Measurement of Diffusion Coefficients of DNA in Agarose Gel

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ABSTRACT: Diffusion coefficients of three linearized plasmid DNAs, sizes from 2686 to 5996 base pairs, have been measured in 0.5% agarose gel. Diffusion of these DNAs in the gel is about 3 times slower than in free solution. The diffusion coefficients vary as the reciprocal of the molecular weight, indicating that these DNAs are moving through the gel by a mechanism intermediate between free diffusion and reptation. This is in accord with the electrophoretic mobility reported in the literature for DNA samples of similar size in agarose.

Introduction

Dilute aqueous agarose gels are widely used as a medium for separating DNA molecules according to size by electrophoresis. Theoretical interpretation of gel electrophoresis usually relies either on reptation theory[1-6] for large DNA sizes or, for smaller sizes, on some variation of Ogston's calculation of the open space available in a gel of infinite straight fibers.[7,8] In this paper we examine the diffusion of a few DNA samples in agarose to see if the same ideas apply both to diffusion and to electrophoresis.

Since de Gennes[1] first proposed the theory of reptation for polymer melts in 1971, much work has been pursued in an effort to verify the theory's predictions. One prediction is that the diffusion coefficient of the center of mass is proportional to molecular weight to the −2 power. Klein[9] was the first to observe this dependence experimentally in a polymer melt; however, later experiments,[10-17] including some on gels, have yielded a variety of exponents. In later work de Gennes[18] considered a gel of cross-linked chains, X monomers between cross-links, swollen by uncross-linked chains, M monomers in length. For the tracer diffusion of labeled probe M-chains he distinguished several regimes in which the diffusion coefficient scaled as either the −1 or −2 power of M depending on the sizes of M and X.

If we adapt these ideas to a gel highly swollen with a small-molecule solvent, we can imagine two regimes: (1) the length of the probe is shorter than the mesh size of the gel and (2) the length of the probe is greater than the mesh size. Regime 1 is often called the Ogston regime, referring to Ogston's open-space calculation already mentioned. When the DNA probe is small, its diffusion coefficient, D, varies as the free-solution diffusion coefficient, D_0, which scales as length to approximately the −0.6 power if the probe is a random coil with excluded volume. Regime 2 is, of course, the reptation regime where the −2 exponent is expected.

In another approach Baumgärtner and Muthukumar[19] brought the randomness of the gel more into consideration; they did a computer simulation of a chain diffusing through a bed of randomly placed cubic obstacles and found that the scaling exponent could exceed −2 when the probe was large as a result of the variations in the entropy of the chain as it diffused among the obstacles.

In contrast to the previously studied systems, the DNA–agarose system is characterized by the low concentration of the gel, in this case 0.5% mass per volume, the high value of the persistence length of the DNA, approximately 50 nm, and the low value of the DNA concentration necessary to avoid DNA–DNA interactions.

Materials and Methods

We could not use dynamic light scattering because of the overwhelming scattering from the agarose; instead we relied on the change in ultraviolet transmission of a thin band of DNA in the gel as it spread with time.

The system was designed to restrict the diffusion of this band to a single axis, x. An expression for the concentration profile of such a configuration as a function of time, $C(x,t)$, is well known.[20] The basic derivation is outlined below. Consider initial conditions to be such that a band is centered at $x = 0$ with concentration $C(x,0) = C_0$ for $-w \le x \le w$ and zero elsewhere. For such an initial step function in concentration the solution of the diffusion equation is

$$C(x,t) = \frac{C_0}{2}\left[\mathrm{erf}\left(\frac{w+x}{2(Dt)^{1/2}}\right) + \mathrm{erf}\left(\frac{w-x}{2(Dt)^{1/2}}\right)\right] \quad (1)$$

where erf is the error function,

$$\mathrm{erf}(z) = \frac{2}{\pi^{1/2}}\int_0^z e^{\xi^2}\,d\xi \quad (2)$$

By applying Beer's law to eq 1 and converting to transmission, an expression for the transmission profile, $T(x,t)$, can be written

$$T(x,t) = e^{-2.303\epsilon l C(x,t)} \quad (3)$$

where ϵ is the extinction coefficient and l is the path length.

