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ROTOR SPEED DEPENDENT SEDIMENTATION OF CIRCULAR AND LINEAR DNA[☆]

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The sedimentation rate of large, linear DNA molecules has been shown to be rotor speed dependent (Rubenstein and Leighton, Biophys. Chem. 1 (1974)). In this communication we report the first studies designed to measure the rotor speed effect with a homogenous, linear viral DNA larger than bacteriophage T2 DNA. We also report the first studies using a homogenous, circular episomal DNA of known molecular weight. For this circular DNA a small rotor speed effect, previously unsuspected, was discovered.

1. Introduction

In 1971, Rubenstein and Leighton [1] reported a curious observation: at high rotor speeds, there appeared to be a small decrease in the sedimentation rate of T2 DNA (9% at 65 000 rpm in a SW 65 rotor [2]). In response to this observation, Zimm developed a theory which predicted that this rotor speed effect would produce a dramatic decrease in sedimentation rate for very large DNA molecules [3,4]. The free ends of the DNA chain tend to be near the outside of the randomly coiled DNA molecule simply because the time-averaged probability of finding DNA segments decreases beyond an end. When such a molecule moves with a relatively high velocity through a solvent, these ends will be pulled away from the rest of the molecule because the drag on chain elements at the outside of the coil is greater than the average drag on all the elements. This dragging behind of the ends leads to a distortion in shape of the entire coiled molecule and, thus, an enhancement of the translational frictional coefficient. Macroscopically, this increase in frictional coefficient is observed as a drop in sedimentation coefficient. It is interesting to note that according to this argument, circular DNA, which has no free ends, would be predicted not to be distorted at high rotor speeds and, hence, not to exhibit this kind of rotor speed effect.

The change in the sedimentation rate with rotor speed described above occurs even at very low DNA concentrations and is properly described as a concentration-independent rotor speed effect. At high DNA concentrations, however, other concentration-dependent sedimentation anomalies can mask this effect [5,6,7]. Therefore, the concentration-independent rotor speed effect is best studied at very low concentrations.

The theory developed by Zimm predicts that the magnitude of the rotor speed effect varies approximately with the 3.8 power of the molecular weight of double helical DNA. For an accurate test of his theory, we have selected the DNA from the bacteriophage SP3 which is 1.3 times larger than T2 DNA and, thus, should exhibit a fractional decrease in sedimentation rate over two and a half times greater than that already found for T2 DNA by Rubenstein and Leighton. An additional advantage of working with SP3 DNA is that it is sufficiently small so that with careful handling, homogenous preparations can be obtained.

One of the more unusual aspects of Zimm's theory is the unique role played by the ends of the DNA molecule. According to Zimm, one "could never predict speed dependence of the sedimentation coefficient for a symmetrical circle. Perhaps an effect would appear from the consideration of momentary fluctuations

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in conformation, but one would expect it to be much smaller than that discussed" [3] for linear DNA. To test this critical aspect of the theory we used DNA from episome 1338 which is a large, circular episome first isolated by Silverman [8]. His genetic studies indicate it should contain approximately one tenth of the *E. coli* genome.

2. Materials and methods

2.1. Preparation of ^{32}P labeled SP3 DNA

Bacillus subtilis strain BS19 was grown with aeration at 37° to a density of 10^8 cells per ml in a media containing MOPS salts supplemented with 0.2 g/l Casamino acids (Difco, Detroit, Michigan), 0.3% glucose, 0.2 mM phosphate, and 0.1 mM magnesium. The composition of MOPS salts was as follows: 20.9 g/l 3-(N-morpholino)propanesulfonic acid (MOPS, ULTROL grade, Calbiochem, La Jolla, California), 3 g/l NaCl, 2 g/l KCl, 1 g/l NH_4SO_4, and 10 ml/l "trace salts". The pH was adjusted to 7.2–7.4 before autoclaving. "Trace salts" contained 0.3 g $FeSO_4 \cdot 7H_2O$, 0.18 g $MnCl_2 \cdot 4H_2O$, 0.135 g $CoCl_2 \cdot 6H_2O$, 0.04 g $ZnSO_4 \cdot 7H_2O$, 0.018 g MoO_3, 0.001 g $CuSO_4 \cdot H_2O$, 1 g $CaCl_2 \cdot H_2O$, and 1 g KNO_3 dissolved in 1 liter of 0.1 N HCl.

