Determination of Hydration and Partial Specific Volume of Proteins with the Spring Balance*

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ABSTRACT: The buoyant weight of a macromolecular component (2) enclosed within a semipermeable membrane and kept at equilibrium with solvent (water) and diffusible solutes depends upon the partial specific volume and on the hydration of the macromolecules, as long as binding of other components besides the pure solvent can be neglected. In an aqueous solvent with a density close to the density of water or in water itself the partial specific volume of the macromolecular solute determines the buoyant weight. Increased density of the solvent (*e.g.*, by addition of glycerol) leads to an increase of the buoyancy attributable to the "hydration" water "bound" to the macromolecules. By the use of a quartz spring balance the buoyant weights of bovine serum albumin, tobacco mosaic virus, and tobacco mosaic virus protein in water, aqueous buffer, and glycerol–buffer mixtures were determined directly. Values for the partial specific volumes in buffer at 20° were determined independently by means of pycnometry. The results, V_2 = 0.727, 0.733, and 0.734 cc per g for tobacco mosaic virus, tobacco mosaic virus protein, and bovine serum albumin, respectively, are in good agreement with data in the literature. Values obtained with the spring balance agree within about 1%. Increasing glycerol concentration ($c < 30\%$ w/v) unveils a linear relationship between density and buoyant weight. The slope, $-dW/d\rho = \gamma_2 G V_1 + \gamma_2 V_2$, as a measure of the hydration G (γ_2 being the amount of solute 2, and V_1, V_2, the partial specific volumes of water and the macromolecular component (2)) leads to G = 0.16, 0.20, and 0.28 g of water per g of protein for tobacco mosaic virus, tobacco mosaic virus protein, and bovine serum albumin, respectively, in fair agreement with previous data from other determinations.

The extent of hydration of proteins in solution in a multicomponent system cannot be defined in terms of a definite amount of "bound" or "free" water. A rigorous thermodynamic treatment, however, enables us to define "hydrate" solvent as the amount of solvent which has to be added to a solution along with anhydrous protein so that the *ratio* of the activity of the solvent in the protein solution to that in the original solution is equal to the *ratio* of the activity of the "free" solute in the protein solution to that in the original solution (Lauffer, 1964). Correlating the formal treatment to measurable quantities, this definition leads to an expression for the buoyant weight of the protein in a mixed (incompressible) solvent

$$W = \gamma_2 G(1 - V_1\rho) + \gamma_2 G'(1 - V_4\rho) + \gamma_2(1 - V_2\rho) + \frac{M_3\gamma_1}{1000} m_3''(H_3 - 1)(1 - V_3\rho) + \text{constant}(1 - V_4\rho) \quad (1)$$

in which the first two terms reflect the buoyant weight of "bound" water and salt, respectively, while the fourth and fifth terms represent the influence of the redistribution of a solvent component of high density and the Donnan equilibrium. W means the weight corrected for total buoyancy of the system, γ_i means the mass and V_i the partial specific volume of the ith component in the sac, and ρ is the density of the external solution. M_i refers to the molecular weight and m_i'' refers to the molality in the external solution of the ith component, while G' and G, respectively, are the weight of salt and of water "bound" by 1 g of protein. H_3 is a constant which depends upon the osmotic pressure difference between the inside and the outside of the semipermeable container of the protein at osmotic equilibrium, the activity coefficients of the third solvent component and "free" water inside and outside the sac, and the partial molal volumes of both water and the third component. 1, 2, 3, and 4 signify water, protein, the third solvent component (*e.g.*, glycerol), and salt, respectively.

In theory, eq 1 can be used to determine the extent of hydration as well as the partial specific volume of the solute by measuring the buoyant weight of a protein solution under conditions of equilibrium dialysis in a spring balance of high sensitivity. In this method the protein solution is contained in a cellophane sac suspended from the end of a delicate quartz spring in media of different solvent densities. At equilibrium the weight of all diffusible components in the sac will be completely cancelled by buoyancy. However, protein, "hydrate" water, and "bound" salt[1] cannot pass the membrane and, therefore, will contribute to the weight according to eq 1.

