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DEPENDENCE OF THE SEDIMENTATION OF HIGH MOLECULAR WEIGHT DNA ON CENTRIFUGE SPEED

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A theory by Zimm [B.H. Zimm, Biophys. Chem. 1 (1974) 279] predicts that for a given centrifuge speed, there is a broad maximum in a plot of the sedimentation coefficient of DNA against molecular weight. Experimental measurements of these maxima for various centrifuge speeds were made for double-helical DNA in neutral sucrose gradients and single-strand DNA in alkaline gradients. The measurements are in quantitative agreement with the theory, providing good evidence for its validity. The existence of the maximum shows that there is a limit to the sedimentation rate under specified conditions for DNA in the linear form. By implication, DNA observed to sediment faster than this limit is not in the linear form to which most sedimentation theory is applicable.

1. Introduction

In 1971, Rubenstein and Leighton [1] discovered that at high centrifuge speeds long linear DNA molecules sedimented slower than would be expected from data at lower speeds. Zimm [2] developed a theory which accounted for this effect as a consequence of the distortion of the DNA random coil by viscous forces. Some experimental evidence for the phenomenon has been published [3–8], mostly of a qualitative nature.

Zimm's theory makes the unexpected prediction that as the DNA length (or molecular weight) increases, there is a maximum in the sedimentation coefficient at a given centrifuge speed [4, 5, 9]. This paper presents experimental measurements of this maximum sedimentation coefficient under a variety of conditions, and shows that the measurements are in quantitative agreement with theory.

2. Theory

Theory [2, 9] predicts that

$$S = S^0/(1 + 0.1155y^2)^{1/4}, \qquad (1)$$

where

S = observed sedimentation coefficient of DNA of mass M daltons,
S^0 = sedimentation coefficient of DNA of mass M daltons at sufficiently low speed,
$y = 8.374 \times 10^{-24} M^2 (1-\bar{V}\rho)^2 (\mathrm{rpm})^2 x/T\eta S^0$, (2)
$\bar{V}$ = partial specific volume of DNA,
ρ = density of gradient at point x,
rpm = centrifuge speed in rev/min,
x = distance (cm) from axis of rotation to sedimenting molecule,
T = absolute temperature,
η = viscosity of gradient at point x, 1.005×10^{-2} poise for water at 20°C.

By the well known Burgi–Hershey relation [10]

$$S^0 = S_R (M/M_R)^\alpha \qquad (3)$$

where the subscript R refers to a reference DNA and $\alpha = 0.38$ [4, 11]. With increasing M, eq. (1) will go through a maximum

$$(S/S_R)_{max} = \left(\frac{2-3\alpha}{2-\alpha}\right)^{1/4} (M/M_R)^\alpha_{max} \qquad (4)$$

when

$$0.1155y^2_{max} = 2\alpha/(2-3\alpha), \quad (5)$$

and $(M/M_R)_{max}$ is found from eqs. (5), (3) and (2).

The parameter y depends on x and also on ρ and η, which vary down the gradient. The dependence is most clearly seen if we write

$$S^0 = S_{20,W}\,\frac{1-\bar{V}\rho}{1-\bar{V}\rho_{20,W}}\,\frac{\eta_{20,W}}{\eta} \quad (6)$$

where the subscripts (20, W) refer to the value of a parameter in a standard state, water at 20°C. Inserting eqs. (6) and (3) in (2) yields

$$y = \frac{8.374\times10^{-24}}{T}\,\frac{(1-\bar{V}\rho_{20,W})M_R^2}{\eta_{20,W}S^R_{20,W}}$$

$$\times (M/M_R)^{2-\alpha}(1-\bar{V}\rho)(\mathrm{rpm})^2 x\,, \quad (7)$$

where $S^R_{20,W}$ is the sedimentation coefficient of a reference DNA at standard conditions.

In sucrose gradients, both $(1-\bar{V}\rho)$ and x vary as the molecule sediments, but it is easy to show that using mean values in eq. (7) of $(1-\bar{V}\rho)$ and x for sucrose gradients and rotors used in this paper leads to errors of less than 1% in S (eq. 1).

