*EQUILIBRIUM SEDIMENTATION OF MACROMOLECULES IN DENSITY GRADIENTS**

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I. QUALITATIVE DESCRIPTION

This communication presents a new method for the study of the molecular weight and partial specific volume of macromolecules, with some illustrations based on results with deoxyribonucleic acid (DNA) and several viruses. The method involves observation of the equilibrium distribution of macromolecular material in a density gradient itself at equilibrium. The density gradient is established by the sedimentation of a low-molecular-weight solute in a solution subject to a constant centrifugal field.

A solution of a low-molecular-weight solute is centrifuged until equilibrium is closely approached. The opposing tendencies of sedimentation and diffusion have then produced a stable concentration gradient of the low-molecular-weight solute (Fig. 1). The concentration gradient and compression of the liquid result in a continuously increasing density along the direction of centrifugal force. Consider the distribution of a small amount of a single macromolecular species in this density gradient. The initial concentration of the low-molecular-weight solute, the centrifugal field strength, and the length of the liquid column may be chosen so that the range of density at equilibrium encompasses the effective density of the macromolecular material. The centrifugal field tends to drive the macromolecules into the region where the sum of the forces acting on a given molecule is zero. (The effective density of the macromolecular material is here defined as the density of the solution in this region.) This concentrating tendency is opposed by Brownian motion, with the result that at equilibrium the macromolecules are distributed with respect to concentration in a band of width inversely related to their molecular weight.

CONCENTRATION DISTRIBUTIONS

1. *Gaussian Bands.*—It is shown in Section II that in a constant density gradient under certain attainable conditions, the concentration distribution at equilibrium of a single macromolecular species is Gaussian. The standard deviation of this Gaussian band is inversely proportional to the square root of the macromolecular weight. The band is centered about the cylindrical surface corresponding to the effective density of the macromolecular material. Figure 2 is a photometric record showing the equilibrium distribution of bacteriophage DNA in a density gradient of cesium chloride in water.

If the macromolecular material is composed of species with various molecular weights and effective densities, the observed equilibrium distribution is the sum of the separate Gaussian distributions with standard deviations and means corresponding to the molecular weights and effective densities, respectively, of the vari-

ous molecular species. When heterogeneities in molecular weight and effective density are both present, it is possible, but a priori unlikely, that the separate distributions would conspire to produce a single observed band of essentially Gaussian form.[1]

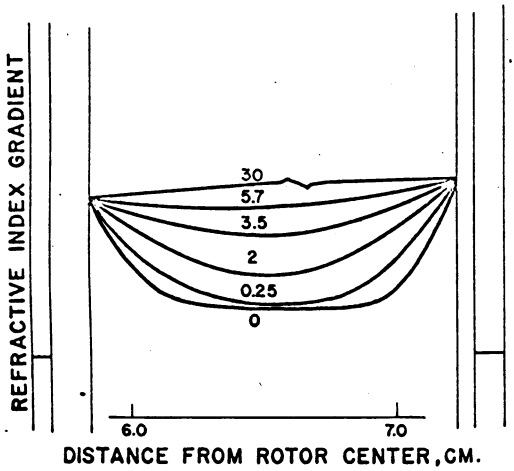

FIG. 1.—Superimposed schlieren diagrams of successive stages in the development of a cesium chloride equilibrium gradient. The numbers above each curve are the times in hours that the centrifuge has been running at 31,410 rpm. These diagrams are from an experiment in which 8 μg. of DNA was present in the cell; the banded DNA produces a diphasic pip in the final schlieren diagram.