In aqueous media with $\rho \approx 1/V_1$ the first and fourth terms on the right in eq 1 vanish, *i.e.*, the weight depends on the partial specific volume of the protein, V_2, and the binding of salt alone. Increasing the density of the solvent by addition of a component (3) of high density (*i.e.*, glycerol) introduces the "hydration term" and the "redistribution term" for the third component. As a consequence, one would expect the two limiting

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[1] Salt excluded by Donnan equilibrium can be included as "negative binding" in the second term of eq 1. G'' has to be defined under this condition as grams of salt "bound" by adsorption as well as excluded by the Donnan equilibrium.

cases, $\rho \approx 1/V_1$ and $\rho \gg 1/V_1$ to offer possibilities to measure V_2 as well as G.

The following results demonstrate the validity of this hypothesis for tobacco mosaic virus, tobacco mosaic virus protein, and bovine serum albumin.

Materials and Methods

Bovine serum albumin (crystallized and lyophilized) was purchased from Sigma (lot no. 37B-0930).

Tobacco mosaic virus (common strain) was isolated by differential centrifugation with two to three depigmentation steps according to Ginoza *et al.* (1954) and Boedtker and Simmons (1958). The stock solutions in 10^{-2} M EDTA (pH 7.5) were kept at 0°.

TMVP was extracted from the virus by the acetic acid method (Fraenkel-Conrat, 1957). The isoelectric precipitate was dissolved at pH ≦9 (KOH) and clarified (after dialysis with the solvent used in the spring balance experiment) by centrifugation at 100,000*g* for 2 hr. Individual samples were prepared for each experiment. The concentrations of protein and virus were determined both from ultraviolet spectra (Cary 14 M) in the range between 420 and 230 mμ (corrected for scattering) and from dry weight determination at 106° after exhaustive dialysis against doubly distilled water. The extinction coefficients and purity ratios are given in Table I.

TABLE I: Characteristics of Proteins and Virus.

	λ_{max} (mμ)	ε (ml/mg cm)	$\epsilon_{max}/\epsilon_{min}$
Bovine serum albumin	280	0.69	1.64
TMV	262	2.7	1.26 ± 0.04
TMVP	281	1.3	2.45 ± 0.02

Buffer solutions of different densities but identical pH and ionic strength were prepared by adding calculated amounts of glycerol to a concentrated stock solution of the buffers (phosphate and acetate) and filling up to the final volume. pH values were checked with a Beckman Research pH meter using Beckman glass and calomel electrodes. All solutions contained 3×10^{-3} M EDTA as bactericide. Chemicals were analytical reagent grade. Doubly distilled water was used throughout. Solvents were carefully deaerated by heating at low pressure.

Equilibration with the solvents was provided by equilibrium dialysis over a period of ≧48 hr at *ca.* 0° after the sac with the protein or virus solution was tied. To avoid changes of concentration from osmotic shrinkage and to provide sacs completely free of air bubbles, changes of the solvent were always performed starting from the solvent with maximum osmotic effect (*e.g.*, 25% glycerol) and changing to lower concentrations of the third component.

Spring balance experiments were performed as described earlier (Stevens and Lauffer, 1965; Jaenicke and Lauffer, 1969). Quartz springs (Worden, Houston, Texas) of 100- and 200-mg maximum load and 250-mm maximum extension were calibrated with the highest possible accuracy using platinum weights. The influence of solvent density on the first- and second-order coefficients of the springs is negligible. Influences of the buoyant weight of the dialysis sac are eliminated by weighing the empty sac under identical conditions as in the protein experiment. To prevent errors from air bubbles in the sac after complete removal of the protein solution, the sacs were dipped into the solvent used for the preequilibration during the mounting procedure. After the sac was attached to the lower hook of the spring, the sac was kept in a small tube with the respective solvent upon transferring it into the spring balance. The equilibration was followed cathetometrically by measuring the extension, h, of the spring as a function of time. The final state (defined by $\delta h < 0.005$ mm) was reached after 48 hr.

