Calculation of Particle Mass from Sedimentation Field-Flow Fractionation Data: The Buoyancy Factor

Samuel M. Mozersky[a]

[a] UNITED STATES DEPARTMENT OF AGRICULTURE, AGRICULTURAL RESEARCH SERVICE, EASTERN REGIONAL RESEARCH CENTER, PHILADELPHIA, PENNSYLVANIA

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SAMUEL M. MOZERSKY

AGRICULTURAL RESEARCH SERVICE, EASTERN REGIONAL RESEARCH CENTER
UNITED STATES DEPARTMENT OF AGRICULTURE
600 E. MERMAID LANE, PHILADELPHIA, PENNSYLVANIA 19118

Abstract

Sedimentation field-flow fractionation equations commonly used for calculation of molecular weight are not valid for multicomponent systems in which there is preferential interaction because the buoyancy term (B) contained therein does not take preferential interaction into account. Correct molecular weights can be obtained by replacing that term with the value of B calculated from equations available in the preferential interaction and ultracentrifugation literature, in which the dependence of B on measured quantities (densities and solute concentration) is expressed indirectly through the partial specific volume at constant chemical potential (ϕ_2'). In the present work an equation for B in the presence of preferential interaction is derived heuristically by applying Archimedes' buoyancy principle to the solvated solute, and B is expressed explicitly in terms of the measured quantities. Equations for ϕ_2', for the partial specific volume at constant molality ($\bar{v}_2$), and for B in the absence of preferential interaction are also derived heuristically. The relationships of ϕ_2' and $\bar{v}_2$ to each other and to the values of B in the presence and absence of preferential interaction are discussed. These considerations provide an easily acquired and intuitively satisfying understanding of the basic concepts involved in dealing with the influence of preferential interaction on the buoyancy term and the molecular weight, and indicate the supplemental experimental measurements needed for calculating correct values of molecular weight from sedimentation field-flow fractionation data.

INTRODUCTION

Previous publications from this laboratory have dealt with determination of the particle size distribution of casein micelles by sedimentation field-flow fractionation (SdFFF) (*1-3*). (Casein micelles are colloidal Ca^{2+} –

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*Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

1505

caseinate complexes containing 10^4 to 10^6 molecules of casein. Various species of casein are present in a micelle, but the monomer molecular weight of all of them is ca. 23×10^3.) To permit adequate control of various factors affecting micelle size, the micelles were made by adding Ca^{2+} to casein. However, these micelles are similar to the casein micelles in milk.

Figure 1 (1) shows the effect of disaccharides, sucrose and lactose, on the apparent molar micellar protein mass, M_{Prot} (the apparent "molecular weight" of the micelles), at the maximum of the SdFFF elution peak. For the micelles of casein preparation H, the presence of lactose during micelle formation gave rise to a 50% reduction in M_{Prot}; sucrose had a small effect in the opposite direction. For the micelles of preparation J, M_{Prot} in the
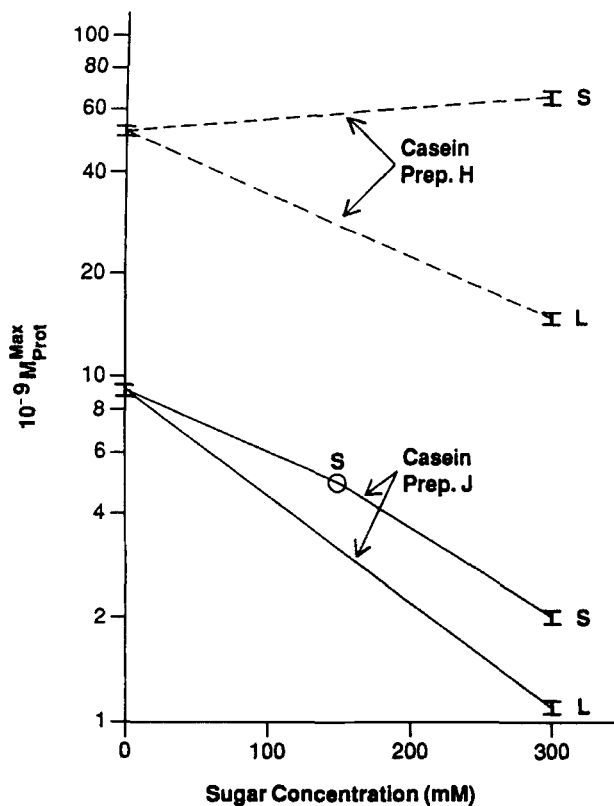

FIG. 1. The apparent micellar protein mass at the peak maximum (M_{Prot}^{Max}) as a function of the concentration of disaccharide for two caseinate preparations, H and J. All but one of the runs were made in triplicate; a vertical bar indicates the standard deviation for a set of 3 runs. The circle indicates the result of a single run. S, sucrose; L, lactose.

presence of lactose was 1/10th as large as in the absence of sugar; here sucrose also had a large effect on micelle size.