Fig. 1 shows eq. (1) plotted as the expected position of DNA of mass M/M_{T2} in a gradient of 30 fractions under sedimentation conditions such that T2 DNA has moved 5 fractions. It is seen that even for quite low rotor speeds, DNAs of a wide range of molecular weight sediment at essentially the same position in the gradient. Sedimentation of DNA with a range of sizes (and there is no source of monodisperse DNA large enough to test Zimm's theory easily) leads to the DNA "piling up" at such a position. The peak so produced in the DNA sedimentation pattern is the parameter measured in the following experiments.

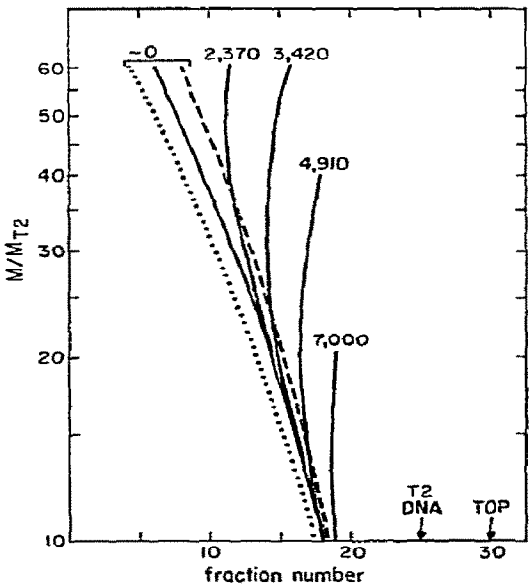

Fig. 1. In this figure the sedimentation of DNA of mass M/M_{T2} (M_{T2} is the mass of T2 DNA) is plotted as the position of such a molecule in a neutral 5–20% sucrose gradient of 30 fractions (sedimentation from right to left) in which T2 DNA sediments 5 fractions (see arrow). An SW50.1 rotor is assumed. The solid lines show the calculated distance moved by DNA of mass M/M_{T2} (shown on the vertical axis) for very low speed (curve marked 0), and for centrifuge speeds of 2370, 3420, 4910 and 7000 rpm as marked. These curves were calculated using eqs. (1), (3) and (7), with $\alpha = 0.38$ [4]. Since the value of α is not firmly established, the low speed curves are also plotted for $\alpha = 0.36$ (– – –) and $\alpha = 0.40$ (.....). The following values of the parameters in eq. (7) were used; $M_{T2} = 1.1\times10^8$ dalton [11]; $S^{T2}_{20,W} = 57.5$ svedberg [4]; $\bar{V} = 0.556$ cm^3/g [15]; $(1-\bar{V}\rho) = 0.424$; $\eta_{20,W} = 1.005\times10^{-2}$ poise; $T = 293$ K; x_0, distance from axis of rotation to meniscus, 6.58 cm; length of 4.8 ml gradient, 3.92 cm.

3. Experimental methods

E. coli AB2497 *thy*$^-$ cells were grown in K medium (19.4 mM NH_4Cl, 43.6 mM Na_2HPO_4, 23 mM KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 μg/ml thiamine, 1% (W/V) Casamino acids, and 1% (W/V) glucose plus 5 μg/ml ^{14}C-thymine at a specific activity of 46 Ci/mol (New England Nuclear). While still in exponential growth the cells were chilled and resuspended at 10^7 cells/ml in 50 mM Tris–2 mM EDTA containing 200 μg/ml eggwhite lysosyme (Calbiochem.). After 10 min incubation at ice temperature, 0.1 ml of the spheroplasts were layered on to 4.8 ml 5–20% (W/V) linear sucrose gradients at 20°C in Siliclad-treated cellulose nitrate tubes. The gradients also contained 5 mM Tris, 1 mM sodium citrate, 10 mM NaCl, 1 mM EDTA, 0.5% (W/V) sodium dodecyl sulfate, and were saturated (at room temperature) with chloroform. Just before the spheroplasts were layered on, 0.01 ml

