SEDIMENTATION EQUILIBRIUM IN A DENSITY GRADIENT

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ABSTRACT

Sedimentation equilibrium in a density gradient has recently been developed into a reliable method for the determination of molecular weights of homogeneous DNA samples. The procedure and calibrations required for this method are outlined in this paper.

In 1957, Meselson, Stahl and Vinograd[1] introduced a remarkable analytical technique for studying the properties of DNA and viruses. Sedimentation equilibrium in a density gradient has since that time been a most important tool of the molecular biologist, playing a role in many of the definitive experiments of the past decade. The resolution and the experimental accuracy which it provides in the determination of buoyant density and amount of DNA in a band have placed this method in a singularly important role for molecular biology. Before discussing some of the detailed features of this method, I would like to list just a small number of the important experiments which have used this technique.

Meselson and Stahl[2] proved in 1957 that the replication of DNA in *E. coli* was semi-conservative by following the history of density labelled (^{15}N) DNA in cells which had been transferred to light medium. This classical experiment was apparently the major motivational factor in the discovery of the density gradient method. The method provided something unique in labelling techniques in that it made possible the physical separation of isotopically labelled material from unlabelled material. This feature of density gradient sedimentation has made numerous similar transfer experiments on other cell components possible as well.

Weigle, Meselson and Paigen[3] in 1959 showed that a whole class of λ transducing phages have different densities indicating their different DNA content.

The controversy between the copy choice and the breakage and rejoin mechanisms of crossing over was resolved in 1961 when it was demonstrated by Meselson and Weigle[4] and by Kellenberger, Zichichi and Weigle[5] that some phage which arose from a genetic cross contained primarily parental DNA. This showed that DNA replication was not required in large amounts for a cross and that mechanism was likely to be breakage and rejoin.

The first demonstration of DNA renaturation and hybridization was made using density gradient sedimentation equilibrium by Schildkraut, Marmur

and Doty[6] in 1961. This phenomenon is of crucial importance today for those of us who are studying the DNA of the higher organism.

Brenner, Jacob and Meselson[7] in 1961 presented definitive evidence for rapidly turning over messenger RNA in the T2 phage infection process and for the absence of the production of new ribosomes in this process. Sinsheimer, Starmen, Nagler and Guthrie[8] a year later proved the existence of the replicative form of ϕX 174 DNA during the infection process, again using sedimentation equilibrium in a density gradient.

More recently, Birnsteil, Speirs, Purdom and Jones[9] in 1968 isolated ribosomal DNA from *X. laevis* and showed it to have G–C rich spacers. This DNA was found in a heavy satellite in a CsCl gradient.

Finally, the pure lac Operon DNA was isolated by Shapiro, Machattie, Eron, Ihler, Ippen and Beckwith[10] in 1969. Most of these experiments would not have been possible without the technique of density gradient sedimentation equilibrium.

The more formal aspects of the development of the density gradient technique as a reliable analytical tool from the point of view of the physical chemist are the topic of this paper. This development is largely the result of the work of Hearst and Vinograd[11] and of Hearst, Ifft and Vinograd[12] in 1961. The use of the bandwidth as a measure of molecular weight has been fraught with problems and therefore subject to much commentary. These problems have been finally resolved, the two major sources of difficulty in the past being: (1) the inadequacies of the photographic record, which has been cured by the double beam optics and the photoelectric scanner now available for the ultracentrifuge, and (2) the failure to extrapolate to zero polymer concentrations, correcting for virial effects. This last factor was demonstrated by Schmid and Hearst[13, 14] and with its inclusion and a recalibration of the necessary density gradients, some very good numbers for the molecular weights of homogeneous DNAs have been obtained.

The description of the equilibrium distribution of DNA in a density gradient is readily calculated from thermodynamics. Since the choice of neutral components in the three-component electrolyte solution is arbitrary we arbitrarily choose Cs DNA as our neutral macromolecular component. This choice in no way influences the conclusions regarding molecular weight or distribution. Although thermodynamics is more general than the following descriptive approach, it is useful to visualize a neutral Cs DNA molecule with

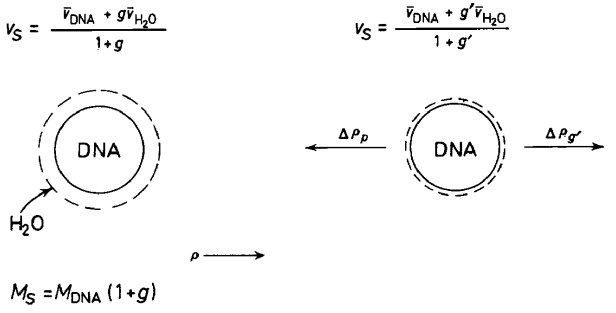

Figure 1. Schematic of DNA in a density gradient.

a thermodynamically defined amount of hydration. This hydration is not necessarily structural, some of it arising from the charge separation between the Cs^+ ions and the phosphate groups of the DNA.