When a bacterial density of 10^8 cells/ml was reached, SP3 phage were added at a multiplicity of 0.2 phage ber bacterium along with 0.2 mc ^{32}P (carrier-free, ICN, Irvine, California). This amount of ^{32}P was calculated to provide about 0.5 radioactive atoms per SP3 DNA molecule. The culture (100 ml) was allowed to grow overnight with aeration. The phage were isolated by differential sedimentation followed by banding in a CsCl step gradient containing MOPS salts with CsCl to give densities of 1.7, 1.55, 1.45, and 1.3 g/ml. This was spun for one hour at 20° and 30 000 rpm in a Beckman SW 60Ti rotor. The phage band (at a density of 1.55 g/ml) was removed through the side of the tube with a syringe and an 18 gauge needle.

The concentrated phage solution was dialyzed overnight into the extraction buffer (0.2 M Phosphate, 10 mM EDTA, pH 8), adjusted to 5 ml, and extracted five times with freshly distilled, buffer-saturated phenol, followed by two extractions with chloroform : Isoamylalcohol, 24 : 1. During extraction, care was taken to avoid breakage of the DNA due to shear. The DNA solution was then dialyzed against four changes of SSC-EDTA, consisting of 0.15 M NaCl, 0.015 M Na Citrate, 1 mM EDTA.

2.2. Preparation of ^{32}P labeled episome 1338 DNA

An *E. coli* K strain which was his^-, leu^-, arg^-, Bl^-, recA, and $strep^r$ and which contained the 1338 episome was kindly provided by Draper and Silverman, University of California, San Diego [8]. Since this episome carries the genes for histidine production, only bacteria containing the episome could grown in a media with no histidine. Therefore, these bacteria were grown in a media containing MOPS salts supplemented with 0.3% glucose, 0.2 mM phosphate, 0.01 mM magnesium, 1 mg/l thiamine, and 100 mg/l of leucine, arginine, and methionine, and 200 mg/l streptomycin sulfate. In addition, 0.8 mc ^{32}P/100 ml of media was added; this amount of radioactivity was calculated to give one decay/week per episome. The cells were grown with aeration at 30° until a cell density of 10^9 was reached. Then the cells were pelleted in a GSA rotor in a Sorvall centrifuge.

The cells were washed in buffer, then resuspended and lysed using the procedure of Worcel and Burgi [9]. After this type of lysis, the *E. coli* genome still exists as a particle with a sedimentation rate greater than 1000 S and could be easily removed by centrifugation. To separate the closed circular episome from the remaining DNA, the solution was made 500 μg/ml in ethidium bromide, adjusted to a density of 1.55 g/ml and spun in a Beckman 50Ti rotor at 38 000 rpm and 20°. After 40 hours, two bands were present. The lower band containing the covalently closed circular DNA, was removed and rebanded in a fresh CsCl, ethidium solution. After the second banding, the episome was dialyzed against four changes of SSC-EDTA.

2.3. Sedimentation rate markers

Tritium labeled M13 was a kind gift from Sid Suggs (UCLA). The sedimentation rate of this phage was found to be 40.6 S. The phage F2 was grown as described by Overby et al. [10] except that a five-fold lower concentration of uridine was used. The sedimentation rate of the phage was taken to be 79 S [10,11].

2.4. Preparative zone centrifugation

Most runs were performed in a Beckman L5-65 centrifuge with a SW 60Ti rotor at 20°. Sucrose gradients were made with a Beckman density gradient engine to correspond to nearly isokinetic gradients [7]. Gradients made with this machine normally weighed the same within 0.02 g and could be produced three at a time. After the gradients were formed, 0.1 ml of a mixture of 0.1 μg/ml DNA labeled with ^{32}P together with whole bacteria phages F2 and M13 labeled with ^{3}H, was carefully pipetted on top of the gradient using a 1 ml plastic pipette with the tip cut-off, and a screw-type pipetting aid. To avoid any possible mixing as the rotor buckets swung into the horizontal position, the centrifuge was started at its lowest possible acceleration. The acceleration rate was increased once the rotor was past 1000 rpm. At the end of the run the rotor was decelerated with the brake on setting 5 until 5000 rpm was reached, at which point the break knob was turned to its lowest setting. The duration of each experiment was adjusted to yield approximately the same final $\int \omega^2 dt$.

