EQUILIBRIUM SEDIMENTATION IN A DENSITY GRADIENT OF MATERIALS HAVING A CONTINUOUS DISTRIBUTION OF EFFECTIVE DENSITIES

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Recent work[1, 2] has shown that equilibrium sedimentation in a density gradient will separate solutes whose effective densities[3] differ very slightly and will provide information from which molecular weights can be computed. This is of particular interest in the study of deoxyribonucleic acid (DNA), as Meselson, Stahl and Vinograd[1] have shown, not only because one can study the replication of DNA[2] but also because there is disagreement between the molecular weights measured for DNA samples by other methods.[4] Since the method is known to be sensitive to small differences in effective density (θ), one would like to know the effects of a continuous distribution in θ. On the one hand it would be useful to measure such distributions, and on the other hand it is important to know how a distribution of θ affects the calculation of molecular weights.

For a single solute, the shape of the curve of concentration versus distance (the "band") is Gaussian,[1] to a first approximation, and the band width is inversely proportional to the square root of the molecular weight, M. Thus a linear plot of $\log c$ versus $(r - r_0)^2$ (where c is concentration, r is distance from the center of rotation, and r_0 marks the center of the band) has been used[1, 2] to test for homogeneity, and M has been found from the slope of this graph. It will be shown below that if the material has a Gaussian distribution of effective densities *the band will still be Gaussian in shape* and the width of the band will be markedly increased by narrow distributions of θ. Thus a Gaussian shape for the band is a necessary but not sufficient condition for homogeneity in θ, and the presence of a Gaussian distribution of θ will cause M to be seriously underestimated.

When the material being studied contains several fractions, we may express the total concentration C as the sum of the individual concentrations (which are given on a weight per volume scale)

$$C = \sum_i c_i \tag{1}$$

and denote the weight fraction of each by

$$w_i = c_i^\circ / C^\circ \tag{2}$$

where c_i° is the initial concentration of species i. Combination of equations (1) and (2) gives

$$C_r = C^\circ \sum_i w_i (c_i / c_i^\circ)_r \tag{3}$$

If the material has a continuous distribution, $g(\theta)$ of species each with an effective density θ the following equation takes the place of equation (3).

$$C_r = C^\circ \int_0^\infty g(\theta)\ (c/c^\circ)_{r,\theta}\, d\theta \tag{4}$$

It has been shown by Meselson *et al.*[1] that under certain conditions discussed in the following section, c is given by

$$\ln\ (c/c_{r_0}) = -(r - r_0)^2/2\sigma^2 + (0)\ (r - r_0)^3 \tag{5}$$

$$\sigma^2 = RT/M\bar{v}\omega^2 r_0 \left(\frac{d\rho}{dr}\right)_{r_0} \tag{5a}$$

$$\theta \equiv \rho_{r_0} = 1/\bar{v} \tag{5b}$$

where (0) $(r - r_0)^3$ means that (minor) terms of order higher than $(r - r_0)^2$ are omitted. Here R is the gas constant, T the absolute temperature, ρ the density of the solution, ω the angular velocity of the rotor, and $\bar{v}$ the partial specific volume (cc/gm) of the solute. In the next section it will be shown that equation (5) retains this form, but that a different significance must be given to σ^2 and θ, when the effects of preferential interaction are considered. The relation of c_{r_0} to the initial concentration c° depends on the type of cell used. In general

$$\int_a^b Ac\, dr = c^\circ \int_a^b A\, dr \tag{6}$$

where A is the cross-sectional area of the cell and the limits b, a mark the ends of the column of solution. For a rectangular cell, use of equation (6) gives

$$c_{r_0} = c^\circ(b - a)/\sigma\sqrt{2\pi} \tag{7a}$$

and for a sector-shaped cell the result is

$$c_{r_0} = c^\circ(b^2 - a^2)/2r_0\sigma\sqrt{2\pi}. \tag{7b}$$

When the distribution of effective densities is Gaussian

$$g(\theta) = \frac{1}{\gamma\sqrt{2\pi}}\, e^{-(\theta - \theta m)^2/2\gamma^2} \tag{8}$$

equation (4) can be integrated readily by completing the square, provided that σ is treated as a constant and ρ is expressed by[5]

$$\rho = \rho_m + \left(\frac{d\rho}{dr}\right)_{rm} (r - r_m) + (0)\ (r - r_m)^2 \tag{9}$$