Figure 1 is a schematic representation of two such DNA molecules at different densities in the CsCl gradient. The molecule at the lower density has a net hydration of g grammes of H_2O/gramme Cs DNA. The molecule at the higher density has a net hydration g' which is less than g[11, 14]. Therefore, a hydrated molecule in the heavy region of an equilibrium band has a higher density than one in the light region of a band and this change in hydration broadens the band. There is also a compression gradient in the solution, adding to the density gradient calculated just from the distribution of CsCl in the cell. Since the solvated DNA is less compressible than the CsCl solution[12],

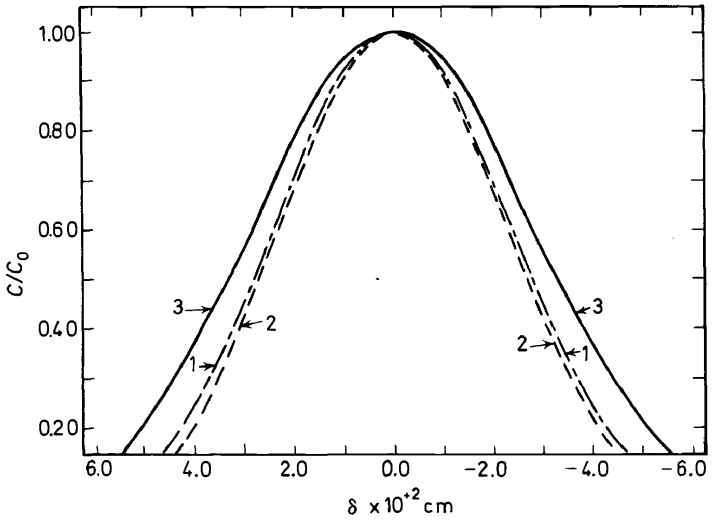

Figure 2. Influence of the effective density gradient on band width. The various effects modifying the density gradient are shown as: 1—the concentration distribution expected from the composition density gradient, 2 — the effect of pressure on the composition density gradient, and 3—the effective density gradient, including the variation of buoyant density with water concentration; this corresponds to the observed concentration distribution. The calculation is performed for 33.3×10^6 daltons Cs DNA, (25.0×10^6 dalton Na DNA) banding in CsCl 1.700 g/ml, 25°C, 6.50 cm from the centre of rotation, at 25 000 rev/min. The following parameters, explained in the later text, were taken to have the numerical values $(1 + \Gamma')/\beta_{\text{eff.}} = 7.87 \times 10^{-10}$, $(1 + \Gamma')/\beta^0 = 11.1 \times 10^{-10}$, $(1 + \Gamma')/\beta_B = 11.9 \times 10^{-10}$, and $1 + \Gamma' = 1.275$.

the effect of compression is to force molecules at high density and pressure to lower densities relative to those at low pressure. The compressibility phenomenon sharpens the actual DNA band. *Figure 2* shows the theoretical changes in hydration. The compressibility makes the band about five per cent narrower, the changes in hydration broaden the band by roughly fifteen per cent.

In calculating a molecular weight from the distribution in the band an effective density gradient must be used. This effective gradient can be measured experimentally by observing the spacing between ^{14}N DNA and ^{15}N

DNA[14, 15]. We have done this for a series of caesium salt solutions[14] and temperatures, and the results are shown in *Figure 3*.

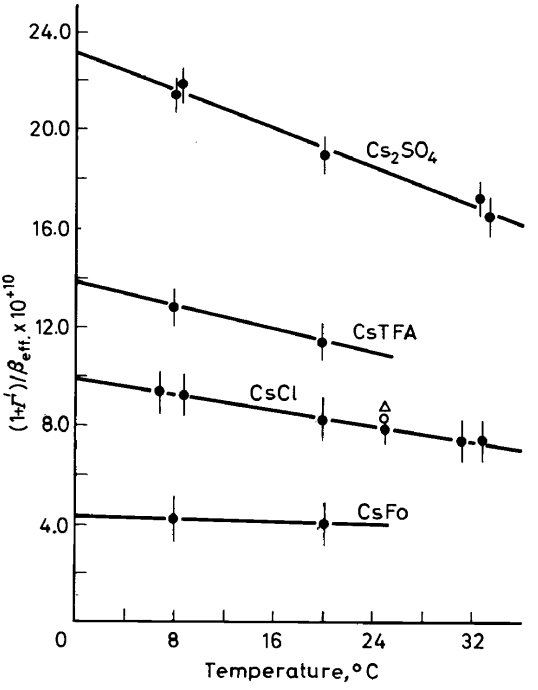

Figure 3. The behaviour of $(1 + \Gamma')/\beta_{eff.}$ is plotted against temperature. The individual determinations at a given temperature are shown as averages with the maximum or standard deviations indicated. The CsCl and Cs_2SO_4 lines are least squared for 40 individuals determinations. The △ for CsCl at 25°C is the value reported by Hearst *et al.*[11, 12], used in a previous publication[13]. The ○ is for coliphage 186P DNA 95 per cent labelled for which $(1 + \Gamma')/\beta_{eff.} = 8.3 \pm 0.3 \times 10^{-10}$.