The tubes were removed from the centrifuge and fractionated with an ISCO (Lincoln, Nebraska) density gradient fractionator, model 640, with a modified tube rack that held 45 mini-scintillation vials. The sucrose gradient was displaced from the bottom with a heavy, inert fluorocarbon, Fluorinert (ISCO). Normally, a drop counter was used to collect 6 drops per fraction. This produced 34 fractions of 0.115 ± 0.005 ml. Four ml of scintillation cocktail, made by mixing 2 l toluene, 1 l Triton X 100, 15 g PPO (Sigma, St. Louis), 0.3 g POPOP (New England Nuclear, Boston, Massachusetts), and 180 ml water, were added to each vial. Then the vials were capped and counted in a Packard Tri-carb liquid scintillation spectrometer, model 3310. The machine was calibrated so that the amount of ^{32}P spill-over into the ^{3}H channel could be calculated and subtracted from the net ^{3}H counts to yield corrected cpm.

A plot of corrected cpm of ^{3}H and ^{32}P as a function of fraction number was constructed to locate peak position. A second plot of the cumulative sum over each peak was used to find the position reached by 50% of the mass, and this position was used to determine the sedimentation rate [12]. The SP3 DNA or episomal DNA peaks were always located between two known sedimentation rate markers.

3. Results

3.1. Molecular weight of SP3 and episome 1338 DNA

Poon [13] and Poon, Schumaker and Romig [14] have measured a value for $s^0_{20,w}$ for SP3 DNA of 72.6 svedbergs, using the analytical ultracentrifuge. They also measured the length of this DNA with an electron microscope, using λDNA as an internal standard. The equation of Freifelder [15] was used to estimate the molecular weight from the sedimentation rate determined with the analytical centrifuge.

$$s^0_{20,w} = 2.8 + 0.00834\, M^{0.479}.$$

This equation yielded a molecular weight for SP3 DNA of 144.9×10^6 daltons. The length measurements gave an average molecular weight of 145×10^6 daltons for SP3 DNA, assuming the molecular weight of λDNA was 30×10^6 daltons [15]. These estimates of the molecular weight of SP3 differed from those published by Poon [13,14] due to uncertainties in the molecular weight of λDNA and the molecular weights of the DNA molecules used to calibrate earlier equations relating the sedimentation rate and the molecular weight of DNA [15].

The sedimentation rate of the episomal DNA at low concentrations and extrapolated to zero rotor speed was found to be 81S in the preparative centrifuge. This corresponded to a molecular weight of approximately 220×10^6 daltons using the formula of Freifelder for sucrose gradients [15]

$$s_1/s_{ref} = (M_1/M_{ref})^{0.38}.$$

In this equation s_1 and M_1 are the sedimentation rate and molecular weight of an unknown DNA and s_{ref} and M_{ref} are the sedimentation rate and molecular weight of a reference DNA. SP3 DNA with a molecular weight of 145×10^6 daltons and an apparent sedimentation rate of 68S on the same type of sucrose gradient, was used as a reference.

3.2. The sedimentation behavior of SP3 DNA as a function of rotor speed

For sedimentation studies, 0.1 μg/ml of SP3 DNA labeled with ^{32}P was layered on top of a 7–23% sucrose gradient, as described above, and spun at various rotor speeds in a Beckman SW 60Ti rotor. At all speeds