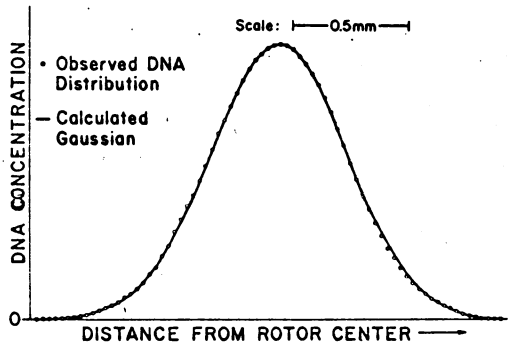

FIG. 2.—The equilibrium distribution of DNA from bacteriophage T4. An aliquot of osmotically shocked T4*r* containing 3 μg. of DNA was centrifuged at 27,690 rpm for 80 hours in 7.7 molal cesium chloride at pH 8.4. Evidence for the attainment of equilibrium was provided by the essential identity of the final band shapes, whether the DNA was initially distributed uniformly in the cell or in an extremely tight band. At equilibrium the observed DNA distribution does not depart appreciably from Gaussian form, indicating essentially uniform molecular weight and effective density. The mean of the distribution corresponds to an effective density of 1.70, and the standard deviation corresponds to a molecular weight for the Cs·DNA salt of 18×10^6. Assuming the base composition for T4 reported by G. R. Wyatt and S. S. Cohen (*Nature*, 170, 1072, 1952) and the glucose content reported by R. L. Sinsheimer (these PROCEEDINGS, 42, 502, 1956), this corresponds to a molecular weight of 14×10^6 for the sodium deoxyribonucleate. The density gradient is essentially constant over the band and is 0.046 gm/cm.^{4}. The concentration of DNA at the maximum is 20 μg/ml.

2. *Bi- or Polymodal Distribution of Banded Material.*—If the effective densities of the macromolecular species are sufficiently distinct, a distribution with more than one mode will be observed. In extreme cases this may lead to the formation of discrete bands. An example is the separation of normal DNA from DNA which contains, instead of thymine, the analogue 5-bromouracil. This unusual DNA is considerably more dense than normal DNA, and prepared mixtures of the two give rise to well-resolved bands in a cesium chloride gradient (Fig. 3). With DNA in cesium chloride, density differences of less than 0.001 gm/cm^3 may be detected.

3. *Skewed Unimodal Bands.*—A skewed band indicates the presence of material heterogeneous with respect to effective density. Such bands are shown in Figures 3 and 4 for bacteriophage DNA containing 5-bromouracil and calf thymus DNA, respectively. The skewness of the former band is the result of compositional

heterogeneity; i.e., some molecules contain more 5-bromouracil (in place of thymine) than others. Effective density heterogeneity may in general be compositional or structural in origin.

4. *Symmetric Unimodal Non-Gaussian Bands.*—For the Gaussian function $y = \exp(-x^2/2\sigma^2)$ a plot of ln y against x^2 yields a straight line of slope $-1/2\sigma^2$. A plot of the logarithm of the concentration in a band against the square of the distance from the maximum provides a convenient test for heterogeneity. Downward concavity anywhere in this plot signifies heterogeneity in effective density. Such a case may be rare in view of the a priori unlikelihood that the effective densities would be distributed in just such a way as to give rise to a symmetrical band.[1] The absence of downward concavity coupled with the observed symmetry of the concentration distribution is strong presumptive evidence for density homogeneity. Under this presumption, upward concavity is evidence for heterogeneity in molecular weight, and the slope at any point is inversely proportional to the weight mean molecular weight of the material at the corresponding position in the band (see Sec. II).

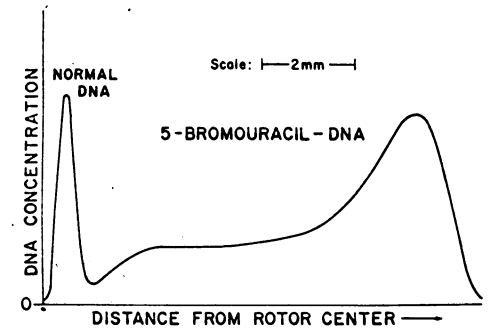

FIG. 3.—The concentration distribution of a mixture of normal and 5-bromouracil-containing DNA from bacteriophage T4. A mixture of osmotically shocked normal and 5-bromouracil-containing T4 (prepared by the method of R. M. Litman and A. B. Pardee, *Nature*, 178, 529, 1956) was centrifuged at 44,700 rpm in 8.9 molal cesium chloride at pH 8.4. The density gradient is 0.12 gm/cm.[4]. The position of the normal DNA indicates an effective density of 1.70; the maximum effective density of the substituted DNA is 1.80.

