[5] Magnetic Densimetry: Partial Specific Volume and Other Applications

By D. W. KUPKE and J. W. BEAMS

This chapter consists of three parts. Part I is a summary of the principles and current development of the magnetic densimeter, with which density values may be obtained conveniently on small volumes of solution with the speed and accuracy required for present-day applications in protein chemistry. In Part II, the applications, including definitions, are outlined whereby the density property may be utilized for the study of protein solutions. Finally, in Part III, some practical aspects on the routine determination of densities of protein solutions by magnetic densimetry are listed.

Part I. Magnetic Densimeter

The magnetic densimeter measures by electromagnetic methods the vertical (up or down) force on a totally immersed buoy. From these measured forces together with the known mass and volume of the buoy, the density of the solution can be obtained by employing Archimedes' principle. In practice, it is convenient to eliminate evaluation of the mass and volume of the buoy and to determine the relation of the magnetic to the mechanical forces on the buoy by direct calibration of the latter when it is immersed in liquids of known density.

Several workers have devised magnetic float methods for determining densities, but the method first described by Lamb and Lee[1] and later improved by MacInnes, Dayhoff, and Ray[2] is perhaps the most accurate means ($\sim$1 part in 10^6) devised up to that time for determining the densities of solutions. The magnetic method was not widely used, first because it was tedious and required considerable manipulative skill; second, the buoy or float was never stationary for periods long enough to allow ruling out of wall effects, viscosity perturbations, etc.; third, the technique required comparatively large volumes of the solution (350 ml) for accurate measurements. The latter requirement is often the most difficult to satisfy because biologically important substances are usually tedious and expensive to purify even in small quantities. The servo-controlled magnetic densimeter, first devised at the University of

[1] A. B. Lamb and R. E. Lee, *J. Amer. Chem. Soc.* **35**, 1666 (1913).
[2] D. A. MacInnes, O. M. Dayhoff, and B. R. Ray, *Rev. Sci. Instrum.* **22**, 642 (1951). *J. Amer. Chem. Soc.* **74**, 1017 (1952).

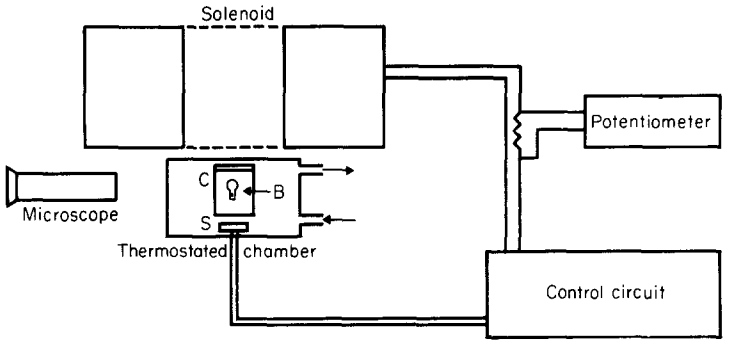

FIG. 1. Schematic diagram of the basic components for a magnetic densimeter—see text for details. B, Buoy; C, cell; S, sensing coil. From D. V. Ulrich, D. W. Kupke, and J. W. Beams, *Proc. Nat. Acad. Sci. U.S.* **52**, 349 (1964).

Virginia[3-7] overcomes the above difficulties and at the same time equals or may exceed the accuracy of the previous methods. Figure 1 shows a schematic diagram of the simplest type of servo-controlled densimeter. The buoy, B, is a small ferromagnetic body covered with an inert coating or mounted rigidly inside a small glass or plastic bulb. The cell, C, containing B is filled to a suitable level with the solution whose density is to be determined; with present buoys, 0.25–0.3 ml of solution is adequate in C—however, precise volumes are unimportant. The cell, C, is surrounded by a water or liquid bath which maintains the temperature constant on the order of 0.001°. The buoy, B, is freely suspended magnetically below the surface of the solution by the air-core solenoid situated above C (for the case shown in Fig. 1). The position of the buoy is sensed by the coil, S, located just below C, and its signal is applied to the electronic servo circuit in such a way that B is maintained automatically at a precise vertical position. A light beam–photoelectric height sensor has been used instead of the sensing coil in many experiments.[3,7] The axially symmetrical magnetic field of the solenoid automatically holds B in the desired horizontal position, since the ferromagnetic buoy will seek the strongest part of the field which is along the axis. When properly adjusted, no vertical or horizontal motion is observed in the microscope (with which a movement of at least 10^{-4} cm is detectable).

[3] J. W. Beams, C. W. Hulburt, W. E. Lotz, Jr., and R. Montague, *Rev. Sci. Instrum.* **26**, 1181 (1955).
[4] J. W. Beams and A. M. Clarke, *Rev. Sci. Instrum.* **33**, 750 (1962).
[5] A. M. Clarke, D. W. Kupke, and J. W. Beams, *J. Phys. Chem.* **67**, 929 (1963).
[6] D. V. Ulrich, D. W. Kupke, and J. W. Beams, *Proc. Nat. Acad. Sci. U.S.* **52**, 349 (1964).
[7] J. P. Senter, *Rev. Sci. Instrum.* **40**, 334 (1969).

The vertical forces on the buoy consist of the downward force of gravity, $m_B g$, and the upward buoyant force $\rho V_B g$, plus the lifting force of the solenoid, $M(dH/dz)$; m_B is the mass of B, g is the acceleration of gravity, ρ is the density of the solution, V_B is the volume and M is the magnetic moment of B and dH/dz is the vertical gradient of the magnetic field, H, at the solenoid where z is the vertical coordinate. Hence,

$$M \frac{dH}{dz} + \rho V_B g = m_B g \tag{1}$$

where ρ is mass per unit of volume for this purpose. The servo circuit maintains the upward forces on the left side of the equation to equal the opposing force on the right very accurately; it can be shown that the buoy may be held stationary to better than one-tenth the wavelength of light. It is essential, of course, that B remains stationary if Eq. (1) is to be valid. The gradient of the magnetic field is exactly proportional to the current, I, in the support solenoid, i.e., $dH/dz = K_1 I$. On the other hand, the magnetic moment, M, of the ferromagnetic material in the buoy is, in general, made up of two parts, i.e., $M = M_0 + f(H)$, where M_0 is a permanent moment and $f(H)$ is the magnetic moment induced in B by the magnetic field H. If the ferromagnetic material in B is "very soft" magnetically, as is the case for properly annealed "Hy-Mu 80" (an alloy of nickel and iron, Carpenter Steel Co.), M_0 is very small and can be neglected in most cases; thus, the total magnetic moment can be written as $M = f(H)$. Furthermore, if the densimeter is used only for measuring small ranges of densities where the changes in H are not large and B is not saturated, $M \cong K_2 I$, and the total upward force, F, on B due to this solenoid is $F = K_3 I^2$, where $K_3 = K_1 K_2$. Equation (1) then becomes

$$K_3 I^2 \cong m_B g - \rho V_B g = V_B g(\rho_B - \rho) \tag{2}$$

where ρ_B is the average density of the buoy in terms of mass per unit of volume. Since the current, I, can be measured to 1 part in 10^6, it is evident from Eq. (2) that by calibrating the buoy with liquids of known density, ρ_B, V_B and K_3 can be determined if the weight of the buoy, $m_B g$ is known. Ordinarily, neither the weight nor the individual values of V_B and K_3 are evaluated, because the equation for the least-squares line of the calibration data is used directly for the calculation of the densities of unknowns; i.e., $\rho = -(K_3/V_B g) I^2 + \rho_B$.

When the buoy contains "very hard" magnetic material, or a permanent magnetic moment, $M = M_0 + K_4 I$, so that Eq. (1) becomes

$$K_5 I^2 + K_6 I = m_B g - \rho V_B g = V_B g(\rho_B - \rho) \tag{3}$$

Consequently, by calibrating with liquids of known densities, K_5, K_6, and V_B can be determined so that the densities of unknowns can be evaluated over the range of the calibration. Recently, Haynes and Stewart[8] have found that with very hard barium ferrite ($BaFe_{12}0_{19}$) in B, $f(H)$ can be neglected over the range used for calibration so that Eq. (3) becomes simply

$$K_6 I \cong m_B g - \rho V_B g = V_B g(\rho_B - \rho) \tag{4}$$

which has the advantage of being linear in I with respect to ρ. It should be noted that in general for air-core solenoids, $H = I \cdot f_1(z)$ and $dH/dz = I \cdot f_2(z)$, so that if B is held at a constant vertical position, $f_1(z)$ and $f_2(z)$ are constants.

As discussed above, Eqs. (2–4) are special cases of Eq. (1), and they hold strictly only over moderate ranges of density. This limitation requires that the calibration values should be over small density intervals and also that the density of the buoy should be as close as possible to that of the solutions to be measured; the latter requirement necessitates the construction and calibration of a number of separate buoys if various density ranges are to be covered (see Part III). A given buoy is sufficient over a range of 0.03 g/ml in density for the purposes currently pursued in the study of proteins. In a prototype model, the above problems have been eliminated by effecting a change in the design of the densimeter.[9] For this purpose M in Eq. (1) is maintained constant at a given vertical position of the buoy and only the gradient, dH/dz, is varied (which is strictly proportional to the current). A schematic diagram of the apparatus is shown in Fig. 2. The buoy is freely supported inside the solution by the air-core solenoids, S_1, S_2 and S_3, which have the same vertical axis. It is convenient, although not necessary, if S_2 and S_3 have the same number of turns and are as nearly identical as possible. Also, it is advantageous to make the radii of S_2 and S_3 larger than that of S_1, but with a fewer number of turns. The buoy, B, is located on the common axis of S_1, S_2, and S_3 and is equidistant from S_2 and S_3. The distance along the axis between B and S_2 and S_3 is usually made equal to the radius of these coils in the manner of Helmholtz coils. In order to support B, a constant current, I_1, is passed through S_1, which is not quite sufficient to support B in the desired vertical position in the field of the microscope (which, of course, is of nonferromagnetic material). Next, a current I_2, is passed through S_2 and S_3, which are connected in series in such a way that their magnetic fields cancel while

[8] W. M. Haynes, Dissertation, University of Virginia (1970); W. M. Haynes and J. W. Stewart, unpublished results.

[9] J. W. Beams, *Rev. Sci. Instrum.* **40**, 167 (1969).