In order to minimize errors from protein concentration and spring calibration, the weights in different solvents were measured each time with the same spring, transferring the sac with the material from one solvent to the one with the next lower density (see above). After final equilibration the sac was emptied and again weighed in the same series of solvents. Temperature was kept constant at 20° with an accuracy of ±0.008° (Jaenicke and Lauffer, 1969).

Densities were measured pycometrically using 50-cc Pyrex volumetric flasks with a capillary of 1-mm diameter as neck and ground-glass stoppers.

Results

Results with the spring balance at different densities are given in Table II. As shown elsewhere (Jaenike and Lauffer, 1969) addition of glycerol in the concentration range up to 25% (w/v) does not change the molecular structure of TMVP considerably, as far as ultraviolet difference spectra and optical rotatory dispersion allow this conclusion. For bovine serum albumin the same holds true (R. Jaenicke, unpublished results). In the case of TMVP, the pycnometric determination of the partial specific volume in the presence and absence of glycerol confirms this result, both values being identical within the range of error (Table III). Addition of glycerol can, therefore, be understood basically in terms of increased density of the medium without structural changes.

At zero glycerol concentration, *i.e.*, under the condition $\rho \approx 1/V_1$ or in pure water the value for V_2 of TMV and TMVP from the spring balance is in good agreement with the pycnometric results and with earlier determinations (Lauffer, 1944).

For bovine serum albumin the values given in the literature are in the range 0.734 ± 0.002 cc per g (Dayhoff *et al.*, 1952) as compared to 0.728 ± 0.003 cc per g in the case of the spring balance determination.

Increasing glycerol concentration up to about 30% glycerol leads to a strictly linear relationship between density and buoyant weight (Figure 1). This allows us to determine G from eq 1 in the differentiated form

$$-\frac{\partial W}{\partial \rho_1} = \gamma_2 G V_1 + \gamma_2 V_2 + (\gamma_3 - \Gamma_3)V_3 + \gamma_2 G'' V_4 \quad (2)$$

At zero osmotic pressure which holds with sufficient accuracy for TMV as well as for TMVP under the given conditions of

TABLE II: Determination of Buoyant Weight, Partial Specific Volume, and $dW/d\rho$ from Spring Balance Experiments, 20°.

	Medium[a]	% Glycerol	γ_2 (g) Dry Wt	ϵ_{max}	$\Delta\Delta h$ (mm)	W (g/100 mg)	ρ (g/cc)	$-\partial W/\partial\rho$	V_2 (cc/g)	
Bovine serum albumin	aq_1	0	0.2457	0.2412	47.602	0.026220	0.99821		0.730	
		25			57.320	0.020192	1.05644	0.1035		
	A	0	0.1444		47.399	0.026965	1.00775		0.725	
		25	0.2435		57.562	0.022000	1.05644	0.1020		
		30	0.1500	0.1524	57.058	0.02000	1.07744	0.1000		
TMV	aq_1	0	0.1425		46.122	0.027888	0.99821		0.7224	
			0.1427		48.568	0.027952			0.7218	
			0.1419		75.608	0.027947			0.7218	
		~25	0.1210	0.118	51.171	0.022639	1.05644	0.09085		
			0.1850	0.1862	78.150	0.022856		0.08713		
	P	0	0.2130	0.2112	72.031	0.027770	1.00179	0.0996	0.7218	
		6	0.2130		68.154	0.02628	1.01675	0.0896		
		10	0.2130		65.865	0.02539	1.02668	0.0885		
		15	0.1427	0.1430	42.554	0.02445	1.03730	0.0894		
		18	0.2130		61.753	0.023807	1.04446	0.0884		
		25	0.2130		58.058	0.022391	1.06048	0.1774		
		30	0.1419		44.498	0.01992	1.07441	⟨0.156⟩		
		50	0.1427	0.1430	34.676	0.012783	1.11813			
					35.406	0.013052				
					36.427	0.013428				
TMVP	aq_1	0	0.2034		103.488	0.026773	0.99821		0.7336	Gel
		25	0.2934	0.291	72.543	0.021387	1.05644	0.09250		Gel
	aq_2	25	0.1208		48.802	0.021300	1.05652	0.09384		Gel[b]
	P	0	0.2101	0.211	106.825	0.026659	1.00173		0.7321	
			0.2139		105.164	0.026612			0.7326	
		12.5	0.2101		95.769	0.023853	1.03142	0.09457		
		25	0.2139		83.684	0.021083	1.06078	0.09365		