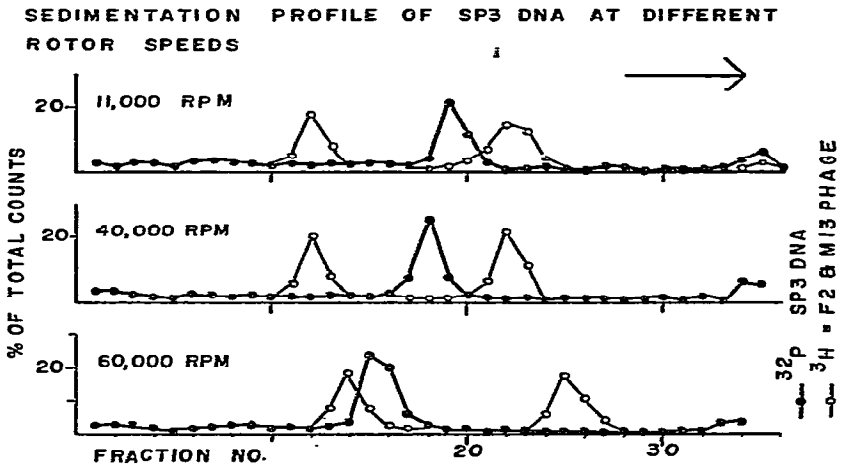

Fig. 1. Sedimentation profiles for SP3 DNA and two sedimentation markers at different speeds. SP3 DNA labeled with ^{32}P, •, was mixed with ^{3}H labeled intact M13 and F2 bacteriophage, ○, and centrifuged in a Beckman SW 60Ti Rotor at different speeds. Direction of sedimentation was from left to right. The SP3 peak was sharp with a half width not much greater than its initial volume, indicating that the DNA was homogeneous in size.

the SP3 DNA and the phage sedimentation markers produced sharp peaks; however, the SP3 DNA did not sediment to the same relative position at the different speeds although the $\int\omega^2 dt$ were similar for each run (fig. 1). Evidently, the sedimentation coefficient was lower at high rotor speeds, as suggested from the work of Rubenstein and Leighton. The plot of the observed sedimentation rate of SP3 DNA as a function of rotor speed showed a sharp decrease in the sedimentation rate at high speeds (fig. 2).

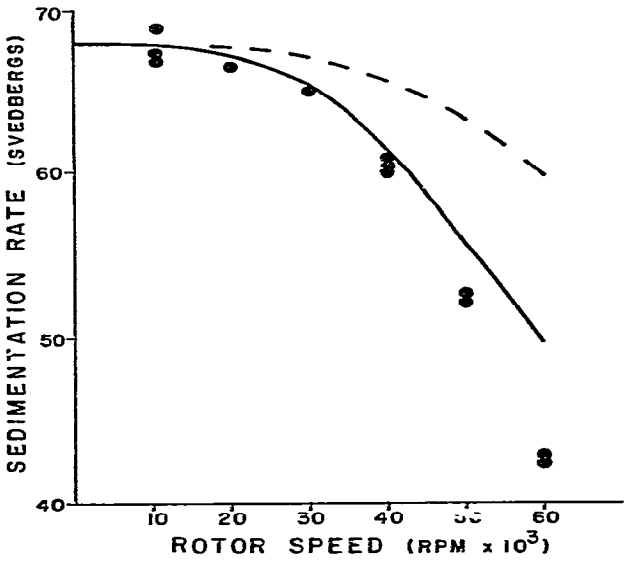

Fig. 2. Sedimentation rate of SP3 DNA as a function of rotor speed. SP3 DNA was spun in a Beckman SW 60Ti Rotor at different speeds. The apparent sedimentation rate of the DNA, •, was measured as described in the text. The solid curve represents the theoretical sedimentation rate using eq. (5) from Zimm and Schumaker [4]. The dashed line represents the theoretical sedimentation rate when the misprint in that paper was corrected, as mentioned in the last paragraph of our discussion, below. The average density, viscosity, and radius through which the DNA moved, and the observed sedimentation rate of 68S for SP3 DNA at low rotor speeds and low DNA concentrations were used to calculate both theoretical curves.

