[CONTRIBUTION FROM THE VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Free-volume Theory of Three Component Systems with Special Reference to Sedimentation in the Ultracentrifuge[1]

BY SIDNEY KATZ

RECEIVED MAY 19, 1955

An interaction coefficient of relevance to the thermodynamics of three-component systems has been calculated by the use of free-volume theory. The system examined consists of a macromolecular component in a solution of two smaller molecules. The results are of particular significance in connection with the attempts made to measure the hydration of biological macromolecules by their sedimentation in mixed solvents. An equation for the density of zero sedimentation rate is developed which predicts values of this density lower than the reciprocal of the partial specific volume of the macromolecular component, even though only the relative sizes of the molecules are taken into account. The relation of the results to other physical methods currently used in the study of macromolecules is also briefly discussed.

The sedimentation of macromolecules of biological origin in binary solvents, chiefly sucrose–water solutions, is frequently used as a measure of the hydration of the macromolecule.[2] The experiments consist of measuring the sedimentation constant of the macromolecule, *e.g.*, tobacco mosaic virus,[3] in a series of sucrose solutions of increasing density. A linear relationship is usually obtained when ηs, the product of the sedimentation coefficient and the viscosity of the solution, is plotted *vs.* ρ, the density of the solution, enabling extrapolation to a density ρ^0 corresponding to $s = 0$. The density ρ^0 is frequently assumed to be the "density" of the hydrated macromolecule, allowing calculation of the amount of water of solvation by comparison with the dry "density" of the macromolecule, *i.e.*, the reciprocal of the partial specific volume of the dry material. It has recently been proved,[4] however, that one can only measure what might be called the "preferential adsorption" of water by such experiments. Thus, suppose one sediments a virus in a mixture of sucrose and water and a mole of virus binds $(kn_1 + w)$ moles of water and kn_3 moles of sucrose, where n_1 is the number of moles of water in the centrifuge cell, n_3 is the number of moles of sucrose and k is a proportionality constant. For w and $k > 0$ this represents a situation in which the macromolecule "binds" some water and sucrose in the ratio making up the bulk solution (*i.e.*, kn_1 and kn_3) and some additional water, w, in excess of this ratio. The value of ρ^0, however, is a measure only of w, no matter how large k is, hence, only if the value of k were known would it be meaningful to speak of the total hydration of the macromolecule.

Ultimately, hydration is a *defined* quantity[2] dependent—whether specifically stated or only implied—on the experimental method used to define it. This is the source of most of the ambiguities encountered in discussions of hydration. The nature of the ambiguity is easily illustrated. In principle, one can say that *every* molecule of water in a beaker of water containing a single virus molecule is "bound" to the virus since it is known that dipole–dipole forces of attraction exist between the virus and water. These forces, to be sure, are significant only at short ranges and for this reason any such definition of hydration could well be considered misleading. On the other hand, it can be equally misleading to speak of water in the immediate neighborhood of a virus particle as hydration unless the neighborhood is well defined.

The dilemma can be avoided by discarding the question "How can one measure the hydration of large molecules and asking, instead, "What does the experiment measure?" In the case of equilibrium or quasi-equilibrium studies of macromolecules in binary solvents recent developments in the theories of sedimentation,[5,6] light scattering,[7,8] osmotic pressure[9] and equilibrium dialysis permit the second question to be answered readily. In particular, the sedimentation velocity theory developed by Wales[5] allows analysis of three-component systems in general thermodynamic terms without explicit use of the concept of hydration. In this paper, Kauzmann's suggestion[10] that the observed effects may arise from the relative sizes of the molecules will be explored with the help of free volume theory.[11]

Theory

The behavior of a sedimenting macromolecule at infinite dilution in a binary solvent is described by[5]

$$f'\eta s = M_2(1 - \bar{V}_2\rho) + \alpha M_3(1 - \bar{V}_3\rho) \quad (1)$$

where

$$\alpha \equiv (\partial m_3/\partial m_2)_{T,P,\mu_3} = -(\partial\mu_3/\partial m_2)_{T,P}/(\partial\mu_3/\partial m_3)_{T,P} \quad (2)$$

The subscript two refers to the unsolvated macromolecule, the subscript three to either of the other components (conveniently, to the one present in the least amount), and the $\bar{V}_i$, M_i and m_i are the corresponding partial specific volumes, molecular weights and molalities. The subscript one will refer to the solvent usually present in greatest amount, in our case, water. In partial derivatives differentiated with respect to the molality or the number of moles of one of the components it will be

(1) This work was supported by contract NR 121-175 between the Office of Naval Research and the University of California.