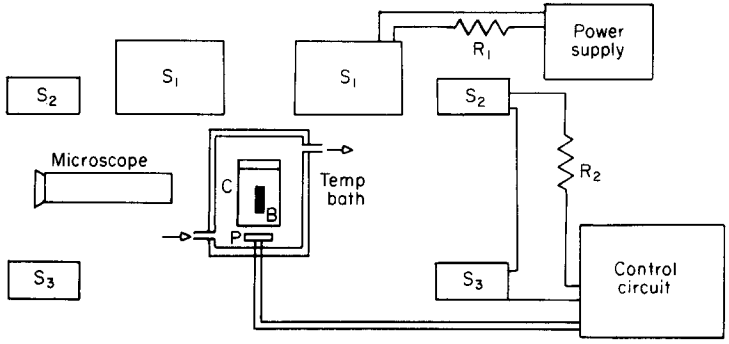

FIG. 2. Schematic diagram of the components for a magnetic densimeter where (a) the current and solution density are in direct proportion and (b) a single buoy is utilized over the total density range for aqueous solution (P = sensing coil)—see text for details. From J. W. Beams, *Rev. Sci. Instrum.* **40**, 167 (1969).

their magnetic field gradients add. As the common current, I_2, in S_2 and S_3 is increased, the resultant magnetic field at B due to I_2 remains unchanged provided that the current, I_1, in S_1 is held constant. If, at the same time, I_2 is in the proper direction, the field gradient, dH/dz, at B increases and, as shown in Eq. (1), the upward force on B increases. If now, the current, I_2, through S_2 and S_3 is regulated by the sensing coil–servocontrol circuit, the buoy B is automatically held at the desired height in the field of view of the microscope. The current, I_1, in S_1 can be maintained constant to 1 part in 10^6 by commercial, constant current power supplies and is determined with the same precision by measuring the potential drop across a standard resistor, R_1, via a potentiometer or differential voltmeter. I_2 also is determined with the same accuracy by measuring the potential drop across the standard resistor, R_2. Since I_1 and M are held constant, the upward force on the buoy due to S_1 is constant and can be set equal to a constant A. Then, from Eq. (1),

$$A + K_7 I_2 = V_B g(\rho_B - \rho) \tag{5}$$

Since K_7 is a constant, the density, ρ, of the solution is a linear function of I_2 only. Consequently, the densimeter can be calibrated with a minimum number of solutions of known densities (with the present "on-line" instrument of the original design, however, the density is already strictly linear with the *square* of the potential drop over the density range used for a given buoy; see Part III). Unlike the original design (Fig. 1), however, the same buoy can be used over a wide range of densities without appreciable change in the precision since the relation between ρ and I_2 (Eq. 5) is strictly linear. As long as it is not necessary to change

I_1 appreciably, which is usually the case in practice, the densimeter need not be recalibrated. It should be noted that this kind of densimeter will function equally well when I_1 in S_1 is slightly larger than necessary to support the buoy at the desired vertical position; in this case, I_2 must be reversed through S_2 and S_3. It is more convenient for some purposes to place the thermostated cell above the solenoid, S_1, and employ a buoy which floats on the solution with no currents applied (i.e., $\rho_B - \rho$ is negative, as in our "on-line" instrument with the simple magnetic support, Fig. 3). In this case, the force on the buoy is down instead of up during a measurement. The precision with the solenoid, S_1, in either position is essentially the same. It has also been found that the solenoid, S_1, may be replaced by a hard ceramic cylindrical magnet (barium ferrite) having a large permanent moment. Instead of varying I_1 in S_1 in order to produce the best operating range for I_2 in S_2 and S_3, the permanent magnet is moved vertically along the axis of S_2 and S_3 until the buoy is not quite supported. (This modification holds if changes in the magnetic field produced at the permanent magnet by S_2 and S_3 are relatively small compared to the total field of the ferrite magnet itself.)

In examining Eqs. (2), (4), and (5), which represent the three most popular methods of operation in magnetic densimetry at the present time, it will be observed that the relation between the proportional changes in densities and the proportional changes in servo-circuit currents are related as follows:

$$\Delta(\rho_B - \rho)/(\rho_B - \rho) = (2K_3/V_B g)\Delta I/I \quad \text{for Eq. (2)}$$
$$\Delta(\rho_B - \rho)/(\rho_B - \rho) = (K_6/V_B g)\Delta I/I \quad \text{for Eq. (4)}$$
$$\Delta(\rho_B - \rho)/(\rho_B - \rho) = (K_7/V_B g)\Delta I_2/I_2 \quad \text{for Eq. (5)}$$

The sensitivity depends upon making $\Delta I/\Delta\rho$ as large as possible while the precision depends upon making $\Delta I/I$ as large as possible, consistent with the determination of K_3, K_6, and K_7. It is clear that in all three cases above, it is advantageous to make $(\rho_B - \rho)$ as small as possible. This is especially true in the first of the two equations above. On the other hand, for the densimeter of Fig. 2, K_7 can be made much larger than K_3 or K_6, so that both the precision and the sensitivity is much greater, especially over wide density ranges. The choice of the above types of densimeters will depend upon the size of the density range normally covered in experiments together with the degree of sensitivity and precision desired. For example, if the density range of the usual experiments is only a few hundredths of a gram per milliliter, the simpler method of Fig. 1 (or as modified in Fig. 3, see Part III) is satisfactory;

this type has been used the most in the study of proteins. The smallest value of ΔI which can be measured accurately in any of these densimeters, is limited by the general "noise" in the circuits; in practice this is about 1 part in 10^6 of I. If the other factors can be held constant, the variation in ρ can be measured with at least this same precision. The control of the temperature and of evaporation, however, may be found to limit the precision more than the electronic noise.

The question of temperature control systems, such as the introduction of thermistor probes at the side of the cell, is beyond the scope of this article; each investigator tends to incorporate his own preferences with the means available. It is perhaps sufficient to note that although the volume, and hence the density, of the glass-jacketed buoy is subject to change with temperature, the uncertainty from this effect appears to be well within the overall operating precision of 1 part in 10^5 when temperatures do not vary more than $\pm 0.005°$. In fact, since the change in the bulk expansion of glass with temperature is much less than that for aqueous solutions, it is observed that the change in the current over small changes in temperature ($\Delta T = 0.01°$) virtually corresponds to the known change in the density of water at that temperature range. Also, since the sample volume can be made small (because the buoy is stationary) and has a relatively large ratio of surface to volume, the sample rapidly comes to the temperature of the circulating thermostated liquid. Since temperature surges from the poorer types of temperature controllers are then seen, owing to the rapid response by the small volume of solution, a small sealed Dewar flask in line with the circulating thermostated liquid between controller and the cell has served to damp out the surges effectively.

As has been mentioned, it is necessary to make all the measurements at the *same* vertical position of the buoy with respect to the solenoid. This is not difficult if the microscope is sufficient for detecting changes in height of the order of 10^{-4} cm. If opaque samples or solutions which must not receive light are to be studied, a second sensing coil (similar to P in Fig. 2) is placed above the buoy. This coil actuates a sensing circuit which when calibrated gives the position of the buoy to 10^{-5} cm. For the usual transparent and semitransparent solutions which are studied in protein chemistry, a light beam, photodiode sensor assembly has often been used instead of the "pick-up" coil sensors.[3,7] An all solid-state model utilizing optical sensing and the inverted solenoid design[7] (which is generally more convenient in biochemistry), has been used in the routine work with proteins (see Fig. 3). Since the magnetic suspension principle readily lends itself to the study of density changes as a function of pressure (as well as temperature and of time in slow

reactions), some experiments with a pressure bomb adapted to the design in Fig. 1 have been made.[10]

The handling of the buoy and the problem of small bubbles growing upon it during measurements are noted in Part III.

Finally, a small modification of the magnetic densimeter has been installed by which the coefficient of viscosity, η, of the solutions can be measured.[11] The changes consist first in making the cell accurately cylindrical on the inside and in making the buoy a carefully balanced cylinder containing an electrically conducting, ferromagnetic material (e.g., "Hy-Mu 80", but not barium ferrite). Second, around the thermostated jacket of the cell, small field drive coils are placed through which an alternating current is passed in such a way that a rapidly rotating field produces a torque on the buoy and causes it to turn. The power input to the drive coils is accurately controlled and measured. When properly calibrated with liquids of known viscosities, the viscosities of unknown solutions are found. The speed of rotation ($\sim$1 rpm) is an accurately repeatable function of the power input to the coils for a given viscosity. This work is still in the preliminary stages, but the precision approaches 1 part in 10^4. The buoy rotates slowly enough that Taylor vortices are not established. By this means shearing is minimized and the corrections for kinetic energy and, of course, for density are eliminated. Also, the problems associated with forcing protein solutions through long, narrow capillaries are avoided. Thus, the density, ρ (and, hence, the apparent specific volume, ϕ_i, of a component i) and the coefficient of viscosity, η, of a single, small sample of solution are measured simultaneously.

Part II. Applications and Definitions

Partial and Apparent Specific Volumes

The partial specific volume, $\bar{v}_i$, of a component i is the derivative, or tangent, at a particular composition of a solution as the total volume, V, varies as a function of the mass in grams, g_i, of the component at constant temperature, T, pressure, P, and grams of all other components, g_j. Thus,

$$\bar{v}_i = \left(\frac{\partial V}{\partial g_i}\right)_{T,P,g_j} \qquad (j \neq i) \tag{6}$$

where V is in milliliters. Hence, $\bar{v}_i$ occurs as the partial derivative in the

[10] P. F. Fahey, Jr., D. W. Kupke, and J. W. Beams, *Proc. Nat. Acad. Sci. U.S.* **63**, 548 (1969).

[11] J. W. Beams and M. G. Hodgins, *Bull. Amer. Phys. Soc., Ser. II*, **15**, 189 (1970).

total differential of the volume, where $V = f(T, P, g_i)$. The total volume of a solution of N components at constant temperature and pressure is given by

$$V = \sum_{i=1}^{N} \bar{v}_i g_i \tag{7}$$

which results from integration of $dV = \Sigma \bar{v}_i dg_i$, provided that the composition is held constant on summing over each successive infinitesimal of the volume, dV. The apparent specific volume, ϕ_i, of a component is simply the difference in volume between a solution and the corresponding solvent medium divided by the number of grams of the component; the solution contains a measured amount of the component i but is otherwise of precisely the same composition as the solvent medium. Accordingly

$$\phi_i = \frac{V - V'}{g_i} = \frac{V - \sum_{j=1}^{N} \bar{v}'_j g'_j}{g_i} \qquad (j \neq i) \tag{8}$$

where single primes refer to the solvent medium of volume, V'; for the general case of N components, j, in the solvent medium, $V' = \Sigma \bar{v}'_j g'_j$ (i.e., Eq. 7). Thus, the definition of ϕ_i arbitrarily assigns a definite volume to the solvent medium within the total solution equal to that of the solvent volume in the absence of component i. Experience has shown that in the case of proteins (and macromolecules generally), differences between $\bar{v}_i$ and ϕ_i ordinarily are not detectable over the rather low range of concentrations usually employed (viz., <5% protein). Self-associating systems, highly asymmetric macromolecules and polypeptides exhibiting random-coil behavior may prove to be exceptions to this empirical generalization, but sufficient data are lacking on this point. Small amounts of titrant, such as H^+, however, are known to bring about changes in $\bar{v}$ of proteins as the titrant is consumed when the protein component is added. Hence, the distinction between $\bar{v}$ and ϕ should be kept in view when studying any new system.