The "molecular weights" calculated in the work referred to above were used for comparative purposes, viz., to demonstrate differences between different preparations of casein in their response to the presence of sucrose and lactose during micelle formation. However, these values, which were calculated in the usual way, i.e., using $\bar{v}_2$, the partial specific volume (at constant molality), cannot be taken to be valid in an absolute sense. The use of $\bar{v}_2$ instead of ϕ'_2, the partial specific volume at constant chemical potential, gives an apparent molecular weight (Ref. 4, Eqs. 6 and 15):

$$M_{2.app} = M_2\left[1 + \frac{(1 - \bar{v}_3\rho_0)}{(1 - \bar{v}_2\rho_0)}\xi_3\right]$$

Here M_2 is the correct molecular weight, $\bar{v}_2$ and $\bar{v}_3$ are the partial specific volumes of the solute (Component 2, e.g., micellar casein) and the cosolvent (Component 3, e.g., lactose or sucrose), ρ_0 is the solvent density, and ξ_3 is the preferential interaction parameter for the cosolvent. ξ_3 is given (Ref. 4, Eq. 11) by

$$\xi_3 = A_3 - g_3A_1$$

where A_3 is the total amount (g) of cosolvent bound per gram of solute, A_1 is the total amount (g) of the principal solvent (Component 1, e.g., H_2O) bound per gram of solute, and g_3 is the cosolvent concentration in grams of cosolvent per gram of principal solvent. ["Binding" of Component i ($i = 1$ or 3) includes any interacton of the solute with Component i which limits its freedom to participate fully as part of the bulk solvent. It includes the whole range of interactions, from irreversible binding to occasional fleeting attractions between the solute and Component i. A_i is thus an average value for the binding of Component i; it represents the amount of Component i which, if it were bound irreversibly to 1 gram of solute, would have the same net effect as does the actual interaction between 1 gram of solute and the total amount ($>A_i$ grams) of Component i with which it interacts in any way, however weakly.] If the composition of the bound solvent were identical to that of the bulk solvent, 1 gram of solute would bind g_3A_1 gram of cosolvent. ξ_3 is therefore the excess of cosolvent in the bound solvent, relative to the bulk solvent. If the bound solvent is richer in the principal solvent than the bulk solvent, as illustrated in Fig. 2 (5), $g_3A_1 > A_3$, and $\xi_3 < 0$. Since $1 - \bar{v}_3\rho_0$ and $1 - \bar{v}_2\rho_0$ are positive, a negative value of the interaction parameter ξ_3 means that $M_{2.app} < M_2$.
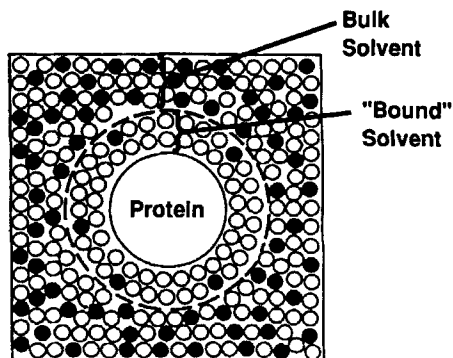
FIG. 2. Preferential hydration of solute (protein) in the presence of sugar. This is sometimes referred to as preferential exclusion of the cosolvent (sugar). In other cases, such as DNA and salt, the reverse would occur, with preferential binding of the cosolvent, as indicated in Table 3. For the definition of "bound" solvent, see the text explaining the equation for ξ_3 in the Introduction. (○) Water, (●) sugar.

Now, in the absence of cosolvent, $A_3 = g_3 = 0$, $\xi_3 = 0$, and $M_{2,app} = M_2$. Therefore, if $\xi_3 < 0$ (in the presence of cosolvent), $M_{2,app}$ is less in the presence of cosolvent than in its absence. Furthermore, if ξ_3 becomes more negative with increasing cosolvent concentration, a plot of $M_{2,app}$ vs cosolvent concentration would have a negative slope, as was obtained in three of the four curves in Fig. 1. Exactly this situation is known to occur for proteins in solutions containing lactose or sucrose (6, 7). The negative slopes in Fig. 1 can thus be attributed, at least partially, to preferential interaction of micellar casein with water in the presence of lactose or sucrose, as depicted in Fig. 2.

The casein system is used here only as an illustration. The same considerations apply to the calculation of SdFFF data for any particle whose composition is affected by its environment. This includes cells, subcellular particles, any particle with a semipermeable membrane, and porous particles, such as those used as chromatographic support media, as well as nonmicellar and molecularly dispersed proteins, such as caseins in the absence of Ca^{2+}. The problem appears to have been almost completely overlooked or ignored by practitioners of field-flow fractionation (FFF). The discussion below will show what must be done to obtain valid "molecular weights."

THEORY

Micelle mass is frequently referred to in the literature as a "molecular weight" (MW). In view of the definition of molecular weight, the term

"MW of casein micelles" ought to mean the mass, in grams, of N micelles, where N is Avogadro's number. Or, the number of daltons per micelle. But, for reasons that will become apparent below, the term MW, when applied to a particle containing a macromolecule, is by convention taken to mean the mass of the macromolecule (in grams) contained in a mole of the particles. Thus, the MW of casein micelles is the mass of protein, in grams, in N micelles (a mole of micelles). To avoid confusion, this was called the "molar micellar protein mass" and was designated as M_{Prot} (*1*). When the context leaves no room for ambiguity, M_{Prot} may be referred to simply as "size."

