*PRESSURE AND HYDRATION EFFECTS ON CHEMICALLY REACTING SYSTEMS IN THE ULTRACENTRIFUGE**

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The theories of sedimentation equilibrium of reacting systems in the ultracentrifuge that have been developed to date do not take account of the pressure dependence of the equilibrium constants for the reactions. For the typical equilibrium experiment, carried out at low angular velocity or with a short fluid column in the ultracentrifuge cell, this effect is small. In this paper it is shown that in density gradient equilibrium experiments, which are carried out at high angular velocities, the pressure gradient and changes in the hydration in the reacting system can, through their influence on the equilibrium constant, significantly affect the distribution of macromolecules throughout the cell. Josephs and Harrington[1] have already pointed out the important effects that hydrostatic pressure developed in centrifuge cells can have on the behavior of reacting systems in sedimentation velocity experiments.

Basic Equilibrium Relations.—For a centrifuge cell of fixed volume the requirement for equilibrium is that the sum of the Helmholtz free energy and the centrifugal potential energy be minimized for the entire cell.[2] Let the cell be divided into equal subcells 1, 2, ..., k, ..., N of constant volume, the number of moles of species i in subcell k being n_i^k. Then the equilibrium condition with respect to changes in the n_i^k is given by

$$\sum_{\text{cells } k} \sum_{\text{species } i} (\mu_i^k + M_i\phi_k)dn_i^k = 0 \tag{1}$$

where μ_i^k is the chemical potential of species i as it exists in subcell k, M_i is the molecular weight of species i, and ϕ_k is the centrifugal potential in subcell k ($\phi_k = -\omega^2 r_k^2/2$ where ω is the angular velocity of the centrifuge rotor and r_k is the distance of the kth subcell from the axis of rotation). Applying the equilibrium condition to the transport of dn_i moles of species i from any subcell to any other subcell leads to the requirement that the total potential of i, $\mu_i + M_i\phi$, is constant throughout the centrifuge cell. That is,

$$\frac{d}{dr}(\mu_i + M_i\phi) = 0. \tag{2}$$

Suppose that there is a chemical reaction

$$\Sigma \nu_i X_i = 0 \tag{3}$$

involving two or more species, where the stoichiometric coefficients ν_i are defined as positive if species X_i is a product and negative if X_i is a reactant. Then, if a small amount of reaction is allowed to take place in any particular subcell k (i.e., at constant r), we must have from equation (1)

$$\sum_{\text{species } i} (\mu_i + M_i\phi)dn_i = 0. \tag{4}$$

But

$$dn_i = (\nu_i/\nu_1)dn_1 \tag{5}$$

so that

$$\sum_{\text{species } i} (\nu_i\mu_i + \nu_i M_i\phi)dn_1 = 0. \tag{6}$$

The term $\Sigma\nu_i M_i$ represents the mass change due to the reaction, which vanishes. Therefore, the equilibrium condition for chemical change at any position in the centrifuge cell is

$$\Sigma\nu_i\mu_i = 0, \tag{7}$$

which is the usual requirement for chemical equilibrium.

Goldberg[3] has shown that equation (2) leads directly to

$$M_i(1 - \bar{v}_i\rho)\omega^2 r = \Sigma(\partial\mu_i/\partial m_j)_{P,T,m_{k\neq j}} \; dm_j/dr, \tag{8}$$

in which $\bar{v}_i$ is the partial specific volume of species i, ρ is the solution density at radius r, and m_j is the concentration of species j. Equation (8) is exact if the m_j are expressed on any mass ratio scale (e.g., molality, weight fraction, or mole fraction), or if the system is incompressible. No approximations are made in deriving equation (8) from equation (2). As Adams and Fujita[4] have pointed out, equation (8) must hold for each species even in the presence of a chemical reaction.

