*EFFECT OF PRESSURE ON THE APPARENT SPECIFIC VOLUME OF PROTEINS**

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Abstract.—The magnetic densimeter has been employed to measure the densities and apparent specific volumes of certain proteins in aqueous solutions as a function of pressure. The method gave values in satisfactory agreement with those found in the literature for aqueous electrolyte solutions. A change in apparent specific volume of the monomeric proteins, ribonuclease and turnip yellow mosaic virus and its capsid protein, at pressures up to 400 atmospheres at 20°C was not observed within the precision of the measurements. Also, no change in the apparent specific volume of tobacco mosaic virus protein was observed as a function of these pressures whether the protein was predominantly in the polymerized or unpolymerized state. The magnetic densimeter was found to be a convenient instrument for measuring compressibilities of very small samples of solutions.

The effect of pressure on protein solutions has been of concern for many years, particularly in ultracentrifugation. The variety and surprising degree of pressure effects on associating protein systems in centrifugal fields have been demonstrated and discussed recently.[1–3] Undoubtedly, technical difficulties are cause for the obvious dearth of pressure-partial volume data on protein systems. Since pressure can be applied directly to small volumes of solution in which the density is measured by the magnetic balancing of a float or buoy within the liquid,[4] it was convenient to explore the potential of this approach for the routine determination of the function $\partial\phi/\partial p$, where ϕ is the apparent specific volume of the solute and p = pressure. We now report our initial attempts with a modified densimeter to measure compressibilities of solutions and to determine ϕ for standard electrolytes, sucrose, and biopolymers in low concentration, at pressures encountered under ultracentrifuge conditions (<400 atm). The biopolymers tested were two relatively nonassociating proteins, the single peptide, ribonuclease, and the multi-subunit icosahedron, turnip yellow mosaic virus, and its RNA-less capsid. A provisional result on tobacco mosaic virus protein, which is known to associate as a function of temperature, concentration, and pH, is also presented.

The densimeter built for this study was similar to that described earlier,[5] except that the solenoid was placed below the solution cell.[6] This modification lends spatial flexibility and greater ease of access to the sample area. Accordingly, the density of the buoy is less than that of the liquid sample and the downward force from the solenoid adds to that of gravitation to counter the buoyant force as described by the relation

$$M \frac{dH}{dz} = V_b g (\rho_s - \rho_b), \tag{1}$$

where g = acceleration of gravity, V = volume, ρ = density, M is the magnetic moment, H is the magnetic field intensity, z is distance along the verticle axis through the solenoid, and the subscripts b and s refer to the buoy and liquid sample, respectively. The force $M(dH/dz)$ is approximately equal to a constant times the square of the current in the solenoid under the conditions of the experiment. The position of the buoy along z is held invariant by the sensing coil and servo system;[4, 5] hence, the current to the solenoid becomes a function of the solution density only. By suitable calibration of the instrument with known reference liquids, the density of an unknown solution is a simple quadratic function of the difference in current between that for the sample and the reference liquid since the density of the latter replaces ρ_b in equation (1). The ferromagnetic material (permalloy) for the buoy was encased in glass instead of Kel-F, as heretofore, to provide for better reversibility when varying temperature or pressure.[7] A schematic of the over-all magnetic piezometer and of the pressure bomb are shown in Figures 1 and 2, respectively. (For specifications of this solenoid and details of the servo circuitry, cf. refs. 4, 5, and 7.) The windows in the bomb for sighting the buoy were fashioned out of $^1/_4$-inch Lucite plate. The leads from the sensing coil were brought out of the bomb through a pressure-tight conical well filled with epoxy. Pressure was generated with a Blackhawk P-76 hand-operated pump and transmitted via the oil in the pump through a stainless steel capillary to the pressure bomb. A dead-weight pressure gauge of 0.635-cm bore was arranged in parallel with the system. The pressures listed in the results correspond to 9-kg additions to the pan and were repeatable to better than the precisions indicated for the over-all measurement at each pressure.