[a] aq_1, water, pH 6.0; aq_2, water, pH 3.9; A, acetate buffer, pH 4.58, $\mu = 0.1$; P, phosphate buffer (KH_2PO_4–K_2HPO_4), pH 6.7, $\mu = 0.05$. [b] Isoelectric.

solvent and protein concentration (Jaenicke and Lauffer, 1969), the third term in eq 2 is zero.[2]

The fourth term can be omitted in the case of the isoelectric protein. As shown in Table II the buoyant weight of TMVP and bovine serum albumin in 25% glycerol remains unchanged within the limits of error when the pH is changed from 6 to 4; in both cases TMVP is fully polymerized. Including the finding of Shalaby *et al.* (1968) that in the case of TMVP there seems to be no ion binding in the given pH range, we can conclude that the fourth term in eq 2 is negligible under the given conditions of the experiment.[3]

Equation 2, therefore, is reduced to

$$-\frac{\partial W}{\partial \rho} = \gamma_2 G V_1 + \gamma_2 V_2 \qquad (3)$$

Discussion

In order to be sure that data as plotted in Figure 1 can be interpreted in terms of hydration, it is necessary to examine carefully the definition of hydration as presented originally by Lauffer (1964). Water of hydration was defined as that amount of water, Δn_1, which must be added along with n_2 moles of anhydrous protein to a solution of component 3 (such as glycerol) in component 1 (such as water) which will lead to the re-

[2] Measurements of the osmotic pressure of the given solutions in a membrane osmometer (Mechrolab Model 503, Hewlett Packard, Avondale, Pa.) give values close to the limits of reproducibility ($P - P_0 <$ 0.2 cm $\sim$ $<2 \times 10^{-4}$ atm for TMV and TMVP, $c \approx 25$ mg/ml in 25% glycerol).

[3] An alternative realization of the same limiting case would be $m_4 = 0$, *i.e.*, protein or virus in pure water. The transition to the gel state, however, which eventually parallels the transition $\mu \rightarrow 0$ might cause anomalies which are equally observed as a consequence of heat aggregation in the isoelectric region (R. Jaenicke, unpublished results).

TABLE III: Apparent Partial Specific Volume of TMV and TMV Protein from Pycnometry, 20°.

	TMV			TMVP		
	C_2 (mg/ml)			C_2 (mg/ml)		
Medium[a]	Dry Wt	ϵ_{max}	V_2 (cc/g)	Dry Wt	ϵ_{max}	V_2 (cc/g)
aq	4.59	4.56	0.7280 ± 0.0020	7.19	7.24	0.7362 ± 0.0015[b]
P	4.39	4.50	0.7275 ± 0.0015	5.02	5.05	0.7340 ± 0.0012
25% glycerol [P]	8.88	8.84	0.7284 ± 0.002	6.48	6.54	0.7421 ± 0.0020[c]

[a] aq water, pH 8.0; P, phosphate buffer, pH 6.80, $\mu = 0.05$. [b] TMVP partially polymerized. [c] TMVP fully polymerized at 20°. Correcting for polymerization $(\Delta V_2)_{polymerization} \approx 0.005$ cc/g; *cf.* Jaenicke and Lauffer (1969)) gives $V_2 \cong 0.737$.

TABLE IV: Comparison of V_2 and G with Data in the Literature.

