Reports Concerning the Subunit Structure of Rabbit Muscle Aldolase.

No. of Subunits	Method	Dissociating Medium	Reference
3	C-terminal analysis		Drechsler *et al.* (1959)
3	C-terminal analysis		Kowalsky and Boyer (1960)
3	Sedimentation equilibrium	Acid	Stellwagen and Schachman (1962)
3	Sedimentation equilibrium	8.0 M urea	Stellwagen and Schachman (1962)
3	Sedimentation equilibrium	Acid	Deal *et al.* (1963)
3	C-terminal analysis		Winstead and Wold (1964b)
3	N-terminal analysis		Edelstein and Schachman (1966)
3	Sedimentation equilibrium	GuHCl	Schachman and Edelstein (1966)
4	Sedimentation equilibrium	GuHCl–MSH (0.1)	Kawahara and Tanford (1966)
4	Hybrid set produced from aldolases A and C	Acid	Penhoet *et al.* (1966)
4	C-terminal analysis		Morse *et al.* (1967)
4	Sedimentation equilibrium	Acid	Sia and Horecker (1968)

tern for aldolase in the dissociating medium is symmetrical, indicating that the subunits are reasonably similar in molecular weight.

Enolase. The value of M_n obtained for the native enzyme is in good agreement with the value of 82,000 obtained by Holt and Wold (1961). The value of M_w is slightly low but does not significantly alter the number of subunits obtained. When this enzyme was dissolved in 20% dioxane or 20% acetone, a molecular weight of 41,000 was obtained (Winstead and Wold, 1965) indicating two subunits. The two-subunit model is supported by the finding that two carboxyl-terminal lysine residues are liberated upon treatment of the native enzyme with carboxypeptidase (Winstead and Wold, 1964a). However, uncertainties are inherent in this method, *e.g.*, steric exclusion of carboxyl-terminal residues to the action of carboxypeptidase (Morse *et al.*, 1967). The data obtained in the present study confirm that rabbit muscle enolase contains two subunits of similar size.

Alcohol Dehydrogenase. The molecular weight of the native enzyme was determined to be 84,000 by sedimentation and diffusion studies of two-subunit structures has been proposed for this enzyme based on the fact that 2 moles of DPNH is bound/mole of native enzyme. A molecular weight of 40,000 was obtained for alcohol dehydrogenase in 8.0 M urea (Drum *et al.*, 1967). The molecular weights obtained in this study under normal conditions and in the presence of GuHCl are consistent with the findings of Drum *et al.*

Lactate Dehydrogenase. The values for M_n and M_w in phosphate buffer and GuHCl–MSH are in good agreement with the value reported by Appella and Markert (1963) and are clearly consistent with a four-subunit structure for this enzyme.

Methemoglobin. The molecular weight of the native protein is well established. Osmotic pressure measurements (Adair, 1928) indicated a molecular weight of 67,000. Schachman and Edelstein (1966) have determined a molecular weight of 64,000 by sedimentation equilibrium. Hemoglobin is known to dissociate under conditions of high ionic strength (Kawahara *et al.*, 1965) and dilution below 0.08 mg/ml (Schachman and Edelstein, 1966). Therefore, methemoglobin experiments were performed at low ionic strength, high initial concentrations (0.2 mg/ml), and low rotor speeds. As can be seen in Figure 6A, the lowest concentration measured corresponded to ln 4.654. This represents a fringe displacement of 105 μ and an absolute concentration of 0.09 mg/ml. Dissociation was not evident under these conditions.

The protein has four subunits of molecular weight 15,500 as determined by sedimentation equilibrium (Tanford *et al.*, 1966) and membrane osmometry (Lapanje and Tanford, 1967). Our sedimentation equilibrium values are consistent with this four-subunit model although a spread of values is obtainable depending on the value used for $\bar{v}$. The osmometry data agrees well with the four-subunit structure.

Bovine Serum Albumin. The molecular weight of the native protein was determined to be 66,900 by sedimentation equilibrium (Schachman and Edelstein, 1966) and 68,000 by osmometry (Scatchard *et al.*, 1946). The molecular weight of serum albumin in GuHCl–MSH (0.1) was found to be 71,000 by membrane osmometry (Lapanje and Tanford, 1967) and 69,000 by sedimentation equilibrium (Tanford *et al.*, 1966). These data are consistent with bovine serum albumin having a single polypeptide chain. The molecular weights from both sedimentation equilibrium and osmometry presented in Tables I and II agree with a one-subunit model.