The authors would like to thank Dr James C. Wang for making his ^{15}N isotopic substitution data on this DNA available to us.

The parameter $(1 + \Gamma')/\beta_{eff.}$ is calculated from equation 1

$$(1 + \Gamma')/\beta_{eff.} = \Delta m \rho_{s,0}/m\Delta r\omega^2 r_0 \qquad (1)$$

where Δm is the change in mass per nucleotide upon isotopic substitution of ^{15}N for ^{14}N, $\rho_{s,0}$ is the buoyant density, m is the mass of DNA per Cs nucleotide, Δr is the distance between the two peaks, ω^2 is the square of the angular velocity of the rotor, and r_0 is the average position of the bands relative to the centre of the rotor. The parameter[11] Γ' is the thermodynamic net hydration analogous to g in our previous example but more rigorously defined. The effective gradient is related to $\beta_{eff.}$ by the equation

$$(\partial\rho/\partial r)_{eff.} = \omega^2 r_0/\beta_{eff.} \qquad (2)$$

Having calibrated all these caesium salt gradients it is easy to determine

the molecular weight of a homogenous Cs DNA using equation 3,

$$\frac{1}{M_3} = \left(\frac{1 + \Gamma'}{\beta_{\text{eff.}}}\right)\left(\frac{\omega^4 r_0^2}{RT\rho_{s,0}}\right)\sigma^2 \tag{3}$$

where the only new parameter is σ^2 which is the mean square standard deviation of the band. This bandwidth must be extrapolated to zero DNA concentration as described by Schmid and Hearst[13]. An example of such an extrapolation is presented in *Figure 4*. The molecular weights obtained for

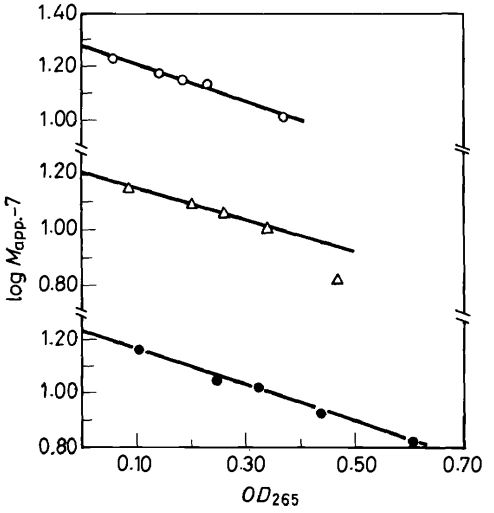

Figure 4. T4 DNA moment analysis log $M_{app.}$ versus concentration with least square lines, ○ 25000 rev/min, △ 30000 rev/min high concentration point omitted, ● 35000 rev/min. Concentrations are the average $OD_{265}^{1\,cm}$ in the band.

homogeneous phage DNAs and corrected to a Na DNA basis are presented in *Table 1*. These values are in excellent agreement with other estimates of the molecular weight of these DNAs.

Table 1. Molecular weight of *E. coli* phage DNAs

DNA	T-7	T-5$^+$	T-4
$M \times 10^{-6}$	24.8 ± 0.4	68.7 ± 6	113 ± 6

Another useful parameter measured by density gradient sedimentation equilibrium is the buoyant density. The most accurate way to do this is to measure the distance of an unknown band from a reference DNA. We choose our reference DNA as *E. coli* and assign it a buoyant density at zero pressure in CsCl of $\rho = 1.710$. The buoyancy gradient[15] (distinct from the effective gradient) must be used to calculate the density difference between these two

bands. The buoyancy β_B is related to the gradient by equation 4.

$$\left(\frac{\partial \rho}{\partial r}\right)_{\text{buoyancy}} = \frac{\omega^2 r_0}{\beta_B} \tag{4}$$

It has been measured by Schmid and Hearst[14] from the difference in position of two bands of identical DNA in different solutions of equal column height but different initial density. *Table 2* presents the calibration of this gradient for a series of caesium salt solutions.

*Table 2**

Salt	$\rho_{s,0}$	$(1 + \Gamma')/\beta_{\text{eff.}} \times 10^{10}$	$1/\beta_B \times 10^{10}$
Cs_2SO_4	1.423	18.3	16.8
$CsOOCCF_3$	1.600	10.9	10.0
CsBr	1.637	15.6	14.4
CsCl	1.709	7.87	9.5
CsOOCH	1.781	3.95	4.2

* From Schmid and Hearst, ref. 14.

Our conclusion is that all of the calibrations required for the use of sedimentation equilibrium in a density gradient as an analytical tool are completed and the technique may be used with reliability.

ACKNOWLEDGEMENT

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