The effective mass M_{eff} (g/mol) of a particle undergoing SdFFF at constant field strength G_0 (cm/s^2) is given (*1, 2*) by

$$\lambda[\coth(1/2\lambda) - 2\lambda] = t_0/6t_e \tag{1a}$$

$$M_{eff} = R_0 T / \lambda w G_0 \tag{1b}$$

where λ is the dimensionless basic retention parameter of FFF (*8*), t_e (min) is the elution (retention) time, t_0 is the value of t_e for unretained particles, R_0 (erg·Kelvin^{-1}·mol^{-1}) is the gas constant, w (cm) is the channel width (thickness), and T (Kelvin) is the absolute temperature.

If the field is held constant for t_c (min) and then allowed to decay exponentially with a field decay constant τ_g (min), M_{eff} for a particle eluting during the decay phase (Phase II) is given (*1, 2*) by

$$M_{eff} = \frac{6R_0 T}{wG_0 t_0}\{t_c + \tau_g[e^{(t_e - t_c)/\tau_g} - 1]\} \tag{2}$$

where symbols common to Eqs. (1) and (2) have the same meaning in both.

If exponential field decay is interrupted at time t_{Hold} (min) before the particle elutes and the rotational velocity of the rotor ω' (rpm) is held constant at the value ω'_{Hold} which it had at that time, then (*1, 2*), for a particle eluting at time $t_e \geq t_{Hold}$,

$$M_{eff} = \frac{6R_0 T}{wG_0 t_0}\left\{t_c + \tau_g[e^{(t_{Hold} - t_c)/\tau_g} - 1] + \left(\frac{\omega'_0}{\omega'_{Hold}}\right)^2(t_e - t_{Hold})\right\} \tag{3}$$

where ω'_0 (rpm) is the initial rotor velocity.

If field decay is not exponential, then Eqs. (2) and (3) must, of course, be modified, the term τ_g[   ] being replaced.

The mass M (g/mol) of the particle, exclusive of any solvent components bound to it, is related to M_{eff} by the buoyancy factor B (*1, 2, 8*):

$$M = M_{eff}/B \tag{4}$$

B is usually taken as being

$$B = 1 - \rho\bar{v} \tag{5}$$

where $\bar{v}$ is the partial specific volume of the particle and ρ is the density of the solution or suspension of which the particle is a part. B is sometimes given as $\Delta\rho/\rho_p$ (*1, 8*), the difference between the densities of the particle and the solution divided by the density of the particle.

Equations (1a) and (1b), in conjunction with Eqs. (4) and (5), are based on the fundamental SdFFF equations derived by Giddings et al. (Eqs. 7 and 9 of Ref. *8*). To emphasize concepts dealt with here, $M\Delta\rho/\rho_s$ of Giddings was set equal to M_{eff}, and $\Delta\rho/\rho_s$ was set equal to B. R of Giddings et al. was replaced by t_0/t_e, and the molecular quantities k and m were replaced by the molar equivalents R_0 and M. Equation (2), in conjunction with Eqs. (4) and (5), is based on Eqs. (14) and (15) of Yau and Kirkland (*9*). Equation (3) was derived by the author (*1*) as an extension of Eq. (2).

Equation (4) is a general expression for the particle mass. The numerator, M_{eff}, contains the information provided by the SdFFF experiment; it is given by one of the first three equations. Which equation is used depends on when the particle in question elutes, during the initial constant field phase, the exponential decay phase, or the terminal constant field (hold) phase. The denominator of Eq. (4), the buoyancy factor B, depends on the density ρ_p (or partial specific volume $\bar{v}$) of the particle in question and the density ρ of the solution of which the particle is a part.

Whereas Eqs. (1) to (3) are specific to SdFFF, Eqs. (4) and (5) also apply to sedimentation equilibrium (SdE). Indeed, SdFFF is, in essence, a combination of sedimentation equilibrium and elution. For SdE, M_{eff} is obtained from the SdE data. For both SdFFF and SdE, B is obtained from separate measurements, usually density measurements.

In this discussion, attention will be directed primarily to the buoyancy factor B of Eqs. (4) and (5). Many of the particles dealt with in SdFFF experiments are complexes of macromolecules which bind or interact with solvent components. Thus, casein micelles are complexes of caseins which interact with water and disaccharides. Now M_{eff} is the effective mass of the eluting particle, including bound solvent components, in the solution or suspension of which it is a part. If $\bar{v}$ for the particle were known, one could then calculate its weight by Eqs. (4) and (5). This would be a true particle

weight, total mass (including bound solvent components) per mole of particles. But $\bar{v}$ is, generally, not known for the whole particle. Consequently, the particle mass cannot be determined. What is known is the $\bar{v}$ of the naked macromolecule, e.g., the caseins in the micelle. If the effective mass of the solvent-free macromolecular complex were known, we could calculate its mass (assuming that $\bar{v}$ for the macromolecular complex is the same as that of its component molecules). But FFF measurements give us M_{eff} for the eluting particle, e.g., the micelle, not the effective mass of the solvent-free macromolecular complex in the particle. Thus, what is known (Eq. 4) is the numerator for the particle as a whole and the denominator for part of the particle. We therefore cannot calculate either the mass of the particle as a whole or that of its (solvent-free) macromolecular complex.