In the experiments thin DNA bands were formed by pouring three layers of gel into a siliconized quartz cuvette (Figure 1). In this case the vertical axis is the diffusion axis and the DNA band which is centered at $x = 0$ has a thickness of $2w$, typically about 1 mm. The faces of the cuvettes were masked to restrict the spectrophotometer beam to a known height, $b - a$, typically about 5 mm. Thus the average transmission over the spatial interval a,b at a time t is measured and can be described by integrating eq 3 over this interval for times $t > 0$ with the assumption that the gel boundaries are distant enough from the DNA band to be ignored.

$$\langle T(x,t)\rangle = \frac{1}{b-a}\int_a^b \exp\left\{C'\left[\mathrm{erf}\left(\frac{w+x}{2(Dt)^{1/2}}\right) + \mathrm{erf}\left(\frac{w-x}{2(Dt)^{1/2}}\right)\right]\right\} dx \quad (4)$$

where $C' = -2.303\epsilon C_0 l/2$. This expression is valid for any placement of the initial band and beam height; however, to increase the experimental accuracy a configuration was chosen to maximize the changes in transmission. This occurs when the sample band is centered in the beam. Beam heights of 2–5 times that of the initial DNA band width were found to be convenient. The curves in Figure 2 show plots of the average transmission from eq 4 as a function of time. Initially one sees a decrease in the transmission; this is a Beer's law effect due to the "unshielding" of absorbing particles as the band spreads. The transmission reaches a minimum followed by a continuous increase as the particles diffuse out of the spectrophotometer beam. The points in the figure are data on the transmission of several linear DNAs.

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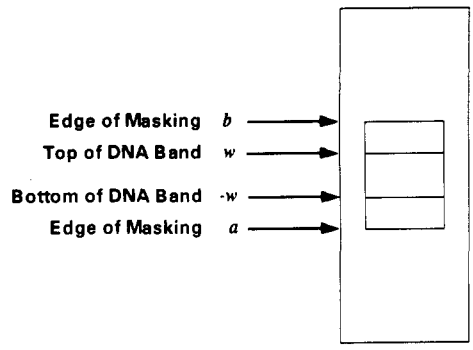

Figure 1. The dichroism cuvette. Three layers of gel are poured into a 10-mm quartz cuvette. The bottom and top layers consist of agarose gel only. The middle band which contains the diffusing species in agarose gel lies at the approximate center of impingement by the spectrophotometer beam and lies between $-w$ and $+w$. The cuvette is masked to provide a well-defined area of the sample, interval a,b, over which the transmission is measured as a function of time.

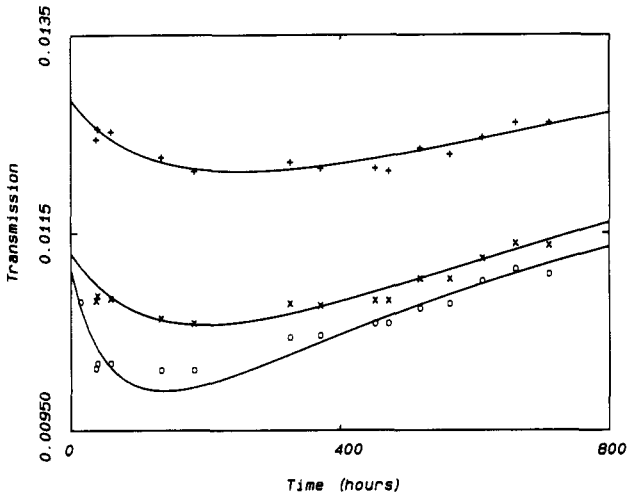

Figure 2. Transmission of diffusing bands of three linear DNAs at 260 nm as a function of time: (O) pUC19; (×) pBR322; (+) pBR325; curves, eq 4. The gel was 0.5% agarose in TE buffer at 22 °C. The curves were fit to the data by the simplex algorithm.

Diffusion coefficients were calculated by fitting eq 4 to the data as described below.

Siliconization of the cuvettes was necessary to provide reasonably flat interfaces between the gel layers. Quartz cuvettes of path length 1 cm with caps were prepared by first soaking in concentrated hydrochloric acid followed by thorough rinsing with deionized water and drying. The cuvettes were then placed in a sealed jar along with a small beaker containing a few drops of dichloromethylsilane whose vapor siliconized the cuvette surfaces. After a few days the cuvettes were removed from the jar, rinsed with deionized water, and baked overnight in a 145 °C oven.

A small rectangular piece of tape of known dimensions was applied to the front face of each cuvette at the approximate center of impingement by the spectrophotometer beam. After the entire face had been painted with flat black enamel, the tape was carefully removed with forceps, leaving a well-defined mask.