In equation (8), θ_m is the mean of the distribution of θ and γ is the standard deviation in equation (9), r_m is the value of r at which $\rho = \theta_m$. For a rectangular cell the result of integration is

$$C = \frac{C^0(b - a)}{\varphi\sqrt{2\pi}}\, e^{-(r - rm)^2/2\varphi^2} \tag{10}$$

$$\varphi^2 = \sigma^2 + \left(\gamma/\frac{d\rho}{dr}\right)^2 \tag{10a}$$

Thus a material with a Gaussian $g(\theta)$ will form a Gaussian band. As equation (10a) shows, the width of the band will be markedly increased by small values of γ. For example, a value for γ of 0.003 gm cm^{-3} would double φ^2 for each of the

DNA bands shown in Figure 2 of the article by Meselson and Stahl[2] and cause M to be underestimated by a factor of 2. Since $\theta_m = 1.7$ gm cm^{-3} for this case, such a value for γ would represent a standard deviation equal only to 0.2 per cent of the mean. Narrow distributions of θ such as this could arise both from chemical and from configurational differences among the molecules. Heating DNA was found[2] to change θ_m by 5 times this amount ($\Delta\theta_m = 0.016$ gm cm^{-3}). Differences in base composition produce sufficient variation in θ that DNA samples from different organisms actually produce separate bands in a cesium chloride gradient.[6] It would not be surprising if differences in base composition among the DNA molecules of one organism were sufficient to cause broadening of this band.

There is a close analogy between the equations given above and the ones which describe boundary spreading in electrophoresis for a material with a distribution of mobilities. In the latter case, the concentration gradient curve is Gaussian for a single solute and also for a material with a Gaussian distribution of mobilities,[7] provided the diffusion coefficient is the same for all species and the field strength and mobilities can be assumed constant. One can measure $g(\theta)$ when the band is not Gaussian in shape by the equation of Brown and Cann.[8] Their equation, which gives the distribution of mobilities in terms of moments of the experimental curve, can be adapted to the sedimentation case simply by changing the notation. However, one must have an independent estimate of σ^2 (equations 5a, 17a). One would like to measure $g(\theta)$ without knowing σ^2 and, by analogy with the measurement of mobility distributions by an extrapolation to infinite time,[9, 10] one might hope to do this by varying ω. However this is not possible, as equations 5a and 10a show: the relative contributions to φ^2 of σ^2 and $\left(\gamma/\frac{d\rho}{dr}\right)^2$ do not vary with ω, since $d\rho/dr$ is proportional to ω^2.

Alternatively one might hope that, when the material is examined both in a rectangular cell and in a sector cell, the band shapes would be sufficiently different to detect the heterogeneity in effective density.[11] The result of integrating equation (4) for the case of a sector-shaped cell is

$$C = \frac{C^0(b^2 - a^2)}{2r_m\varphi\sqrt{2\pi}}\, e^{-(r-r_m)^2/2\varphi^2}\left\{1 + \frac{\epsilon\sigma^2}{r_m} - \epsilon(r - r_m) + \epsilon^2(r - r_m)^2 + \ldots\right\} \tag{11}$$

$$\epsilon = 1/r_m\left[1 + \left(\sigma\frac{d\rho}{dr}/\gamma\right)^2\right] \tag{11a}$$

The additional terms of equation (11) are only slightly larger than the ones omitted from equation (5), and both are outside present experimental error when light-absorption optics are used. Consequently, examining a material in these two types of cells offers little promise of detecting heterogeneity in θ.

A method of detecting heterogeneity in θ which does appear promising (Meselson *et al.*,[1] footnote 1) is the use of a partition cell to isolate material on either side of the band center, followed by rebanding of the isolated materials. The new bands should differ from that of the starting material both in shape and position. One might be able to detect differences in position by using schlieren optics to find the

positions of the bands, and by examining both materials simultaneously in a twin cell experiment.