Ovalbumin. The molecular weight of ovalbumin has been determined to be 43,500 by sedimentation equilibrium centrifugation (Svedberg and Pedersen, 1940) and 45,000 by membrane osmometry (Güntelberg and Linderström-Lang, 1949). That ovalbumin consists of a single polypeptide chain was demonstrated by its be-

havior in 8.5 M urea (Simpson and Kauzmann, 1953; Frensdorff *et al.*, 1953). The molecular weight data reported in this study agree with these findings.

Partial Specific Volumes. A precise knowledge of $\bar{v}$ for proteins is particularly important in computing values of M_w in dissociating media of high density and conflicting values of $\bar{v}$ have been reported for some proteins. It is possible to utilize the molecular weights obtained by osmometry to calculate values of $\bar{v}$. If the molecular weight determined by osmometry for a dissociated protein is assumed to be correct it can be inserted in eq 1 along with the experimental results from the centrifuge and a value of $\bar{v}$ calculated. This approach can be used only if the protein dissociates into subunits of the same molecular weight (*i.e.*, $M_w = M_n$). The values of $\bar{v}$ calculated in this manner are given in Table V and are compared with values of $\bar{v}$ for native proteins. The deviations were calculated assuming that both M_n and d ln D/dx^2 could be in error by as much as 5%.

TABLE V: Comparison of Values of $\bar{v}$ for Native Proteins with Those Calculated from Osmometry Data for GuHCl–MSH (0.5)-Treated Proteins.

Protein	Native $\bar{v}$ at 20°	Calculated $\bar{v}$ at 20°[a]
Serum albumin	0.729	0.726 ± 0.002
Ovalbumin	0.744	0.746 ± 0.002
Alcohol dehydrogenase	0.750	0.749 ± 0.001
Enolase	0.747	0.723 ± 0.001
Methemoglobin	0.750	0.746 ± 0.002
Lactate dehydrogenase	0.740	0.732 ± 0.001
Aldolase	0.742	0.743 ± 0.002

[a] Represents apparent $\bar{v}$ (ϕ).

Hade and Tanford (1967) conclude that specific binding of guanidine hydrochloride to proteins in 6 M GuHCl–MSH (0.1) requires that an apparent specific volume having a value 0.01–0.02 below $\bar{v}$ for the native protein be used to obtain accurate values of M_w. The small differences between the values of $\bar{v}$ for most native and dissociated proteins calculated from osmometry data indicate that no such general correction is required.

In the case of aldolase if the value of $\bar{v}$ calculated from osmometry data is used the protein appears to have 3.7 ± 0.2 subunits; if $\bar{v}$ is corrected according to Hade and Tanford (1967) then the protein appears to have 3.95 ± 0.3 subunits.

Enolase is the only protein which shows a large decrease in $\bar{v}$ in GuHCl–MSH (0.5). However the value of the native $\bar{v}$ at 20° is calculated from data obtained at 1° using a correction of 0.001 cc/g per deg. The temperature dependence of $\bar{v}$ may be substantially smaller (0.0004 cc/g), as suggested by Hunter (1966). In this case $\bar{v}$ at 20° is 0.736 and the difference between the native value and that in GuHCl–MSH is decreased to 0.013. If the lower values of $\bar{v}$ are used, molecular weights, M_w, for native and dissociated enolase of 75,000 and 37,000 are obtained. The discrepancy between the M_w and M_n values for the native enzyme become appreciably greater than those for the other proteins, and the value of M_w is significantly lower than that reported by Holt and Wold (1961). A value of $\bar{v}$ = 0.755 is calculated for native enolase at 20° from the value of M_n obtained by osmometry. Since essentially the same molecular weight was obtained by Holt and Wold (1961) using $\bar{v}$ = 0.728 at 1° the dependency of $\bar{v}$ on temperature must approximate 0.001 cc/g per deg for this protein.

[η], B, and $s_{20,w}$. The number of subunits in a protein can be ascertained by comparison of the molecular weights in normal and dissociating media with certainty only if the dissociating medium completely disrupts the noncovalent structure of the protein and produces complete separation of the subunits. In such media the protein should behave as a random coil. Tanford and coworkers have shown that GuHCl–MSH (0.1) causes a number of proteins to behave as random coils as estimated from values of B, [η], and root-mean-square end-to-end distances, $\langle L^2 \rangle_0^{1/2}$ (Tanford *et al.*, 1966; Lapanje and Tanford, 1967).