All experiments used TE buffer (1 mM Tris, 0.1 mM Na_2-EDTA, pH 7.9). Agarose gels were prepared by adding the desired weight of agarose powder (BRL LE agarose) to a measured volume of TE buffer. This mixture was weighed before being heated to dissolve the agarose. After dissolution and cooling to 60 °C, the solution weight was brought back to the preheating weight with deionized water to replace that lost through evaporation. All agarose concentrations are given as weight of agarose per volume of solvent.

Three linearized plasmid DNAs, pUC19 (2686 bp), pBR322 (4363 bp), and pBR325 (5996 bp), were prepared essentially as described by Maniatis et al.[21] The three plasmids and *E. coli* HB101 were obtained from Bethesda Research Laboratories. The plasmids were then linearized with *Sma*I, *Pvu*II, and *Eco*RV, respectively; these are blunt-cutting enzymes with only one sensitive site on each plasmid.

DNA and agarose solutions in TE buffer were prepared at twice the final desired concentrations and kept at 55 °C in closed Eppendorf tubes throughout the mixing and pouring procedures. Gel and DNA/gel solutions of desired concentration were prepared by mixing equal volumes of the 2× gel with either buffer or 2× DNA solutions. Meanwhile, the cuvettes were also maintained at 55 °C in an oven. This eliminated the quick gelation that occurs when small volumes are poured into cool cuvettes and also helped in obtaining flat interfaces between successive gel layers.

The bottom layer of gel without DNA was quickly pipetted into a warm cuvette to about 0.5 mm below the center of the masking. Gentle tapping of the cuvette on the benchtop helped to flatten the gel surface quickly. The cuvette was then capped and placed on a level surface in the 55 °C oven to equilibrate for about 10 min. The cuvette was then placed at room temperature for about 20 min to allow for gelation during which time a cathetometer was used to measure the position of the top gel surface relative to the cuvette masking. The cuvette was then returned to the oven to reheat before pouring the next gel layer. It should be noted that reheating does not melt the gel already poured as it normally melts at temperatures above 90 °C. A thin DNA/gel layer of about 1-mm thickness was poured next and the relative position of its top surface measured in the same manner. Finally a top layer of gel without DNA and of the same volume as the bottom layer was poured. After the top layer had solidified, a few drops of mineral oil were placed on the gel, caps coated with vacuum grease were inserted, and the cap area was wrapped tightly with Parafilm. Such precautions were taken to prevent drying of the sample and were quite important due to the long duration of the experiments. A cuvette containing only agarose gel was poured as a blank. The final DNA concentration in the band was usually about 40 μg/mL and was determined by comparing the absorbance of a portion of the sample in a 1-mm cuvette against an agarose blank.

The transmission at 260 nm was recorded as a function of time with a Beckman DUR spectrophotometer equipped with a Gilford automatic recorder.

Calculations

Before fitting eq 4 to the data, it was necessary to rearrange the data. First, eq 4 applies to the transmission from the diffusing species only; however, scattering from the gel at 260 nm and blocking of the beam by the masking also have a significant effect which becomes constant after gelation is complete. This can be expressed as follows:

$$\langle T(x,t)\rangle_a^b = \frac{T_{gel}}{b-a}\int_a^b \exp\left\{C'\left[\operatorname{erf}\left(\frac{w+x}{2(Dt)^{1/2}}\right) + \operatorname{erf}\left(\frac{w-x}{2(Dt)^{1/2}}\right)\right]\right\} dx \quad (5)$$

where T_{gel} is the transmission of the combination of the gel and maskings. This constant must either be determined by independent measurement of a blank or eliminated entirely from consideration by taking ratios of pairs of transmission data points separated by a known time interval, $t_m - t_n = \Delta t$

$$\frac{\langle T(x,t_m)\rangle_a^b}{\langle T(x,t_n)\rangle_a^b} \quad (6)$$

T_{gel} and $b-a$ are thus eliminated. We found this tactic preferable to measurement of a blank gel because of the risk of error due to differences between the gels. The method is similar to the Guggenheim method of determining rate constants.[22]

During gelation the scattering from the gel increases to its final constant value over about 12 h. During this period