In practice, it would be very inconvenient to measure absolute volumes as a function of the mass of the pure protein component in order to obtain values of $\bar{v}$ or ϕ with the accuracy often required (e.g., ± 0.002 ml/g, or better). Highly accurate measurements of the volume change, ΔV (see Dilatometry, this volume [18]), may be employed to obtain differences in ϕ; however, the determination of absolute values of $\bar{v}$ or ϕ for proteins is usually not attempted with the dilatometric

techniques available.[12] Instead, the measurement of the density is employed routinely for the determination of specific volumes.

Density, by definition, is the mass per unit of volume; for the study of protein solutions, the density defined in grams per milliliter is standard usage, and these units will be used throughout this discussion.[13] With the density given in these units, and $\bar{v}$ and ϕ given in units of milliliters per gram of a component, it will be most convenient to use concentrations defined in terms of grams per milliliter, c. For a mixture of N number of components[14] in solution, the density, ρ is given very simply by

$$\rho = \frac{\sum_{i=1}^{N} g_i}{V} = \sum_{i=1}^{N} c_i \tag{9}$$

where the total volume, V, is in milliliters. Densities of solutions can be measured with a routine accuracy of 1 to 2 parts in 10^5 with ordinary care; *differences* in density between solutions in a series are obtainable with precisions on the order of 10^{-6} g/ml. As rule of thumb, densities should be accurate to about 10^{-5} g/ml in order to obtain values of $\bar{v}$ for proteins good to 10^{-3} ml/g in aqueous systems. For this purpose, however, the precision by which protein concentrations are measured is the limiting factor rather than the density (see later).

The partial specific volume of a protein is obtained from a plot of the density versus the concentration of the "defined" protein component. (Whatever definition is chosen, it must be adhered to consistently in all solutions of the series; e.g., the component may be the anhydrous, isoionic protein or a charged protein ion including a stoichiometric number of counterions or, for this purpose, simply a *reproducible* substance which may contain known and unknown ingredients such that the properties of

[12] N. Bauer and S. Z. Lewin, *in* "Physical Methods of Organic Chemistry" (A. Weissberger, ed.), 3rd ed., Vol. I, Part I, Chapter IV. Wiley (Interscience), New York, 1959.

[13] The milliliter (ml) is a defined volume on the basis of a weight of pure water at 3.98°, whereas the cubic centimeter (cc) is an absolute volume based on an arbitrarily selected standard of length. The latter standard is essentially unavailable to the investigator compared with pure water; i.e., the maintenance of an absolute volume in terms of length for routine reference is highly impractical. Note also that, 1 cc = 0.999973 ml; this difference can be important when using reference standards from density tables; therefore, the units employed in such tables must be taken into account.

[14] A component is a definable material, not necessarily a pure compound or single chemical species, which can be added quantitatively to a system so that the composition of the system in terms of the masses of each component can be known.

the substance are maintained constant. The value of $\bar{v}$ which is obtained is a property of whatever material is added independently to a solvent medium of fixed composition. That is, all other components of the solution, which we define here as the *solvent medium*, must be maintained in precisely the same proportion to one another in each solution of the series so that only the substance called the protein component is varied.) For most purposes, the partial specific volume of the protein at infinite dilution is desired. This quantity is denoted as $\bar{v}_2^0$, where subscript 2 refers to the protein component and superscript zero to vanishing protein concentration—or, on occasions, to an *initial* condition which may contain the protein at some specified concentration (i.e., the solvent medium can contain a known amount of the protein as a part of this medium). $\bar{v}_2^0$ is obtained via density measurements from the relation

$$\bar{v}_2^0 = \frac{1}{\rho'}\left[1 - \left(\frac{\partial \rho}{\partial c_2}\right)_m^0\right] \tag{10}$$

where ρ' is the density of the protein-free solvent medium (in this case) and subscript m refers to constant molality of all other components. Thus, if the data describe a curve, the limiting slope, $(\partial\rho/\partial c_2)_m{}^0$, must be evaluated, such as by polynomial fitting in order to derive a curve representing the least-squares deviation from the data points. [If the concentration is desired in terms of the weight fraction, W_2, the limiting slope, $(\partial \ln \rho/W_2)_m{}^0$, is evaluated where $W_2 = g_2/\Sigma g_i$; Eq. (10) is otherwise identical.] The quantity, ρ', is usually the most accurate measurement in the entire procedure, being independent of the protein component, and serves to anchor the curve when a statistical weighting procedure is considered. Most frequently, however, the data are described by a straight line. It is then a simple matter to apply a linear, least-squares fit to the data with a desk calculator. Often, correlation coefficients (r) are found to be better than 0.99999 for these linear representations. Hence, in the usual case, $\bar{v}_2^0 = \bar{v}_2$ at any concentration of the protein; also then $\bar{v}_2 = \phi_2$, the apparent specific volume, since these latter two parameters are related by

$$\bar{v}_2 = \phi_2 + g_2\left(\frac{\partial \phi_2}{\partial g_2}\right)_m \tag{11}$$

Thus, if the density increment per unit of concentration, $(\Delta\rho/c_2)_m$, is constant as the mass of component 2 is varied, the derivative in the above equation is zero. If ρ versus c_2 is a curved function and the partial specific volume is desired at a particular concentration, $\bar{v}_2$ is calculated from the value of the derivative, $(\partial\rho/\partial c_2)_m$, at this concentration by the expression for the derivative

$$\left(\frac{\partial\rho}{\partial c_2}\right)_m = \left(\frac{1 - \bar{v}_2\rho}{1 - \bar{v}_2 c_2}\right) \tag{12}$$

where ρ is the density of the solution at c_2. For this purpose, the data must necessarily be extensive for evaluation of the tangent at c_2 with confidence. Obviously, the value of ϕ_2 at c_2 may be quite different from $\bar{v}_2$ because the former value is obtained simply by extending a straight line from the density of the solvent to the density of the solution at c_2, the slope of which, $(\rho - \rho')/c_2 = \Delta\rho/c_2$, along with the value of ρ' yields ϕ_2 by

$$\phi_2 = \frac{1}{\rho'}\left[1 - \left(\frac{\Delta\rho}{c_2}\right)_m\right] \tag{13}$$

This equation is consistent with the definition of ϕ_i given in Eq. (8). It is seen that the value of the slope is the familiar buoyancy term $(1 - \phi_2\rho')$. Although in the study of proteins, $\bar{v}_2$ and ϕ_2 often appear to be identical over the dilute range in c_2 which is covered experimentally, the casual intermingling of these parameters no doubt has contributed to the vagueness surrounding their distinction. ϕ_2 can be a very useful, practical quantity in handling a number of problems, where $\bar{v}_2$ is difficult to evaluate, if it is recognized that the solvent medium is arbitrarily assigned ideality with respect to volume. A component or group of components cannot be given an exact 3-dimensional space in a mixture with other components, but an assigned volume can lead to strict relations for useful application. In terms of $\bar{v}_2$, a thermodynamic volume can be assigned; e.g., the thermodynamic volume of the nonprotein components is $V - \bar{v}_2 g_2$, or in 1 ml of the protein solution the quantity $(1 - \bar{v}_2 c_2)$ describes the fraction of a milliliter which is contributed, thermodynamically, by these components. If $(\partial\rho/\partial c_2)_m$ is not a constant, then clearly nonideality with respect to solvent volume is evident, and the fractional volumes per milliliter $(1 - \bar{v}_2 c_2)$ and $(1 - \phi_2 c_2)$ will not be equal. Although proteins and macromolecules, generally, exhibit positive values for the density increment in aqueous media, it should be recognized that neither $\bar{v}_2$ nor ϕ_2 are necessarily positive and may be zero or negative.

In practice, it is inconvenient to add the anhydrous protein component to the solvent medium in order to make up a series of solutions upon which to determine ϕ_2 or $\bar{v}_2$ (unless one is specially set up for carrying out weighings in an anhydrous atmosphere). Dried protein when opened to room air quickly takes up water, the amount varying with the relative humidity and temperature. Since water is usually the major solvent component, the protein component can be added in the

form of a water solution of known composition. For this purpose, the protein preparation (preferably the isoionic product where convenient) is dissolved in water to make a stock solution from which the composition is determined by accurate dry weight analysis. If the protein preparation is not soluble in water, the solubilizing aqueous medium of known composition, such as may be used for the density measurements, should contain only components that are nonvolatile (e.g., salts) or are completely volatile—otherwise the dry weight analysis will be meaningless. With muticomponent solvents, of course, the weight of the protein preparation added to a weight of the solvent must be known in order to ascertain the new weight fraction of the nonvolatile solvent component(s) for the dry weight calculations. After the composition of the stock solution is known, aliquots may be weighed into weighed aliquots of the solvent medium on an analytical balance for the density series. If the solvent is not pure water, the other solvent components must be added in their pure state to each solution of the series to compensate for the amount of water, and any other components, which were added along with the protein in the stock solution. Alternatively, the protein stock solution may be adjusted on the analytical balance with known amounts of the other component(s) so that the adjusted stock solution is isomolal with respect to the solvent medium. The series of solutions, increasing in c_2, are then prepared by simply weighing aliquots of the adjusted stock solution into weighed aliquots of the solvent medium. The concentration in terms of c_2 is easily calculated after the density of the solutions in the series is determined by the definition

$$c_i = \rho W_i \tag{14}$$

where W_2 for each solution is obtained from its weight fraction in the stock solution along with the weight of this solution and that of the solvent medium used in each dilution.

The dry weight analysis on the protein solution used for the dilution series must be very carefully done because the evaluation of c_2 is the major source of error in estimating ϕ_2 or $\bar{v}_2$. [As a rule of thumb, a $\pm 1\%$ error in c_2 causes an uncertainty in ϕ_2 of ± 0.003 ml/g, whereas densities which are accurate to ± 1 part in 10^5 give rise to about ± 0.001 ml/g error.] A comparison of protocols for obtaining the dry weight of proteins is given by Hunter.[15] The precisions which we have obtained by applying the procedures described by Hunter, such as extrapolation

[15] M. J. Hunter, *J. Phys. Chem.* **70**, 3285 (1966).

of the weights to zero time after removal of the dried samples from an anhydrous atmosphere, are within 1–2 parts in 10^3.