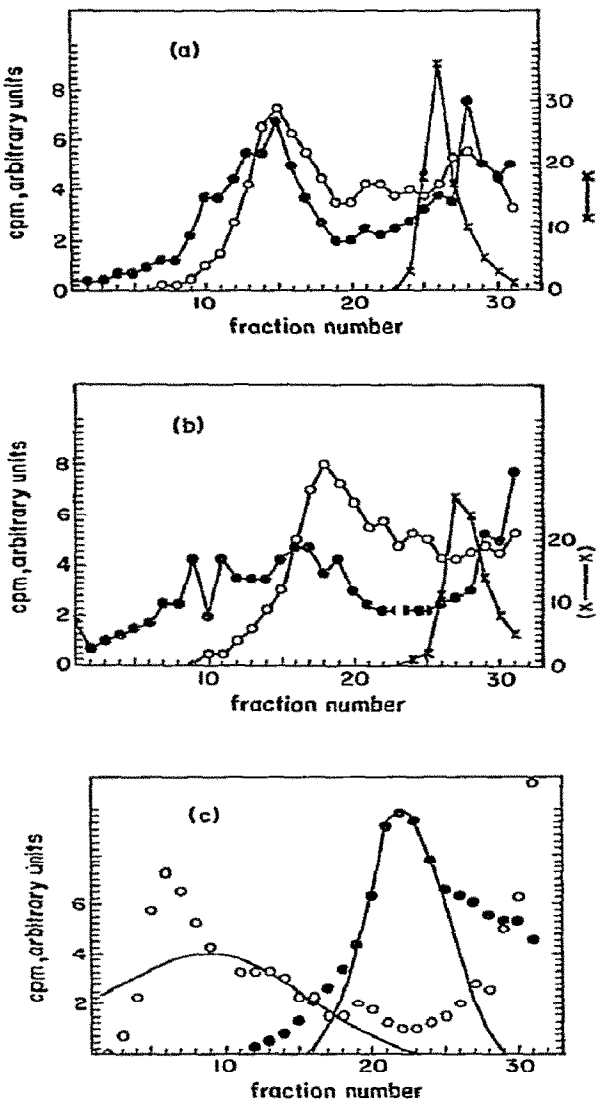

Fig. 2. Neutral sucrose gradients of large DNA from *E. coli* cells lysed on top of the gradient. Sedimentation is from right to left.

(a) Profiles from gradients centrifuged at 3460 rpm for 43 hr in an SW50.1 rotor. (•) *E. coli* DNA from unirradiated cells. (o) *E. coli* DNA from cells given 3 kilorads of ^{60}Co gamma rays. (×) T2 DNA.

(b) Gradients for unirradiated cells sometimes showed only DNA sedimenting several times further than T2 DNA, without any clear peaks. Irradiation of these cells with a low dose of gamma rays always produced a clear peak, as shown.

of 50 mM Tris–2mM EDTA containing about 0.05 μg of T2 DNA labeled with ^{3}H-thymine was put on the gradients. The gradients were allowed to sit for 90 min at 20°C before the centrifuge was started.

Alkaline 5–20% (W/V) sucrose gradients contained 0.8 M NaCl, 0.2 M NaOH and 4.5 mM EDTA in 5 ml cellulose nitrate tubes at 20°C. To the top of each 4.8 ml gradient were added in order: 0.1 ml containing 0.5% (W/V) Sarkosyl, 0.5 M NaOH and 10 mM EDTA; 0.01 ml of T2 DNA solution with ^{3}H-thymine, and 0.05 ml of cells (2×10^{7} cells/ml) in 50 mM Tris–2 mM EDTA. The gradients were allowed to sit 30 min at 20°C before centrifugation.

Most centrifugations were in a Beckman SW50.1 rotor, but some experiments used SW50L and SW65 rotors. All centrifugations were at 20°C. In all cases, speeds were determined by counting numbers of revolutions and measuring elapsed time. For low speed (less than 7000 rpm), frequent checks were made during the centrifugation period to establish that the centrifuge speed did not vary significantly.