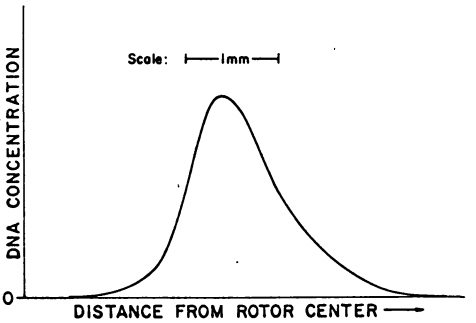

FIG. 4.—The concentration distribution of calf thymus DNA banded in cesium chloride. 2.7 μg. of calf thymus DNA (prepared by a detergent method by Dr. C. Jardetzky) was centrifuged at 44,700 rpm in 7.7 molal cesium chloride at pH 8.4. The skewness in the resultant band indicates heterogeneity in effective density.

MEASUREMENTS OF EFFECTIVE DENSITY

The mean effective density of macromolecular material (distributed in any manner within the density range of the solution) may be found from the mean of the mass distribution evaluated from the observed concentration distribution. If effective density is influenced by composition, the distribution provides a basis for the analysis of the composition of the material. This application is illustrated by the results with phage DNA containing 5-bromouracil (Fig. 3). The effective density of this DNA is found to be related to the degree of substitution of thymine by 5-bromouracil as determined chromatographically.

MOLECULAR-WEIGHT DETERMINATIONS

In the absence of density heterogeneity, both the number and weight mean molecular weights may be calculated from the observed shape of the band at equilibrium, as is shown in Section II. Molecular weights may be calculated from concentration distributions influenced by density heterogeneity; however, these should be considered minimal values. In the examples presented, only a few micrograms of the macromolecular material were present in the cell. The usefulness of the method for biological studies is further illustrated by the study of intact viruses in cesium chloride gradients. Both bacteriophage and tobacco mosaic virus[2] have been banded without loss of infectivity. Preliminary work with bovine albumin and human hemoglobin suggests the applicability of this method to smaller macromolecules. Details of the experimental procedures and results with several materials will be published elsewhere.

II. QUANTITATIVE RELATIONS

The total potential of any component at equilibrium in a closed system at constant temperature must be uniform throughout the system. In a centrifugal field this requirement results in the rigorous condition[3]

$$M_i(1 - \bar{v}_i\rho(r))\omega^2 r\, dr - \sum_K \frac{\partial \mu_i}{\partial C_k} dC_k = 0, \tag{1}$$

where M_i, $\bar{v}_i$, μ_i, C_i are molecular weight, partial specific volume, partial molal (Gibbs) free energy, and concentration of the ith component. The summation extends over a complete set of independently variable components. The angular velocity is given by ω, the radial co-ordinate is r, and the density of the solution at r is ρ. We shall consider a system containing water, a low-molecular-weight electrolyte XY, and a macromolecular electrolyte PX_n. The discussion will apply equally well to positive or negative polymeric ions and, with $n = 0$, to neutral polymer molecules. In the case of neutral polymers the low-molecular-weight solute may be a nonelectrolyte.

