A rheological separator for very large DNA molecules

Ken A. Dill[†] and Bruno H. Zimm

Department of Chemistry, B-017, University of California (San Diego), La Jolla, CA 92093, USA

Received 16 July 1979

ABSTRACT We present a rheological separation method for DNA molecules in which their deformability is used to advantage. This is the "radial migration method"; here we present experimental verification of the principle, theory having been reported elsewhere. The main conclusions are: (1) the theory is reasonably good; (2) radial migration is highly sensitive to the molecular weight, as predicted, and (3) intact T2 DNA (1.25×10^8 daltons) can be made to migrate about three centimeters in less than three hours.

INTRODUCTION

We present here a novel separation technique applicable to large DNA molecules. This method makes use of the migration of the DNA molecules in the radial direction that occurs when solutions are subjected to flow between rotating concentric cylinders or cones. This radial velocity depends on the molecular weight to the $5/2$ power. Substantial separation can be achieved in times of a few hours with a simple instrument. Here we demonstrate experimental verification of the principle of radial migration with T2 DNA; a detailed theory has been presented elsewhere.¹⁻⁴

The device is shown in figure 1. It is a pair of concentric cones, made of Plexiglas, the top one of which is turned by a motor. The DNA solution is in the gap between them. The relative motion of the top cone produces a shear stress in the solution which stretches the molecule along the circular flow lines. The stretching force has a small component which acts radially inward at the center of the molecule, drawing it toward the center of the cones. The larger the molecule, the more of the curvature it sees, and the faster it migrates toward the center. Thus, in principle, DNA molecules of different molecular weight which start in a band at the outside edge at time $t = 0$ will migrate at different rates, and thereby separate, and so fractions can be removed through the hole in the center of the bottom cone. The largest molecules will be in the earliest fractions.

Figure 1a. The Radial Migration DNA Separator. The cones are made of Plexiglas. The DNA solution is put between them. The apparatus is mounted on a drill press, and the top cone is turned by a slow, variable-speed motor. After radial migration has occurred, fractions are collected by dripping slowly through the hole in the center of the bottom cone.

Figure 1b. Cut-away view of radial migration separator. Theoretical model for polymer is as frictional beads connected by entropic springs. The shear stress in the solution stretches the springs, which results in a small component of the force acting radially inward. Thus the molecules migrate toward the center.

Experimental Protocol

T2 bacteriophage is first grown up on *E. coli* B, by the procedure of Doermann *et al.*⁷ To get the phage DNA, we extract using the hot phenol method of Massie and Zimm.⁸ The stock solutions were made to be 400 - 700 $\mu\text{g/ml}$ in T2 DNA, in a buffer of 0.195 M Na^+ (BPES buffer: 0.006 M Na_2HPO_4 , 0.002 M NaH_2PO_4 , 0.001 M Na_2EDTA , 0.179 M NaCl , pH 6.8). To prepare a solution for the separator we added 0.075 ml of 10 x BPES buffer to an appropriate volume (typically 0.1 - 0.2 ml) of DNA stock solution (so as to give 5 - 30 $\mu\text{g/ml}$ final DNA concentration). This was then brought to 3 ml final volume by the addition of optically pure (J.T. Baker "Photrex") glycerol. Therefore, depending on the DNA concentration, there was about 92% glycerol by volume. The final Na^+ concentration is 0.05 M. The viscosity of the solvent was measured to be 2.9 poise, in a Cannon-Fenske capillary viscometer at 25°C. The solution was typically mixed in a polystyrene test tube at 37°C for 15 minutes at a rotation rate of about one revolution per 10 sec. None of these details of mixing was critical. The retardation times of the DNA solutions were about 300 seconds, which, in this solvent, indicates most of the molecules are full-sized T2 DNA.⁶

