Simultaneous Determination of Viscosity and Density of Protein Solutions by Magnetic Suspension

(intrinsic viscosity/specific volumes/virus/hydration)

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ABSTRACT The first results are reported with a magnetic suspension instrument for determination of the viscosity and density concurrently on small volumes (0.2 ml) of protein solution. Reasonable agreement was obtained with literature values for the intrinsic viscosities and specific volumes (partial or isopotential) of serum albumin and ribonuclease in native solvents, and in 6 M guanidinium chloride with ' or without 2-mercaptoethanol. Turnip Yellow Mosaic virus and myosin were also studied, the results with the virus being related to hydration and structure data and those with myosin to the dissociative character of the protein. The possibility of using this approach to follow the time course of viscosity and density changes during reactions is shown.

The feasibility of determining the density on small volumes (<0.3 ml) of protein solution accurately and rapidly by the magnetic suspension principle has been described in detail elsewhere (1–6). The principal feature of this method lies in stably balancing a small buoy or float, containing a soft ferromagnetic core, at a reproducible position within the solution by feedback control circuitry. At a fixed height in the column of solution (i.e., a given vertical distance from a solenoid) the total magnetic force is the difference between the opposing forces of gravity and buoyancy. In practice, the magnetic force is given in terms of the electric current to a solenoid, and this current is a function of the density of the liquid. With appropriate standard solutions, the instrument can be calibrated to yield a standard curve of the current, in terms of voltage, versus the density, so that the volume and mass of a buoy need not be determined. When the temperature is held constant within $\pm 0.01°$ and care is taken to prevent bubbles from collecting on the buoy, the measurements are conveniently performed to give a routine, overall precision of better than ± 5 μg/ml in the density of aqueous solutions. The magnetic method readily lends itself to variation of the temperature, pressure (7), and masses of the components and to the monitoring of systems undergoing change.

Recently, an attempt has been made to increase the versatility of the magnetic suspension approach to include evaluation of the viscosity (8). By the use of remote drive coils situated around the solution cell (to provide a rapidly rotating magnetic field), a sufficiently constant torque on the suspended buoy can be applied with negligible heating. At slow rotations (e.g., 100 sec/rev), it was observed that the time per revolution is directly proportional to the relative viscosity of standard Newtonian solutions, such as mixtures of H_2O–D_2O. For this purpose, the buoy is shaped in the form of a smooth cylinder. Since the height of the buoy in the solution remains constant during the rotation, by virtue of the controlled solenoid, the density can be determined at the same time. Hence, it becomes possible to make measurements relating to both the hydrodynamic and partial volume properties of a macromolecule simultaneously on the same small sample.

The intrinsic viscosity, $[\eta]$, is the parameter of particular interest in the viscometric study of macromolecules, and its evaluation affords a much more stringent test of the magnetic method than comparisons on Newtonian standards. For this parameter, the generally small difference between the viscosity of the solution, η, and that of the solvent, $\eta°$, is divided by $\eta°$ to give the specific viscosity, η_{sp}, which in turn is reduced to that per unit of concentration, c, of the macromolecule, η_{sp}/c (i.e., the reduced viscosity or the viscosity number). The reduced viscosities are then plotted as a function of c, where $[\eta]$ is the extrapolated value at the intercept, $c = 0$. $[\eta]$ reflects the contribution to the viscosity by the macromolecule in the absence of its own concentration effects and may be regarded as a measure of the effective hydrodynamic volume per unit of mass of the macromolecule. In this paper, the units for $[\eta]$ are given in milliliters per gram (ml/g).