Three components are necessary and sufficient[4] to describe the composition of the system. They are chosen here as water, XY, and the neutral unsolvated molecule PX_n. Other choices are of course permissible, but this one is especially convenient. For definiteness in making certain approximations and for comparison with experiment, we shall refer to the system *water–cesium chloride–cesium deoxyribonucleate.* The total amount of polymer will be made so small as to have a negligible effect on the potentials of the salt and water. Therefore, we may first calculate the concentration distribution of XY from equation (1), ignoring the polymer, and then, again using equation (1), find the distribution of the polymer in the salt gradient. This gradient is

$$\frac{dC_{XY}}{dr} = \frac{\partial C_{XY}}{\partial a_{XY}} \cdot \frac{da_{XY}}{dr} = \frac{\partial C_{XY}}{\partial a_{XY}} \cdot \frac{a_{XY} M_{XY}(1 - \bar{v}_{XY}\rho(r))\omega^2 r}{RT}. \tag{2}$$

The sum of equation (1) has been replaced by its equivalent, $RT\ d\ ln\ a_{XY}$, where a_{XY} is the activity. Values of a_{XY}, ρ, and $\bar{v}_{XY}$ as functions of concentration and

pressure are experimentally determinable and may be found in the literature for some systems. The salt concentration as a function of r may be found by numerical integration of expression (2). Alternatively, it may be measured by optical methods in the centrifuge itself.

In many cases it is possible to select XY so that the density and concentration gradients are essentially constant over regions sufficiently long to encompass a polymer band. For example, it is found, both by calculation from equation (2) and by direct observation of the refractive index gradient in the centrifuge, that the cesium chloride concentration and density gradients are essentially constant over the region of a DNA band. For computational simplicity, we shall consider only these linear systems, so that upon choosing r_0 in the region of a band, we may write C_{XY} and ρ as

$$C_{XY}(r) = C_{XY}(r_0) + \left(\frac{dC_{XY}}{dr}\right)_{r_0} (r - r_0), \tag{3}$$

$$\rho(r) = \rho(r_0) + \left(\frac{d\rho}{dr}\right)_{r_0} (r - r_0). \tag{4}$$

Having found the distribution of XY, we may now employ equation (1) to determine the distribution of PX_n. Making use of equation (4), we write the first term of equation (1), which represents the work per mole done against the centrifugal field moving PX_n from r to $r + dr$, as

$$M_{PX_n} (1 - \bar{v}_{PX_n}\rho(r_0) - \bar{v}_{PX_n}\left(\frac{d\rho}{dr}\right)_{r_0} (r - r_0)) \omega^2 r\, dr. \tag{5}$$

It should be emphasized that $\bar{v}_{PX_n}$ is the partial specific volume of PX_n in a solution of XY at a concentration $C_{XY}(r)$. In order to evaluate the remaining term in equation (1), we consider it to be composed entirely of the osmotic work $RT\, d \ln C_{PX_n} + zRT\, d \ln \gamma_X C_X$, where z is the effective number of counter-ions which must be moved along with the charged polymer molecule PX_{n-z} in order to maintain electrical neutrality and γ_X is the activity coefficient of the ion X. We thereby neglect any other change in the free energy of the polymeric component as it is moved through the solution. This should be a valid approximation if the fractional change in the concentration of XY across a polymer band is small. It is especially plausible for the case of DNA in cesium chloride, for which the cesium chloride concentration changes by less than one part in one hundred over the region of a band. Also, over a small concentration range, the term $d \ln \gamma_X$ will be negligible in comparison to $d \ln C_X$. Incorporating these approximations in the limit of low polymer concentration, we have

$$M_{PX_n}\left(1 - \bar{v}_{PX_n}\rho(r_0) - \bar{v}_{PX_n}\left(\frac{d\rho}{dr}\right)_{r_0} (r - r_0)\right)\omega^2 r\, dr - RT\, d \ln C_{PX_n} - zRT\, d \ln \left(C_{XY}(r_0) + \left(\frac{dC_{XY}}{dr}\right)_{r_0} (r - r_0)\right) = 0. \tag{6}$$

Assuming $\bar{v}_{PX_n}$ and z to be independent of r over the region of a band, this may be integrated with respect to $(r - r_0)$ from $r = r_0$ to $r = r$, yielding

$$M_{PX_n}(1 - \bar{v}_{PX_n}\rho(r_0))\,\omega^2(r - r_0)\left(\frac{r - r_0}{2} + r_0\right) - M_{PX_n}\bar{v}_{PX_n}\left(\frac{d\rho}{dr}\right)_{r_0}$$
$$\omega^2(r - r_0)^2\left(\frac{r - r_0}{3} + \frac{r_0}{2}\right) - RT \ln \frac{C_{PX_n}(r)}{C_{PX_n}(r_0)} - zRT \ln$$
$$\left(1 + \left(\frac{dC_{XY}}{dr}\right)_{r_0} \cdot \frac{(r - r_0)}{C_{XY}(r_0)}\right) = 0. \quad (7)$$