Table I
Diffusion Coefficients in 0.5% Agarose and Free-Solution Estimates (cm^2/s)

	length, bp	agarose	free solution
linear pUC19	2686	1.25×10^{-8}	3.8×10^{-8}
linear pBR322	4363	6.92×10^{-9}	2.7×10^{-8}
linear pBR325	5996	5.69×10^{-9}	2.2×10^{-8}

it is difficult to separate contributions to the transmission due to scattering by the gel from those due to DNA absorption. Furthermore, the structure of the gel is changing during gelation, and the diffusion coefficient of the DNA may be varying over time as well. Therefore, only data taken after 12 h were used and the experimental times inserted into eq 6 were adjusted by a constant, t_0, to reflect the times that would have been recorded had the experiment begun with complete gelation and the final diffusion coefficient, D, at time $t = 0$,

$$t_n = t_{n,\mathrm{exp}} + t_0 \tag{7}$$

where $t_{n,\mathrm{exp}}$ is the experimental time and t_0 is a constant to be determined. The combined eqs 5 and 6 must be numerically integrated by computer using trial values for D in the quantity $D\Delta t$.

The data were fit by a least-squares method using the "simplex" algorithm which is described in detail in refs 23 and 24. This program finds the best values of a function's unknown parameters by minimizing the sum of the squared residuals:

$$S = \sum_i (y_i - y'_i)^2 \tag{8}$$

where y_i are the experimental data (dependent variable) and y'_i are the corresponding function values generated with a particular choice of parameters. The trial parameters are accepted when user-set criteria for the fractional errors in the parameters and in S are met. This algorithm was used to find best-fit values for D and t_0. The data points were the ratios of the transmissions of pairs of experimental points, eq 6.

In order to plot the computed curves along with the actual data, the value of T_{gel} in eq 5 had to be determined. The simplex algorithm was used again with the values for D and t_0 previously found inserted into eq 5, thus eliminating the need for measuring T_{gel}.

Results

We first attempted to obtain a diffusion coefficient for T7 phage DNA (MW about 26×10^6), but this DNA was much too large, and its diffusion was too slow for reasonable data collection. Attention was turned to smaller DNA, namely, the linearized plasmid DNA described above. Even these experiments were quite lengthy, taking 6–8 weeks. Preliminary repeated experiments on the same DNA but with different gel preparations yielded slightly different diffusion coefficients, presumably because of differences in the gels. Therefore simultaneous experiments using identical gels were done using the three linearized plasmid DNAs mentioned above. Figure 2 displays the results. Table I summarizes the diffusion coefficients found.

The advantage of this method lies mainly in its simplicity. Only an ordinary UV–visible spectrophotometer and standard quartz cuvettes are needed. The method is useful where approximate values of diffusion coefficients are desired without using specialized equipment. A disadvantage is the long time required for large molecules.

Discussion of Results

For comparison, the diffusion coefficients of the three linearized plasmids in free solution were estimated. Eigner and Doty[25] obtained the following empirical relationship between the sedimentation coefficient, s_0, and the molecular weight, M, for DNA as large as four million daltons:

$$s_0 = 0.116M^{0.325} \tag{9}$$

where the sedimentation coefficient is that extrapolated to zero concentration in water at 20 °C. This equation was used to estimate the sedimentation coefficients of the three linearized plasmids. Estimates of the diffusion coefficient at zero concentration, D_0, in water at 20 °C can then be obtained by the Svedberg equation,

$$D = \frac{s_0 RT}{M(1 - \bar{\nu}\rho)} \tag{10}$$

where $\bar{\nu}$ is the partial specific volume of DNA and ρ is the density of water at 20 °C. The partial specific volume of DNA was taken as 0.55 mL/g and the density of water as 0.9982 g/mL. This yields the estimates for the D_0 shown in Table I.

Diffusion constants of several DNA samples in our size range were measured directly by Sorlie and Pecora[26] and by Soda and Wada.[27] Their results fit eqs 9 and 10 within 5%, thus supporting the use of these equations.

These calculated free-solution diffusion coefficients are 3–4 times those obtained experimentally in the gel; see Table I. It was expected that the diffusion coefficient of DNA in a gel would be smaller than in free solution, but as far as the authors know, this is the first time that it has been actually measured.

The question arises regarding the conformation of DNA as it diffuses through the gel. In one extreme case the radius of gyration could be small enough in comparison to the pore size of the gel to allow DNA to pass through without interacting with the gel. In this case the diffusion coefficient should be proportional to $M^{-0.675}$ as in free solution. In the other extreme the DNA would have to pass through the pores in an end-on fashion by reptation. In this case the diffusion coefficient is proportional to M^{-2}. Thus a comparison of the pore size of the gel and the radii of gyration of the DNAs in the buffer used and the molecular-weight dependence of the diffusion coefficients are important.