2.3. Sedimentation behavior of episomal DNA 1338 as a function of rotor speed

Isolated covalently closed, circular DNA labeled with ^{32}P was allowed to decay for 4 to 7 days at

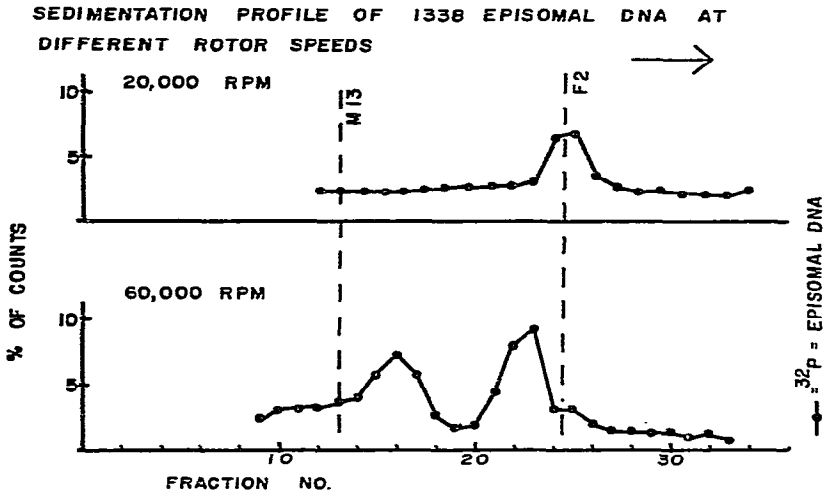

Fig. 3. Sedimentation profile of 1338 episomal DNA at different rotor speeds. Episome 1338 DNA labeled with ^{32}P, which contains both linear DNA molecules and relaxed circular molecules, was mixed with ^{3}H labeled intact M13 and F2 bacteriophage and was spun in a Beckman SW 60Ti Rotor at different speeds. The position of the phage markers are shown by vertical dashed lines. The direction of sedimentation was from left to right. At 20 000 rpm the two forms of the episome sedimented as a single zone. At 60 000 rpm the episome band split into two peaks.

which point most of the molecules should have been broken by radioactive decay of the ^{32}P. Since decay of ^{32}P produces both single strand and double strand breaks in DNA, the covalently closed circular DNA should have been converted to a mixture of relaxed circular and linear molecules. This mixture was then studied at different rotor speeds in the preparative centrifuge, as described previously. Fig. 3 shows data for episomal DNA centrifuged at two speeds; 20 000 and 60 000 rpm. At the low speed the DNA sedimented as one sharp peak close to the 79S marker. At higher speeds the DNA sedimented as two peaks; one sedi-

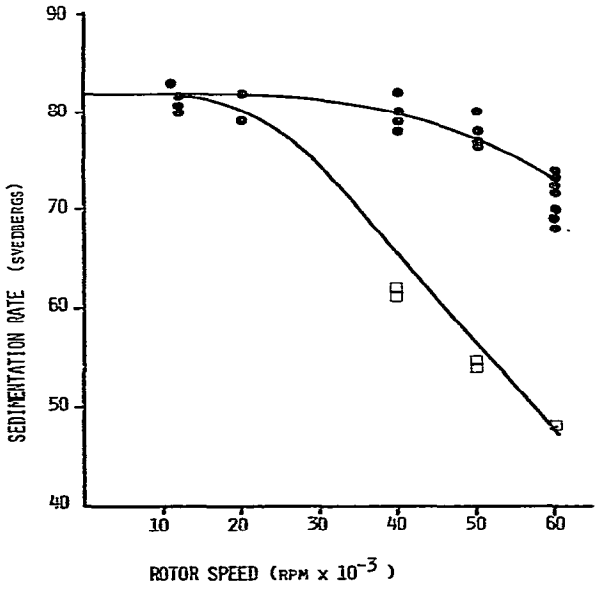

Fig. 4. Sedimentation rate of episome 1338 as a function of rotor speed. The apparent sedimentation rate of episome 1338 DNA was measured at different speeds as described in the text. At high rotor speeds two peaks were observed. The slow moving peak, probably linear DNA, is indicated by □; the faster peak, relaxed circular DNA, is indicated by •. Lower curve represents the theoretical sedimentation rate calculated from eq. (5) of Zimm and Schumaker [4] with a molecular weight for the DNA of 220×10^6 daltons. We found that a good fit through the data representing relaxed circular DNA could be obtained using the Zimm and Schumaker equation if a molecular weight of 110×10^6 daltons was used. The average density, viscosity, and radius through which the DNA DNA moved, and the observed sedimentation rate of 81S for the episomal DNA at low rotor speeds and low DNA concentrations were used to calculate both theoretical curves.

mented close to the 79S marker, but noticeably slower, and the other sedimented close to the 40S marker.