(2) For a recent comprehensive review, *cf.* the article "The Hydration of Viruses," by M. A. Lauffer and I. J. Bendet in "Advances in Virus Research," Vol. II, Academic Press, Inc., New York, N. Y., 1954, p. 241.

(3) H. K. Schachman and M. A. Lauffer, THIS JOURNAL, **71**, 536 (1949).

(4) S. Katz and H. K. Schachman, *Biochim. Biophys. Acta*, **18**, 28 (1955).

(5) M. Wales and J. W. Williams, *J. Polymer Sci.*, **8**, 449 (1952).

(6) R. J. Goldberg, *J. Phys. Chem.*, **57**, 194 (1953).

(7) J. G. Kirkwood and R. J. Goldberg, *J. Chem. Phys.*, **18**, 54 (1950).

(8) W. H. Stockmayer, *ibid.*, **18**, 54 (1950).

(9) G. Scatchard, THIS JOURNAL, **68**, 2315 (1946).

(10) Discussed in ref. 3.

(11) J. H. Hildebrand and R. L. Scott, "Solubility of Non-Electrolytes," Reinhold Publishing Corp., New York, N. Y., 1950, pp. 198, *et seq.*

understood that the corresponding quantities for the other components are to be held constant. T is temperature, P, pressure, μ_3 is the chemical potential of component three and $f\eta'$ is the friction constant of the macromolecular hydrodynamic unit. Alternatively, $f\eta'$ may be regarded as the friction constant of the macromolecule subject to whatever forces exist between it and the other components of the solution. Equations 1 and 2 make it obvious that any kind of interaction between the components which affects the chemical potential of component three, whether it influences the partial molar entropy or heat of mixing (or both) will affect the sedimentation of the macromolecule. Using a superscript zero to denote $s = 0$, eq. 1 gives

$$-\alpha^0 = M_2(1 - \bar{V}_2\rho^0)/M_3(1 - \bar{V}_3\rho^0) \quad (3)$$

from which it can be seen that if the M_i and $\bar{V}_i$ are available the problem of calculating ρ^0 is reduced (with an exception to be discussed) to the problem of finding a suitable form for the chemical potential of component three.

The experimental results of Schachman and Lauffer[3] on centrifugation of tobacco mosaic virus in sucrose–water solutions and serum albumin–water solutions showed that ρ^0 in the serum albumin water solutions was about 11% lower than the result in sucrose–water solutions. Kauzmann suggested that this difference arose from the relative sizes of sucrose, serum albumin and water and constructed what amounted to a rough distribution function of the two non-virus components in the vicinity of the virus indicating that the "average density" close to the surface of the virus could be less than the macroscopic density of the solution. Kauzmann's suggestion can be implemented by the use of the free volume theory of multicomponent systems.[11] For the entropy of mixing of n_i moles each of three components of free volume V_i^f one can write

$$\Delta S_M = -R\Sigma n_i \ln \phi_i \quad (4)$$

where

$$\phi_i = n_i V_i^f / \Sigma n_i V_i^f \quad (5)$$

and the sums extend over all components.

By ignoring any relative size differences among the molecules and taking all the free volumes to be equal one obtains the entropy of mixing of an ideal solution. More generally, although the effects of relative sizes tend to be exaggerated,[12] one may take V_i^f proportional to V_i, the molar volume. Differentiation of eq. 4 with respect to n_3 then gives the partial molal entropy of component three and, therefore, the chemical potential of component three, if the partial molal heat contents are known, since

$$\Delta\mu_3 = \Delta\bar{H}_3 - T\Delta\bar{S}_3 \quad (6)$$

The result is

$$\Delta\mu_3 = \Delta\bar{H}_3 + RT[\ln \varphi_3 + 1 - (\varphi_3/x_3)] \quad (7)$$

where $\Delta\mu_3 = \mu_3 - \mu_3^0$; μ_3 is the chemical potential of component three in the solution and μ_3^0 the chemical potential of the pure component; φ_3 is its volume fraction (defined by $n_3V_3/\Sigma n_iV_i$) and x_3 its mole fraction in solution and $\Delta H_3 = [\partial(\Delta H_M)/\partial n_3]_{T,P}$ where ΔH_M is the heat of mixing of the three components. To assess the effects of size alone on α we can impose the condition $\Delta H_M = 0$. Less restrictive conditions giving the same results are

$$(\partial\Delta H_3/\partial m_2)_{T,P} \ll T(\partial\Delta S_3/\partial m_2)_{T,P}$$

and

$$(\partial\Delta H_3/\partial m_3)_{T,P} \ll T(\partial\Delta S_3/\partial m_3)_{T,P}$$