The experimentally determined intrinsic viscosities [η] of proteins in GuHCl-MSH and values of [η] calculated according to the equation of Tanford *et al.* (1966) which was derived for random coiled polypeptide chains in GuHCl–MSH are given in Table III. The agreement is excellent indicating the applicability of the method to a number of proteins not previously examined. The data also support the contention that this solvent produces random coils whose properties are largely determined by the number of amino acid residues they contain.

The second virial coefficient, B, is a function of the actual volume occupied by the polypeptide chain. For randomly coiled molecules, B has a value 10–100 times greater than for globular molecules and should approximate 5×10^{-4} cc mole g^{-2} (Tanford, 1966). The values of B obtained from the slopes of the plots of π/c *vs.* c (Figures 1 and 2) are given in Table III. All values are in close agreement with those predicted for random coils. Those for serum albumin and aldolase are in good agreement with the values reported by Lapanje and Tanford (1966) for these proteins in GuHCl–MSH (0.1).

All proteins show a decrease in $s_{20,w}$ when dissolved in GuHCl–MSH (0.5) when compared with native values. In the case of ovalbumin and serum albumin this is due to unfolding of the protein and in the case of all other proteins this is due to unfolding with concomitant dissociation into subunits.

References

Adair, G. S. (1928), *Proc. Roy. Soc.* (*London*) *A120*, 573.

Appella, E., and Markert, C. L. (1963), *Biochem. Biophys. Res. Commun. 6*, 171.

Dayhoff, M. O., Perlmann, G. E., and MacInnes, D. A. (1952), *J. Am. Chem. Soc. 74*, 2515.
Deal, W. C., Rutter, W. J., and Van Holde, K. E. (1963), *Biochemistry 2*, 246.
Drechsler, E. R., Boyer, P. D., and Kowalsky, A. G. (1959), *J. Biol. Chem. 234*, 2627.
Drum, D. E., Harrison, J. H., Li, T. K., Bethune, J. L., and Vallee, B. L. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1434.
Edelstein, S. J., and Schachman, H. K. (1966), *Federation Proc. 23*, 1248.
Frensdorff, H. K., Watson, M. T., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5157.
Güntelberg, A. W., and Linderström-Lang, K. (1949), *Compt. Rend. Trav. Lab. Carlsberg 27*, 1.
Hade, E. P. K., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 5034.
Holt, A., and Wold, F. (1961), *J. Biol. Chem. 236*, 3227.
Huggins, M. L. (1942), *J. Am. Chem. Soc. 64*, 2716.
Hunter, M. J. (1966), *J. Phys. Chem. 70*, 3285.
Kawahara, K., Kirshner, A., and Tanford, C. (1965), *Biochemistry 4*, 1203.
Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Kirshner, A., and Tanford, C. (1964), *Biochemistry 3*, 291.
Kowalsky, A., and Boyer, P. D. (1960), *J. Biol. Chem. 235*, 604.
Lapanje, S., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 5030.
Morse, D. E., Chan, W., and Horecker, B. L. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 628.
Noelken, M. E., and Timasheff, S. N. (1967), *J. Biol. Chem. 242*, 6080.
Nozaki, Y., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 742.
Penhoet, E., Rajkumar, T. V., and Rutter, W. J. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1275.
Reithel, F. J., and Sakura, J. D. (1963), *J. Phys. Chem. 67*, 2497.
Scatchard, G., Batchelder, A. C., and Brown, A. (1946), *J. Am. Chem. Soc. 68*, 2320.
Schachman, H. K., and Edelstein, S. J. (1966), *Biochemistry 5*, 2681.
Sia, C. L., and Horecker, B. L. (1968), *Arch. Biochem. Biophys. 123*, 186.
Simpson, R. B., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5139.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University, Reprinted by the Johnson Reprint Corp., New York, N. Y.
Tanford, C. (1966), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 210.
Tanford, C., Kawahara, K., and Lapanje, S. (1966), *J. Biol. Chem. 241*, 1921.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Am. Chem. Soc. 89*, 729.
Taylor, J. F., and Lowry, C. (1956), *Biochim. Biophys. Acta 20*, 109.
Vallee, B. L., and Hoch, F. L. (1957), *J. Biol. Chem. 225*, 185.
Winstead, J. A., and Wold, F. (1964a), *Biochemistry 3*, 791.
Winstead, J. A., and Wold, F. (1964b), *J. Biol. Chem. 239*, 4212.
Winstead, J. A., and Wold, F. (1965), *Biochemistry 4*, 2145.
Woodfin, B. M. (1967), *Biochem. Biophys. Res. Commun. 29*, 288.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.