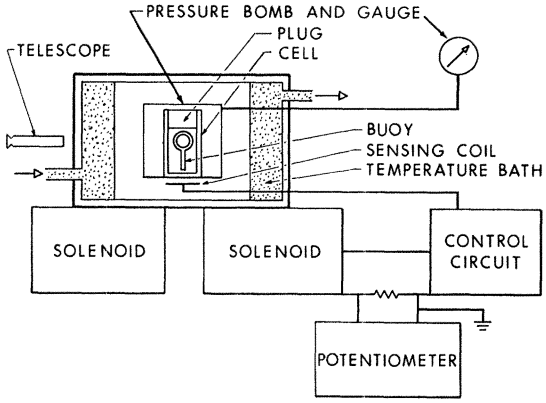

FIG. 1.—Schematic diagram of apparatus.

Procedure and Materials.—Solution (0.5 ml) is inserted via Lang-Levy type micropipet into the glass cell, and the Teflon plug is fitted into the cell until solution extrudes through the small axial channel. The small brass plug is then pushed into this channel,

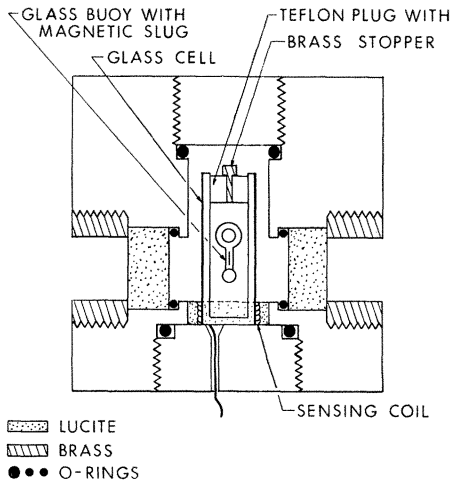

FIG. 2.—Pressure chamber. Scale is 2 inches on each side.

and the space outside of the cell, in contact with the pressure line, is filled with water. The thermostated bomb is sealed and the density function (current) is measured at each pressure setting when the buoy remains at a fixed height with respect to the cross hairs in the microscope; approximately 10 min was allowed to attain thermal equilibrium. As is indicated in the schematics, pressure is transmitted both outside and inside the cell to prevent shattering and/or distortion of the glass cell. Contamination of the solution with the outside water and oil was not observed, and the Teflon plug exhibited negligible hysteresis upon cycling with respect to pressure. Leaks were easily detected because pressure is transmitted throughout via liquid media.

The salts used were of reagent-grade quality and were recrystallized, pulverized, and dried *in vacuo* over activated alumina. Sucrose was from the National Bureau of Standards (Lot 6340) and was similarly pulverized and dried. Standardized solutions of hydrochloric acid and of alkali were employed whenever these materials were required. All other water for solutions was redistilled from an all-glass apparatus prior to use. The ribonuclease was type XII-A purchased from Sigma Chemical Co. (essentially phosphate-free). The pH of the protein solutions when dialyzed against water was usually below 6. For these experiments, stock solutions of approximately 6% in ribonuclease were dialyzed versus about 200 vol of 1 *M* NaCl–0.1 *M* glycine (adjusted to a pH of 9.4–9.5 with NaOH) in the cold room for 48 hr with one or two changes of the solvent during this period. The dialysis bag (Union Carbide Corp., size 16 casing or size 18, dry-heated at 90° for 1–2 weeks—boiled inside out for 5 min prior to use) containing the solution was further dialyzed for several days against glass-distilled water which was changed five or more times. The pH of the final solution ranged from 9.1 to 9.4, which is sufficiently close to the accepted isoionic point for purified ribonuclease in water. These solutions were filtered through a prewashed, size 0.22-μ Millipore filter, and they remained clear thereafter. Dry weight analyses were performed in quadruplicate paired with dialyzate samples in microweighing bottles (400-μl capacity), using the procedure of Hunter[8] for final drying *in vacuo* at 110° and extrapolation of weights to zero time; the bulk of the water, however, was removed at <50° in an atmosphere of nitrogen gas. The agreement among replicate protein samples was better than 1 part in 500, and no residue was detected in any of the dialyzate samples. The absorbance at 277.5 mμ (1-cm path length) of 1 mg ribonuclease/ml in water at 27° was 0.737 liter/gm. The specific refractive increment, $\Delta n/c$ (where c is grams protein per ml), at 546 mμ and 20° was 0.189$_1$ ml/gm. The samples of turnip yellow mosaic virus and the RNA-less capsids therefrom were prepared by Dr. W. Godschalk according to the method of Kaper.[9] An absorptivity of 8.5 liters/gm at 260 mμ was used for the virus and a value of 1.28 liters/gm at 275 mμ was used for the capsids.[9] Tobacco mosaic virus protein was prepared by Dr. G. K. Ackers according to Fraenkel-Conrat[10] (absorptivity of 1.27 liters/gm at 280 mμ).