Equation 7 then gives, at infinite dilution of the macromolecule

$$\alpha = \frac{n_1\varphi_3V_2(V_1 - V_3) + n_3V_3^2}{n_1V_3\varphi_1(V_1 - V_3) + n_1V_3^2} \quad (8)$$

from which the dependence of α and s (eq. 1) on the relative sizes of the molecules and the dependence of α on the concentration of component three are apparent. In order to solve for ρ^0 one can use equation 2 and the relations

$$n_1/n_3 = -M_3(1 - \bar{V}_3\rho^0)/M_1(1 - \bar{V}_1\rho^0) \text{ and}$$
$$\varphi_3 = \bar{V}_3(1 - \bar{V}_1\rho^0)/(\bar{V}_3 - \bar{V}_1) \quad (9)$$

valid at infinite dilution of the macromolecule. These give, ignoring the difference between specific and partial specific volumes

$$\rho^0 = \frac{M_1M_2\bar{V}_1(\bar{V}_1 - \bar{V}_2) + M_2M_3\bar{V}_3(\bar{V}_2 - \bar{V}_3) + M_3M_1\bar{V}_3(\bar{V}_3 - \bar{V}_1)}{\bar{V}_1\bar{V}_3[\bar{V}_3(M_1 - M_2) + \bar{V}_2(M_3 - M_1) + \bar{V}_1(M_2 - M_3)]} \quad (10)$$

For the systems with which we are concerned, M_3, $M_1 \ll M_2$ while the $\bar{V}_i$ are all of the same order of magnitude. Hence, to excellent approximation

$$\rho^0 = \frac{(M_3/\bar{V}_1)(\bar{V}_2 - \bar{V}_3) + (M_1/\bar{V}_3)(\bar{V}_1 - \bar{V}_2)}{M_3(\bar{V}_2 - \bar{V}_3) + M_1(\bar{V}_1 - \bar{V}_2)} \quad (11)$$

a result dependent on the "density," $1/\bar{V}_2$, of the macromolecule but not on its molecular weight. Had the chemical potentials of ideal solutions been used the result would have been

$$\rho^0 = (M_2 - M_1)/(V_2 - V_1) \sim M_2/V_2 = 1/\bar{V}_2 \quad (12)$$

which can also be obtained from equation 10 by assuming $V_1 = V_3$.

Application of equations 11 and 12 to the data recently compiled by Lauffer and Bendet[2] gives the results shown in Table I. Sucrose was used as the third component in all cases; and the value $\bar{V}_3 = 0.645$ used in the calculations. With the exception of T-2 bacteriophage where agreement between ρ^0, (eq. 11) and experiment is satisfactory, the table shows that the experimental results lie between ρ^0 calculated from ideal solution laws and ρ^0 calculated from free volume theory. Although the relative size effects tend to be exaggerated it is clear that the simple free volume theory in which free volumes are considered proportional to molar vol-

TABLE I

COMPARISON OF THE PREDICTIONS OF FREE VOLUME THEORY AND EXPERIMENT[a]

Macromolecule	$\bar{V}_3$	ρ^0 eq. 12	ρ^0 expt.	ρ^0 eq. 11
Tobacco mosaic virus	0.73	1.37	1.27	1.08
Southern bean mosaic virus	.69	1.45	1.23, 1.26	1.12
Influenza A virus	.82, 0.75	1.22, 1.33	1.18	1.02, 1.06
Vaccinia virus	.75	1.33	1.17	1.06
T-2 bacteriophage	.66	1.52	1.27	1.30

[a] The data are taken from the article previously cited, ref. 2. Double entries appear when two values were given in this article.

(12) J. H. Hildebrand and R. L. Scott, ref. 11, chap. 6.

umes is successful in predicting values of ρ^0 which are less than $1/\bar{V}_2$. In the case of T-2 phage, agreement between theory and experiment is good and the fact that $\rho^0 \sim 1/\bar{V}_2$ is anticipated by equation 11 for, when $\bar{V}_2 \sim \bar{V}_3$, $\rho^0 = 1/\bar{V}_3 = 1/\bar{V}_2$.