It was of interest to determine $[\eta]$ not only for the more routine case of native protein in compatible aqueous solvents, but also to evaluate $[\eta]$ in denaturing media and upon reduction of disulfide bonds. In addition, preliminary experiments were done to test the feasibility of observing the time course of viscosity and density changes after the addition of the disulfide cleavage agent. Finally, a test with the stiff rod-like protein, myosin, was attempted; however, this protein underwent aggregation in our hands (e.g., the temperature could not be adequately controlled below 10° with the available equipment, whereas 4–5° is usually used for work on myosin). Hence, this study was limited simply to noting whether the known dissociation of myosin at very high dilutions is manifested by a downward trend in the reduced viscosities as $c \rightarrow 0$. For example, if the dissociable species are in a staggered parallel arrangement at higher concentrations, a greater decrease in the reduced viscosity compared to stable species should be seen on dilution, as has been observed by Burke and Harrington (9).

EXPERIMENTAL

The magnetic visco-densimeter used for these experiments was described (8). Approximately 0.2 ml of the liquid sample, inserted via a gas-tight syringe, was used for each measurement; this amount virtually filled the cell chamber, which

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Abbreviations: TYMV, Turnip Yellow Mosaic virus; GuCl, guanidinium chloride.

was sealed with a tightly-fitting silicone stopper. Since the rotating buoy is fully submerged, the existence of a small air gap above the liquid was assumed to have no significant effect on our present study, e.g., there were no measurable differences in rotation rate over a rather wide variation in settings for the height of the buoy. All protein solutions and solvents to be tested had been filtered through 0.22-μm Millipore filters, except that 8-μm and 0.8-μm filters and filter pads were used with myosin and Turnip Yellow Mosaic virus (TYMV) solutions, respectively. Sufficient rinsings of the filter were made in the case of fixed components (ribonuclease studies), so that the final concentration of these components remained essentially unchanged according to density determinations. Samples were not deaerated to eliminate bubbles, because the density changes measurably during short evacuation periods; instead, the solutions were warmed a few degrees above the operating temperature before measurement. The precision of the density measurements was somewhat less than that with our standard magnetic densimeter (5); a different visual index for buoy height was utilized with the visco-densimeter because of the cylindrical shape of the buoy. An automatic height-sensing device is currently under study in order to eliminate the small uncertainty entailed with reproducing the vertical position of the buoy by visual inspection through a microscope. The forces applied in the experiments were all of a calculated shear stress on the order of 0.0015 dynes cm^{-2}. The rates of shear ranged from 10^{-1} sec^{-1} for dilute aqueous solvents to 10^{-3} sec^{-1} for the most concentrated myosin solutions. Timing was done with electric timers (to 0.05 sec) by observation of the coincidence of one of the vertical imperfection lines on the buoy with the crosshair of a microscope. A second timer was activated after one or more complete revolutions with the first timer turned on; the second timer was shut off after the buoy had rotated the same number of times as with the first timer. The mean of the two readings was used for the viscosity calculations and was almost always less than 0.05 sec from either of the two extremes.

Protein solutions for the visco-densimeter were prepared as follows: Solutions of ribonuclease-A (RNase) were made up by adding together known weights of the components on the analytical balance (see below), in order to determine the partial specific volume, $\bar{v}$, for comparison purposes. A water solution of RNase of known composition based on dry weight analysis was added to a weight of KCl or guanidinium chloride (GuCl) on the balance in such proportions that the water and salt bore the same weight ratio to that in the corresponding solvent. Dilutions of the protein–water–salt stock solution were prepared by weighing together aliquots of the salt with aliquots of the solvent. Hence, only the mass of the anhydrous, isoionic protein component was varied in a concentration series. The concentration, c, (in g/ml) of each diluted solution was calculated by multiplying the weight fraction of RNase with the observed density. All other protein solutions were prepared by dialysis and diluted with dialyzate. Hence, only the isopotential specific volumes, v_μ, (6) were obtainable, which values may not correspond closely with the thermodynamically defined partial specific volumes of these proteins in the two-component (or more) solvents chosen. Where 2-mercaptoethanol was added, the amount was comparatively small, so that volume additions to both protein solution and solvent were made via gas-tight syringes; e.g.,

0.1–1.3 μl of 2-mercaptoethanol might be introduced into 200 μl of a solution in the visco-densimeter. Some error in the density difference between solution and solvent may be incurred in this way, but no additional error of significance should obtain in the relative viscosity.