Theory and Test of Method.—For these studies the apparent specific volume was determined at a fixed concentration of solute at various pressures up to about 400 atm. φ for the ith solute component in solution is defined as

$$V = g_o\phi_o + g_i\phi_i, \tag{2}$$

where g is grams and the subscript "o" refers to the pure solvent. ϕ in terms of the densities of the solvent and of the solvent + solute for a given weight fraction W_i of the ith solute component in the solution is

$$\phi_i = \frac{1}{\rho_o}\left(1 - \frac{\rho - \rho_o}{\rho W_i}\right). \tag{3}$$

Because W_i is pressure-independent, the change in ϕ_i with pressure is given by the change in density. Since V_b in equation (1) is not pressure-independent, it may be expressed as

$$V_b = V_b^{p=1} [1 - K_b(p - 1)], \quad (4)$$

where p is pressure in bars (1 bar = 0.987 atm) and K_b is the compressibility of the buoy. Since the magnetic force is equal to a constant k times I^2, then from equations (1) and (4)

$$kI^2 = V_b^{p=1} [1 - K_b(p - 1)] g (\rho_s - \rho_b), \quad (5)$$

and at any pressure, where $A = k/(gV_b^{p=1})$,

$$\rho = \frac{AI^2 + \rho_b^{p=1}}{1 - K_b(p - 1)}. \quad (6)$$

Accordingly, ϕ_i at any pressure becomes

$$\phi_i = \frac{1}{\rho_o}\left[1 - \frac{I^2 - I_o^2}{W_i(I^2 + \rho_b/A)}\right], \quad (7)$$

where I refers to the current required for balancing the buoy in the solution and I_o to that required for balancing in the corresponding solvent.

The function $\partial\rho/\partial p$ for water, the primary reference standard, was obtained from a quadratic fit to the data of Kell and Whalley at 20°.[11] The calculated values based on the polynomial $\rho = 0.998160 + 4.5817 \times 10^{-5}p - 4.4336 \times 10^{-9}p^2$ agreed, on the average, with the observed values to 3 ppm and with a maximum deviation of 7 ppm between 5.30 and 443.49 bars. The densities of water at the pressures used in this study, calculated by means of the foregoing polynomial, are shown in Table 1. These densities were used in the calculation of ϕ for NaCl, KCl, HCl, and sucrose at the several pressures. Where the solvent was not pure water, as in some of the experiments with proteins, the density of the solvent (ρ_o) was obtained from the relation

$$\rho_o = \rho_{H_2O}\left[\frac{I_o^2 + (\rho_b/A)}{I_{H_2O}^2 + (\rho_b/A)}\right]. \quad (8)$$