In the case of the casein micelle, its structure is a very open one (10–12); solvent inside the micelle exchanges rapidly with solvent in the environment of the micelle. There is even some exchange between proteins inside and outside the micelle (10). Now, any free solvent inside the micelle has virtually no effect on the buoyancy of the particle. (Free solvent inside the micelle does have a slight effect on the buoyancy, because of the small difference in density between the solvent and the micelle-containing solution as a whole.) M_{eff} of the micelle should therefore be equal to the M_{eff} of the protein in the micelle. Consequently, both numerator and denominator of Eq. (4) are known for the protein in the micelle, and one might expect to be able to calculate $M = M_{Prot}$, the molar micellar protein mass.

This kind of reasoning is the justification for the values of particle mass (e.g., M_{Prot}) usually calculated. However, although the bulk (free) solvent inside the micelle can be disregarded, this doesn't mean that one can deal with the naked protein alone. Proteins and other macromolecules bind solvent components. Thus, for example, the portion of the micelle responsible for its sedimentation is the solvated protein. And, in general, binding is selective, i.e., preferential. For example, when the solvent consists of an aqueous solution of sucrose or lactose, there is preferential binding of water to the protein, i.e., the water/sugar ratio is higher for the protein-bound solvent than for the free (bulk) solvent. An equation is therefore needed for calculating the buoyancy factor B for the solvated macromolecule in a particle. To provide a basic understanding of the concepts involved, a heuristic derivation of B and related quantities will be given. Rigorous treatments of the thermodynamic quantities have been provided by others in connection with discussions of preferential interactions, the stabilization of protein structure by sugars, and the interpretation of sedimentation equilibrium data (4, 13–17).

The simplest system to be considered consists of two components, a macromolecule and a solvent, which do not interact. Consider m_2 grams

of a macromolecule (Component 2), e.g., a protein, dissolved in m_1 grams of water (Component 1). In principle (but not in practice), the partial specific volume of the solute can be obtained as follows. Knowing the density of the solvent (g/mL) and its mass, its volume $v_1 = m_1/\rho_1$ (mL). The difference $v - v_1$ between the volume of the solution (v) and that of the solvent (v_1) can be assigned to the solute. The partial specific volume of the solute $\bar{v}_2 = (v - v_1)/m_2$ (mL protein/g protein). The concentration of macromolecule $c_2 = m_2/v$ (g protein/mL solution). And the density of the solution $\rho = m/v$ (g/mL solution), where $m = m_1 + m_2$.

The m_2 grams of solute are buoyed up by the mass of solution displaced, i.e., by $\rho(v - v_1)$ grams of solution. The effective mass of macromolecule is therefore

$$m_{\text{eff}} = m_2 - \rho(v - v_1) \tag{6}$$

and the buoyancy factor

$$B = m_{\text{eff}}/m_2 \tag{7}$$

$$= \frac{m_2 - \rho(v - v_1)}{m_2}$$

$$= 1 - \rho\bar{v}_2 \tag{8}$$

Therefore,

$$m_2 = \frac{m_{\text{eff}}}{1 - \rho\bar{v}_2} \tag{9}$$

An example is shown in Table 1. The (fictitious) solution was made by dissolving exactly 20 g protein (dry) in exactly 1020 g water. All figures are assumed to be known to 6 significant figures, but zeros used only to specify precision are omitted for simplicity. And, for the sake of simplicity, the density of the solvent, water, is taken to be exactly 1. (Water of this

TABLE 1

Example of a (hypothetical) Two-Component System

(1) Water (solvent)	1020. g	1020. mL
(2) Protein (solute)	20. g	15. mL
Solution	1040. g	1035. L

density at, say, 25°C, could be obtained by adding a small amount of D_2O to H_2O.) Subtracting the volume of the water (1020 mL) from the volume of the solution (1035 mL), we get a volume of 15 mL for the protein. Therefore,

$$\bar{v}_2 = 15 \text{ mL}/20 \text{ g} = 0.75 \text{ mL/g protein}$$

The concentration of the protein $c_2 = 20$ g/1035 mL, and the density of the solution $\rho = 1040$ g/1035 mL. The 20 g of protein are buoyed up by 15ρ g of solution. Hence $m_{\text{eff}} = 20 - 15\rho = 4.928$ g, and $B = m_{\text{eff}}/m_2$ (Eq. 7) is just under 0.25.