REAGENTS AND PROTEINS

GuCl was "ultrapure," obtained from Mann Research Laboratories, Inc.; 6 M solutions of GuCl gave about 0.02 absorbancy units at 280 nm in a 1-cm light path. All other reagents were of analytical grade and were not purified further. Fresh, deionized water was used for all solutions. Bovine pancreatic RNase was purchased from Sigma Chemical Co. (Type XII-A, essentially phosphate free). This product was deionized by rapid flow (3–6 ml/min) through a 2 × 30 cm mixed-bed ion-exchange column of washed Amberlite MB-1 resin overlayed with about 5 cm of the weaker MB-3 resin. The pH of the pooled effluent fractions was 9.5 ± 0.1, compared to about pH 7–8 for various lots of the undeionized material in water. The deionized solution was freeze-dried and stored at −30° until used. The freeze-dried protein was dissolved in deionized water (about 4% solution) and filtered through a 0.22-μm filter. The concentration of RNase was assayed by measurement of the absorbance at the maximum at 277.5 nm in triplicate; an extinction coefficient of 0.722 ml/mg was used (1-cm path, 20°)—it was determined under identical circumstances from dry weight analyses at 105–110° under reduced pressure, (10). Dilutions for the absorption measurements were made by weight with water on the analytical balance, and these weight concentrations were then converted into grams per unit volume ("c" scale) by use of the observed density of the stock protein solution and the value 0.998234 g/ml (11) for the density of water at 20°. Bovine serum albumin (albumin) was supplied by Armour and Co. (Type F, essentially fatty-acid free). This product was similarly deionized, freezed-dried, and filtered after it was dissolved in water. The pH of the deionized albumin-water stock solutions was 5.1 (5.6 in 0.15 M KCl), compared with a pH of 6.1 for the undeionized material in water. Concentrations were estimated by light absorption in the manner described for RNase, but an extinction coefficient of 0.667 ml/mg was used at the 278.5-nm maximum (12). TYMV was isolated (13) by Dr. W. Godschalk from infected leaves of Chinese cabbage plants, grown for 21 days after inoculation in an artificially-lighted growth chamber at 20° (by Dr. J. M. Kaper, U.S. Dept. of Agr., Beltsville, Md.). The top component (capsid material) was removed from the virus by means of density-gradient centrifugation (25,000 rpm for 3 hr in a Spinco SW-27 rotor in a 14–56% sucrose gradient in 0.01 M phosphate, pH 7). This preparation was repurified by density gradient centrifugation before use, so that schlieren patterns from sedimentation velocity experiments showed only traces of the capsid material and RNA fragments remaining. For the concentration assays, the absorbance at the 261.5-nm maximum was measured (1-cm path, 20°). An extinction coefficient of 8.5 ml/mg was applied, in conjunction with a correction for scattering determined empirically from dry weight analyses. The scatter correction that corresponded most precisely with the dry weights was (A_{320}–A_{400}) (320/261.5)^2; this result was substracted from the total absorbance. Rabbit muscle myosin was a gift from Dr. S. M. Mozersky (U.S. Dept. of Agr., Eastern Regional Labo-
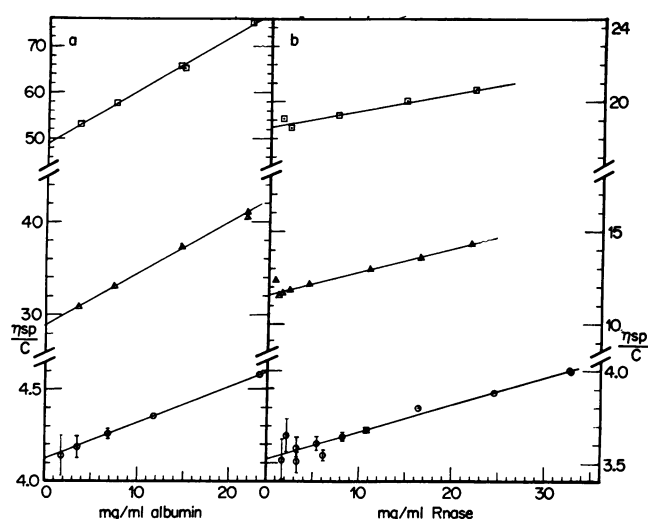
FIG. 1. Reduced viscosity against protein concentration at 25°. (a) Bovine-serum albumin (deionized); (b) Pancreatic RNase (deionized). ○, in 0.15 M KCl; △, in 6 M GuCl; □, 6 M GuCl–0.1 M 2-mercaptoethanol. *Error bars* reflect the uncertainty in timing for a fixed number of rotations of the buoy per determination in each experimental curve (usually 4 or 5 rotations, or about 500 sec) without regard to the decrease in the value of η_{sp} with dilution. *Data points* are the average of two or more samples. [η] was calculated by linear least-squares fitting to each set of data. In ml/g, [η] = 4.13 (albumin–KCl), 29.0 (albumin–GuCl), 49.0 (albumin–GuCl–2-mercaptoethanol), 3.52 (RNase–KCl), 11.6 (RNase–GuCl), 18.7 (RNase–GuCl–2-mercaptoethanol).