At this point we will consider certain of the assumptions made in deriving equation (5), in order to see what factors might cause the band for a single solute with the properties of DNA to deviate from Gaussian shape. First, there is the question of the interaction of the solute with itself: equation (5) applies to the limiting case in which the concentration of solute approaches zero. Since one can study DNA at very low concentrations (*ca.* 0.001 gm/100 ml) with light absorption optics, the assumption seems reasonable. Secondly, there is the question of charge effects, caused by dissociation of the polyelectrolyte into ions. These have been considered by Meselson *et al.*[1] and by Yeandle,[12] and the conclusion has been reached that the shape of the band remains Gaussian. Thirdly, there is the question of preferential interaction of the solute with one of the components of the mixed solvent, a situation which is known to affect markedly the light scattering behavior of such a system.[13] An equation has already been given for the effect of this on the position of the center of the band.[14]

Consider a system of three nonionizing components: a mixed solvent whose components are labeled 0, 1, and a macromolecular solute labeled 2. In order to obtain a simple equation which applies rigorously to compressible systems, it is convenient to use a molal, or weight per weight, concentration scale. Equation (43) of Williams *et al.*[14] provides a convenient and rigorous starting equation.

$$\lim_{W_2 \to 0} \frac{1}{W_2} \frac{dW_2}{dr} = \frac{\omega^2 r M_2 (1 - \bar{v}_2 \rho)}{RT} \left\{ 1 + \frac{\Gamma'(1 - \bar{v}_1 \rho)}{(1 - \bar{v}_2 \rho)} \right\} \tag{12}$$

$$\Gamma' = -(m_1 \beta_{21} M_1 / M_2)/(1 + m_1 \beta_{11}) \tag{12a}$$

$$\beta_{ik} = \left(\frac{\partial \ln \gamma_i}{\partial m_k} \right)_{T, P, m} \tag{12b}$$

Here W is the number of grams and m is the number of moles per 1000 gm of component 0, and γ_i is the activity coefficient of component i on the molal scale. If we again define r_0 to be the position of the maximum concentration

$$\frac{dW_2}{dr} = 0, \qquad r = r_0 \tag{13a}$$

and define θ, as before by the value of ρ at which $r = r_0$, we have[14]

$$\theta \equiv \rho_{r_0} = \left[\frac{1 + \Gamma'}{\bar{v}_2 + \Gamma' \bar{v}_1} \right]_{r_0} \tag{13b}$$

When the variables on the right-hand side of equation (12) are expressed in series form as functions of $(r - r_0)$

$$r = r_0 \left[1 + \left(\frac{r - r_0}{r_0} \right) \right] \tag{14a}$$

$$X = X_{r_0} + \left(\frac{dX}{dr} \right)_{r_0} (r - r_0) + (0)\,(r - r_0)^2 \tag{14b}$$

$$\lim_{W_2 \to 0} \left(\frac{dX}{dr}\right)_{r_0} = \left[\left(\frac{\partial X}{\partial m_1}\right)_P \frac{dm_1}{dr} + \left(\frac{\partial X}{\partial P}\right)_{m_1} \frac{dP}{dr}\right]_{r_0} \tag{14c}$$

where X = either ρ, $\bar{v}_2$, $\bar{v}_1$, or Γ', and then these expressions are substituted into equation (12), the result is

$$\lim_{W_2 \to 0} \frac{1}{W_2} \frac{dW_2}{dr} = -\frac{(r - r_0)}{\sigma_W^2} + (0)\,(r - r_0)^2 \tag{15}$$

$$\sigma_W^2 = RT/(M_2\omega^2 r_0) \left\{\bar{v}_2 \frac{d\rho}{dr} + \rho \frac{d\bar{v}_2}{dr} - \frac{d\Gamma'}{dr}(1 - \bar{v}_1\rho) + \Gamma'\left(\bar{v}_1 \frac{d\rho}{dr} + \rho \frac{d\bar{v}_1}{dr}\right)\right\}_{r_0} \tag{15a}$$

Integration with respect to $(r - r_0)$ gives, as before, a Gaussian curve.

$$\lim_{W_2 \to 0} \ln\,[W_2/(W_2)_{r_0}] = -\frac{(r - r_0)^2}{2\sigma_W^2} + (0)\,(r - r_0)^3 \tag{16}$$