The Isopotential Specific Volume

When proteins are dialyzed in a solvent medium which is not pure water, the density versus concentration plot will not be necessarily identical to that obtained as outlined under the preceding heading wherein the solvent components were isomolal at all values of c_2. When dialysis is employed, it is assumed that osmotic equilibrium has been attained. Hence, the chemical potentials, μ, of all diffusible components (1, 3, 5 . . . , etc.) must be identical on the two sides, but it is not necessary that the molalities of the diffusible components be the same (except in the case of a one-component solvent, such as pure water). As a matter of experimental record, the molalities are hardly ever equal. This difference in distribution becomes strikingly evident after dialyzing a moderately low concentration of protein in a solvent medium containing relatively high concentrations of a diffusible component, such as salt, sugar, urea. Thus, the values of the derivative in the isopotential versus the isomolal density series, $(\partial\rho/\partial c_2)_\mu$ and $(\partial\rho/\partial c_2)_m$, respectively, at a particular value of c_2 may be not only different, but appreciably so. The partial specific volume, via Eq. (10), may be sufficiently different from the corresponding specific volume obtained from density measurements after dialysis equilibrium so that serious discrepancies may arise when applying these quantities indiscriminantly toward some purpose (e.g., sedimentation equilibrium). Quite generally, the isopotential curve is linear over the usual dilute range in c_2 so that $(\partial\rho/\partial c_2)_\mu$ is a constant and equals $(\partial\rho/\partial c_2)_\mu{}^0$ at vanishing protein concentration. A specific volume, $v_\mu{}^0$ derived from $(\partial\rho/\partial c_2)_\mu{}^0$ may be calculated with the use of a relation analogous to that for the partial specific volume. Thus,

$$v_\mu{}^0 = \frac{1}{\rho'}\left[1 - \left(\frac{\partial\rho}{\partial c_2}\right)_\mu^0\right] \tag{15}$$

where subscript 2 is dropped because this specific volume can only refer to the nondiffusible component. For the present discussion, we refer to $v_\mu{}^0$ (or v_μ) as the "isopotential specific volume." [It is clear that we cannot call this quantity a partial specific volume, because the masses of the other components are not being held constant as component 2 is varied, an absolute requirement for defining any partial molal or partial specific property of a component. The diffusible components have access to the dialyzate and their distribution on the protein side of the membrane may change as c_2 varies.] The quantity, $(\partial\rho/\partial c_2)_\mu{}^0$, is

utilized in the study of multicomponent systems, e.g., by low-angle X-ray scattering[16] and by equilibrium sedimentation[17] where this derivative replaces the familiar buoyancy term, $(1 - \phi_2 \rho')$, derived for two-component systems. Following the dialysis of the protein solution to equilibrium, a dilution series in c_2 is prepared by weighing together appropriate aliquots of each solution on the two sides of the membrane. The mixture of dialyzate and equilibrated protein solution, in effect, simulates what the composition of the diffusible components would have been if this lower concentration of protein had actually been dialyzed against a large volume of this solvent [provided that $(\partial\rho/\partial c_2)_\mu$ is essentially a constant].[18] The densities are then determined on the dialyzate, the equilibrated protein solution and the weighed dilution series. [Six to eight dilutions in addition to dialyzate and undiluted protein solution seem to be adequate where linearity in ρ versus c_2 is obvious. This can easily be done with the magnetic densimeter, which requires about 1 ml of each solution for triplicate measurements ($\sim$250 μl per sample); this number of measurements (24–30) can be made within 3 hours if a calibration curve is already available.]

The assignment of c_2 in an isopotential series also requires the highest possible accuracy in the determination of the protein concentration of the stock (dialyzed) solution. In this case, the method for evaluation of c_2 must be one which can be made insensitive to the concentration of the diffusible components (e.g., the molality of a component 3 may be different on the two sides of the membrane). Hence, refractive index or dry weight analysis would tend to reflect differences in the concentration of the diffusible components if the dialyzate is used as a reference medium. Ordinarily, direct light absorption methods are employed because these are faster and can be about as accurate as other methods, such as the determinaiton of the nitrogen content. Frequently, the necessary measurements can be made on dilutions of the dialyzed solution at the maximum absorbance in the 280-nm region of the ultraviolet spectrum. For this analysis, an accurate value for the specific absorbance of the protein component must be available. This value is obtained with best confidence by performing careful dry weight analyses on the same protein preparation as that used for the density experiments. That is, a stock solution of the protein in essentially the same solvent medium as that used in the dialysis experiments is prepared by weight so that the weight fraction of nonvolatile components, other than the protein, is known. In this way the dry weight and the absorbance may be de-

[16] H. Eisenberg and G. Cohen, *J. Mol. Biol.* **37**, 355 (1968).
[17] E. Reisler and H. Eisenberg, *Biochemistry* **8**, 4572 (1969).
[18] E. F. Casassa and H. Eisenberg, *J. Phys. Chem.* **65**, 427 (1961).

termined concurrently and related together to give the proper specific absorbance at a particular temperature and wavelength. Generally, dilutions of a stock protein solution are required for measuring the absorbance in the ultraviolet region. For best accuracy, the dilutions are made by weight on the analytical balance with the dialyzate as the diluent (or the isomolal solvent medium if this is known). These weight dilutions are then converted to volume dilutions via the known densities of the protein solution and the solvent. The volume dilution factor is applied because we are interested in the value of c_2, and also the specific absorbance is given in terms of volume per unit weight of the substance. A given weight of the protein solution may be represented by, Σg_i, the sum of the grams of each of the N components in the solution which is on the balance, and the weight of the diluent is represented by, $\Sigma g'_j$ $(j \neq 2)$, the sum of the grams of the N number of nonprotein components, which are added to the protein solution. The volume dilution is then given almost exactly by

$$\text{Volume dilution} = \frac{\left(\sum_{i=1}^{N} g_i\right)/\rho + \left(\sum_{j=1}^{N} g'_j\right)/\rho'}{\left(\sum_{i=1}^{N} g_i\right)/\rho} \qquad (j \neq 2) \qquad (16)$$

where ρ' is the density of the dialyzate or diluent. This equation assumes only that $\bar{v}_2$ does not change with dilution; however, the effect would be trivial for any reasonable change in $\bar{v}_2$. In some cases dilution with dialyzate may not be sufficiently accurate because of significant light absorption by one of the diffusible components. Another diluent of known density, such as water, may be employed to dilute equal aliquots of the protein solution and dialyzate, the latter acting as a blank. In general, the proportion of diluent should be large in order to minimize error as a result of slightly unequal amounts of the absorbing diffusible component in the paired-weight samples of solution and dialyzate. With the volume dilution known, the observed absorbance is immediately converted to the value of c_2 for the undiluted solution by use of the specific absorbance (or absorptivity in ml/g). The dilutions for determination of the specific absorbance value itself are treated similarly except that the isomolal solvent medium is usually prepared as the diluent after W_2 is known from the dry weight analysis.

The volume dilutions for the solutions made up with the dialyzed protein solution and dialyzate for the density series are also calculated by Eq. (16). In this way the value obtained for c_2 on the dialyzed solution may be directly converted to the corresponding value of c_2 of each

diluted sample. As in the case of the isomolal experiments for $\bar{v}_2$, correlation coefficients of linear, least-squares fits to the observed density versus c_2 values are found to be on the order of 0.99999—indicating that the weight-dilution operations at the balance can be carried out with a precision similar to that for the density determinations by the magnetic densimeter. Little is to be gained by performing ultraviolet absorption measurements on each diluted solution of the series because the error of the absorption measurement is much larger than that for the dilution procedure. Hence, replicate analyses for c_2 on the undiluted protein solution suffice to yield a weighted value for use with the volume dilutions in order to obtain c_2 for each diluted sample. If the density determinations are sufficiently accurate, any inconstancy in $(\partial\rho/\partial c_2)_\mu$ with dilution is most clearly seen by a volume dilution series based on a given value of c_2 for the stock solution. It is obvious, of course that replicate dialysis experiments are to be performed by which a weighted value of $(\partial\rho/\partial c_2)_\mu$ is obtained so that more confidence is given to the value of this derivative for a defined protein component in a specified multicomponent solvent medium.

Since $(\partial\rho/\partial c_2)_\mu$ appears not to vary appreciably with c_2, the apparent quantity, ϕ_μ, at a finite value of c_2 may be determined in practice.[18] This parameter is evaluated instead of $v_\mu{}^0$ if many density measurements decreasing in c_2 cannot be conveniently carried out. Thus, by analogy to Eq. (13), ϕ_μ is related to the difference in density between solution and the equilibrated solvent by

$$\phi_\mu = \frac{1}{\rho'}\left[1 - \left(\frac{\Delta\rho}{c_2}\right)_\mu\right] \tag{17}$$

The difference between ϕ_μ and $v_\mu{}^0$, in most cases, will be a result of the larger uncertainty in the former value because fewer density determinations are performed. Sufficient examples are lacking, however, for one to be able to predict the circumstances in which curvature in ρ_μ versus c_2 may be expected; hence, the equivalence of a determined value of ϕ_μ with $v_\mu{}^0$ in a given case must be considered as provisional. In this connection, it should be observed that ϕ_μ does not bear the same relationship to v_μ at a particular value of c_2 as ϕ_2 does to $\bar{v}_2$ (Eq. 11). If the diffusible components in the protein solution redistribute as a function of c_2 during the dialysis, a hypothetical solvent medium of the same composition as that in the protein solution would have to be changed each time c_2 is changed; thus ϕ_μ and v_μ are not simply related as in the experiments where the solvent composition does not change as component 2 is varied. Finally, $(\Delta\rho/c_2)_\mu$ may be greater or less than $(\Delta\rho/c_2)_m$ and neither the difference in the value of the slope nor the sign of the differ-

ence can be predicted from a value of either ϕ_μ or ϕ_2 alone in the absence of additional information. The difference between these slopes obtained by density under isopotential and isomolal conditions has important application to the study of multicomponent systems. This application is discussed separately under the heading "Preferential Interaction" (Part II).

It will be noted that we have ignored in this discussion of the isopotential specific volume the fact that a small pressure difference usually exists between the two phases at osmotic equilibrium. Ordinarily, such differences in pressure are less than 0.02 atm for average-sized proteins at concentrations under 5%. An increase in the pressure of 0.02 atm increases the density of aqueous solutions by about 1×10^{-6} g/ml, which is well within the overall precision of the method.