In terms of isothermal compressibilities, K_t, a comparison of our results for NaCl, KCl, and HCl with those calculated from Harned and Owen[12] is shown in Table 2. The experimental K_t was found by curve fitting the measured densities to a polynomial of the form $\rho = A + B(p - 1) + C(p - 1)^2 + D(p - 1)^3$. K_t then becomes

$$K_t = \frac{1}{\rho}\left(\frac{\partial\rho}{\partial p}\right)_T^{p=1} = \frac{B}{A} \quad (9)$$

or essentially the limiting slope over the intercept in these cases. The change in apparent specific volume for NaCl also may be compared to the results of Gibson and Loeffler.[13] Their values were interpolated with respect to both pressure and weight fraction and were extrapolated to 20°. At 412.5 bars (our highest experimental pressure), where $W = 0.043187$, the experimental value obtained for $(\phi^{p=412.5} - \phi^{p=1}) = 0.3379$ ml/gm $- 0.3070$ ml/gm $= 0.0309$ ml/gm, whereas the corresponding value derived from Gibson and Loeffler is 0.0305 ml/gm. A similar correspondence was found at lower pressures in all three

TABLE 1. *Density of water (20°) at experimental pressures of this study.**

Pressure (bars)	Density of water (gm/cc)	Pressure (bars)	Density of water (gm/cc)
1.0	0.998207	219.0	1.007984
25.4	0.999323	246.6	1.009195
53.1	1.000581	274.3	1.010398
80.7	1.001831	301.9	1.011595
108.4	1.003076	329.6	1.012785
136.0	1.004313	357.2	1.013968
163.7	1.005544	384.9	1.015144
191.3	1.006767	412.5	1.016314

* Calculated from a curve fitted to the data of Kell and Whalley[11] according to the polynomial $\rho = 0.998160 + 4.58167 \times 10^{-5} p - 4.43362 \times 10^{-9} p^2$. The isothermal compressibility, $K_t = 4.58167 \times 10^{-5}/0.998207 = 45.92 \times 10^{-6}$/bar. The curve was fitted to the data between 5.30 and 443.49 bars, resulting in a small extrapolation to the value shown for 1 bar.

experiments with this solution. For KCl and HCl solutions, apparent specific volumes as a function of pressure for making comparisons appear to be lacking. We find an increase in the apparent specific volume of KCl ($W = 0.041463$) at 20° to be $0.3955 - 0.3740 = 0.0215$ ml/gm in proceeding to 412.5 bars, and an increase of $0.5214 - 0.4983 = 0.0231$ ml/gm in this pressure range for HCl ($W = 0.00233$). For sucrose in water ($W = 0.04229$) we obtain for the increase in ϕ, between 1 and 412.5 bars at 20°, values of 0.0022 to 0.0028 ml/gm over a period of $2^1/_2$ months. Comparison with literature values was very difficult. From the adiabatic compressibility data of Bachem[14] we obtain for $\Delta\phi$ (412.5 — 1 bars) a value of 0.0019 ml/gm at 25°. According to Perman and Urry,[15] the temperature dependence for sucrose below $W = 0.0512$ is negligible between 30 and 80°; however, the isothermal value would tend to be higher by the ratio of specific heats, C_v/C_p, at constant volume to that at constant pressure, i.e., $K_t(C_v/C_p) = K_s$, where K_s is the adiabatic compressibility. Another comparison may be made with Glucker's[16] fit of Perman and Urry's data. At 30° the compressibility value leads to $\Delta\phi$ (412.5 — 1 bars) = 0.0020 ml/gm. The much earlier (1898) work of Tait[17] done at 12.4° gives $\Delta\phi$ (412.5 — 1 bars) = 0.0066 ml/gm, after interpolation of his compressibility data at pressures up to 546.9 atm. In all these comparisons for sucrose the concentrations that were employed were closely similar to the one we have used. The most feasible compressibility comparison which can be made on sucrose is with Bachem's data. The ratio of K_t ($\times 10^5$ bar) for sucrose solution to that of water at 20° by the magnetic piezometer is $44.58/45.92 = 0.9712$, while the ratio of adiabatic compressibilities from Bachem at 25° ($K_s \times 10^6$ bar) is $43.35/44.73 = 0.9624$. An approximate correction to our isothermal data can

TABLE 2. *Comparison of isothermal compressibilities (25°) for electrolytes in water.*