The association of cellular material with the sedimenting DNA was measured by growing cells in medium with ^{14}C-glucose and ^{3}H-thymine. DNA from one portion of these cells was extensively purified by repeated elution from hydroxyapatite, and measurements in a liquid scintillation counter showed 15 cpm ^{3}H/cpm ^{14}C. Another part of these cells was sedimented in both neutral sucrose and alkaline gradients by exactly the procedures outlined above. In each case

The profiles are from gradients centrifugated at 330 rpm in an SW65 rotor for 46 hr. The key is the same as for fig. 2(a).

(c) Two gradients of DNA from *E. coli* cells which received 12.5 kilorads of ^{60}Co gamma rays. (•) Profile for centrifugation in an SW50.1 rotor at 3460 rpm for 43 hr. (o) Profile for centrifugation in an SW50.1 rotor at 10640 rpm for 13.5 hr (Note that the DNA sediments farther because $(\text{rpm})^2 t$ was three times larger than for the low speed experiment). The two smooth curves are theoretical distributions (see [4] for details) calculated for 0.35 randomly located double-strand breaks per mass of DNA equal to that of T2 DNA, which would give a weight-average molecular weight of 6.3×10^{8} dalton. At the lower centrifuge speed, there is more material sedimenting on the high molecular weight side than expected from degradation to random linear polymers, but no peak. At the higher centrifuge speed, a considerable amount of material has piled up at a position which is just about that calculated from the theory as a limit for these centrifugation conditions. The sedimentation coefficient for this peak is plotted in fig. 3 with the symbol □.

the ^{14}C activity sedimenting with the ^{3}H-thymine label was determined. After correcting for the ^{14}C label known to be associated with the DNA, no detectible activity from cell material sedimenting with the DNA was found, the uncertainty being a mass of cell material less than about 7% of the DNA mass. Thus it is reasonable to assume that adhering debris did not have significant effect on DNA sedimentation. More detail on these experiments is given elsewhere [12].

4. Experimental results

When unirradiated cells are converted to spheroplasts and lysed on top of neutral sucrose gradients, the typical DNA sedimentation pattern is like that in fig. 2a. Irradiation of the cells with small gamma ray doses before spheroplasting quickly decreases the amount of the fastest sedimenting DNA, but still leaves a peak at fraction 15 (see the 3 kilorad profile in fig. 2a). The fact that 3 kilorads does not alter the position of the peak, although this dose produces 1–2 double-strand breaks per cell genome of 2.7×10^9 daltons [12], suggests that the peak represents DNA piling up against a sedimentation limit, rather than a large number of DNA molecules with a specific size.

T2 DNA was sedimented in the same gradient, and moved 5 fractions as shown; thus the peak in the distribution moved about 16/5 = 3.2 times faster than T2 DNA. The major source of error in this ratio is measuring the distance moved by the marker T2 DNA, since it moved so little.

A more accurate value for the distance moved by the T2 DNA can be determined by using the fact that the sedimentation properties of T2 DNA are well established. In a number of experiments carried out in the same neutral gradients described under Experimental Methods, it was established that the distance D_{T2} (cm) that T2 DNA sediments in t seconds is accurately given by

$$D_{T2} = (S^{T2}_{20,W}/f)[(2\pi/60)\,\mathrm{rpm}]^2 t\,, \tag{8}$$

$$f = \frac{1}{D}\int_{x_0}^{x_0+D} \frac{1}{x}\,\frac{1-\bar{V}\rho_{20,W}}{1-\bar{V}\rho}\,\frac{\eta}{\eta_{20,W}}\,dx\,, \tag{9}$$

where η and ρ are the measured viscosity and density of the gradients at x cm from the axis of rotation, x_0 is the distance from the meniscus to the axis of rotation, D is the distance the molecule sediments, and f is essentially constant in many 5–20% sucrose gradients [13, 14]. The parameter $S^{T2}_{20,W}$ for T2 DNA was found to be 57.5 svedberg, in agreement with previous measurements [4]. The details are given elsewhere [12]. Thus $S/S_{T2} = D/D_{T2}$, where D_{T2} was calculated from the time and centrifuge speed using eq. (8). In no case did the value of D_{T2} so calculated differ from the experimental one by as much as one fraction.