	V_2 (cc/g)			G (g of Water/g of Protein)	
	From ρ	From W_{aq}	Lit.		Lit.
Bovine serum albumin	0.734[a]	0.725–0.730	0.734[b]	0.28	0.2[c–h] 0.25–0.359[u] 0.3[i] 0.38[j,k] 0.48[l] 0.5[m]
TMV	0.727	0.722	0.727[n] 0.73–0.77[o] 0.74[p] 0.743[q] 0.77[r] 0.73[s]	0.16	0.27[t]
TMVP	0.734	0.732		0.205	

[a] From magnetic float measurements (Stauff and Jaenicke, 1961). [b] Dayhoff *et al.* (1952). [c] Oncley (1942). [d] Oncley *et al.* (1947). [e] Baldwin *et al.* (1955). [f] Wagner and Scheraga (1956). [g] Tanford and Buzzell (1956). [h] Tanford (1961). [i] Ritland *et al.* (1950). [j] Taylor (1950). [k] Stauff and Mehrotra (1961). [l] Anderegg *et al.* (1955). [m] Krause and O'Konski (1959). [n] Lauffer and Stanley (1939). [o] Bawden and Pirie (1937). [p] Harrington and Schachman (1956). [q] Schramm and Bergold (1947). [r] Stanley (1938). [s] Lauffer (1944). [t] Lauffer and Bendet (1954). [u] Ifft and Vinograd (1962, 1966).

sult that f_1'/f_3' (both on "free-water" basis) equals f_1''/f_3'' (reference solution). The symbol, f_i, means the activity coefficient on the rational basis of the ith component. Equation 4 can be derived[4] from this definition.

$$h_m = \frac{\frac{1000}{18}(\partial \ln f_3/\partial m_2 - \partial \ln f_1/\partial m_2)}{1 + \left(1 + \frac{18}{1000}m_3\right)m_3(\partial \ln f_3''/\partial m_3) + \frac{18}{1000}m_2 h_m} \quad (4)$$

The derivation of this equation involves the definition, $\Delta n_1 \equiv h_m n_2$, where h_m is the "hydration" in terms of moles of water per mole of anhydrous protein. The fact that the positions of the experimental points on the lines in Figure 1 are not sensitive to the protein concentration in the particular experiment represented is evidence for the validity of this definition. The final term in the denominator on the right of eq 4 is usually so small that it can be neglected. The denominator of eq 4 shows that hydration depends on the concentration of component 3 and, therefore, on the activity of solvent, as pointed out originally by Lauffer (1964). For example, in a 5 *m* glycerol solution, the activity of the water is approximately 0.90 and the denominator has a value of approximately 1.2, indicating that the hydration is approximately 20% less than the value at zero glycerol concentration where the water activity is near unity. When the protein concentration is sufficiently low so that $\partial \ln f_3/\partial m_2$ and $\partial \ln f_1/\partial m_2$ are independent of protein concentration, eq 4 shows that a limiting value of hydration is approached as the concentration of component 3 ap-

[4] Equation 4 can be derived by considering the addition of n_2 moles of anhydrous protein to a solution containing n_1 moles of solvent and n_3 moles of solute (glycerol, etc.) as a two-step operation. The first step is to add n_2 moles of anhydrous 2 plus Δn_1 moles of 1 to the solution of 1 and 3. Equations 5 and 6 follow from the definition of Lauffer (1964). Consult the appendix for the derivation of these equations (5–16).

proaches zero. Thus, in terms of our definition of hydration, the concept of hydration in infinitely dilute glycerol (or infinitely dilute sucrose, etc.) solutions has meaning. Equation 4 emphasizes, however, that the concept of hydration as defined by us depends upon the nature of all of the low molecular weight constituents, since $\partial \ln f_3/\partial m_2$ and $\partial \ln f_1/\partial m_2$ should have different values, for example, in sucrose and in glycerol solutions. The relationship between h_m of eq 4 and G of eq 2 and 3 is, $G = 18h_m/M_2$.

From the slopes of the curves in Figure 1 the values of G summarized in Table IV were calculated. This table also includes the data for V_2 at 20°, interpolated from Figure 8a of Jaenicke and Lauffer (1969). In the case of TMV the deviation from the line in Figure 1 of the data at zero glycerol concentration in buffer, which is outside the expected range of error attributable to protein concentration and extension of the spring, was neglected since all values at zero glycerol concentration in pure water coincide on the line and since our theory predicts a straight line at low glycerol concentration. Since the solutions in pure water were not deionized, TMV at the given pH values consists of weakly charged macroions with their counterions. There is no reason to postulate a strong influence of protein–protein interaction on the hydration of the macromolecular component as long as gel formation can be excluded. Therefore, it seems to be justified to include the data at zero ionic strength. However, TMVP, which is in the polymerized form under the given conditions of solvent and protein concentration (Jaenicke and Lauffer, 1969), forms a gel at zero ionic strength. As in the case with isoelectric coagulation of proteins (Jaenicke, 1966) or other gelation reactions (*cf.* Heyman, 1935; Cassel and Christensen, 1967) the gel formation is accompanied by a volume increase of about 0.001 cc/g. Nevertheless, $-\partial W/\partial \rho$, from which G is calculated, is nearly the same in the presence of and in the absence of buffer ions (Table II).