The cones were mounted on a drill press. The shaft for the top cone was fitted into the chuck (see the next section for design details). To run an experiment, the top cone of the separator was first raised, using the drill press handle. Both cones were washed well with 0.5% SDS detergent in water, then completely rinsed with distilled water. The chamber was dried either with a lint-free cloth, or suction by a vacuum line. The efflux tube (see figure 1) was also dried by vacuum line suction. The 3 ml solution was gently poured into the chamber at the center. The small size of the efflux tube and the high viscosity prevented the solution from coming out the efflux tube quickly. The top cone was lowered gently to the point that both cones touched at the center. (Where shear sensitivity of the DNA has been a problem, we have used a syringe pump to slowly input solution through the efflux tube, with the cones already preset). A machinist's displacement dial indicator was moved into place on the top of the top cone, and was adjusted to indicate zero displacement. The top cone was then raised 0.002 inches, so as to allow flow out the efflux hole. Once the cones have been set in place, the first fraction—a reference fraction—is allowed to drip by gravity. We collect about 0.4 ml of solution, and this takes about 30 minutes, due to the high viscosity of the solution. This has been found to be the most reliable procedure for getting a good reference fraction. Once this fraction is collected, the

efflux tube is plugged at the end with a syringe needle which has been sealed. The top cone is then set to spinning at a slow speed at first, increasing to the final speed within ten to 30 minutes. The top cone is spun at that constant, final speed for the appropriate migration time, then the procedure of collecting fractions is begun. To do that, the speed of the top cone is reduced to less than 10% of the running speed. This prevents further radial migration, but the slow spinning maintains an even circularly symmetric distribution of material during the collection of fractions. A series of 0.4 ml fractions are then collected with a small Gilson FC-80K microfractionator, set in the drop counting mode. This fraction collection is not completely satisfactory, however, because the solution is elastic and stringy, resulting in multiple counts for each "drop" from the efflux tube. Therefore the operator has to watch the collection to be sure that the fractions have about the same volumes. The choice for the size of the volumes will be discussed in the next section.

To determine the DNA concentrations of each fraction, the absorbance at 260 nm was read in a Gilford Spectrophotometer. We found that it was most convenient and reproducible to dilute each fraction with a low viscosity solvent. The procedure was to withdraw 0.3 ml of the 0.4 ml sample, using a Pipettman 1000 with a tip which had been cut off with scissors. This increases the bore of the tip and allows for reproducible volumetric withdrawals of the highly viscoelastic solution. To this was added 0.6 ml of the 1 x BPES buffer, and the sample was mixed well. These 0.9 ml samples were then read in the spectrophotometer, and these are the data we have plotted.

For some of the experiments, we sheared the T2 whole DNA to half-sized molecules according to the shear degradation method of Adam and Zimm.⁹ The procedure was to gently pour the solution into the annulus between the concentric cylinders in a Rao flow birefringence machine, which was used for its constant and high shear rate. The radius of the outside cylinder is 1.85 cm, and of the inside is 1.80 cm. The solution was sheared at a rate of 384 sec^{-1} for 1500 seconds. The measurement of retardation times on these sheared solutions showed less than about 1-2% still full-sized, the majority of the DNA (20-70%) was half-sized, and the rest was quarter-sized and smaller. In principle, the distribution of these sizes could be determined by a careful study of viscoelastic retardation and relaxation parameters,¹⁰ but we have not done that.

The Separation Apparatus

According to the theory, a radial separation of molecules should occur

in any circular shearing flow apparatus. We require a device in which the molecules to be separated can be layered in a ring around the outside, and from which fractions can be collected when the DNA has migrated to the center. We have experimented with several such devices, and the present design is shown in figure 1. The two cone surfaces are not parallel to each other, there is a small relative angle between them. This gives a shear rate which is constant everywhere throughout the gap, and is easy to calculate. These coaxial cones allow for a density gradient to be layered in the vertical direction, the reasons for which are the same as in density gradient band sedimentation in the centrifuge. First, the molecules can migrate as independent bands, rather than as overlapping boundaries, and second the gradient stabilizes against convection. The convection problem here is analogous to "overloading a gradient" in sucrose sedimentation. However, the destabilizing force is different. Overloading a sedimentation gradient occurs when the bulk density of the layer of macromolecules is too high. Overloading a gradient in the radial migration separator occurs when the bulk normal stress pressure (Weissenberg pressure) in the macromolecule layer is too high. We have studied this problem extensively, and will present that work elsewhere. The conclusion, however, is that most of the convection can be avoided either with the proper use of gradients, or by doing boundary migration experiments, such as we present here.