From equation (7) we can write

$$K(r) = \prod_{j=1}^{n} a_j^{\nu_i} = K_0 \exp\left(-\int_{P_0}^{P_r} \Delta V_0 dP/RT\right), \tag{9}$$

where P_r is the pressure at radius r, P_0 is the standard state pressure, a_j is the activity of species j at pressure P_r, and $\Delta V_0 = \Sigma\nu_i(\partial\mu^0{}_i/\partial P)$ is the standard volume change for the reaction. If we rewrite (9) in a more useful approximate form by replacing activities at the standard pressure by concentrations on a mass ratio scale, we obtain

$$K'(r) = \prod_{j=1}^{n} m_j^{\nu_i} = K_0 \exp\left(-\int_{P_0}^{P_r} \Delta\bar{V} dP/RT\right), \tag{10}$$

where $\Delta\bar{V} = \Sigma\nu_i(\partial\mu_i/\partial P) = \Sigma\nu_i M_i\bar{v}_i$, which is the volume change which would be measured in a dilatometer under the conditions at radius r in the cell. Different volume changes are necessary in equations (9) and (10) because activities may vary with pressure, but concentrations on a mass ratio scale are independent of pressure. To the extent that activities at the standard pressure P_0 can be replaced by concentrations, equation (10) is exact.

Application to Buoyant Density Gradients.—Hearst and Vinograd[5] have considered the application of equation (8) to macromolecules in a buoyant density gradient—that is, a system in which a dense low-molecular-weight solute has been added so that the factor $1 - \bar{v}\rho$ vanishes for the macromolecular species at some point within the cell. By introducing a solvation parameter

$$\Gamma_i \equiv -\left(\frac{\partial\mu_s}{\partial m_i}\right)_{m_s}\left(\frac{\partial\mu_s}{\partial m_s}\right)_{m_i} = \left(\frac{\partial m_s}{\partial m_i}\right)_{\mu_s},$$

where component s is the solvent, they were able to eliminate the solvent and gradient-forming solute from the system of equations and derive a set of equations functionally identical with the ordinary case (8) but in which the species i refer to solvated macromolecules. Thus

$$M_{i,s}(1 - \bar{v}_{i,s}\rho)\omega^2 r = \Sigma(\partial\mu_i/\partial m_j)dm_j/dr, \tag{11}$$

$$M_{i,s} = M_i + \Gamma_i M_s, \tag{12}$$

$$\bar{v}_{i,s} = \frac{M_i\bar{v}_i + \Gamma_i M_s\bar{v}_s}{M_i + \Gamma_i M_s}. \tag{13}$$

Hearst and Vinograd were further able to show that each macromolecular species i will form a band about the radius in the centrifuge cell at which the term $1 - \bar{v}_{i,s}\rho$ vanishes, and that if the bands are sufficiently narrow, they will be very close to Gaussian in shape. Kegeles[6] has pointed out, however, that even in a perfectly linear density gradient wide bands will be somewhat skewed.

The application of equations (10) and (11) to a system of chemically reacting macromolecules in a buoyant density gradient has some interesting consequences. Equation (11) is a differential equation describing the shape of the band for each species present and, as has been pointed out above, is unaffected by the presence of the reaction. Equation (10), which describes how the concentrations of the various species are related at a given radius, determines the relationship between the integration constants for the set of equations (11). Thus the reaction affects only the total amounts of each species present in the centrifuge cell.

Equation (10) is not directly suitable for reactions involving solvated species since it requires that the solvent be written explicitly into the reaction. This is contrary to customary practice, which is to include the solvent activity in the equilibrium "constant." The solvent can be included in either the chemical potential term or the potential energy term of equation (6). To include the solvation change in the centrifugal potential energy we may define $\Delta\Gamma = \Sigma\nu_i\Gamma_i$ as the solvation change for the reaction. If the reactants and products are considered only as hydrated macromolecules, the solvation change can be regarded as a change in mass between reactants and products, and we have (since $\Delta\Gamma = -\nu_s$)

$$\sum_{i\neq s}\nu_i\mu_i = \phi_r\Delta\Gamma M_s. \tag{14}$$

Rederiving (10) with (14) instead of (7) gives

$$K'(r) = \prod_{j\neq s} m_j^{\nu_j} = K_0' \exp\left[\left(-\int_{P_0}^{P_r}\Delta\bar{V}dP + {}^1/_2\Delta\Gamma M_s\omega^2 r^2\right)/RT\right], \tag{15}$$

in which K_0' is the customary equilibrium "constant" (i.e., including the solvent activity) at the standard pressure P_0 and zero centrifugal potential.