Solution	$K_t(\times 10^6$ bar) (observed at 20°; corr. to 25°)*	$K_t(\times 10^6$ bar) (calculated from Harned and Owen[12])
NaCl ($W = 0.043187$)	41.38	41.65
KCl ($W = 0.041463$)	42.60	42.72
KCl ($W = 0.007135$)	44.81	44.96
HCl ($W = 0.00233$)	45.80	45.40

* A correction of -0.06×10^{-6}/bar was applied to the observed K_t at 20° to compensate for the compressibility of pure water at 25° (i.e., 45.86×10^{-6}/bar at 25° minus 45.92×10^{-6}/bar at 20°).

be employed to yield an adiabatic compressibility for the sucrose solution at 25°. Thus, $K_s^{25} = K_t^{20} - [K_t^{20}\text{ (water)} - K_s^{25}\text{ (water)}] = 43.38 \times 10^{-6}$/bar, which agrees closely with the above value from Bachem of 43.35×10^{-6}/bar.

Results with Proteins.—The data for experiments on ribonuclease, turnip yellow mosaic virus, and its capsids are shown in Table 3. It is apparent that no change in ϕ is discernible for these monomeric proteins by pressures ordinarily generated in the ultracentrifuge. It should be noted that absolute values of ϕ at 1 atmosphere were usually not determined, because, for best accuracy, this required an additional calibration curve with standard sucrose solutions and several determinations on samples of the protein preparation to be performed prior to each pressure run; such data, while of some interest in the case of ribonuclease at varied pH, were unnecessary for the purpose at hand. The experiment on

TABLE 3. *Apparent specific volume (ml/gm)* of proteins at various pressures.*

Pressure (bars) 20°C	Ribonuclease In water pH = 9.1 W = 0.0602	Ribonuclease In HCl pH = 2.03 W = 0.0471	Turnip yellow mosaic virus In water pH ~ 7 W = 0.0342	Turnip yellow mosaic virus In 0.1 M KCl pH = 7.0 W = 0.0375	Viral capsid in water pH ~ 7 W = 0.0045
1	0.7075	0.7075	0.6631	0.6631	0.7271
25.4	0.7077	0.7069	0.6635	0.6640	0.7310
53.1	0.7074	0.7075	0.6633	0.6639	
80.7	0.7071	0.7077	0.6636	0.6635	0.7297
108.4	0.7069	0.7071	0.6638	0.6642	0.7308
136.0	0.7073	0.7065	0.6638	0.6628	
163.7	0.7073	0.7070	0.6631	0.6638	0.7232
191.3	0.7075	0.7071		0.6628	0.7259
219.0	0.7075	0.7071		0.6634	
246.6	0.7074	0.7066	0.6634	0.6641	0.7262
274.3	0.7070	0.7070		0.6642	
301.9	0.7069	0.7068		0.6639	0.7305
329.6	0.7073	0.7075		0.6637	
357.2	0.7075	0.7075		0.6643	0.7252
384.9	0.7063	0.7064		0.6640	
412.5	0.7077	0.7067		0.6642	0.7256

* The indicated apparent specific volumes are relative to the values at atmospheric pressure (≈1 bar). The values for the virus and its capsid at 1 bar are averages taken from previous determinations in these laboratories; the value used for ribonuclease at 1 bar is the average from determinations on this preparation (Sigma, Lot 27B-8550) *in water* after the dialysis treatment described in the text; therefore, the value assumed at pH 2.03 must not be taken to mean that ϕ is the same at pH 9.1 and at 2.03.

ribonuclease at pH 2.03 was done because Brandts[18] has noted that this protein undergoes a marked conformational change around pH 2, suggesting an expanded molecule, at temperatures as low as 20° as used in this study. Additional experiments with ribonuclease (not shown) in 0.01 M NaOH–0.02 M glycine, pH 9.7, and in 0.01 M HCl, pH 2.16, also exhibited no $\partial\phi/\partial p$ function in either solvent over this pressure range. Two other virus experiments in water and one in dilute phosphate buffer (pH 7) showed the same precision and lack of pressure dependence as that indicated in Table 3 for this virus. Since the virus capsids are generally less stable than the virus and are presumed to be hollow, icosahedral shells, the solution after the pressure run was observed by velocity sedimentation (33,450 rpm), but no evidence of aggregation or of dissociation into subunits was noted.