Composition Analysis by Density

It is sometimes important to be able to evaluate small changes in the composition of a two-component solvent medium, such as water (component 1) and a salt, saccharide, urea, etc. (component 3), after an equilibration procedure (e.g., equilibrium dialysis or column experiments). As previously noted, water and component 3 may redistribute relative to some initial composition after equilibrating with a protein solution via a semipermeable membrane. Experiments on multicomponent systems are frequently carried out in a solvent medium of water plus relatively large amounts of a diffusible solute (as component 3). Almost invariably, the partial specific volume of component 3 is much lower than that of the water. Hence, an accurate density determination can be used to fix the composition of the solvent mixture with very little uncertainty. If density-composition tables or relations are available at the desired temperature for a particular component 3 in water, the composition is obtained by simply referring the observed density to the table. Density-composition tables are frequently accurate to 1 part in 10^5 (cf., International Critical Tables, Vol. III); in some instances, the accuracy is even better, e.g., the data on sucrose–water mixtures which are often used for density calibration purposes.[19] Usually such tables, or relations based upon them, are given in terms of the weight fraction, W, of the solute (i.e., W'_3 in our notation). The concentrations in grams per milliliter, c_i, of both the solute and the water component are easily calculated with Eq. (14); hence,

[19] F. Plato, *Kaiserlichen Normal-Eichungs-Kommission, Wiss. Abl.* **2**, 153 (1900). Quoted in "Polarimetry, Saccharimetry and the Sugars" (F. J. Bates *et al.*, eds.), *Nat. Bur. Stand. U.S. Circ.* 440, p. 626 ff. (1942).

$$c'_3 = \rho' W'_3$$
$$c'_1 = \rho'(1 - W'_3) = \rho' - c'_3 \tag{18}$$

where primes refer to the solvent medium (usually containing no protein) as applied throughout this article. If density-composition data are not available for the desired two-component solvent medium at the temperature required, density measurements must be performed on mixtures of the weighed-in pure components covering the composition range of interest. For this purpose, considerable care must be given to the work at the analytical balance so that sources of error are minimized and appropriate corrections to the apparent weights are applied.[20,21] Obviously, the correction of the apparent weights to vacuum conditions becomes more important as the weight fraction of component 3 increases, because of the difference in density between the components. The work at the balance should exceed an accuracy of 1 part in 10^5 if densities good to about 10^{-5} g/ml are desired. Thus, the use of an average value for the density of air (1.2 mg/ml) for the vacuum correction may not be sufficiently accurate in uncontrolled rooms where weighings are performed (the temperature, relative humidity, and barometric pressure readings are employed to assess the air density at the time of weighing[12]). It is safest to determine the density on the freshly prepared solutions. In order to remove gas without evacuating, the tightly sealed bottles may be centrifuged at low speed for a few minutes, or may be simply warmed at a temperature well above that being used for the density measurements (e.g., in clothing pockets). With the magnetic densimeter, a host of density values, in triplicate or quadruplicate, can be run out in a single day (8–12 samples per hour of <0.3 ml each). With the use of an appropriate computer program, a density-composition curve can be fitted to the data from compositional increments of about 0.005 in W'_3 ($\sim 0.5\%$), which is adequate for most purposes. One procedure is to program a curve by polynominal fitting[22] to the reciprocal of the observed density versus the weight fraction, W'_3, of solute.[23] The generated curve may be utilized in the same program to calculate not only the density at small intervals of composition (e.g., $\Delta W'_3 = 0.001$), but also

[20] A. H. Corwin, *in* "Physical Methods of Organic Chemistry" (A. Weissberger, ed.), 3rd ed., Vol. 1, Part I, Chapter III. Wiley (Interscience), New York, 1959.

[21] L. B. Macurdy, *in* "Treatise on Analytical Chemistry" (I. M. Kolthoff, P. J. Elving, and E. B. Sandell, eds.), Vol. 7, Part I, Chapter 74. Wiley, New York, 1967.

[22] W. Godschalk, "VBARTAB", a program in Algol (1968). Computer Sciences Center, University of Virginia, Charlottesville, Virginia.

[23] G. N. Lewis and M. Randall, *in* "Thermodynamics" (revised by K. S. Pitzer and L. Brewer), p. 207. McGraw-Hill, New York, 1961.

the partial specific volumes, $\bar{v}'_1$ and $\bar{v}'_3$, at these intervals for both water and component 3, respectively; $\bar{v}'_1$ and $\bar{v}'_3$ are given by the intercepts at $W'_3 = 0$, $W'_3 = 1$, from the linear extension of the derivatives, $(\partial 1/\rho'/\partial W'_3)_T$, at each value of W'_3.[22] The densities from such fitted curves can be made more accurate through the averaging procedure than that indicated by the precision of the data from which the curves are drawn; comparison experiments in our laboratory have shown that the densities from fitted curves derived from data in the literature, which were given only to 10^{-4} g/ml, are accurate to 10^{-5} g/ml. The availability of partial specific volumes throughout the soluble composition range of two-component aqueous solvents is a valuable library for various purposes. With values of $\bar{v}'_1$ and $\bar{v}'_3$ at hand for any density determined on such solvents, another means of calculating c'_1 and c'_3 may be noted. From the definition of density (Eq. 9) and of volume (Eq. 7) which in terms of c_i *is* $\Sigma_{i=1}\ \bar{v}_i c_i = 1$, it is seen that

$$\begin{aligned} c'_3 &= -\left(\frac{1 - \bar{v}'_1\rho'}{\bar{v}'_1 - \bar{v}'_3}\right) \\ c'_1 &= \left(\frac{1 - \bar{v}'_3\rho'}{\bar{v}'_1 - \bar{v}'_3}\right) = \rho' - c'_3 \end{aligned} \tag{19}$$

These alternative relations, while less direct for calculating c'_1 and c'_3 than by Eqs. (18), are shown to underscore the fact that the sensitivity of the method depends on the difference in the partial specific volumes of the two components in the mixture. The denominator, $(\bar{v}'_1 - \bar{v}'_3)$, of most two component solvents used for the study of proteins lies between 0.25 and 0.75 ml/g, which is sufficient to establish differences of 10^{-3} g/ml in the concentration of a component with little uncertainty; denominators as low as 0.01 ml/g can still be used to give reasonably accurate concentration differences of a component ($\Delta c'_i \cong 10^{-2}$ g/ml). Obviously, as $(\bar{v}'_1 - \bar{v}'_3) \to 0$, the density method for evaluating the composition of two-component solutions becomes useless.

A unique value for the composition is not given by a density determination if the solution contains more than two components. Hence, for a protein which has been dialyzed in a two-component solvent medium, additional information is required in order that the distribution of the diffusible components may be determined by density. In this case, an independent evaluation of the protein concentration is carried out, which along with the value of $\bar{v}_2$ or ϕ_2, can give values of c_1 and c_3 with reasonable accuracy. Where the plot of ρ versus c_2 is found to be linear during the determination of $\bar{v}_2^0$ for the protein, as is usually observed, a derived density, ρ'_D for the combined diffusible components

in the protein solution may be calculated. The derived density is obtained by subtracting the protein concentration, c_2, from the density of the dialyzed protein solution (which gives $c_1 + c_3$) and dividing this mass per milliliter by the apparent volume of the 2 diffusible components in 1 ml (i.e., $1 - \phi_2 c_2$). Thus,

$$\rho'_D = \left(\frac{\rho - c_2}{1 - \phi_2 c_2}\right) \tag{20}$$

The derived density of the nonprotein components in the dialyzed protein solution is then related to the density-composition curve for the particular two-component solvent in order to obtain c'_1 and c'_3 via Eqs. (18) or (19). The latter concentrations are converted to the values for c_1 and c_3 in the protein solution by

$$\begin{aligned} c_3 &= c'_3(1 - \phi_2 c_2) \\ c_1 &= c'_1(1 - \phi_2 c_2) \end{aligned} \tag{21}$$

which can be derived from the definition of density and the apparent specific volume (Eqs. 9 and 8). It is assumed that the presence of protein does not cause chemical changes of significance in the species of components 1 and 3.

In the unusual case where $\phi_2 \neq \bar{v}_2^0$ at a given value of c_2, i.e., $(\partial\rho/\partial c_2)_m$ is not a constant, another equation is used for c_1, c_3. If $\bar{v}_2$ varies with c_2, then either or both, $\bar{v}'_1$, $\bar{v}'_3$, of the two-component solvent medium, which are utilized in the definition of ρ'_D (i.e., $1 - \phi_2 c_2 = \bar{v}'_1 c_1 + \bar{v}'_3 c_3$), are not identical to $\bar{v}_1$, $\bar{v}_3$, respectively, of the protein solution. [In fact, we only assume when $\phi_2 = \bar{v}_2^0$ that $\bar{v}'_1$ and $\bar{v}'_3$ for the pure solvent medium are identical to $\bar{v}_1$ and $\bar{v}_3$, respectively, in the protein solution; changes in $\bar{v}_1$ and $\bar{v}_3$, however, might compensate exactly to still satisfy, $1 - \phi_2 c_2 = \bar{v}'_1 c_1 + \bar{v}'_3 c_3$, the volume relation for apparent quantities (see Eq. 8, and assign $V = 1$ ml). Such coincidences, however, seem very unlikely.] Hence, when $\bar{v}'_1$ and $\bar{v}'_3$ cannot be substituted for $\bar{v}_1$ and $\bar{v}_3$ with sufficient accuracy, then by $\rho = \Sigma c_i$ and $\Sigma \bar{v}_i c_i = 1$, the concentrations c_1 and c_3 are given with

$$\begin{aligned} c_3 &= -\frac{(1 - \bar{v}_2 c_2) - \bar{v}_1(\rho - c_2)}{(\bar{v}_1 - \bar{v}_3)} \\ c_1 &= \frac{(1 - \bar{v}_2 c_2) - \bar{v}_3(\rho - c_2)}{(\bar{v}_1 - \bar{v}_3)} \end{aligned} \tag{22}$$