Experimental values of S/S_{T2} for DNA from unirradiated cells for various values of $(\mathrm{rpm})^2 x$ are plotted as filled-in circles in fig. 3. Also shown are plots of eq. (1) (see caption).

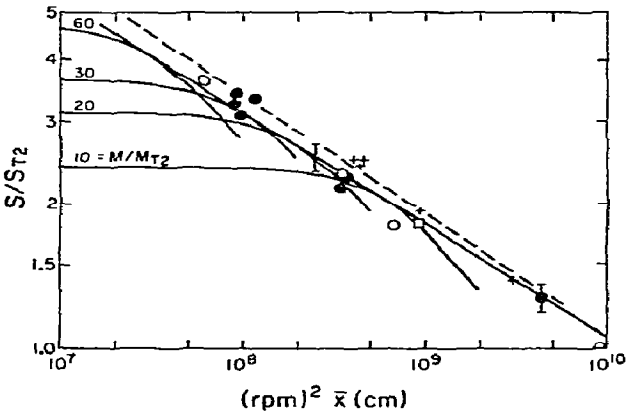

Fig. 3. A plot of eq. (1) for various values of M/M_{T2} for neutral sucrose gradients. For y given by eq. (7) the following values were used: $S_{20,W}$ (for T2 DNA) = 57.5 svedberg [4]; T = 293 K; $\eta_{20,W} = 1.005 \times 10^{-2}$ poise; $\bar{V}$ = 0.556 [15, 16]; $M_{T2} = 1.1 \times 10^8$ dalton [11]; $(1-\bar{V}\rho) = 0.424$, the value one quarter way down the gradient – $(1-\bar{V}\rho) = 0.416$ at the top of the gradient and 0.431 at the middle, so little error is introduced by using 0.424 as a mean value.

The solid curves were calculated with $\alpha = 0.38$. The solid straight line tangent to the curves for various values of M/M_{T2} marks the limit at which linear DNA of any mass can sediment for a given value of $(\mathrm{rpm})^2\bar{x}$. The dashed line is the tangent line for the case $\alpha = 0.40$.

Also shown are various experimental measurements of S/S_{T2}: (•) S/S_{T2} for DNA from unirradiated *E. coli* AB2497 cells. (○) S/S_{T2} for DNA from AB2497 cells given 3–5 kilorads of gamma rays. (▫) S/S_{T2} for the peak in the DNA profile at 10640 rpm shown in fig. 2c. (+) values for S/S_{T2} for large DNA from *B. subtilis* cells [4].

The vertical bar gives the error associated with ± 1 fraction uncertainty in the measurement of the sedimentation position of the large DNA.

It is clear from fig. 3 that the experimental points do not fit a curve for a particular value of M/M_{T2}. Rather, they appear to fall along the tangent to all the curves. Also shown (in crosses) are similar data for large DNA from *B. subtilis* [4], which show a similar dependence of S/S_{T2} on $(\text{rpm})^2x$.

When unirradiated cells are lysed, the sedimentation pattern of the DNA is sometimes a rather broad pattern as shown in fig. 2b. When these cells are irradiated with 3–5 kilorads before lysis, a distinguishable peak appears; the open circles in fig. 3 show the positions of some such peaks, which again appear to fall on the limiting tangent line.

A clear example of the effect of centrifuge speed is shown in fig. 2c for DNA from cells irradiated with 12.5 kilorads. For DNA sedimented 43 hr at 3460 rpm, the peak moved about 9 fractions (profile at right), and could be reasonably fitted by a theoretical distribution curve for 0.35 breaks per mass M_{T2} of T2 DNA. The same *E. coli* DNA sedimented at 10460 rpm for 13.5 hr (about three-fold larger $(\text{rpm})^2t$) showed a distribution which could be well fitted by a theoretical curve for the same 0.35 breaks/M_{T2} at the low molecular weight side, but which also showed a sharp peak on the high molecular weight side of the distribution. The position of this peak is plotted on fig. 3 with a square, right on the line marking the limit at which linear DNA with free ends should sediment.