ratory, Philadelphia, Pa.). The myosin was extracted (14) from Longissimus dorsi muscle, purified twice by dilution precipitation, and then passed through a column of cellulose phosphate (15). The myosin fraction of the column effluent was further precipitated by dilution, redissolved, and then chromatographed on hydroxapatite (16). The major component associated with ATPase activity was used for this study. These protein solutions were maintained at 0° and were used within 2 weeks after the final chromatography. Just prior to use, the solutions were centrifuged at 58,000 × *g* for 45 min and filtered. Concentrations were determined by the absorbance at 278 nm, corrected for scattering (17); an extinction coefficient of 0.497 ml/mg in 0.6 M KCl (determined by dry weight analysis on weighed aliquots of myosin–water suspensions to which known amounts of KCl were added in order to solubilize the myosin) was used. Dilutions were made with a similar final concentration of KCl (0.6 M) of known density. The myosin solutions for the visco-densimeter were prepared from weighed portions of a dialyzed and filtered stock solution and of dialyzate containing either 0.6 M KCl–1 mM dithiothreitol, pH 7.2, or 0.5 M KCl–0.2 M (K)PO₄–2 mM EDTA, pH 7.15.

RESULTS

In Fig. 1 are shown viscosity data on isoionic albumin and RNase in native and denaturing solvents, and in denaturant plus the disulfide cleaving agent, 2-mercaptoethanol. For the albumin preparation the value of [η] (4.13 ml/g) in dilute KCl is somewhat higher than the lowest values reported, but is lower than several others (3.7–4.9 ml/g) (18). Apparently, these discrepancies reflect differences in the prepara-