Since the partial specific volumes of water and of component 3 must be determined on such a protein solution, the density method for evaluating c_1 and c_3 would seem to be impractical. As a general note, however, if densities can be determined quickly and accurately, it becomes

feasible to define the volume of any solution of interest in terms of all the partial volumes (Eq. 7)—an informative operation which has seldom been accomplished for systems of more than two components and which has fundamental importance for our understanding of protein solutions during conformational transitions where all $\bar{v}$ probably change. The evaluation of $\bar{v}_1$ and $\bar{v}_3$ for the present purpose is straightforward. $\bar{v}_1$ may be determined by weighing in increments of water to aliquots of the dialyzed protein solution and then measuring the densities of these solutions. The limiting slope at c_2^0 (i.e., at zero water addition) is evaluated from a plot of the observed densities versus the masses of added water. For $\bar{v}_3$, a second curve is prepared by weighing in component 3 to other aliquots of the protein solution and by determining these densities. The values of $\bar{v}_1$ and $\bar{v}_3$ are then computed from the limiting slopes at c_2^0 with the use of Eq. (10), where the appropriate subscripts are substituted for subscript 2 and the density of the protein solution at c_2^0 is substituted for ρ'. [After calculating c_1 and c_3 with Eq. (22), a value of $\bar{v}_2$ may be calculated from the relation $\Sigma \bar{v}_i c_i = 1$, to be compared with the value of $\bar{v}_2$ determined directly. A discrepancy here reflects error primarily in the value assigned to c_2 via an indirect method, such as by light absorption; $\bar{v}_2$ which is determined directly should be the more accurate, being based on a dry weight analysis.] As is now evident, the accuracy of the values of c_1 and c_3 obtained by Eqs. (20) and (21), or by Eq. (22), will depend primarily on the value assigned to the protein concentration. An error of 1% in c_2 will affect the values of c_1 and c_3 by about 10^{-4} g/ml; at relatively high values of c_2 (e.g., 0.1 g_2/ml), a 1% error changes c_1, c_3 about 5×10^{-4} g/ml. Also, an effect equivalent to an error of 1% in c_2 is caused by an error of 0.003 ml/g in $\bar{v}_2$.

Small amounts of other substances, such as a sulfhydryl compound or a buffer pair, are sometimes added to the two-component solvent medium, when necessary, for the study of a particular protein. In this event, approximate corrections can be applied to the observed densities of both solvent and protein solution to account for the additive. The effect on the density difference between solution and dialyzate as a result of any redistribution of such additives during dialysis is very small if their concentrations are kept low and if these substances do not bind to the protein.

Preferential Interaction

The unequal distribution of diffusible components on two sides of the membrane (other than from Donnan effects) was demonstrated long ago by S. P. L. Sørensen and co-workers in their many osmotic

experiments on proteins in high concentrations of salt.[24] The difference in the molality of a diffusible component 3 on the two sides at equilibrium is a manifestation of the more general term "preferential interaction." In molecular terms, it is assumed that the protein, in, say, a two-component solvent medium, has a stronger affinity for one of these components than for another; hence, one might conclude that in the immediate surroundings of the protein, the composition of these solvent components is different than in the bulk solvent regions having properties like that of the pure solvent medium, or dialyzate. Thermodynamically, we simply mean by the term preferential interaction that the presence of protein affects the chemical potentials of water and of component 3 differently. Güntelberg and Linderstrøm-Lang,[25] presented the thermodynamic formalism for the case of protein in a two-component medium of water and salt. In this treatment, the protein component is redefined to include the amount of excess (or deficient) diffusible component relative to dialyzate composition, so that the remainder of the diffusible components in the protein solution has a composition like that of the dialyzate (see also[18,26,27]).

In two-component solvents, the excess mass of a diffusible component in the protein solution relative to the composition of the dialyzate (or deficient mass of the other diffusible component) appears to be a linear function of the protein concentration. Hence, the change in grams, g_j, $(j = 1,3)$ of either component 1 or component 3 with grams of protein, $(\partial g_j/\partial g_2)_\mu$, may be treated as a constant in redefining the protein component to account for the unequal distribution at osmotic equilibrium. Using the notation of Casassa and Eisenberg,[18] where $\xi_j = (\partial g_j/\partial g_2)_\mu$, the concentration of the revised protein component, c^*_2, defined in this way is

$$c^*_2 = c_2 + \xi_j c_2 \tag{23}$$

A positive value of ξ_1, therefore, is a measure of the preferential hydration of the protein, whereas a positive value of ξ_3 (with ξ_1 necessarily negative) is a measure of the preferential affinity for component 3 by the protein. A negative value of ξ_j does not mean that this component is repelled by the protein relative to the noninteracting case; both water and component 3 may be "bound" in some undefined sense. The puzzling feature of the past, where a positive value of ξ_3 was observed with pro-

[24] S. P. L. Sørensen and co-workers, *C. R. Trav. Lab. Carlsberg* **12**, 1 (1917).
[25] A. V. Güntelberg and K. Linderstrøm-Lang, *C. R. Trav. Lab. Carlsberg, Ser. Chim.* **27**, 1 (1949).
[26] G. Scatchard, Y. V. Wu, and A. L. Shen, *J. Amer. Chem. Soc.* **81**, 6104 (1959).
[27] E. F. Casassa and H. Eisenberg, *J. Phys. Chem.* **64**, 753 (1960).

tein in low concentrations of component 3, whereas a positive value of ξ_1 was seen at high concentrations of component 3, appears now to be a manifestation of two exclusions; the net exclusion of component 3 by component 1 in some regions of the solution and a net exclusion of component 1 by component 3 in other regions (includes binding).[28,29] In other words, a positive value of ξ_1 represents the total net exclusion of component 3 by water from all volume elements (in the protein solution) which do not have a composition like that of the dialyzate (or of a bulk solvent phase in the protein solution with properties like that of dialyzate). Both components 1 and 3, however, may exclude each other in different volume elements relative to dialyzate composition, so that

$$\xi_1 = -\xi^*_3(1/W'_3) + (\xi^*_1 + \xi^*_3) = -\xi^*_3(c'_1/c'_3) + \xi^*_1 \tag{24}$$

where ξ^*_j $(j = 1,3)$ is the grams of component j which excludes from some volume elements the other diffusible component per gram of protein.[29] [Eq. (24) may be cast in terms of ξ_3 by substituting with Eq. (26) and noting that $1/W'_3 = (1 + c'_1/c'_3)$ for a two-component solvent.]

ξ_j is readily defined in terms of the "c" scale of concentration (i.e., in grams per milliliter). For ξ_3, one simply subtracts the grams of component 3 relative to its proportion with the grams of water per milliliter of dialyzate from the corresponding mass ratio in 1 ml of the dialyzed protein solution. This difference in grams of component 3 relative to grams of water on the two sides of the membrane is reduced to that per gram of the dry protein relative to water. Thus,

$$\xi_3 = \left(\frac{c_3}{c_1} - \frac{c'_3}{c'_1}\right) \Big/ \frac{c_2}{c_1} = \frac{1}{c_2}\left[c_3 - c'_3\left(\frac{c_1}{c'_1}\right)\right] \tag{25}$$

where c_j, c'_j may be evaluated from densities by Eqs. (18), (20) and (21). The relation for ξ_1 is identical except that all subscripts 1 and 3 are interchanged. Hence, ξ_1 and ξ_3 are easily interconverted; by setting the respective equations for ξ_1 and ξ_3 (Eq. 25) equal to c_2, it is seen that

$$\xi_1 = -\xi_3(c'_1/c'_3) \tag{26}$$

which is a relation equivalent to that used by Güntelberg and Linderstrøm-Lang[25] to treat preferential hydration of the protein in terms of $(\partial g_3/\partial g_2)_\mu$.

In practice, if accurate density values are not convenient to deter-

[28] V. N. Schumaker and D. J. Cox, *J. Amer. Chem. Soc.* **83**, 2445 (1961).

[29] D. W. Kupke, *in* "Physical Principles and Techniques of Protein Chemistry" (S. J. Leach, ed.), Part C, Chapter 1. Academic Press, New York, in press.

mine routinely and when ξ_j is assumed to be constant in c_2, it can be shown that ξ_j may be obtained by

$$\left(\frac{\Delta\rho}{c_2}\right)_\mu - \left(\frac{\Delta\rho}{c_2}\right)_m = \xi_j(1 - \bar{v}'_j\rho') = \xi_1(1 - \bar{v}'_1\rho') = \xi_3(1 - \bar{v}'_3\rho') \quad (27)$$

Hence, density values for the protein solution both before and after dialysis and a density value for the solvent medium (= dialyzate) gives immediately the value of ξ_j if accurate values for c_2 in both protein solutions are obtainable. The dialyzate should be exchanged during the dialysis so that the final composition is identical to that of the original (isomolal) solvent medium. The values of $\bar{v}'_j$ are taken from the programmed density-composition tables for the two-component solvent medium as noted in the previous subsection.[22] The casting of ξ_j in terms of the density method in order to avoid the tedious and often imprecise evaluation of the diffusible components on both sides of the membrane by standard methods is easily accomplished beginning with the definition $\rho = \Sigma c_i$ and $\Sigma\bar{v}_i c_i = 1$; these details are given elsewhere.[29]

Obviously, much more confidence in the values of ξ_j is given if the slopes, $(\partial\rho/\partial c_2)_\mu{}^0$ and $(\partial\rho/\partial c_2)_m{}^0$, are determined concurrently on the same protein preparation and solvent system. The evaluation of c_2 is carried out on both the isopotential and isomolal stock solutions by the same indirect method, based on dry weight, so that the effect of systematic errors tends to cancel in evaluating ξ_j. This is seen from the fact that $\xi_j{}^0$ is related to the difference in the two limiting slopes, such that

$$\left(\frac{\partial\rho}{\partial c_2}\right)_\mu^0 - \left(\frac{\partial\rho}{\partial c_2}\right)_m^0 = \xi_j{}^0(1 - \bar{v}'_j\rho') \quad (28)$$

Hence, plots of ρ versus c_2 from the dilution series on each experiment (isopotential and isomolal) will tend to show up any curvature or show whether the slopes of each are constant as noted from the correlation coefficients by linear least-squares analysis; ordinarily, $\xi_j{}^0 = \xi_j$ over the usual range of protein concentration covered. As pointed out in the first 2 subsections of Part II, a number of density determinations are required to define each of the curves of the dilution series (preferably with triplicate measurements at each dilution). Hence, a rapid densimeter for the accurate measurement of small volumes of solution is necessary for this purpose.