As previously mentioned, these calculations apply in principle, but not in practice; the volume of the solution cannot be measured precisely, especially when, as in practice, the amounts of solvent, solute, and solution are approximately one-hundredth as large as those in the example of Table 1. However, the densities of small volumes of the solvent (ρ_0) and the solution (ρ) can be measured with high precision, and the concentration of Component 2 can be measured with adequate precision. And it can be shown (see Section 1 of the Appendix) that the buoyancy factor is given, to a good approximation, by the equation

$$B = \left(\frac{\rho - \rho_0}{\rho_0}\right)\left(\frac{\rho - c_2}{c_2}\right) \tag{10}$$

and (Section 2 of the Appendix) that

$$\bar{v}_2 = \frac{1}{\rho_0}\left(1 - \frac{\rho - \rho_0}{c_2}\right) \tag{11}$$

Here the subscript 0 refers to the solvent. Since, in the present case, the solvent consists of a single component, water, $\rho_0 = \rho_1$.

As will be shown below, particle weights $M = M_2$ calculated for a 2-component system using Eq. (10) and (4) are not seriously in error even when the macromolecule is hydrated. Serious errors arise in some 3-component systems, i.e., systems in which the solute (Component 2), a macromolecule, a particle, or a macromolecule-containing particle, is dissolved or suspended in a 2-component solvent consisting of a material of relatively low molecular weight (Component 3) dissolved in the principal solvent (Component 1). Component 3 is sometimes referred to as the cosolvent. For a 3-component system in which the solute does not interact with either of the 2 components of the solvent, the solution consists of m_2 grams of

naked solute (i.e., solute having no bound solvent components) dissolved in m_0 grams of solvent having the composition m_3/m_1. Such a system is, for our purposes, indistinguishable from a 2-component (single-component solvent) system, and the particle weight can (again) be calculated using Eqs. (10) and (4). In general, though, the solute does interact with the components of the solvent; the naked solute is hydrated (i.e., it interacts with Component 1) and cosolvated (i.e., it interacts with Component 3). The sedimenting particle is thus solvated solute. (The term solvation will be used to mean interaction of the macromolecule with the principal solvent and with the cosolvent.) The composition of the solvent in equilibrium with the solvated solute is, in general, different from that of the solvent in which the solute wad dissolved. As an example (Table 2), suppose that 20 g of pure (unhydrated, salt-free) DNA are dissolved in a solvent consisting of 1020 g water (of density exactly 1) and 200 g of a hypothetical low molecular weight salt. Before addition of the DNA, the solvent occupied 1145 mL. After addition of the DNA, the solution occupies 1160 mL, an increase of 15 mL. A volume of 15 mL can therefore be assigned to the 20 g of DNA, so $\bar{v}_2 = 0.75$ mL/g. It should be noted that the unit mL/g refers here to mL of naked DNA per g naked DNA. This value is calculated without any information about DNA solvation. When the DNA is solvated, the solvent components are redistributed (Table 3). In the case shown (Tables 2 and 3) the hydration $h = 0.5$ g water/g DNA and the cosolvation $c = 0.5$ g salt/g DNA. There are, therefore, 40 g of solvated DNA in equilibrium with 1200 g of solvent having the composition shown (Table 3). It should be noted that the DNA bound solvent (Table 3, b and c) is 50% (w/w) salt, whereas the free solvent is slightly less than 16% salt. If this DNA solution were dialyzed against a solvent having the composition shown (Table 3), the composition of the solution would not change (provided that we applied pressure to counteract osmotic pressure). The

TABLE 2
Example of a (hypothetical)
Three-Component System

Solvent:		
Water (1)	1020. g	1020. mL
Salt (3)	200. g	125. mL
Solvent	1220. g	1145. mL
Solute:		
DNA (2)	20. g	15. mL
Solution	1240. g	1160. mL

TABLE 3
Example of a (hypothetical) Three-Component System
with Solvation

Solvent:		
Free water	1010. g	1010. mL
Salt	190. g	118.75 mL
Solvent	1200. g	1128.75 mL
Solute		
(a) DNA	20. g	15. mL
(b) Bound water	10. g	10. mL
(c) Bound Salt	10. g	6.25 mL
Solvated DNA	40. g	31.25 mL
Solution	1240. g	1160. mL

water inside the dialysis sac would have the same chemical potential, μ_1, as the water outside the membrane, and μ_3, the chemical potential of the salt, would be the same inside and outside. On the other hand, in terms of molal composition, i.e., the ratio of total salt/total water, the solution has the same molality as the solvent in which the DNA was dissolved (Table 2).

The 40 g of solvated DNA are buoyed up by 31.25ρ g solution, ρ being 1240/1160 g/mL (Table 3). The effective mass of the solvated DNA is therefore just under 6.6 g, and the buoyancy factor (Eq. 7)

$$B \equiv m_{\mathrm{eff}}/m_2 = 0.3297$$

This is effective mass of solvated DNA per unit actual mass of naked (unsolvated) DNA.