This method for determining hydration has the advantage over many others in that it is, when carried out under optimal conditions, a purely equilibrium or thermodynamic method. Therefore, it is entirely appropriate for a thermodynamic definition of hydration. The method does yield values of hydration which are of the same order of magnitude as those obtained by other methods on the same proteins. As can be seen from eq 3, the slope, $-\partial W/\partial \rho$, is the sum of two terms, much the smaller of which involves hydration. Therefore, very precise experimental measurements of $\partial W/\partial \rho$ will yield considerably less precise values of hydration.

In the case of TMV, hydration can also be calculated from the results of Schachman and Lauffer (1949) on the sedimentation of TMV in sucrose solutions. While this is not an equilibrium method, the considerations involved are in many ways comparable to those herein discussed. The value of 0.27 g of water/g of TMV is obtained from those measurements (Lauffer and Bendet, 1954) in comparison with 0.16 resulting from the present study. There is in reality no reason to expect the two numbers to be identical because component 3 in the Schachman–Lauffer experiment was sucrose and in the present experiment, glycerol. As discussed by Schachman and Lauffer, one of the factors involved in "hydration" of proteins in ternary systems is the size of the low molecular solute molecules. There must be a space surrounding each colloidal molecule of thickness equal to the radius (or "average" radius in the nonspherical case) of the low molecular weight solute particle into

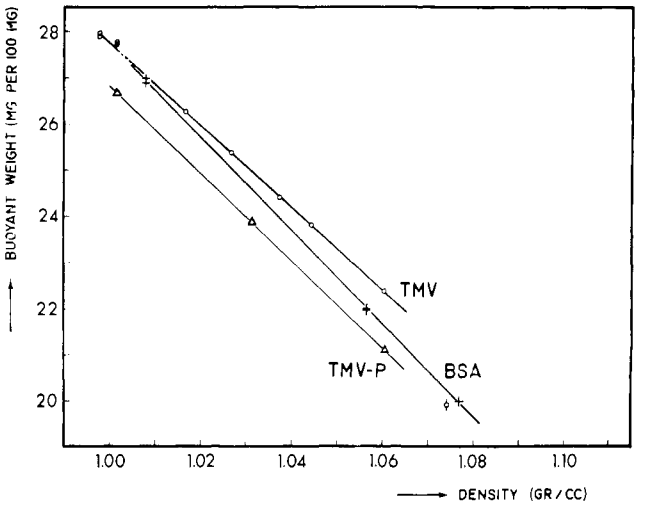

FIGURE 1: Buoyant weight, W (mg/100 mg), as a function of density from spring balance experiments in aqueous buffers and glycerol buffer mixtures, 20°. Data from Table II. (+—+) Bovine serum albumin in acetate buffer, pH 4.58, $\mu = 0.1$. (○—○) TMV in water and phosphate buffer, pH 6.7; $\mu = 0.05$. (△—△) TMVP in phosphate buffer, pH 6.7, $\mu = 0.05$.

which the centers of such solute molecules cannot penetrate. This "excluded volume" must contribute to the calculated hydration. This value should be larger when the solute is sucrose than when it is glycerol. Thus, one should expect higher values for "hydration" in sucrose solutions than in glycerol solutions, other things being equal. In terms of the definition of hydration herein employed, such considerations are involved in $\partial \ln f_3/\partial m_2 - \partial \ln f_1/\partial m_2$. Other factors which must be involved are the difference between the forces of attraction or repulsion between protein and the low molecular constituents of the solution.