In these experiments, we fill the gap between the cones with the DNA solution. Then we spin the top cone at a constant rate, during which the molecules migrate. Finally, we allow fractions to drip from the center hole in the bottom cone. To measure the DNA concentration in each fraction, we read optical densities. This DNA concentration profile can then be compared to the theoretical profile.¹

The device was built in the UCSD machine shop. The cones are made of clear Plexiglas, and the brass rotor shaft on the top cone was fitted directly into the ball bearings of a drill press. Some of the tension from the drill press handle spring was released so that the top cone can be raised or lowered and left at a fixed height. This operation can be performed independently of the rotation of the top cone. The original drill press motor was replaced with a Minarik TR9020 variable-speed precision motor, the speed of which is regulated to better than 1%. The speed is reduced by a 40:1 worm gear (Boston Gear). The drive chain uses the V-belt pulley system of the drill press, with pulley sizes as shown in the figure. We have found radial migration to be reasonably insensitive to bench top vibrations, although in any

case the mass of the drill press probably helps damp them out. The radius of the device is somewhat arbitrary. The device shown will hold about 300 mls of solution. We typically only use about 3 mls. Therefore a much smaller machine would probably suffice. One advantage of a smaller device would be the simplification of the machining. In our case, to reduce vertical wobble at the outside edge (to less than about 0.005 inches), the cones were machined in two stages. First, the rough cuts were made on the lathe to give conical surfaces near their final tolerance. The cones were then warmed in an oven for 48 hours to allow the stresses to relax out, after which the finishing machining and polishing were done. The outlet tube was 1 mm i.d.

Experimental Results

1. Dye Experiments. In order to be sure that the dripping of fractions would not seriously disturb the concentration profile from the chamber, we first did experiments with blue dextran dye (molecular weight = 20,000) in solvent with no DNA. 1.5 ml of 1% dye solution was put at the center apex of the cones, and 1.5 ml of the same solvent, but with no dye, formed a ring around the outside, on top of the other solution. Thus there was a single step dye gradient as a function of radius which had been put into the chamber. The sample volume for each fraction collected was 0.13 ml, and indeed the dye gradient from the fractions (as measured spectrophotometrically at 300 nm) was a faithful reproduction of the input profile. There was a spreading of the boundary, however, and this boundary edge is magnified and displayed in figure 2. Since the dye is of high molecular weight, and the solvent so viscous, diffusion cannot be the cause of this boundary smoothing. However, since there is no density gradient, there will be Poiseuille flow through the efflux tube, which will cause mixing of the sample with some small amount of residual solution from previous fractions. This mixing will occur even at the very slow drip rates we use here (about one drop per 5 - 30 seconds). We can determine the magnitude of this effect as follows. If there were no efflux tube, and therefore no mixing at all, we would collect a series of fractions with concentrations $c(n)$ where $n = 0, 1, 2, \dots, m$ is the index of each fraction. However, each real fraction also contains some small amount of the previous fractions, so that we actually measure concentrations, $c'(n)$, $n = 0, 1, 2, \dots, m$. We let f be the ratio of mixing volume to total volume of each fraction, then

$$c'(n) = c(n)(1 - f) + c'(n-1)(f) \quad (1)$$

Figure 2. Mixing volume measured by dye experiments. The spreading of the dye boundary is described by eqs. 1 - 3 (see text). The mixing volume, fv_s , is found to be 0.050 ml. The numbers on the vertical scale are arbitrary concentration units.

If we divide this equation by Δn , we get a first order difference equation

$$\frac{\Delta c'(n)}{\Delta n} + \alpha c'(n-1) = \alpha c(n) \quad (2)$$

where $\alpha = \frac{1-f}{\Delta n}$ and $\Delta c'(n) = c'(n) - c'(n-1)$

which specifies the shape of the measured concentration distribution for any given concentration gradient in the chamber. For example, a sharp step boundary in the chamber would be smoothed to an exponential decay of concentration in the measured samples, as we found experimentally (see figure 2). To get the actual concentrations in the chamber from the measured samples,