Similar measurements of S/S_{T2} for various sedimentation conditions have been made for large single-strand *E. coli* DNA in alkaline sucrose gradients. The experimental results are shown in fig. 4.

To compare measurements in alkaline gradients with the theory, eqs. (1), (3) and (7), it is necessary to have a value of $S_{20,w}$ for the reference DNA, single-strand T2 DNA in this case. For single-strand DNA the sedimentation coefficient is strongly dependent on pH and ionic strength [17]. Therefore, T2 DNA was sedimented in the alkaline gradients actually used (fig. 5). As described in the figure caption, $S_{20,w}$ was calculated as 62.3 svedberg.

This then enabled the theoretical curves for the alkaline gradients to be calculated as shown in fig. 4. The points at which DNA peaks are formed fall reasonably close to the limit at which linear DNA can sediment, according to the theory.

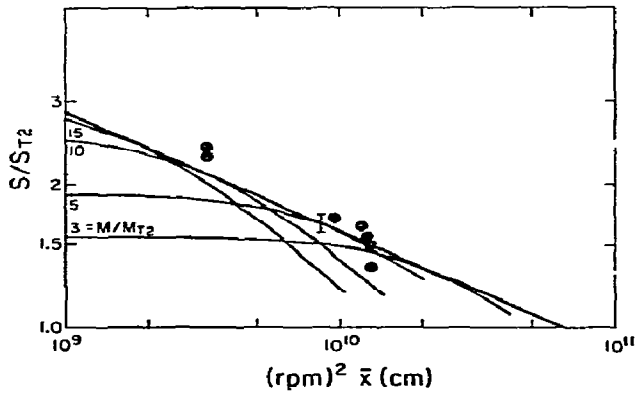

Fig. 4. The solid circles are measurements of the peak in alkaline sucrose gradients of large *E. coli* DNA. For each gradient the position of the T2 DNA used was that from the plot in fig. 5 at the appropriate value of $(\text{rpm})^2t$, representing an averaging of the available data. The vertical bar indicates the error in S/S_{T2} for ± 1 fraction uncertainty in the sedimentation position of the large DNA.

The curves are calculated from eqs. (1), (3) and (7), with the same values of the parameters as in fig. 3, with the following exceptions: $S_{20,w} = 62.3$ svedberg (see fig. 5); $M_{T2} = 0.55 \times 10^8$ dalton (half the double-strand mass); $\bar{V}$ for DNA in alkaline conditions = 0.573 [18], $\alpha = 0.40$ [19].

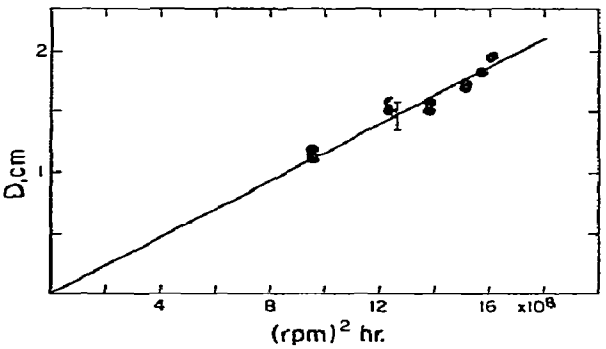

Fig. 5. T2 DNA was sedimented in an SW 50.1 rotor in alkaline sucrose gradients, and the position of the peak for various times and speeds were plotted as shown. The vertical bar shows the error associated with ± one fraction in the position of the measured peak. The parameter characteristic of the sedimentation under these conditions, $S^{T2}_{20,w}$, was calculated using eq. (8), with $f = 0.209$ by extrapolation of the data given by Blattner [13]. For this calculation, $\bar{V}$ for DNA in alkali was equal to 0.573 [18]. This calculation gave, for single-strand T2 DNA in these alkaline gradients, $S^{T2}_{20,w} = 62.3$ svedberg.

5. Discussion

Good experimental tests of the Zimm theory are difficult. The prediction, as shown in fig. 1, is for a decreased sedimentation coefficient as the centrifuge speed increases. Only for DNA several times the size of the largest well-characterized linear DNA, that from T2 bacteriophage, would the effects be large at speeds obtainable with current commercial ultracentrifuges, 65 000 rpm.