In many cases, including that of DNA in cesium chloride solution, the band width may be made quite small compared with the distance of the band from the center of rotation, so that $|r - r_0| \ll r_0$. Further, because of the small magnitude of the gradient of $C_{XY}(r)$, $\ln(1 + (dC_{XY}/dr)_{r_0}(r - r_0)/C_{XY}(r_0))$ may be expanded as $(dC_{XY}/dr)_{r_0}(r - r_0)/C_{XY}(r_0)$. Introducing these approximations in equation (7) and completing the square in the variable $(r - r_0)$, we obtain

$$C_{PX_n}(r) = C_{PX_n}(r_0)\left(\exp\frac{\alpha^2}{2\sigma^2}\right)\left(\exp\frac{-1}{2\sigma^2}((r - r_0) + \alpha)^2\right), \quad (8)$$

where

$$\sigma^2 = \frac{RT}{M_{PX_n}\bar{v}_{PX_n}\left(\frac{d\rho}{dr}\right)_{r_0}\omega^2 r_0} \quad (9)$$

and

$$\alpha = \frac{zRT\,(dC_{XY}/dr)_{r_0}}{M_{PX_n}\bar{v}_{PX_n}\omega^2 r_0 C_{XY}(r_0)} - \frac{(1 - \bar{v}_{PX_n}\rho(r_0))}{\bar{v}_{PX_n}\,(d\rho/dr)_{r_0}}. \quad (10)$$

This is a Gaussian distribution with standard deviation σ. Equation (8) is simplified by choosing r_0 as the mean in which case $\alpha = 0$. Therefore, the density of the medium at the band center is given by the expression

$$\rho(r_0) = \frac{1}{\bar{v}_{PX_n}}\left(1 - \frac{zRT(dC_{XY}/dr)_{r_0}}{M_{PX_n}\omega^2 r_0 C_{XY}(r_0)}\right)\left(\frac{d\rho}{dr}\right)_{r_0}. \quad (11)$$

Thus the pull of the counter-ions displaces the origin of the Gaussian band from the region of density $1/\bar{v}_{PX_n}$ to r_0, where the density is $\rho(r_0)$. Our final result, then, for the distribution of a single polymeric species at equilibrium in a constant density gradient is

$$C_{PX_n}(r) = C_{PX_n}(r_0)\exp\frac{-(r - r_0)^2}{2\sigma^2}. \quad (12)$$

From the observed value of σ, the molecular weight is calculated as

$$M_{PX_n} = \frac{RT}{\bar{v}_{PX_n}(d\rho/dr)_{r_0}\omega^2 r_0\sigma^2}. \quad (13)$$

The molecular weight obtained from equation (13) refers to the dry neutral molecule PX_n whether or not the species actually present is solvated or charged. Because it is much more convenient to measure the effective density $\rho(r_0)$ than to determine $\bar{v}_{PX_n}$ by the usual pycnometric method, one might inquire under what

conditions it is permissible to equate the two quantities. This may be done when

$$\left(1 - \frac{zRT(dC_{XY}/dr)_{r_0}}{M_{PX_n}\omega^2 r_0 C_{XY}(r_0)}\right) \simeq 1. \tag{14}$$

For DNA in cesium chloride solution, even if each primary phosphate carried an effective negative charge (which is surely not the case), the approximation (14) involves an error less than 10 per cent.