The results above are in agreement with observations by velocity sedimentation of these proteins, whereby no clear dependence of s_{20} on angular speed has been reported. Heretofore, however, it has not been shown by direct measurements whether protein monomers undergo changes in ϕ at ultracentrifuge pressures. The lack of an observed dependence on pressure for proteins may be considered, as a first approximation, largely as an effect of surface interactions between solvent and solute and of charge density. The surface to volume (i.e., mass) ratio and charge density are substantially less with most macromolecules than for small-molecular-weight compounds. Very likely $\partial\phi/\partial p$ for proteins are more than an order of magnitude less than that observed for sucrose; our data are not sufficiently precise to detect such small changes, and an impractically high concentration of such solutes would have been required in order to measure this function. That salts, notably NaCl, show $\partial\phi/\partial p$ an order of magnitude larger than that for sucrose is probably not so much a reflection of surface to volume ratios as to charge effects on the near-neighbor water molecules of the bulk solvent. It has been suggested that the lower heat capacity of salt solutions compared to pure water is a result of a competitive ordering tendency on the near-neighbor water dipoles by an ion, and by the tendency of bulk water to form clusters.[19] This competition gives rise to a randomizing effect on the water molecules between the irrotationally bound water about the ion and the free water of the solution. In an analogous way, one may invoke a random region of water in the vicinity of small ions which should be less compressible than bulk water, because the packing within the clusters of bulk water is regarded to be somewhat less dense than in random water.

Preliminary results were obtained with the associating protein subunits of tobacco mosaic virus. This protein undergoes reversible polymerization with increase in temperature, but the association is also sharply pH-dependent.[20] Stevens and Lauffer[21] have reported in a dilatometric study that a positive change of about 0.0074 ml/gm in the apparent specific volume takes place upon polymerizing the protein when the pH was lowered from 7.5 to 5.5 in 0.1 *M* NaCl at 4°. It was not feasible at this point to make measurements at temperatures other than 20°; however, the data of Lauffer *et al.*[20] indicate that this protein is essentially unpolymerized at this temperature and pH. Moreover, our concentration of protein (7 mg/ml) in 0.1 *M* NaCl was only about one-fourth the initial concentration at pH 7.5 as used by Stevens and Lauffer. Undoubtedly, the molecular weight of our preparation was somewhat greater, on the average, than that of the trimer (mol wt $\sim$ 53,000) of the basic subunit according to osmotic pressure data at 15.8° for this concentration and pH,[22] but the protein was clearly not in the highly polymerized or rodlike form as seen at the lower pH. Indeed, the solution, which was clear at pH 7.5, became distinctly opalescent as soon as the pH was lowered to 5.5. Further, 0.14 milliequivalents of HCl per gram of protein was required to effect this change in pH, in precise agreement with that found by Stevens and Lauffer at 4°. Upon application of pressure to the solution at pH 7.5 (to 357.2 bars) and at pH 5.5 (to 412.5 bars), no net change in ϕ at either pH was observed (14 pressure points at pH 7.5 and 16 points at pH 5.5); the extreme deviations about the mean were $\pm$ 0.002 ml/gm. Measurements to

412.5 bars were also made at pH 6.5, since at this pH a temperature transition to the unpolymerized state can be effected.[20, 23] Our results, again, showed no net deviation in ϕ with pressure. In general agreement with Stevens and Lauffer, we noted an increase in ϕ upon polymerization, i.e., approximately + 0.010 ml/gm from pH 7.5 to 5.5 and about +0.009 ml/gm from pH 7.5 to 6.5 at atmospheric pressure (these $\Delta\phi$ are not highly precise since we did not perform dry weight analyses nor independently repetitive experiments). The results suggest that the reaction mechanism under our conditions is relatively independent of pressure below 400 atmospheres as compared to hydrogen-ion concentration or temperature; however, further work is required before a definitive conclusion can be drawn.

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