In this study, the prediction that DNAs of a wide range of molecular weight will sediment at about the same place in a gradient (see fig. 1) is tested. The positions of this "pile up" were measured for double-strand DNA in neutral gradients (fig. 3) and for single-strand DNA in alkaline gradients (fig. 4) and compared with theory. The excellent agreement between the measurements and the calculations is good evidence for the validity of the theory.

Zimm's theory would predict that a linear flexible chain could not have a sedimentation coefficient, for a given $(\text{rpm})^2 x$, greater than that given by the line tangent to the set of curves for various M in figs. 3 or 4. DNA having such sedimentation properties is sometimes observed (fig. 2a and 2b), and it may be asked what form this DNA is in.

The results of this paper strongly imply that such DNA is not the linear random coil to which the theory applies. The DNA may be in some more compact form, held there perhaps by cellular material. Alternatively, the DNA may not have free ends, but be in a circle. The theory [2] suggests that the distortion of the random coil caused by the motion of the molecule through a viscous medium first takes place at free ends, and some evidence that circles are less affected by viscous drag has been published [20].

At the low centrifuge speeds used in these experiments, the accelerative g-forces are smaller than in most band sedimentation experiments, and the possibilities of "streaming" instabilities are increased. Mason [21] has shown that no such instabilities will occur if

$$\frac{1}{\Delta_{\text{suc}}}\left(\frac{\partial \rho}{\partial x}\right)_{\text{suc}} > \frac{1}{\Delta_{\text{DNA}}}\left(\frac{\partial \rho}{\partial x}\right)_{\text{DNA}} \qquad (10)$$

Here Δ is the diffusion coefficient for either the sucrose or the DNA molecules, and $(\partial \rho/\partial x)$ is the density gradient for either sucrose or DNA. All these quantities can readily be determined except $(\partial \rho/\partial x)_{\text{DNA}}$ (Δ for DNA can readily be calculated from the sedimentation coefficient.)

High values of $(\partial \rho/\partial x)_{\text{DNA}}$ can occur when the DNA is layered on the gradients. However, in these experiments the ratio of the density (microgram/ml) to Δ for the marker T2 DNA was always larger than the same ratio for the large *E. coli* DNA. Since no evidence for instability for the sedimentation of T2 DNA was seen, it is unlikely that the concentration gradients for large DNA set up in establishing the gradients would lead to instability.

High concentration gradients could also form as a result of the "piling up" of DNA at the sedimentation limit set by the Zimm theory. For the experiments shown in fig. 2, there is a considerable amount of DNA from cells irradiated with 3 kilorads sedimenting at this limit, with little DNA beyond it. Thus it seems unlikely that for the gradients from unirradiated cells, the DNA sedimenting beyond the limit is there because of instabilities. In experiments with higher DNA concentrations, however, the formation of "streamers" could lead to DNA sedimenting faster than the Zimm theory would predict.

The results also imply that the size of linear DNA greater than $(1-3) \times 10^9$ daltons cannot be determined by sedimentation using current techniques.

The results in neutral gradients show that for the large DNA ($\geq 30\, M_{\text{T2}}$) from unirradiated or lightly irradiated cells, the observed peaks are close to the line tangent to the set of calculated curves for various monodisperse DNAs, and do *not* follow a curve which would correspond to that for some mean molecular weight $\bar{M}$. At first glance this suggests that perhaps the form of the calculated curve is incorrect for sufficiently large values of the parameter y (eqs. 1 and 2); the sedimentation coefficient might not decrease as fast with increasing rotor speed as eq. (1) would predict. Indeed, Zimm and Schumaker [9] point out that a failure of the theory as y increases would not be surprising.

However, it must be pointed out that these experiments were done with DNA that is clearly *not* monodisperse. The peaks observed are those predicted by the flat maximum in the sedimentation coefficient, which is at the limiting tangent as shown in figs. 3 and 4. The demonstration by Chia and Schumaker [5] that sedimentation curves for DNA of different masses

actually cross is evidence that the current theory is at least qualitatively right.

Acknowledgement

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