Fig. 4 shows the measured sedimentation rates of the episomal DNA as a function of rotor speed. The episomal DNA clearly separated into two peaks at high speeds, and the DNA in one of these peaks underwent a large reduction in sedimentation rate at high rotor speeds. Presumably, this DNA was the linear form of the episomal DNA. The other peak then represented the relaxed circular form of the episomal DNA which had been predicted to show essentially no rotor speed effect. It was interesting to find that this DNA also showed a significant reduction in sedimentation rate at high speeds, although a smaller reduction than would be found for linear DNA of the same size.

4. Discussion

Zimm has developed a theoretical treatment for the dependence of the sedimentation rate of very large DNA molecules on the strength of the centrifugal field. Exact experimental verification of this theory has been difficult to produce because only very large, linear DNA molecules were expected to show a substantial dependence and because a convenient source of large, homogeneous DNA has been difficult to find. Several workers have shown that large, *nonhomogeneous* DNA behaved as predicted, at least qualitatively [3, 17–22].

In this work, linear DNA from the bacteriophage SP3 with a molecular weight of 145×10^6 daltons was isolated in a pure and homogeneous state. When this DNA was spun in a SW 60 Ti rotor at high speeds, there occurred a drastic decrease in the sedimentation rate of the DNA — four times that observed by Rubenstein and Leighton for T2 DNA at high speeds [2]. This result is in qualitative agreement with Zimm's theory which predicted that the rotor speed effect on the sedimentation rate would exhibit a large molecular weight dependence. The observed decrease in sedimentation rate of SP3 DNA actually was slightly greater than the decrease predicted by Zimm's theory.

A large circular DNA with a molecular weight of about 220×10^6 daltons was also studied in the centrifuge at high speeds. This circular DNA always contained some linear DNA of the same molecular weight. At low rotor speeds both forms of the DNA sedimented as one peak, while at high speeds two peaks were observed. One of these peaks showed a very large decrease in sedimentation rate as the rotor speed was increased as would be expected for long, linear molecules. The other peak, presumably the relaxed circular molecules, showed a smaller and unpredicted decrease in sedimentation rate at high rotor speeds. The decrease in the sedimentation rate for the presumptive circular DNA was found to be approximately equal to the decrease expected for a linear molecule only half as long as the circular DNA.

In developing a theoretical treatment of the rotor speed effect, Zimm assumed that free ends were necessary for any distortion of the DNA at high rotor speeds. The equations which he developed require that all other interactions cancel out. The results presented here, showing a small speed effect for the circular DNA, suggest that free ends are not necessary and that loops of DNA can also be pulled out and distorted. This conclusion also follows from the work of Hecht [25] and Hecht et al. [26]. These authors found that the sedimentation rate of bacterial nucleoids exhibited a marked rotor speed dependence. Nucleoids are a folded, supercoiled DNA structure with no free ends.

Lange et al. [23] suggested that the rotor speed dependence of the sedimentation rate for large DNA molecules was an artifact of sucrose gradients in swinging bucket rotors. The experiments done in this work with a mixture of circular and linear molecules of the same molecular weight demonstrate that the rotor speed dependence is not an artifact of sedimentation due to the sucrose gradient or to wall effects; both molecular forms experienced the same gradients and collisions with the walls, but the circular and linear DNA molecules demonstrated quite different rotor speed effects. Recent work in Pettijohn's laboratory [24] has also shown that bacterial nucleoids display a marked rotor speed dependence in the zonal rotor where there can be no wall effects and where the sucrose gradient was constructed as suggested by Lange et al.

The fact that the equation published by Zimm and Schumaker [4] agrees well with the data of Rubenstein and Leighton and agrees approximately with the data presented in this work may be fortuitous. Zimm and Konrad have recently found that the published equation is incorrect due to a misprint in earlier work [27].

The rpm term in their equation should be replaced by rpm $\cdot\sqrt{2}$. When this corrected formula is used it predicts (fig. 2, dashed line) a much smaller dependence of the sedimentation rate on rotor speed than was observed for SP3 DNA, as shown by the experimental points of fig. 2.

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