tions, as has been proposed by Tanford and Buzzell (18), because we have performed a direct comparison via capillary viscometry on our preparation and obtained virtually the same intrinsic value and slope as by the magnetic method ([η] = 4.14–4.20 ml/g). By velocity sedimentation, our preparation exhibited a somewhat broader leading than trailing edge near the base line in the schlieren patterns, suggestive of the presence of some material heavier than the principal species. Isoionic RNase in either 0.15 or 1.50 M KCl, repeatedly yielded intrinsic values close to 3.50 ml/g (±0.02), which is marginally higher than the average figure of 3.30 ml/g reported by Buzzell and Tanford (19). The discrepancy may be a result of the different procedures used to assess the dry weight and/or possibly to differences in purity. Both of these proteins exhibited some variability in the values of [η] in 6 M GuCl, a point discussed by Tanford *et al.*, (20). During a given set of measurements, a good extrapolation to *c* = 0 could be obtained (Fig. 1). The reduced viscosities of albumin in particular increased substantially over several days; Tanford *et al.* observed [η] for albumin to increase from 26 to 30 ml/g in 10 days. RNase, on the other hand, yielded intrinsic values ranging from 10.7 to 11.6 ml/g, with only minor changes in slope. Comparison data on RNase under these particular conditions has not come to our attention. The addition of 2-mercaptoethanol to these proteins in 6 M GuCl produced the well-known marked increase in viscosity. The intrinsic value for albumin ([η] = 49 ml/g) is comparable to that reported by Castellino and Barker (21) (51.3 ml/g) and that by Tanford *et al.* (20) (52.2 ml/g). With RNase, the observed value for [η] (18.7 ml/g) is distinctly higher than that reported (20) (16.6 ml/g). No reason for this sizeable discrepancy is apparent; another preparation from a different lot number, similarly deionized and prepared by weight in 6 M GuCl and 0.1 M 2-mercaptoethanol, was somewhat more viscous. Tanford *et al.* (20) have shown that for several proteins in this solvent a straight line can be fitted to the logarithms of their intrinsic viscosities versus the logarithms of the average number of their amino-acid residues, *n*, in the dissociated peptide chains. The equation obtained for this line, with proteins ranging from insulin (average of *n* = 26) to myosin (*n* = 1790) is: [η] = 0.716 $n^{0.66}$. Although the plotted point for RNase with our value of [η] falls a little above this line, the slope is not appreciably altered by the substitution unless the insulin data point is ignored (a long gap exists in the log–log plot between insulin and the next-larger protein).
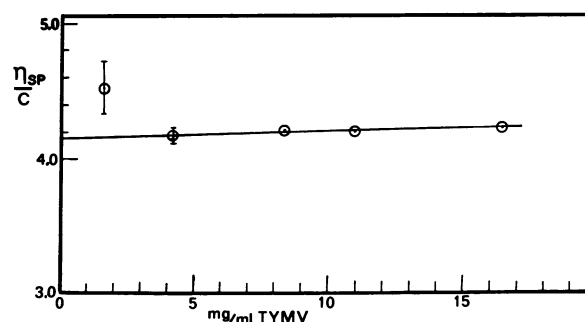

FIG. 2. Reduced viscosity against TYMV concentration at 25° in 0.075 M KCl–0.025 ionic strength (K)PO₄, pH 7.0. A value of 4.18 ml/g for [η] was calculated by linear least-squares fitting, without the data point at 1.7 mg/ml where η_{sp} = 7.55 × 10⁻³.

The average values for the partial specific volume, $\bar{v}$ ($c \rightarrow 0$) of RNase were 0.705 ml/g in 0.15 M KCl, 0.715 ml/g in 6 M GuCl, and 0.712 ml/g in 6 M GuCl + 0.1 M 2-mercaptoethanol ($\bar{v}$ was consistently lower by about 0.001 ml/g at all 2-mercaptoethanol concentrations above 0.1 M in GuCl). Independent experiments with our routine magnetic densimeter have yielded essentially the same values for this source of RNase; a higher value for $\bar{v}$ in GuCl than in native solvents has always been observed. The values for the isopotential specific volumes, v_μ ($c \rightarrow 0$) of this albumin were 0.745 ml/g in 0.15 M KCl, 0.727 ml/g in 6 M GuCl, and 0.721 ml/g in 6 M GuCl + 0.1 M 2-mercaptoethanol. Since $\bar{v}$ was not determined, no conclusions may be drawn on the preferential interaction of albumin with GuCl or for water. Although the differences between the above values for albumin have significance, none of the values are to be taken as accurate, because we have used an arbitrary extinction coefficient with which to estimate the concentrations of this preparation; generally, v_μ in dilute KCl should not be very different from $\bar{v}$, which is about 0.735 ml/g according to the most accurate work noted (10).