$$c(n) = \frac{c'(n) - c'(n-1) f}{1 - f} \quad (3)$$

where $c'(0) = c(0)$

From this expression, it is clear that we only need to measure f from a single dye experiment, then we can always correct our data for any input concentration gradient (of dye or DNA), and for any sample volume. Therefore from figure 2, we calculate that the mixing volume is 50 μ l, which is about half the volume of the efflux tube. It is clear, therefore, that it does us no good to collect fractions of a size smaller than about three to five times the mixing volume, so we have chosen to collect 0.4 ml samples. Since the total volume of solution is 3 ml, this means we usually collect six fractions and one reference. Under these conditions, the correction in equation 3 is small since f is small, so $c'(n) \cong c(n)$, so that the profiles resulting from the collected fractions should not differ much from the original profile in the chamber. To reduce the boundary smoothing to a minimum, we must reduce the mixing volume, and as with similar systems for column fraction collection, the way to do this is to use the smallest practical efflux tubing. For solutions of large DNA, the countervailing consideration is that the shear stress be small enough so as not to break the molecules. Even though the shear stress depends inversely on the cube of the radius of the tube, the shear rate here is about 10 sec^{-1} , and the shear stress about 30 dynes/cm^2 , very much smaller than is required to break the molecules according to the criteria of Adam and Zimm.⁹ We have minimized the tubing volume to the point below which we could no longer get practical flow rates for a solvent of this viscosity.

2. Radial Migration of Single Species. In these experiments, the idea was to fill the gap between the cones with a constant concentration everywhere of a single size of DNA, usually T2 whole molecules. The top cone is then spun for some appropriate time, and fractions are collected thereafter. If radial migration has occurred, then there should be a high concentration of DNA in the first fraction, and lower concentrations in the others. The concentration profile as a function of time of spinning, t , and of the radius, r , should be:¹

$$c(r,t) = \frac{c_o(r_o^2 + 2k_o t)^{1/2}}{r} \quad (4)$$

subject to the constraint that there should be no DNA outside a boundary, r' , which moves inwardly according to:

$$r' = (r_o^2 - 2k_o t)^{1/2} \quad (5)$$

where

$$k_o = \frac{1.026 \times 10^{-32} \eta_o \Omega^2 M^{5/2}}{kT} \quad (6)$$

η_0 is solvent viscosity (poise), Ω = angular velocity of the top cone (radians/sec), kT is Boltzmann's constant times temperature (ergs), M is molecular weight of the DNA in gm/mole, and r_0 is the radius of the outside boundary of solution at time $t=0$. The initial DNA concentration is c_0 . (k_0 is given here for an assumed gaussian random-coil molecule. A better value, gotten from reference 1, takes account of solvent effects. There is about a 30% difference.) Since the molecules accelerate as they approach the center of the device, it is less useful to calculate a velocity than to calculate a time, t^* , after which no further change in the concentration profile should occur. This is the time required for molecules to migrate from the outer meniscus of solution to the center, and is given by $t^* = r_0^2 / 2 k_0$. Under the conditions of these experiments on T2 whole DNA (molecular weight = 1.25×10^8 daltons,⁶ the theoretical prediction is that t^* should be 1.5 hours.¹ In figure 3, we show a typical experimental time series. In each of those experiments, the conditions are identical, except that the migration is allowed to run for different amounts of time. Indeed, if the top cone is spun for 45 minutes or more, the DNA concentration is enhanced at the center of the cones. There appears to be very little change in the shape of the concentration profile after a couple of hours of migration, in reasonable agreement with the t^* calculated from the theory. Therefore the time course of the concentration profile is reasonably consistent, quantitatively, with the theoretical model¹ for radial migration. The predicted value of t^* may be somewhat low. However, we think it is premature to make a more precise comparison of theory with experiment, unless the solution temperature were known or controlled more accurately--there is no temperature bath around this device. The viscosity of this solvent is quite sensitive to temperature. We have done experiments in a temperature-jacketed concentric cylinder device, with which we can make a more accurate comparison with the radial migration theory, and the agreement is good.¹¹ Note, however, that the optical density, and thus DNA concentration, never goes to zero in the outside fractions. We comment further on this under Discussion.

Theoretically, radial migration should be highly sensitive to molecular weight. To test that, we ran calf thymus DNA (molecular weight $\sim 10^7$ daltons) under the same conditions as described above for the T2 DNA. The calf thymus DNA should not migrate in such a short time. That experiment is summarized in figure 4a, which shows that indeed the profile of concentration is practically flat. Also shown in figure 4 is the result of an experiment in which we purposely degraded a solution of T2 DNA by shear between concentric cylinders

Figure 3. Time series of radial migration experiments. Migration is from right to left. Radial migration occurred over the time course indicated above each profile. Fractions were then collected, and the DNA concentrations measured by OD₂₆₀. The dotted line in each profile is the initial concentration. Note that migration is completed in only a few hours. Note also that there is always some residual DNA left at the outside of the chamber (see text). The rate of rotation of the inner cone was 0.42 revolutions per second, giving a shear rate of 40 sec^{-1} .