The alternative derivation is to include the solvent activity as a function of radius in equation (6). For this derivation the reaction is written

$$\sum_{i\neq s}\nu_i X_i - \Delta\Gamma X_s = 0, \tag{16}$$

which causes the equilibrium condition to become

$$\sum_{i\neq s}\nu_i\mu_i - \Delta\Gamma\mu_s = 0. \tag{17}$$

The term $\Delta\Gamma\mu_s$ can be evaluated in terms of the standard pressure and zero centrifugal potential by using equation (2).

$$\mu_s(r) = \mu_{s,0} - M_s \int_0^r d\phi$$

$$= \mu_{s,0} + {}^1/_2\omega^2 M_s r^2. \quad (18)$$

From (17) and (18) we get

$$K'(r) = \prod_{j\neq s} m_j^{\nu_j} = K_0 \exp\left[\left(\Delta\Gamma\mu_{s,0} - \int_{P_0}^{P_r} \Delta\bar{V}dP + {}^1/_2\Delta\Gamma M_s\omega^2 r^2\right)/RT\right], \quad (19)$$

which is identical with (15) with K_0' given by $K_0 \exp(\Delta\Gamma\mu_{s,0}/RT)$.

Discussion.—Some of the consequences of these equations are illustrated in Figures 1–4, which were computed for a simple dimerizing macromolecular system in a

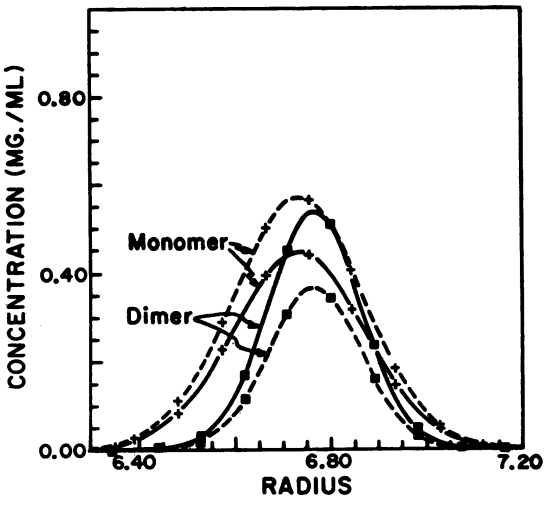

FIG. 1.—Concentrations of monomer and dimer in the density gradient described in the text. *Dashed curves*, concentrations before adding 3-mm layer of immiscible liquid to centrifuge cell. *Solid curves*, concentrations after 3-mm layer.

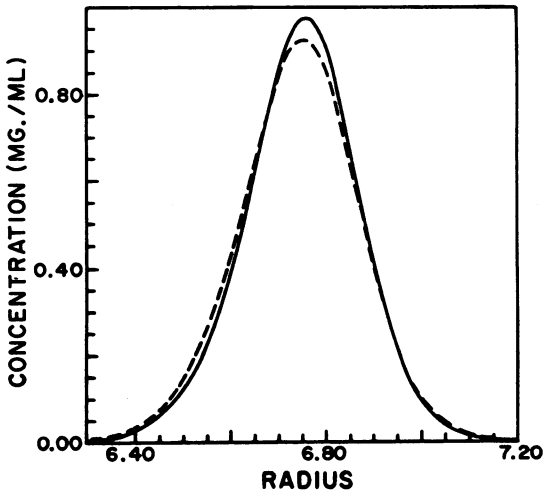

FIG. 2.—Total protein concentrations in the density gradient before (*dashed curve*) and after (*solid curve*) adding 3-mm layer.