It's possible to show (see Section 3 of the Appendix) that B is given, to a good approximation, by an equation strictly analogous to that (Eq. 10) for the 2-component system, viz.,

$$B = \left(\frac{\rho' - \rho_0'}{\rho_0'}\right)\left(\frac{\rho' - c_2'}{c_2'}\right) \tag{12}$$

Here ρ_0' is the density of the solvent in dialysis equilibrium with the solution, whose density is ρ' and whose solute concentration is c_2' (g naked solute/mL solution). In the 2-component system, the solvent in which the solute was dissolved was water and the solvent which would be in dialysis equilibrium with the solution is water. It was therefore unnecessary to distin-

guish the solvent which was isomolal with the solution from the solvent whose components were isopotential with the dialyzable component of the solution. The same is true of the 3-component system when the solute does not interact with either component of the solvent. But in a 3-component system in which the solute interacts preferentially with one of the two solvent components, it is essential to distinguish the isopotential solvent (in dialysis equilibrium with the solution) from the isomolal solvent.

It may be pointed out that as

$$c_2' \rightarrow 0$$

then

$$\rho' - c_2' \rightarrow \rho_0'$$

$$(\rho' - c_2')/\rho_0' \rightarrow 1$$

and

$$B \rightarrow (\rho' - \rho_0')/c_2' \rightarrow (\partial \rho'/\partial c_2')_\mu^0$$

Otherwise stated,

$$B^0 = (\partial \rho'/\partial c_2')_\mu^0 \tag{13}$$

as required (15–17). The superscript 0 indicates $c_2' = 0$, and the subscript μ indicates constant chemical potentials μ_1 and μ_3.

Using the approximate equation for B (Eq. 12), one obtains a value of 0.3352 for the solution given in Table 3. Comparing this with the exact value calculated previously, 0.3297, the approximate value is in error by 1.7%. By contrast, if $1 - \rho'\bar{v}_2$ had been used as the buoyancy factor (Eqs. 5 and 8), the results would have been in error by -40% (Table 5), which would give rise to an error of almost 70% in molecular weight.

The equation for the buoyancy factor (Eq. 12) can be rewritten as

$$B = 1 - \frac{\rho'}{\rho_0'}\left(1 - \frac{\rho' - \rho_0'}{c_2'}\right) \tag{12a}$$

Now, in the simpler cases (Eq. 8), $B = 1 - \rho\bar{v}_2$, $\bar{v}_2$ being the partial specific volume at constant molality. By analogy, we can define an apparent partial

specific volume ϕ_2' by the equation

$$B = 1 - \rho'\phi_2' \tag{14}$$

By "apparent" is meant a quantity replacing the partial specific volume $\bar{v}_2$ which gives the correct value of B (to a close approximation) even for a 3-component system with preferential interaction. Comparing Eqs. (12a) and (14), we obtain

$$\phi_2' = \frac{1}{\rho_0'}\left(1 - \frac{\rho' - \rho_0'}{c_2'}\right) \tag{15}$$

Disregarding the primes, the right-hand side of this equation is identical to the equation (Eq. 11) for $\bar{v}_2$. But there is a crucial difference. When calculating $\bar{v}_2$, ρ_0 means the density of the solvent in which the solute was dissolved, ρ being the density of the resulting solution. Otherwise stated, ρ_0 is the density of the solvent which is isomolal with the solution. When calculating ϕ_2', ρ_0' means the density of the solvent in dialysis equilibrium with the solution, whose density is ρ'. Otherwise stated, ρ_0' is the density of the solvent, each of whose components has the same chemical potential as that component has in the solution. ϕ_2' is sometimes referred to as the partial specific volume at constant chemical potential. For the 2-component system, where the solvent has only one component, usually water, the solvent is both isomolal with the solution and also has the same chemical potential as the water in the solution. $\bar{v}_2$ and ϕ_2' are then identical. The same holds true for a 3-component system in which the solute does not interact with either of the two components of the solvent. It also holds true when the solute-bound solvent has the same composition as the free solvent. But when there is preferential interaction, $\bar{v}_2$ and ϕ_2' can be very different. Returning to our last example, $\bar{v}_2 = 0.75$ (exactly) and $\phi_2' = 0.6219$.

Table 4 summarizes information for (hypothetical) examples of 2-component systems with and without hydration. Here $\phi_2' = \bar{v}_2$ (= 0.75) and $1 - \rho\bar{v}_2 = B$ (Eq. 8). There are no serious errors. Note also the units of B: although technically the unit of B is 1, it is important to remember that B is the effective mass of the solvated solute per unit actual mass of naked solute. And for ϕ_2': although the unit is the same as for $\bar{v}_2$, mL/g, it is important to remember that it refers to mL of solvated solute per gram of naked solute, whereas for $\bar{v}_2$ it refers to mL of naked solute per gram of naked solute.

TABLE 4
Summary of Two-Component Systems

	No hydration	With hydration	
h	0	1	g H_2O/g protein
$\bar{v}_2$	0.75	0.75	mL/g protein
B	0.2464	0.2415	$\dfrac{\text{g effective of solvated protein}}{\text{g protein}}$
$\{B^a$	0.2464	0.2464	$\dfrac{\text{g effective of solvated protein}}{\text{g protein}}$
Error	0	2.0	Percent
$\{1 - \rho\bar{v}_2$	0.2464	0.2464	$\dfrac{\text{g effective of protein}}{\text{g protein}}$
Error in B	0	2.0	Percent
ϕ_2'	0.75	0.75	$\dfrac{\text{mL solvated protein}}{\text{g protein}}$

[a]From Eq. (10).