Having shown that a single polymeric species in a constant density gradient will be distributed at equilibrium in a band of Gaussian shape, we now turn to the more general situation of a polymer heterogeneous with respect to molecular weight although homogeneous with respect to effective density. In the limit of low polymer concentration, interactions between polymer molecules may be presumed absent, so that the observed band will be the sum of many Gaussians with coincident origins, with each Gaussian possessing a standard deviation related to the molecular weight of the corresponding species by equation (9).

It then follows with no further assumptions that the weight and number average molecular weights of the polymeric material at r are given, respectively, by

$$M_W(r) = -2\beta \frac{d \ln C_{PX_n}}{d(r - r_0)^2}, \tag{15}$$

$$M_N(r) = \frac{-\beta C_{PX_n}(r)}{\int_{-\infty}^{r} (r - r_0) C_{PX_n}(r)\, dr}. \tag{16}$$

The corresponding molecular weight means for the material comprising a band are

$$M_W = \frac{-2\beta \int (d \ln C_{PX_n}/d(r - r_0)^2\, C_{PX_n}(r)\, dr}{\int C_{PX_n}(r)\, dr,} \tag{17}$$

$$M_N = \frac{-\beta \int C_{PX_n}(r)\, dr}{\int (r - r_0)^2 C_{PX_n}(r)\, dr,} \tag{18}$$

where

$$\beta = \frac{RT}{\bar{v}_{PX_n}(d\rho/dr)_{r_0}\omega^2 r_0}.$$

The integrations are to extend over the entire band and are written so as to apply to cells having straight walls whether radial or not. In the completely general case of heterogeneity of both density and molecular weight, the molecular weights calculated from the above equations are minimal values.

Finally, we turn to the problem of estimating the time necessary for the distribution of the polymer to approach closely to its equilibrium value. It can be shown[5] that the time required for the concentration of a single species to be within 1 per cent of its equilibrium value from the center of the Gaussian to two standard deviations may be estimated as

$$t^* = \frac{\sigma^2}{D}\left(\ln \frac{L}{\sigma} + 1.26\right), \qquad L \gg \sigma. \tag{19}$$

where σ is the standard deviation at equilibrium, D is the diffusion constant, and L is the length of the liquid column in which the polymer was evenly distributed at the start of centrifugation. This estimate is based on the assumption that the density gradient is fully established at time zero. The time actually required for the equilibration of XY may be estimated theoretically.[6] In the DNA–cesium chloride system, the cesium chloride equilibrium is, in fact, approached much more quickly than that for the polymer and the estimate of equation (19) agrees with observation.

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[1] It should be pointed out that this possibility is subject to experimental test. By means of a partition cell the material on either side of the mean may be isolated and rebanded. The new band will be skewed if there was density heterogeneity in the original band. Alternatively, one may compare the concentration distribution observed in cells of different shape. For a single species, the concentration distribution is independent of cell shape. However, for material heterogeneous with respect to either molecular weight or effective density or both, the observed concentration distribution is dependent on variations in the area of the cell along the radius of rotation. This dependence is such that for material of homogeneous density the symmetry of the band is not disturbed (although its shape may be). For a material with density heterogeneity, however, the band will show various departures from symmetry, depending on cell shape and the particular density distribution present.

[2] A. Siegel, private communication, 1957.

[3] T. Svedberg and K. O. Pedersen, *The Ultracentrifuge* (Oxford, 1940).

[4] Although three components are sufficient for a thermodynamic description of the system in the absence of the centrifugal field, more may be required in its presence. A system subject to a centrifugal field may be regarded as being composed of a continuous sequence of phases of infinitesimal depth in the direction of the field. A number of components sufficient to describe the chemical composition of each phase must be employed. In the present case, more than three components would be required if any product arises with composition not describable in terms of the three chosen components and which sediments differently than any of the components involved in its formation. This complication is explicitly excluded from the present discussion.

[5] G. M. Nazarian and M. Meselson, to be published.

[6] R. A. Pasternak, G. M. Nazarian, and J. R. Vinograd, *Nature*, 179, 92, 1957; G. M. Nazarian, doctoral dissertation, Department of Chemistry, California Institute of Technology, 1957.