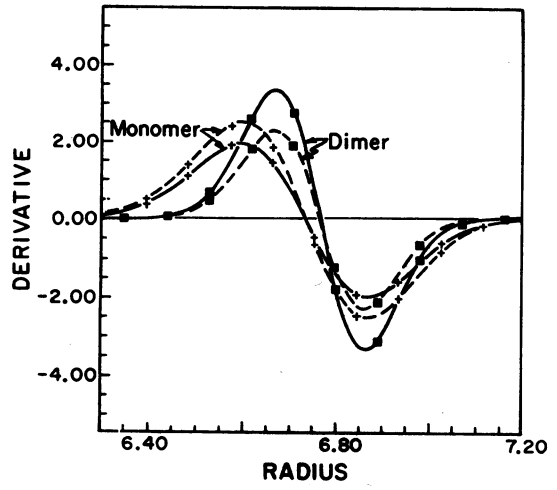

FIG. 3.—Concentration gradients of monomer and dimer from Fig. 1 before (*dashed curves*) and after (*solid curves*) adding 3-mm layer.

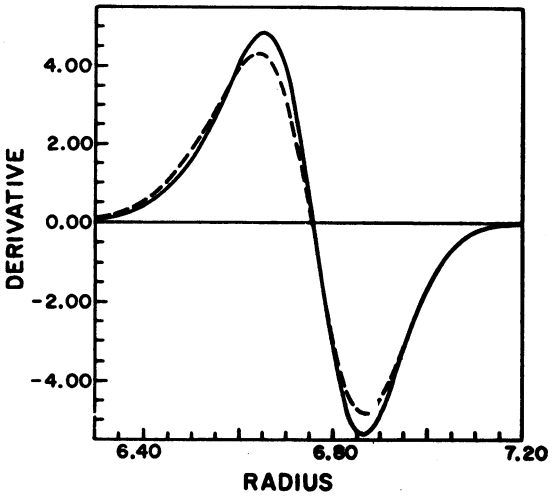

FIG. 4.—Concentration gradients of total protein before (*dashed curve*) and after (*solid curve*) adding 3-mm layer.

linear density gradient. The conditions chosen can be attained in an analytical ultracentrifuge. They correspond to an experiment in which a centrifuge cell is filled to a level 3 ml from the top with a solution which at equilibrium forms a density gradient from 1.237 gm/cc at the top of the solution (r = 6.3 cm) to 1.437 gm/cc at the bottom of the cell (r = 7.2 cm). The protein concentration is initially 0.05 mg/ml. The cell is centrifuged to equilibrium at 60,000 rpm. It is then filled to the top (r = 6.0 cm) with an immiscible liquid of lower density (ρ = 1.2) than the solution, and again centrifuged at 60,000 rpm to equilibrium. The monomer is assumed to have a molecular weight of 30,000 and a partial specific volume of 0.75 ml/gm. For simplicity it was assumed that $\Delta\Gamma = 0$. The association constant was taken as 0.25 $(\text{mg/ml})^{-1}$ at atmospheric pressure; this corresponds to a monomer:dimer ratio of 5.85:1 in the initial solution before centrifugation. The volume change on association was assumed to be −250 ml/mole. This volume change is 0.56 per cent of the molar volume of the product. There is evidence that

some protein reactions have molar volume changes at least this large, and denaturation reactions can have even larger volume changes.[7-9]

Under the postulated conditions of the initial run, the monomer:dimer ratio in the middle of the cell would be 1.43. This ratio is reduced to 0.83 on the addition of the 3-mm layer of liquid. The total concentration curve (Fig. 2) is, however, rather insensitive to this shift—and, of course, in most ultracentrifuge experiments of this type it would not be possible to observe reactants and products separately. The derivative curves (Figs. 3 and 4), which correspond to the photographs obtainable with the schlieren optical system, show the effect of the increased pressure somewhat more clearly. The observable effects are still not large, but they are definitely present and of measurable magnitude. It should be noted that the pressure differential in this case is less than would have occurred had the cell simply been filled to the top with solution; therefore, in reacting systems the band shape can be influenced by the length of the fluid column in the cell.

It is by no means clear how such data can be adequately analyzed for a reacting system. The resolution depends on the molar volume change for the reaction, but a large molar volume change tends greatly to favor the more dense species. In the example given, the peaks for monomer and dimer are separated by $1/3$ mm. To cause a 1-mm separation of peaks in the given density gradient, the volume change on association would have to be −750 ml. Such a volume change would have a drastic effect on the association constant, as can be seen in Table 1. With a volume change large enough to cause a 1-mm peak separation and an association constant of 0.25 at atmospheric pressure, only the dimer would be observed in a density gradient experiment. An association constant of 0.01 and a volume change of −750 ml (which would mean very little dimer at atmospheric pressure; a 1% solution would contain approximately 1% dimer) would produce comparable amounts of monomer and dimer in a density gradient experiment with a full cell.