Fig. 2 shows a result with the isometric virus TYMV. The value of 4.18 ml/g found for $[\eta]$ is similar to that (4.0 ml/g) reported by Schachman (22) for another isometric virus (Bushy Stunt) of somewhat higher anhydrous molecular weight, but compares unfavorably with the value of 6.35 ml/g for Southern Bean Mosaic virus determined by Miller and Price (23). The latter virus is also thought to be a regular polyhedron (but, see ref. 24) having a molecular weight slightly greater than that of TYMV. Miller and Price, however, noted a substantial disagreement between the total hydration values calculated via their value of $[\eta]$ assuming spherical dimensions and that obtained from sedimentation rates in sucrose media (1.07 against 0.76 g H_2O/g dry virus, respectively). Aside from making comparisons with literature values, it was of interest to consider TYMV as a model particle. This virus is described as a stellated icosahedron (25) in its broad detail (5.5×10^6 daltons), and should nearly approximate the hydrodynamic behavior of a sphere except for surface hydration. When a value of 14.5 nm is used as the maximum radius of the particle (i.e., the effective x-ray scattering radius of the protein subunit protuberances) (26), a volume of 12.77×10^{-18} cm^3 is enclosed in a sphere whose surface touches the extremities of the particle. (Note: half the interparticle distance in the crystal is 15 nm, but this probably includes external hydration.) The volume of these spheres per gram of dry virus is 1.398 cm^3. The apparent hydrodynamic volume, however, is 1.672 ml/g dry virus, obtained by dividing 4.18 ml/g, the observed value of $[\eta]$, by 2.5, the viscosity increment for a hard sphere. Hence, surface hydration of the particle amounts to 0.274 ml/g (1.672–1.398 ml/g), assuming that the unhydrated polyhedron contributes to the viscosity identically as a hard sphere of 14.5-nm radius. The values for the total apparent hydration deduced from equilibrium dialysis and density (our unpublished results) and by velocity sedimentation (27) range from 0.68 to 0.71 g H_2O/g dry virus. If surface hydration is 0.274 ml/g, the interior solvent space by difference from an average of 0.70 ml of total water (ρ = 1.0 g/ml) per gram of dry virus is 0.426 ml/g. When the latter value is coupled with the molecular weight and the anhydrous specific volume (0.665 ml/g, see below), into terms of a vol-
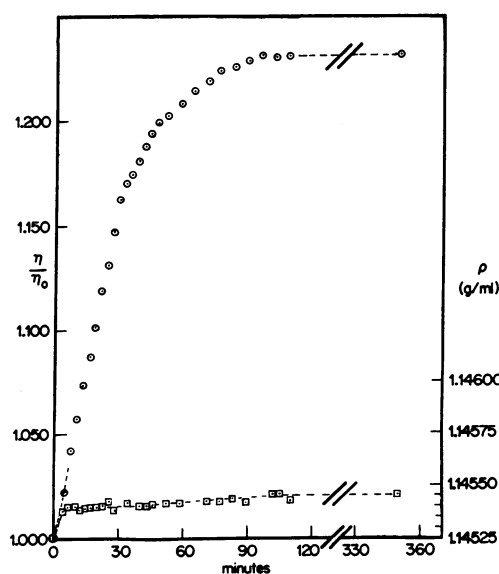

Fig. 3. Relative viscosity and density against time after addition of 1.3 μl of 2-mercaptoethanol (0.1 M) to 200 μl of 14.1 mg/ml of albumin in 6 M GuCl in the visco-densimeter cell, 25°. ⊙, viscosity; ▫, density.

ume-equivalent sphere, a radius of 13.35 nm is obtained. Allowing for the fact that the volume of the polyhedral virus must be substantially less than that of a sphere defined by the maximum observed radius, the radius of the volume-equivalent sphere should be moderately less than the maximum one. Interestingly, a model based on x-ray diffraction suggests an inner radius of about 10.5 nm as the spherically-averaged uniform density approximation of the protein shell or capsid (26). An interior sphere composed of 0.426 ml of H_2O per gram of dry virus has a very similar radius (10.37 nm per particle). (The RNA is thought to be interwoven with the protein subunits of the capsid, and is not segregated as a distinct entity in the interior.) It is easily shown, of course, that all of these derived dimensions and the surface hydration value depend heavily on the figure chosen for the maximum radius of the sphere enclosing the TYMV particle. We merely point out that our intrinsic viscosity result is realistic with respect to current information on this virus.