An example of a simplified kind of preferential interaction experiment may be noted. If a diffusible component 3 is comparatively inert with respect to interactions with protein, it can be shown that the value of ξ_1 will remain essentially constant as the amount of component 3 is

varied in the solvent medium.[30] (The corresponding plot of ξ_3 versus c'_3, however, will exhibit a hyperbolic function tending toward $-\infty$ as c'_3 increases.) Hence, when $(\partial\xi_1/\partial c'_3)_\mu \sim 0$, the value of ξ_1 may be assumed, as a reasonable approximation, to represent the total mass of water per gram of protein from which component 3 is excluded. Complex macromolecules may contain holes or inner solvent spaces (e.g., isometric viruses), and some noninteracting solutes (e.g., sucrose) cannot penetrate these spaces owing to steric exclusion. Thus, relatively large, positive values of ξ_1 may be obtained when impenetrable diffusible solutes are employed in equilibrium dialysis experiments in which ξ_1 is found not to depend on c'_3. Such values of ξ_1 have been used to calculate the volume of these inner spaces for the case of isometric viruses in the presence of sucrose; these volumes, in conjunction with the molecular weight and apparent specific volume of turnip yellow mosaic virus have yielded dimensions for the virus which are in agreement with those deduced by X-ray diffraction and electron microscopy.[31]

Since dry weight analysis is usually necessary to establish any indirect method for the evaluation of c_2, a procedure is at hand by which values for the preferential interaction as determined with Eq. (27) can be checked on occasions. If component 3 is truly nonvolatile, an amount of the dialyzed protein solution is weighed on the analytical balance to give the total grams defined by $(g_1 + g_2 + g_3) = \Sigma g_i$. If the density, ρ, of this solution is determined, the volume V is given by $\Sigma g_i/\rho$. From the drying procedure, we obtain a value for the evaporated mass of water, g_1, and a value $(g_2 + g_3)$ for the residue comprising the masses of protein and component 3. Recalling Eqs. (7) and (8), it is evident that 2 simultaneous equations are available for calculating g_2 and g_3, if the partial specific volumes of the components of the solution before drying are known; it may be assumed that $\phi = \bar{v}_2$. Hence,

$$\sum_{i=1}^{N=3} g_i - g_1 = g_2 + g_3$$

and

$$\Big(\sum_{i=1}^{N=3} g_i\Big)\Big/\rho = \bar{v}'_1 g_1 + \phi_2 g_2 + \bar{v}'_3 g_3 \qquad (29)$$

A similar set of equations may be written for the case where component 3 is completely volatile. The values of $\bar{v}'_1$ and $\bar{v}'_3$ are taken from the

[30] D. W. Kupke, manuscript submitted.

[31] J. P. Senter and D. W. Kupke, unpublished experiments (1966).

programmed density-composition data on the two-component solvent[22] at the composition of the dialyzate, which leads to no significant error. ϕ_2 is determined independently using isomolal conditions (see first section in Part II); this procedure involves the dry weight analysis used in determining a coefficient for an indirect assay method for the protein (such as the absorptivity in ultraviolet absorption measurements). The dry weight of the two-component dialyzate is usually carried out concurrently along with that for the dialyzed protein solution. As noted in the preceding section, the density of the dialyzate fixes its composition very accurately. The dry weight value obtained on the dialyzate then serves as a check on whether the drying and heating protocol is indeed adequate in order to yield quantitative results. For example, the protocol may cause partial volatization of component 3 or changes in its mass through oxidations, polymerization reactions, etc. The difference in the measured values of g_3 and g'_3 per unit weight of water on the two sides of the membrane is then related to g_2 in order to calculate ξ_3 by Eq. (25) in which grams are substituted for the concentrations, c_i.

Volume and Partial Volume Changes; Other Applications

The change in Gibbs free energy, G, characterizing a chemical reaction at constant temperature and pressure is the familiar relation

$$\Delta G = \Delta E + P\Delta V - T\Delta S \tag{30}$$

where E is the internal energy and S the entropy. Ordinarily, chemists are not concerned with the change in volume, ΔV, because such volume changes are often not discernible and seem to be trivial in terms of the total free-energy change. Kauzmann[32,33] has pointed out that the change in volume may reflect a number of structural changes in protein solutions and that the measurement of ΔV can lead to unique information not given by other methods; furthermore, he and his associates have shown that the study of model systems may be utilized to interpret changes in the volume.[34-36] The classical method of measuring ΔV by direct dilatometry is discussed by Katz (see this volume [18]). With suitable control experiments it is possible to measure the change in the apparent molal or specific volume of a component.[29,37]

If densities can be determined conveniently and accurately, it is

[32] W. Kauzmann, *Biochim. Biophys. Acta* **28,** 87 (1958).
[33] W. Kauzmann, *Advan. Protein Chem.* **14,** 1 (1959).
[34] W. Kauzmann, A. Bodansky, and J. Rasper, *J. Amer. Chem. Soc.* **84,** 1777 (1962).
[35] J. Rasper and W. Kauzmann, *J. Amer. Chem. Soc.* **84,** 1771 (1962).
[36] L. M. Krausz and W. Kauzmann, *Arch. Biochem. Biophys.* **139,** 80 (1970).
[37] S. Katz and T. G. Ferris, *Biochemistry* **5,** 3246 (1966).

evident that such measurements may also be used to evaluate ΔV of a process. A description of a simple, yet fairly common, example may be considered. We wish to measure the change in volume on mixing together two aqueous solutions, one containing protein and the other a reactant component, such as a denaturant. The densities of both solutions, a and b, are determined giving the values ρ_a and ρ_b, respectively. An amount of one of the solutions is weighed on the analytical balance and an amount of the second solution is then added and weighed. The two masses are denoted as $(\sum_{i=1} g_i)_a$ and $(\sum_{i=1} g_i)_b$. From Eq. (8), it is evident that the total volume before mixing, V^B, at the temperature of the density measurements is

$$V^B = \frac{\left(\sum_{i=1}^{N} g_i\right)_a}{\rho_a} + \frac{\left(\sum_{i=1}^{N} g_i\right)_b}{\rho_b} \tag{31}$$

The density, ρ_{a+b}, is then determined on the mixture at the same temperature (noting when equilibrium is achieved by the constancy of the density). The new volume after mixing, V^A, is given by

$$V^A = \frac{\left(\sum_{i=1}^{N} g_i\right)_a + \left(\sum_{i=1}^{N} g_i\right)_b}{\rho_{a+b}} \tag{32}$$

The difference, $V^A - V^B$, gives the volume change, ΔV, for the process. The components can be varied in a number of obvious ways so that the difference in the apparent specific volumes of the protein as a result of the reaction can be calculated.[29,37]

Heretofore, density has not been used appreciably for this purpose because of the inconvenience and relatively large amounts of solution which are required for good accuracy with the conventional pycnometric procedures. With the magnetic densimeter, less than 1 ml is required of each solution to be mixed and the duration of the experiment is a matter of minutes if equilibrium is achieved rapidly. A change in density with time is easily observed after mixing since the amount of current needed to hold the buoy at a constant vertical position is automatically monitored as the density changes. A special advantage of the density method resides in the fact that the absolute values for the partial specific volume of any component can be determined in the reactant solutions and in the final mixture. Usually the change only in the partial or apparent specific volume of the protein has been determined. It is clear, however, that the change in $\bar{v}$ for all the components is necessary for a complete

description of the volume change in terms of the partials; moreover, $\Delta\bar{v}$ for the components other than the protein may be highly informative also. For example, one may employ density as a titration method, whereby a particular reactant is added quantitatively to the protein solution in the densimeter. The plot of density versus concentration provides the data by which $\bar{v}$ of the reactant can be followed as its mass is increased. [For this purpose, Eq. (12) may be required for which Eq. (14) is used to convert the grams of added material in terms of the "c" scale of concentration.] For reactions generally, a change in volume may be expected because it is improbable that the volume changes associated with the breaking of structures and formation of new ones should cancel exactly. In the case of simple, stoichiometric reactions, the plot may show a sharp change after the titration is complete; after this point is reached, the curve may be very similar to that obtained when the solution contains no protein (unpublished experiments have borne this out.) More generally, the partial volume spectrum of a component may be determined over a large span of the concentration. In this way it is possible to assess the changes in $\bar{v}_i$ for a denaturant added to a protein solution from a smooth curve of ρ versus c_i over the entire soluble concentration range. Heretofore, only a few isolated points on the total curve have been determined in the cases studied. With the magnetic densimeter (i.e., Fig. 3), it has been possible to add successive 0.1-mg increments of a pure component to a known amount of the solution in the cell (Fig. 3). The density (i.e., the voltage) is measured after each addition when the substance has dissolved. For greater ease in performing this type of experiment, a larger cell can be substituted than that shown in Fig. 3. Also, regulated volumetric inflow mechanisms as well as regulation of the composition of gases and of pressure[10] can be adapted to the magnetic method. Furthermore, since the volume of the solution can be kept very small, it is feasible to study the density as a function of temperature.

Finally, the opportunities for kinetic studies by density are now enhanced. The servo mechanism currently being utilized in the model of Fig. 3 holds the buoy precisely in position as the density of the solution changes. The recorder connected to the differential voltmeter describes the change in voltage with time, t. Since many reactions involve some change in the volume, the parameter, $(d\rho/dt)$, may be informative toward interpreting the mechanisms of a process. Preliminary experiments have shown that changes in the density of 10^{-6} g/ml during a period of close temperature control can be evaluated with good precision. Perturbation and relaxation experiments appear not to have been initiated, but this approach should be compatible with the magnetic suspension principle.

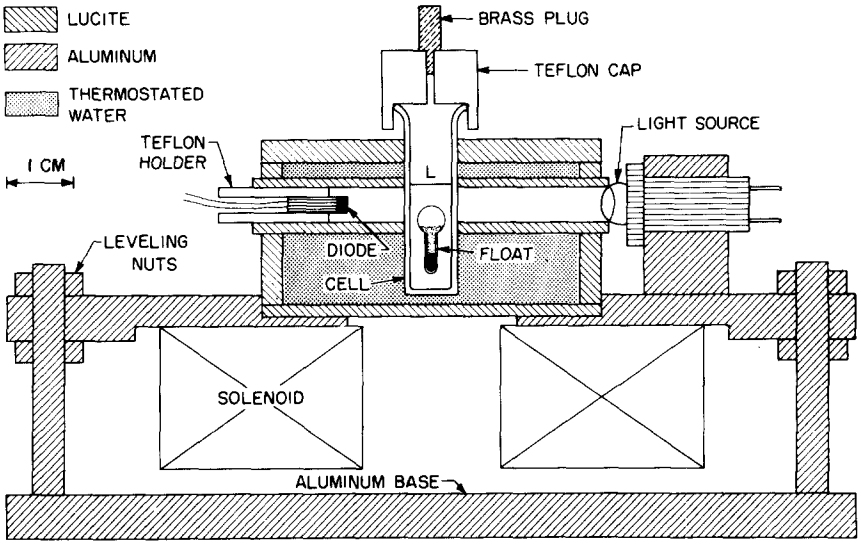

FIG. 3. Side view of constant-temperature cell, optical sensing elements, and solenoid for the solid-state, optical sensing magnetic densimeter. Viewing microscope, not shown, is at right angles to light source; for further details consult original paper. From J. P. Senter, *Rev. Sci. Instrum.* **40**, 334 (1969).