For the case of association brought about by hydrophobic or ionic bonding, $\Delta\bar{V}$ might be of the order of plus 10–20 ml per bond, and $\Delta\Gamma$ would be expected to be positive. If $\Delta\Gamma$ were of the order of five molecules of water per bond, the hydration term in the exponential of equation (15) would be four to eight times larger than the volume change integral, and would have the opposite sign. Hydration effects might therefore produce even larger shifts in the equilibrium constant than those in the examples of Table 1.

TABLE 1

EFFECT OF PRESSURE IN CENTRIFUGE CELL ON DIMERIZATION OF A MACROMOLECULE

	P_r		K_r			
			$\Delta\bar{V} = -250$		$\Delta\bar{V} = -750$	
r	Before	After	Before	After	Before	After
6.3	0.0	86.3	0.25	0.60	0.25	3.46
6.4	30.9	117.2	0.34	0.83	0.62	9.14
6.5	62.8	149.1	0.48	1.15	1.76	24.32
6.6	95.8	182.1	0.67	1.61	4.80	66.77
6.7	129.9	216.2	0.94	2.28	13.28	189.6
6.8	165.0	251.3	1.35	3.26	39.34	554.3
6.9	201.3	287.6	1.96	4.72	120.4	1682.
7.0	238.7	325.0	2.87	6.92	378.1	5301.
7.1	277.3	363.6	4.25	10.26	1228.	17280.
7.2	317.0	403.3	6.38	15.40	4155.	58436.

"Before" and "after" refer to the layering of a liquid of density 1.2 on the sample; r is in centimeters, P_r is in atmospheres, K_r is in ml/mg, and $\Delta\bar{V}$ is in ml/mole.

The pressures listed in the table are only slightly higher than those encountered in sedimentation velocity experiments at the same angular velocity, the difference being due to the higher density used in the density gradient experiments. It is therefore obvious that, for reacting systems with similarly large volume changes, these effects can alter the sedimentation behavior, especially as the boundary moves past the mid-point of the cell. Such behavior has been observed by Josephs and Harrington[1] in sedimentation velocity studies of the formation of myosin polymers. There is no existing theory for the analysis of the sedimentation behavior of a chemically reacting system in which the equilibrium "constant" is variable throughout the cell, although there are theories for the case in which the equilibrium constant does not change.[10, 11]

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[1] Josephs, R., and W. F. Harrington, *Biochemistry*, **5**, 3474 (1966); Josephs, R., and W. F. Harrington, *Federation Proc.*, **26**, 728 (1967).

[2] Denbigh, K., *The Principles of Chemical Equilibrium* (London: Cambridge University Press, 1963), p. 85.

[3] Goldberg, R. J., *J. Phys. Chem.*, **57**, 194 (1953).

[4] Adams, E. T., Jr., and H. Fujita, in *Ultracentrifugal Analysis in Theory and Experiment*, ed. J. W. Williams (New York: Academic Press, 1963), p. 119.

[5] Hearst, J. E., and J. Vinograd, these PROCEEDINGS, **47**, 999 (1961).

[6] Kegeles, G., in *Physical Techniques in Biological Research*, ed. S. Moore (New York: Academic Press, in press).

[7] Kettmann, M. S., A. H. Nishikawa, R. Y. Morita, and R. R. Becker, *Biochem. Biophys. Res. Commun.*, **22**, 262 (1966).

[8] Simpson, R. B., and W. Kauzmann, *J. Am. Chem. Soc.*, **75**, 5139 (1953).

[9] Kauzmann, W., *Biochim. Biophys. Acta*, **28**, 87 (1958).

[10] Gilbert, G. A., *Proc. Roy. Soc.*, **A250**, 377 (1959).

[11] Gilbert, G. A., and R. C. Jenkins, *Proc. Roy. Soc.*, **A253**, 420 (1959).