An isopotential specific volume of 0.665 ml/g (25°) was determined during this study with the visco-densimeter. This value compares with an earlier result of 0.661 ml/g at 20° (28); it is probably equivalent to the anhydrous specific volume at these relatively low-salt concentrations.

As noted at the outset, the experiments with myosin generally showed more aggregation than that of a simple monomer–dimer system. The data taken between 2 and 20 mg/ml exhibited an upward curvature in the plots of η_{sp}/c versus c. Between 0.2 and 2.0 mg/ml, linear segments were obtained. Below 0.2 mg/ml (to 0.04 mg/ml), a downward curvature was clearly evident in all experiments. The latter behavior is consistent with the recent suggestion of Burke and Harrington (9) that myosin monomers associate in a staggered parallel array. In only one experiment was the degree of aggregation low enough to infer by extrapolation an approximate value for $[\eta]$, which was about 250 ml/gram (6.8 sec was the smallest difference in the measured times between a

dilute solution and the solvent.) A more detailed account of myosin, to include preferential interaction by density, is in preparation.

Fig. 3 is a representative time study with albumin in 6 M GuCl after the addition of 2-mercaptoethanol (0.1 M). The relative viscosity was determined after one or more complete revolutions of the buoy (various means could be used in order to measure less arc per unit of time). The curve is not particularly smooth, but this feature has been characteristic of all the albumin experiments. RNase, on the other hand, exhibited a much smoother curve, although considerably lower concentrations of 2-mercaptoethanol were required in order to avoid a very steep rise with time (e.g., the maximum viscosity was achieved within about 5 min after the addition of only 12 mM 2-mercaptoethanol). In general, the time to reach the maximum viscosity with albumin in 6 M GuCl after addition of 0.1 M 2-mercaptoethanol was roughly proportional to the concentration of the protein from 3 to 20 mg/ml. The overall increase in density, after the initial increase from the added 2-mercaptoethanol, was very small (about 5×10^{-5} g/ml, or about -0.005 ml/g in $\bar{v}$), but the interval for this change appears to correspond approximately with that for the viscosity change. The precision of the density changes, as noted before, is poorer with the present visco-densimeter than it need be. The initial increase in the density (about 14×10^{-5} g/ml) upon the addition of 2-mercaptoethanol is about the same increment as seen on a similar addition to the 6 M GuCl solvent, whereas the increase in the specific viscosity on adding 2-mercaptoethanol to the protein-free solvent was only 0.0044, or relatively trivial.

In conclusion, the simultaneous measurements effected via magnetic suspension make possible the accurate and rapid determination of the viscosity increment, $[\eta]/\bar{v}$, a dimensionless constant reflecting both the hydrodynamic and thermodynamic volumes of a dissolved macromolecule (or for the determination of $[\eta]/v_\mu$, which in combination with $\bar{v}$ leads to an increment in which any excess solvent component relative to dialyzate is included with the macromolecule). For kinetic studies, it may be noted that changes in both η_{sp}/c and the apparent specific volume during a process are often so small that independent determination of the change in these quantities could not be expected to yield an accurate index of their changes at a given point in the history of the reaction. As a viscometer alone, the visco-densimeter appears to be as potentially accurate and free of the classic disadvantages of a rotational system as other recent models [for adaptations of remote drive on the inner cylinder, see also (29–32)]. The present instrument incorporates the shear stress capabilities of these other instruments, along with self-centering of a completely submersed inner rotor, which may be used for all liquids within a density range of 0.4 g/ml without interchanging. By virtue of the torque control, this approach should be suitable for the study of mechanically unstable, large macromolecules and for retardation experiments after the cessation of shear stress, while still being convenient and economical for routine viscosity studies on proteins.

NOTE ADDED IN PROOF

20-Fold weight dilutions of the albumin–GuCl solutions as used for concentration analyses were, at most, only 1% lower in specific absorbance than albumin–water solutions.

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