Table 5 summarizes pertinent values for (hypothetical) examples of 3-component systems. When the solute does not interact with either component of the solvent, $1 - \rho\bar{v}_2$ (Eq. 8) gives the correct value for the buoyancy factor. But, when there is preferential interaction, large errors can result, in these examples 53 and -40%. There is nothing wrong with the value of $\bar{v}_2$; it just should not be used to calculate B.

TABLE 5
Summary of Three-Component Systems

	No Solvation	Hydration	Solvation
h^a	0	1	0.5
c^b	0	0	0.5
$\bar{v}_2$	0.75	0.75	0.75
B	0.1983	0.1293	0.3297
$\{B$	0.1983[c]	0.1315[c]	0.3352[d]
Error (%)	0	1.7	1.7
$\{1 - \rho\bar{v}_2$[e]	0.1983	0.1983	0.1983
Error (%)	0	53	-40

[a]g water/g protein or DNA.
[b]g cosolvent/g protein or DNA.
[c]From Eq. (10).
[d]From Eq. (12).
[e]Eqs. (5) and (8).

DISCUSSION

From what has been stated above, it is clear that the buoyancy factor B used (Eq. 4) for calculation of the "molecular weight" $M = M_2$ of a solute (a dissolved or suspended macromolecule or particle) can always be determined from density and concentration measurements. For a 2-component system, and for a 3-component system in which there is no interaction between solute and solvent, B can be obtained from Eq. (10), where ρ is the density of the solution containing the solute in question, c_2 is the solute concentration therein, and ρ_0 is the density of the solvent in which the solute was dissolved or suspended. However, for a 3-component system in which there is preferential interaction of the solute with the solvent components, Eq. (10) (and Eqs. 5 and 8) are not valid. In this case, and, more generally, wherever preferential interaction cannot be ruled out, the solution or suspension in question must be dialyzed to equilibrium against solvent prior to measuring density and concentration. B is then calculated from Eq. (12), where ρ' and c_2' are the density and solute concentration (of the solution or suspension) inside the dialysis sac, and ρ_0' is the density (of the solvent) outside the membrane. Alternatively, B can be obtained from Eqs. (14) and (15). Details as to how to carry out the experimental work are provided in Ref. *4*.

B and ϕ_2' generally vary with the solute concentration c_2'. By obtaining the values of B and ϕ_2' at several values of c_2', one can extrapolate to $c_2' = 0$ to obtain B^0 and $(\phi_2')^0$ *(4)*.

In the examples given in Table 5 for hydration (only) in a 3-component system, $1 - \rho\bar{v}_2$ is 53% higher than the correct buoyancy factor. If $1 - \rho\bar{v}_2$ were used instead of B (Eq. 12), the apparent molecular weight calculated by Eq. (4) would therefore be in error by -35%. More generally, when the bound solvent is richer in Component 1 than the bulk solvent, the calculated molecular weight will be lower than the correct value. Since low molecular weight sugars are known to be preferentially excluded from contact with proteins *(6, 7)*, molecular weights measured in solvents containing such sugars will be underestimated if $1 - \rho\bar{v}_2$ is used instead of B of Eq. (12). Thus, the decrease in apparent M_{Prot}^{Max} observed in three of the four cases in Fig. 1 is partially attributable to such an effect.

INTERPRETIVE SUMMARY

The quality and stability of milk products is significantly affected by the size of the casein micelles, the large, complex particles which contain most of the protein of milk. The molecular weight of such very large particles in solution can be obtained by the relatively new technique of sedimentation field-flow fractionation (SdFFF). Calculation of the molecular weight from

the SdFFF data requires knowledge of the buoyancy factor B of the particle. The equation for B usually used is, in general, not valid when the solution also contains substantial amounts of a low molecular weight substance such as a sugar (in addition to the solvent and the large particles). Unacceptable errors in calculated molecular weights can result if the usual equation for B is used in such cases. An equation of general validity in which B is expressed directly in terms of measurable quantities is heuristically derived and discussed, together with related quantities. The discussion provides an understanding of the concepts involved, so that the reader will know the experimental arrangement required to make the needed measurements and how to calculate the correct values of B and the desired molecular weight.

APPENDIX

(1) Equations required for the derivation of the expression (Eq. 10) for the buoyancy factor B for a 2-component system, with no interaction between solute and solvent.

$$m_{eff} = m_2 - \rho v_2 \text{ (cf. Eq. 6)}$$

$$v_2 = v - v_1$$

$$\rho = m/v, \qquad \rho_1 = m_1/v_1$$

$$B = m_{eff}/m_2 \text{ (Eq. 7)}$$

$$m_2 = c_2 v$$

$$m = m_1 + m_2$$

$$\rho_1 = \rho_0$$

Utilizing the above equations, one obtains

$$B = \left(\frac{\rho - \rho_0}{\rho_0}\right)\left(\frac{\rho - c_2}{c_2}\right) \tag{10}$$

(2) Equations required for the derivation of the expression (Eq. 11) for the partial specific volume $\bar{v}_2$ (at constant molality).