Appendix

In all of the equations below, symbols without superscripts refer to the resulting protein solution considered in terms of total amounts of solvent, solute, and colloid; symbols with single prime superscripts refer to the protein solutions considered on the basis of "hydrated" protein and "free" solvent and solute. Symbols with double prime superscripts refer to a reference solution of 1 and 3 without protein. N_i means mole fraction and n_i means number of moles of the ith constituent. From eq 5 and 6, eq 7 and 8 can be derived. The approximation in eq 8 is highly accurate when $\Delta n_1/n_1$ is less than 0.02.

$$f_1N_1 = f_1 \frac{n_1 + \Delta n_1}{n_1 + \Delta n_1 + n_2 + n_3} = f_1'N_1' = \frac{f_1'n_1}{n_1 + n_2 + n_3} \quad (5)$$

$$f_3N_3 = f_3 \frac{n_3}{n_1 + \Delta n_1 + n_2 + n_3} = f_3'N_3' = f_3' \frac{n_3}{n_1 + n_2 + n_3} \quad (6)$$

$$f_1/f_3 = (f_1'/f_3') \frac{n_1}{n_1 + \Delta n_1} = (f_1''/f_3'') \frac{n_1}{n_1 + \Delta n_1} \quad (7)$$

$$\ln f_1 - \ln f_3 = \ln f_1'' - \ln f_3'' - \ln\left(1 + \frac{\Delta n_1}{n_1}\right) = \ln f_1'' - \ln f_3'' - \frac{\Delta n_1}{n_1} \quad (8)$$

In the second step, Δn_1 moles of 1 are withdrawn. This results in the same situation as if only anhydrous protein had been added in the first place. Equations 9, 10, and 11 follow. The

$$\ln f_1 - \ln f_3 = \left[\ln f_1'' - \left(\frac{\partial \ln f_1}{\partial n_1}\right)\Delta n_1\right] - \left[\ln f_3'' - \left(\frac{\partial \ln f_3}{\partial n_1}\right)\Delta n_1\right] - \Delta n_1/n_1 \quad (9)$$

$$(\ln f_1 - \ln f_1'') - (\ln f_3 - \ln f_3'') = (\partial \ln f_3/\partial n_1 - \partial \ln f_1/\partial n_1)\Delta n_1 - \Delta n_1/n_1 \quad (10)$$

$$n_2(\partial \ln f_1/\partial n_2 - \partial \ln f_3/\partial n_2) = -(\Delta n_1/n_1) \times [1 + n_1(\partial \ln f_1/\partial n_1 - \partial \ln f_3/\partial n_1)] \quad (11)$$

left member of eq 11 is accurate over the range of protein concentration in which $\partial \ln f_1/\partial n_2$ and $\partial \ln f_3/\partial n_2$ are constant. When one introduces the definition of h_m found in the text above, eq 12 is obtained. From eq 8 one can derive eq 13. Equation 14 is the Gibbs–Duham relationship, from which eq 15 follows. When eq 13 and 14 are substituted into 12, eq 16 results. This can be recast in the form of eq 4 by introducing the definition of molality.

$$h_m = \frac{n_1\partial \ln f_3/\partial n_2 - \partial \ln f_1/\partial n_2}{1 + n_1(\partial \ln f_1/\partial n_1 - \partial \ln f_3/\partial n_1)} \quad (12)$$

$$\partial \ln f_1/\partial n_1 - \partial \ln f_3/\partial n_1 = \partial \ln f_1''/\partial n_1 - \partial \ln f_3''/\partial n_1 + h_m n_2/n_1^2 \quad (13)$$

$$n_1\partial \ln f_1'' + n_3\partial \ln f_3'' = 0 \quad (14)$$

$$n_1\partial \ln f_1''/\partial n_1 = -n_3\partial \ln f_3''/\partial n_1 \quad (15)$$

$$h_m = \frac{n_1(\partial \ln f_3/\partial n_2 - \partial \ln f_1/\partial n_2)}{1 - (n_1 + n_3)\partial \ln f_3''/\partial n_1 + (n_2/n_1)h_m} \quad (16)$$

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