until it had no measurable viscoelastic relaxation. In that case, too, we expect no migration. Shown in figure 4c is an experiment in which there was only partial preliminary shearing of the T2 DNA before it was put into the chamber for migration. Indeed, some migration occurred. To further check the sensitivity of radial migration to molecular weight, we sheared T2 whole molecules to the extent that the primary retardation time was that of half-sized molecules (see Experimental Protocol). Complete radial migration of half molecules should take 5.9 times longer than migration for whole molecules, and quarter molecules should take about 34 times longer to migrate. Therefore, for the conditions shown in figure 5, we expected only some very small fraction of the half molecules to have migrated to the center of the

Figure 4. Concentration profiles from small DNA. a. Calf Thymus DNA migrated 10 hours. b. T2 DNA sheared extensively, then migrated for 12 hours. c. T2 DNA partially sheared, then migrated 12 hours. Compare to figure 3, intact T2 DNA.

apparatus, which indeed is what we found.

3. Radial Migration in DNA Mixtures. In these experiments, we prepared each solution so as to contain both T2 and CT DNAs. The experiment was other-

Figure 5. Radial migration of half-sized T2 DNA molecules. In both experiments, migration occurred for 9.5 hours. Dotted line shows the initial DNA concentration. a. Solid line shows migration of T2 half molecules. b. Solid line shows migration of intact, whole T2 DNA. The same solution was then sheared to half molecules, then put into separator to migrate again, which is shown by the dashed line. Conditions were the same as with figure 3.

wise identical to previous ones. During the 9.5 hours over which radial migration was run, the T2 should have migrated so as to be collected in the first fraction, while the non-migrating CT DNA should remain equally distributed in all the fractions. This is verified by the results shown in figure 6. After the optical densities were read, three fractions--the reference, the

Figure 6. Radial migration of intact T2 and calf thymus DNAs. Two typical experiments. Calf thymus DNA concentration is shown with slanted lines, T2 DNA is shown in white. Shading represents samples in which relative proportions of the DNAs are unknown. The figures on the left represent profile of DNA input, on the right is after 9.5 hours of radial migration. (See text for details). The large T2 DNA molecules migrate selectively in relation to the small calf thymus DNA, which does not migrate very much.

first, and the last--were set aside to be run in the Model E Ultracentrifuge, in order to determine the relative concentrations of T2 to CT DNA. We used the scanning absorbance system of the centrifuge which reads optical densities at 265 nm. The slowly-sedimenting species, the CT DNA, was found to be at about the same concentration in all three fractions. The T2 DNA, on the other hand, was most highly concentrated in the first fraction, and was almost non-existent in the last one. Thus selective migration of the T2 DNA occurs in the presence of calf thymus DNA. Comparison of these experiments with the previous ones in which the CT DNA is absent shows in these a much lower level of T2 DNA in the outside fraction. In principle, the ratio of T2 DNA in the last fraction to T2 DNA in the first fraction should be zero. In the previous experiments with T2 DNA alone, that ratio never gets much smaller than 0.20-0.25. However, here we find that ratio to be 0.05-0.15, more close to the ideal. We have verified this by viscoelastometry, which is sensitive only to the T2 DNA concentrations in these mixtures, and not to the CT DNA. The ratio of viscoelastic recoils is 0.13 (for the component of relaxation which is due to full-sized T2 DNA), but because the dependence of these recoils contain both linear and quadratic terms in concentration, we can only say that the ratio of T2 DNA in the last fraction to that of the first fraction is less than 0.13. The conclusion is that the presence of the CT DNA in the mixture makes for a more complete radial migration of the T2 DNA. We think this is due to reduced convective mixing, which is discussed further in the following section.

DISCUSSION

Here we have demonstrated a simple device, inexpensive to build, in which radial migration of T2 DNA molecules occurs. We have shown the effect to be highly sensitive to molecular weight, as predicted by a molecular theory of radial migration. We have also demonstrated that large DNA molecules migrate selectively in the presence of smaller molecules. Furthermore, we have preliminary results, including the original observations on human, *Drosophila*, and *E. coli* DNAs which motivated this work, which suggest that radial migration occurs with molecules much larger than T2 DNA. Therefore, we believe radial migration shows promise as a simple laboratory technique for separating very large DNA molecules.