The simple theory given also supports Svedberg's[13] and Sharp's[14] method of determining the partial specific volume of viruses and proteins by measurement of sedimentation constants in the presence of increasing quantities of D_2O in H_2O. Sharp's tacit assumption of $\alpha \sim 0$ is supported by eq. 1 and 8 since, when $V_1 = V_3$, α reduces to n_3/n_1 making the second term on the right-hand side of eq. 1 negligible compared to the first.

Discussion

Experiments of the kind discussed are commonly interpreted as arising from hydration of the macromolecule. A recent example,[15] in the case of T-2 bacteriophage, is the statement that if sucrose does not change the bacteriophage in any way, the water of hydration (calculated from ρ^0 and the partial specific volume of dry phage) is also the value of the water of hydration of the phage in water. Since free volume theory predicts values of $\rho^0 \leqslant 1/\bar{V}_2$ on the basis of entropy effects arising solely from the relative sizes of the molecules, this statement cannot be considered valid.

Similar effects in three-component systems of the type described can be anticipated in light-scattering and in equilibrium dialysis. Thus, since sucrose has a positive refractive index increment and $\alpha < 0$ at accessible values of sucrose concentration, the intercept of a plot of Kc_2/R_{90} *vs.* c_2 may be expected, according to the theory of Kirkwood and Goldberg[7] and Stockmayer,[8] to be higher in concentrated sucrose solutions than in water or dilute buffer. In equilibrium dialysis the molality of the third component inside the dialysis bag (when the charge on the second component is negligible) is given[16] by $m_3 = m_3' + \bar{\nu}m_2$ where m_3 is its molality outside the dialysis bag, m_2 is the molality of the macromolecular component and $\bar{\nu}$ is often interpreted as the number of moles of component three bound by one mole of component two. The coefficient $\bar{\nu}$ is equal to $(\partial m_3/\partial m_2)_{T,P,\mu_3}$ as can be seen by differentiating the last expression and taking the limit as m_2 approaches zero. (The term $(\partial m_3'/\partial m_2)_{T,P,\mu_3} = 0$ since at equilibrium $\mu_3 = \mu_3'$ and maintaining μ_3' constant requires m_3' constant.) Thus, in an equilibrium dialysis study of, for example, tobacco mosaic virus, sucrose and water one should find the concentration of sucrose greater *outside* the dialysis bag. Such concentration differences could probably be measured by differential refractometry and have a decided advantage over the ultracentrifugal method in that α can be measured as a function of sucrose concentration. This information can, in turn, be used to obtain the dependence of the friction constant on the concentration of sucrose by means of equation 1. If this were known it would be possible to estimate ρ^0 for those systems (*e.g.*, sodium deoxyribonucleate–water–sucrose[4]) in which $\bar{V}_2 < \bar{V}_3$. For systems of this kind, *i.e.*, for systems in which it is impossible to achieve $s = 0$ even when the volume fraction of component three approaches unity, knowledge of the dependence of $f\eta'$ on the concentration of component three is essential in estimating ρ^0.

The theory presented has serious limitations even within its framework of negligible heat effects, since it is well known that the shape of the macromolecule affects the entropy of mixing. As an example of the limitation we can consider the probable effect on ρ^0 of distorting a large spherical molecule into a long thin rod. According to Kauzmann's simple picture, one can anticipate that ρ^0 for the thin rod would be considerably smaller than ρ^0 for the sphere, implying a dependence of ρ^0 on the amount of surface per unit volume of the macromolecule. Such a dependence is ignored by the formulation given here, but more detailed treatments of the heat and entropy of mixing for *coil-like* molecules in binary solvents are currently available.[17,18]

The author is indebted to Mr. Leonard Peller, Dr. Robert Hersh and Dr. Verne Schumaker for many helpful and pleasant discussions.

BERKELEY, CAL.

(13) T. Svedberg and I. B. Eriksson-Quensel, *Nature*, **137**, 400 (1936).

(14) D. G. Sharp, D. Beard and J. W. Beard, *J. Biol. Chem.*, **182**, 279 (1950).

(15) N. W. Taylor, H. T. Epstein and M. A. Lauffer, THIS JOURNAL, **77**, 1270 (1955).

(16) G. Scatchard, *Amer. Scientist*, **40**, 61 (1952).

(17) T. Kawai, *Bull. Chem. Soc. Japan*, **25**, 336 (1952).

(18) A. R. Shultz and P. J. Flory, *J. Polymer Sci.*, **15**, 231 (1955).