Part III. Practical Notes on Measurements

These remarks on certain practical aspects of the measurements relate principally to the solid-state model utilizing optical sensing and the inverted solenoid design (Fig. 3).[7] This model is currently our most convenient one for routine experiments on protein solutions. For this instrument, the buoys are calibrated to float on the liquids of interest so that the force of the solenoid adds to that of gravity (g). Hence, Eq. (2) for balancing of the opposing forces is written

$$K_3 I^2 = V_B g(\rho - \rho_B) \tag{33}$$

where the density of the solution, ρ, is greater than the density of the buoy, ρ_B. Since the sensitivity drops off as the difference between ρ and ρ_B increases, a given buoy is normally used over a range of about 0.02 g/ml in the density; this is sufficient to cover a change in protein concentration of about 70 mg/ml. The sensitivity, or change in current per unit change in the density ($dI/d\rho$), is given for this case by

$$\frac{dI}{d\rho} = \frac{I}{2(\rho - \rho_B)} \tag{34}$$

when the opposing forces on the suspended buoy are equalized. Thus, a series of buoys may need to be constructed if studies are to be made in solvent media which are substantially more dense than dilute aqueous salt or buffer solutions. The buoys are usually catalogued in terms of the composition of sucrose–water mixtures used for their calibration, e.g., 0–5, 5–10, 10–15, etc. weight percent of sucrose (1% w/w sucrose increases the density by about 0.004 g/ml). The buoys are cleaned in mild soap solution of moderate pH and stored in sealed vials containing distilled water. On occasions, a sulfuric acid-dichromate oxidizing solution is employed to remove organic material which has adsorbed to the glass jackets. The buoys are removed from the cell or a container by inserting a thin, bevel-tipped polyethylene tube which is gently forced around the bulb of the buoy. The inside diameter of the tube is approximately the same as that of the bulb. The buoy is then discharged from the beveled edge into another liquid by pushing a Teflon rod through the tube from the other end.

A given buoy is calibrated by alternately introducing and removing known sucrose–water, KCl–water, or other standard solutions into the cell. The values for the density of standard solutions are obtained, as noted before, by curve-fitting procedures[22] on the sucrose data of Plato[19] or on the data for aqueous salts from the International Critical Tables. [As must be evident, the densities of these various standard solutions are not altogether self-consistent, because the temperature scale, refinements in weighing and purification of materials have undergone small improvements since these tables were constructed. Hence, the densities of standard solutions are somewhat relative, and it is important to describe the calibration protocol in detail if densities more accurate than 10^{-5} g/ml are to be compared when better absolute densities become available. Aqueous cesium chloride would appear to be a more ideal calibration standard by virtue of the large density range (viz., 1 to 1.8 g/ml) and low viscosity; the determination of absolute densities on the ultrapure salt now available has not been undertaken to our knowledge. It is also important to note whether a density–composition table is given in terms of grams per milliliter or in grams per cubic centimeter. The density of pure water at 3.98° is 1.000000 g/ml = 0.999973 g/cm^3; hence, 1 ml = 1.000027 cm^3.] Usually 6 to 8 concentrations of a primary standard, each measured in triplicate, are used to calibrate a given buoy. This operation may require 2–3 hours, but a calibration appears to be good for several weeks unless the instrument is altered. Since a precision resistor ($\sim$1 ohm) is employed in the densimeter, the square of the observed voltage, E, is plotted as a function of the known

densities. Linear, least-squares fittings have been found to describe the results over a density span of ~0.03 g/ml with correlation coefficients of better than 0.99999. A least-squares fitting and calculation of the slope, intercept, and correlation coefficient from the calibration data require only a few minutes if a programmable desk calculator is available.

A change of 0.1 mV corresponds approximately to a change in density of 2×10^{-6} g/ml over the density range ordinarily used for a given buoy. The noise level can usually be held to an order of magnitude less than this if a corresponding control of the temperature is achieved; at 20° the density of water changes about 2×10^{-6} g/ml per 0.01°C. Hence, an overall fluctuation within 0.01° during the measurements corresponds to an uncertainty of about 0.1 mV or $\sim 2 \times 10^{-6}$ g/ml in the density. This much uncertainty can be tolerated for most of the purposes outlined here. Repetitive measurements on aliquots of a given protein solution are easily maintained to within 0.2 mV. Samples are introduced slowly from a gastight syringe (e.g., 250 μl) through the small axial hole in the cell cap. (The Teflon cap in Fig. 3 has been replaced with a small silicone stopper, which is forced into the neck of the cell. This stopper makes a much tighter seal around the upper wall of the cell and eliminates creeping by the solution around the lip. A narrow axial hole is drilled through the stopper; during measurements, this hole is kept plugged by a tightly fitting brass insert.) The syringe needle should extend just below the cell cap so that there is no danger of scratching the glass jacket of the buoy. The sample is removed with another syringe via a length of narrow-gauge Teflon tubing instead of a needle; in this case the tubing must reach to the bottom of the cell. As the concentration of salt or other component of the aqueous solvent increases, apparent microcrystallization ensues around the edge of the meniscus during long-term experiments unless the liquid virtually fills the available space in the cell. A slow decrease in the density may be observed in such cases over a period of hours depending on the concentration of the salt. In the usual case where prompt measurements are desired, this long-term effect is not a problem. Temperature equilibration requires about 2–3 minutes if the cell is encased in a brass block through which the thermostating liquid is pumped (the design in Fig. 3, showing a plastic enclosure for thermostating the cell, requires more time for temperature equilibration). Temperature equilibration and normalization of the rotational position of the buoy is hastened by bobbing the buoy with the controls and/or by moving the buoy from side to side with a small magnet. The entire operation for a measure-

ment, including the introduction and removal of a sample, requires about 5 minutes. When a solution is introduced which differs in density by more than 0.002 g/ml from the previous sample, 3 rinsings are usually sufficient. With protein solutions differing in density by about 10^{-4} g/ml, only 1 rinse is needed.

The major problem causing delays in a measurement is the growth of bubbles on the buoy. Warming or briefly centrifuging the solution minimizes bubble formation. Although bubbles cannot always be seen (with the present design, part of the buoy remains out of the field of the microscope), a steady voltage is seldom attained quickly when a bubble forms. The voltage, in any case, is higher than normal, because the buoy becomes lighter and requires more current to hold it at the desired vertical position in the microscope field. Removal and reinsertion of the solution sometimes eliminates the bubble; frequently, however, a bubble repeatedly forms on the same spot and the buoy must then be removed and cleaned. A minor problem arises from small debris in the solution which occasionally falls upon the top of the buoy where the rounded portion of the bulb intercepts the light path. Although the mass of these particles seen by the microscope are insignificant usually, the interference with the light may be sufficient to change the voltage measurably. Removal and immediate reinsertion of the same solution eliminates the offending particle. Wherever possible, the solutions should be filtered prior to use.

Normally, about 1.2 ml of a particular protein solution is used for a density value. This amount allows 200–300 μl to be used as a rinse, leaving three portions ($\sim$300 μl each) available for measurement; the average of the observed voltages is used for calculation of the density from the calibration equation. A dilution series is prepared by weight, as described in Part II, so that 6–8 solutions are available for calculating a value of $(\partial\rho/\partial c_2)_\mu{}^0$ or $(\partial\rho/\partial c_2)_m$, which is generally sufficient if no curvature is apparent. The lowest concentration of the series contains about 0.5 mg/ml of protein, which is still approximately 1.5×10^{-4} g/ml more dense than the solvent medium. The densities calculated from the square of the average voltage with the calibration equation are plotted against the concentration, and a linear least-squares fit to the data is constructed with the programmable calculator. Typically, the correlation coefficients of such dilution series on a variety of proteins (but nonassociating systems) are slightly better or worse than 0.99999, indicating that the variation of density with protein concentration can be expected to show a constant value (i.e., $\bar{v}_2 = \phi_2$ and $v_\mu = \phi_\mu$) over the dilute range of concentrations usually studied (e.g., $\Delta c_2 \sim 0.02$ g$_2$/ml).

Another solid-state model designed for work with protein solutions

has been developed by Goodrich *et al.*[38] Procedures for fabrication of buoys have been outlined by these authors and by Senter.[7]

Acknowledgment

Grants (GB 5569-8284-27331) to the authors by the U.S. National Science Foundation to develop magnetic densimetry for the study of proteins are gratefully acknowledged.

[38] R. Goodrich, D. F. Swinehart, M. J. Kelly, and F. J. Reithel, *Anal. Biochem.* **28**, 25 (1969).

[6] Partial Specific Volume Measurements by the Density Gradient Column Method

By J. D. SAKURA and F. J. REITHEL

Calculation of molecular weights from ultracentrifuge data requires precise values for the $(1 - \bar{v}\rho)$ or $(\partial\rho/\partial c)_\mu$ term, where $(\partial\rho/\partial c)_\mu$ is change in density with respect to concentration of dialyzed protein. In many cases there is uncertainty concerning the value of $\bar{v}$, the partial specific volume of the protein. Even ρ, the density of the buffer used, or the dialyzate, is often not readily available. Furthermore, the use of $\bar{v}$ to calculate the apparent molecular weight of proteins in three-component solutions, such as concentrated urea or guanidine hydrochloride (GuHCl), may lead to serious error if there is preferential binding of a solvent component to the protein.[1]

Two operationally defined relations applied by Casassa and Eisenberg[2,3] to "$\bar{v}$" determinations recognize these preferential solvent interactions. For two-component systems or for proteins in low ionic strength buffer, the apparent partial specific volume is defined by

$$\phi = \frac{1}{\rho_s}\left(1 - \frac{\Delta\rho}{c_2}\right) \tag{1}$$

where c_2 is the protein concentration in grams per milliliter of solution, ρ_s is the density of the solvent, and $\Delta\rho$ is the difference between solution and solvent densities. If protein concentration is low or if ϕ is independent of protein concentration, the apparent partial specific volume, ϕ, reduces to the thermodynamic partial specific volume $(\bar{v})_{T,P,m_3}$, the

[1] H. K. Schachman, *Brookhaven Symp. Biol.* **13**, Brookhaven National Laboratory, Upton, New York, 1960, p. 49.
[2] E. F. Casassa and H. Eisenberg, *J. Phys. Chem.* **65**, 427 (1962).
[3] E. F. Casassa and H. Eisenberg, *Advan. Protein Chem.* **19**, 287 (1964).