In the present experiments, the most notable discrepancy between the experimental results and those expected theoretically is that the ultimate

ratio of DNA concentration in the first fraction to the last fraction is too low. We think there are primarily two reasons for this: convection, and shear degradation. There are two sources of convection. First, thermal gradients; this apparatus has no temperature control. Second, inertial forces; as the top cone spins, small volume elements nearby are thrown outward. These elements mix with solution near the stationary bottom cone. In fact, the rotation rate is near the point of convective instability. For larger DNAs, where lower rotor velocities would be practical, this would be less of a problem. A stabilizing influence is the DNA itself. The source of the migration velocity is an inward radial force, which is also the source of an inward pressure, called the normal-stress pressure. It acts on volume elements of bulk solution in the same way as the inertial pressure, but in the opposite direction, and thereby works against that source of convection. This would account for the more nearly ideal radial migration of T2 DNA in a solvent containing calf-thymus DNA.

The other likely cause for incomplete migration is shear degradation of some of the DNA. By measurement of viscoelastic recoils, we have found the fraction of broken molecules to be small, less than perhaps 5%, in these experiments.

To first order at least, radial migration is reasonably independent of concentration. We have done experiments from 5 to 30 $\mu\text{g/ml}$ of DNA, with no consistent difference in the normalized concentration profile or time course. Radial migration occurs at salt concentrations of a factor of three higher and lower than the 0.05 M Na^+ in which these experiments were done. At lower shear rates and lower solvent viscosities, radial migration occurs, but at a slower rate. We also used other solvents, including solutions of sucrose, CsCl, and those to which detergents were added, and found that, to the precision of these simple experiments, density or solvent chemistry had little effect on migration velocity.

The shear rate here is high enough to substantially deform the DNA. A dimensionless measure of molecular deformation is $K\tau$, where K is the shear rate (sec^{-1}) and τ is the relaxation time (sec) of the molecules. Shear stress phenomena such as intrinsic viscosity and viscoelasticity begin to deviate from ideal behavior when $K\tau$ is in the range of 1 to 10.^{8,10} In our radial migration experiments, $K\tau$ is of the order of 10^4 . Apparently, the theory of radial migration is at least approximately valid up to surprisingly large shear rates.

In summary, selective radial migration of whole T2 DNA molecules occurs

relative to and in the presence of small DNA molecules in the coaxial cone device described herein. Even though the shear rate is high, the experiments are in reasonable agreement with theory, and the amount of shear degradation is found to be small. Some convective mixing occurs, but this mixing can be reduced by the addition of smaller DNA molecules. Possibly a simple density gradient in the vertical direction between the coaxial cones would minimize the problem. One could then layer a band of DNA molecules around the top outside edge of a higher density solvent, making it possible to separate bands of large DNA molecules by radial migration, in analogy with band sedimentation in the centrifuge.

Acknowledgments

We thank Peggy Hansen for technical assistance with the experiments. Tim Lohman helped with the Model E Ultracentrifuge experiments. We thank Jim Brannon for helping construct an early version of the machine. We are grateful to Carol Post, Eric Chase, Dick Shafer, and Mark Troll for useful discussions. This work was supported by American Cancer Society grant #NP-150, and by a grant from the Cancer Research Coordinating Committee, University of California.

†Present address: Department of Chemistry, Stanford University Stanford, CA 94305, USA.

References

1. Dill, K. (submitted to Biophysical Chemistry).
2. Shafer, R., Laiken, N., and Zimm, B. (1974). *Biophys. Chem.* 2, 180.
3. Shafer, R. (1974). *Biophys. Chem.* 2, 185.
4. Dill, K. and Shafer, R. (1976). *Biophys. Chem.* 4, 51.
5. Yamakawa, H. (1971). *Modern Theory of Polymer Solutions*, Harper and Row.
6. Bowen, B. and Zimm, B. (1978). *Biophys. Chem.* 7, 235.
7. Doermann, A., Eiserling, F., and Boehner, L. (1973). *J. Virol.* 12, 374.
8. Massie, H. and Zimm, B. (1965). *Proc. Nat. Acad. Sci.* 54, 1641.
9. Adam, R. and Zimm, B. (1977). *Nucl. Acids Res.* 5, 1513.
10. Dill, K. (1978). Ph.D. thesis, University of California, San Diego.
11. Post, C. and K. Dill. Unpublished observations.