In order to obtain an equation on the c scale, for comparison with equation (5); we consider the case in which $d\bar{v}_1/dr$ and $d\bar{v}_2/dr$ are negligible and λ', the quantity corresponding to Γ', is a function only of the concentrations. Then, following the same procedure outlined in equations 12–16, one obtains the result

$$\lim_{c_2 \to 0} \ln\,[c_2/(c_2)_{r_0}] = -\frac{(r - r_0)^2}{2\sigma^2} + (0)\,(r - r_0)^3 \tag{17}$$

$$\sigma^2 = RT/(M_2\omega^2 r_0) \left[(\bar{v}_2 + \lambda'\bar{v}_1) \frac{d\rho}{dr} - (1 - \bar{v}_1\rho)\left(\frac{\partial\lambda'}{\partial c_1}\right)_P \frac{dc_1}{dr}\right]_{r_0} \tag{17a}$$

$$\lambda' = -\left(\frac{c_1 M_1}{M_2}\right)\left(\frac{\partial \ln \gamma_2}{\partial c_1}\right)_P \Big/ \left[1 + c_1\left(\frac{\partial \ln y_1}{\partial c_1}\right)_P\right] \tag{17b}$$

$$\theta \equiv \rho_{r_0} = \left[\frac{1 + \lambda'}{\bar{v}_2 + \lambda'\bar{v}_1}\right]_{r_0} \tag{17c}$$

If the partial volumes $\bar{v}_2$ and $\bar{v}_1$ are known, one can find λ' by measuring θ and using equation (17c). Then one can find M_2 from σ^2, by means of equation (17a), if $(\partial\lambda'/\partial c_1)$ is approximated by (λ'/c_1). This procedure will probably be accurate enough for measurements made with light absorption optics. More rigorous procedures could be devised by using equations (15) and (16).

In summary, if the macromolecular material examined by this method has a Gaussian distribution of effective densities it will form a Gaussian band and thus appear to be homogeneous. However, the band width will be increased and the apparent molecular weight will be less than the true molecular weight, often by a large amount. When the effects of preferential interaction of the solute with one component of the solvent are considered, one finds that the band for a single solute is still Gaussian but that its position and width are altered. One can take account of this, at least approximately, if the partial volumes are known.

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[1] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957).

[2] Meselson, M., and F. W. Stahl, these PROCEEDINGS, **44**, 671 (1958).

[3] The term "effective density"[1] refers to the density of the solution when the buoyant forces acting on the solute are zero; more precisely, the effective density is defined by equations (5b) and (17c).

[4] Butler, J. A. V., D. J. R. Laurence, A. B. Robins, and K. V. Shooter, *Proc. Roy. Soc.* (Lond.) **A, 250**, 1 (1959).

[5] Terms of order $(r - r_m)^2$ were omitted also in the derivation of equation (5).

[6] Marmur, J., talk presented at the April, 1959, meeting of the Federation of American Societies fo · Experimental Biology.[15]

[7] Alberty, R. A., *J. Am. Chem. Soc.*, **70**, 1675 (1948).

[8] Brown, R. A., and J. R. Cann, *J. Phys. Chem.*, **54**, 364 (1950).

[9] Baldwin, R. L., P. M. Laughton, and R. A. Alberty, *J. Phys. Chem.*, **55**, 111 (1951).

[10] Gosting, L. J., *J. Am. Chem. Soc.*, **74**, 1548 (1952).

[11] See footnote 1 of the article by Meselson *et al.*[1]

[12] Yeandle, S., these PROCEEDINGS, **45**, 184 (1959).

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

[14] Williams, J. W., K. E. Van Holde, R. L. Baldwin, and H. Fujita, *Chem. Rev.*, **58**, 715 (1958).

[15] (Added in proof.) This work is now in print: Sueoka, N., J. Marmur and P. Doty, *Nature* (Lond.) **183**, 1427 (1959). Rolfe and Meselson (private communication) independently have found that DNA samples from different organisms form separate bands in a density gradient, and that there is a relation between the mean effective density and the amount of guanine plus cytosine in the DNA. Work is in progress in Dr. Meselson's laboratory on measurement of the heterogeneity in effective density.

AGGREGATION OF DYES BOUND TO POLYANIONS

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Acridine orange (AO) is one of a number of dyestuffs which aggregate in aqueous solution. It is thought that these flat, aromatic dye molecules aggregate by stacking on top of one another, and are held together by London dispersion forces between their π-electron systems. The argument for aggregation in the case of AO rests upon a quantitative analysis by Zanker[1] of the variation in the dye spectrum with concentration and temperature. As the dye concentration is increased, the absorption band (at 492 mμ) of the monomer falls and is replaced by a new band (at 464 mμ) due to dimers. With further increases in concentration this band shifts further toward shorter wave lengths, corresponding to the formation of higher aggregates. Zanker showed that these changes could be quantitatively expressed in terms of an association equilibrium constant, corresponding to a free energy decrease in forming a dimer of 5.7 kcal/mole.

When AO is used to stain certain polyanionic tissue elements or is mixed with