Let subscript 0 refer to the solvent in which the solute (Component 2) is dissolved.

$$\bar{v}_2 = (v - v_0)/m_2$$

$$\rho = m/v, \qquad \rho_0 = m_0/v_0, \quad \text{and} \quad m_2 + m_0 = m$$

$$m_2/v = c_2$$

Utilizing the above equations, one obtains

$$\bar{v}_2 = \frac{1}{\rho_0}\left(1 - \frac{\rho - \rho_0}{c_2}\right) \tag{11}$$

The derivation is independent of the nature of the solvent, i.e., whether it contains one or more components, and is independent of whether there is interaction between the solvent and solute. It is only assumed that the partial specific volumes of the solvent components are unchanged by binding to the solute.

(3) Equations required for the derivation of the expression (Eq. 12) for the buoyancy factor B for a 3-component system, with interaction between the solute (Component 2) and the components (1 and 3) of the solvent. It is assumed (see text) that dialysis equilibrium has been established between the solution inside the membrane and solvent outside the membrane. To distinguish the densities and solute concentration of this system from that in which the solution is not dialyzed, these quantities will be designated by primes (ρ_0', ρ', c_2', etc.).

Letting m_{2s} = mass of solvated solute

v_{2s} = volume of solvated solute

h = hydration (g water/g solute)

c = cosolvation (g cosolvent/g solute)

$$m_{\text{eff}} = m_{2s} - \rho' v_{2s}$$

$$v_{2s} = v - (v_0)_{\text{f}}$$

where $(v_0)_f$ is the volume of free solvent (i.e., solvent components not bound to the solute)

$$m_{2s} = m_2 + hm_2 + cm_2$$

$$\rho' = (m_1 + m_2 + m_3)/v$$

$m_1 - hm_2 = (m_1)_f = $ the mass of free Component 1 in the solution

$m_3 - cm_2 = (m_3)_f = $ the mass of free Component 3 in the solution

$(m_1)_f + (m_3)_f = (m_0)_f$, the mass of free solvent

Let

$$(m_0)_f/(v_0)_f = (\rho'_0)_f$$

Since the system is in dialysis equilibrium, the chemical potential of each solvent component inside the membrane is the same as that outside the membrane. The composition of free solvent inside the membrane can therefore be taken to be equal to that of the solvent outside the membrane. Therefore

$$(\rho'_0)_f = \rho'_0,$$

where ρ'_0 is the density of the solvent outside the membrane.

$$B = m_{eff}/m_2 \quad (Eq. 7)$$

$$m_2 = c'_2 v$$

$$m_0 = m_1 + m_3$$

$$m_2 \ll m_0$$

$$(h + c)m_2 \ll m_0$$

$$(m_0)_f/m_0 \approx 1$$

Utilizing the above equations, one obtains

$$B = \left(\frac{\rho' - \rho_0'}{\rho_0'}\right)\left(\frac{\rho' - c_2'}{c_2'}\right) \tag{12}$$

SYMBOLS

A_i total amount of component i bound per unit mass of solute (g/g)

B buoyancy [g (effective mass) of solvated solute/g (actual mass) of unsolvated solute]

c cosolvation (g cosolvent/g solute)

c_2 concentration of Component 2 (g/mL solution)

g_3 solvent composition (g cosolvent/g principal solvent)

G gravitational (centrifugal) field strength (acceleration) (cm/s^2)

G_0 value of G during constant field phase (cm/s^2)

h hydration (g water/g solute)

m mass (g)

M molecular weight (g/mol)

M_{eff} effective particle mass in the medium in which the particle is suspended (g/mol particles)

M_{Prot} mass of protein in the particle (molar micellar protein mass) (g protein/mol particles)

R_0 gas constant (erg·Kelvin^{-1}·mol^{-1})

t_c time (from start of eluant flow) when constant field phase ($G = G_0$) terminates and decay begins (min)

t_e elution (retention) time (min)

t_0 value of t_e for unretained particles (min)

t_{Hold} time at which decay phase terminates and hold phase begins (min)

T absolute temperature (Kelvin)

v volume (mL)

$\bar{v}$ partial specific volume of the particle (mL/g)

w width (thickness) of the separation channel (radial distance between outer and inner walls (cm)

Greek Letters

λ basic retention parameter of FFF (dimensionless)

μ chemical potential

ξ_3 preferential interaction parameter (g cosolvent/g solute)

ρ density of the solution (g/mL)

τ_g exponential decay constant of the field G (min)

ϕ' partial specific volume at constant chemical potential (mL/g)

ω' rotational velocity of rotor (rpm)

ω_0' value of ω' during (initial) constant field phase (rpm)

ω_{Hold}' value of ω' during hold phase (rpm)

Subscripts

0	solvent
1	principal solvent (e.g., water)
2	solute (e.g., protein, DNA)
2s	solvated solute
3	cosolvent (e.g., salt or sugar)
f	free
p	particle (solute)
eff	effective
Prot	protein

Superscripts

System in which solution is in dialysis equilibrium with solvent

0      $c_2 = 0$

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