If linear DNA in solution (not in a gel) is assumed to be a random coil, the radius of gyration can be estimated with the following expression (eq 5–36 of Bloomfield et al.[28] with higher order terms neglected):

$$R_G = \left[\frac{a}{3}(L - 3a)\left(\frac{L}{2a}\right)^{\epsilon}\right]^{1/2} \tag{11}$$

where a is the persistence length and L is the contour length of the coil. The factor $(L/2a)^{\epsilon}$ corrects the radius of gyration of an idealized random coil for excluded volume effects. The contour length of DNA is calculated with the assumption of a 0.34-nm rise per base pair. Hagerman[29,30] determined the persistence length of DNA to be 50 nm at a salt concentration of 1 mM. The cation concentration in the diffusion experiments was also about 1 mM. The parameter ϵ was taken to be 0.05 from the expression

$$\epsilon = 0.05 - 0.11[\mathrm{Na}^+] \tag{12}$$

which was proposed by Douthart and Bloomfield.[31] This gives radii of gyration of 119, 160, and 191 nm for linear pUC19, pBR322, and pBR325, respectively. Griess et al.[32]

have determined effective pore radii, P_E, for several types of agarose gels by electrophoresing latex spheres and various spherical bacteriophages. For a given sphere of radius R, the mobility at zero gel concentration, μ_0, was found by extrapolation from a plot of the mobility, μ, versus gel concentration, A. To interpret their measurements Griess et al. imagined the sphere to be moving along the axis of a cylindrical tube and used a formula originally derived by Faxén for the mobility reduction due to viscous resistance:[33,34]

$$\frac{\mu}{\mu_0} = 1 - 2.104\left(\frac{R}{P_E}\right) + 2.09\left(\frac{R}{P_E}\right)^3 - 0.95\left(\frac{R}{P_E}\right)^5 \quad (13)$$

Values for μ, μ_0, and R were inserted into this equation and used to solve for P_E. It was found that P_E was independent of R and that the following general expression was obeyed:

$$P_E = P_E(1)A^{-x} \quad (14)$$

where P_E is the effective radius[32] of a gel with an A of 1.0 and x is an exponent that depends on the gel type. Their data for Seakem LE agarose was used to estimate the effective pore size of the gel used in our diffusion experiments, where A is 0.5. Here, P_E is[32] 148 nm, x is 0.87, and eq 14 yields a pore radius of 270 nm. We can now insert our R_G values in the Faxén equation and calculate the expected mobility reduction, μ/μ_0, for each sample; the results in order of increasing molecular weight are 0.236, 0.118, and 0.0825. These predict values for D of 0.90×10^{-8}, 3.20×10^{-9}, and 1.82×10^{-9}, respectively, smaller than the observed values in Table I, and increasingly so as the molecular weight goes up. This suggests that the larger samples, at least, are moving through the gel not as undistorted random coils but with the help of some other mechanism, presumably reptation.

(Renkin[35] has added a factor of $(1 - R/P_E)^2$ to the Faxén expression to take account of the space excluded to the moving sphere by contact with the tube wall. Brenner and Gaydos[36] have derived a different expression for the case of $R/P_E < 0.3$. These two expressions give similar numerical values. We have not used them here in order to maintain consistency with Griess and Serwer's method of determining P_E; if we had used them, the predicted values of D would have been still smaller.)

Another approach can be made through a formula derived by Cukier,[37] who proposed the formula

$$D/D_0 = \exp(R/\xi) \quad (15)$$

where ξ is a characteristic length describing hydrodynamic screening in the gel; in a very approximate sense ξ can be thought of as the mesh size of the gel. Averaging this formula over our three samples yields a value of 132 nm for ξ. This is smaller than two of our three molecular radii, which suggests again that the molecules cannot maintain spherical shape while moving through the gel.

The best-fit line to a log–log plot of the diffusion coefficient versus molecular length (Figure 3) has a slope equal to −1.000 and an intercept of −4.488. This yields

$$D = 0.0112N^{-1.00} \quad (16)$$

where N is the molecular length. Thus D depends on molecular length with an exponent between the free-solution value of −0.675 and the reptation value of −2. This is in agreement with the conclusion from the Faxén formula.

Our results are also in general agreement with the data of Stellwagen[38] and Hervet and Bean,[39] who studied the dependence of the electrophoretic mobility on molecular

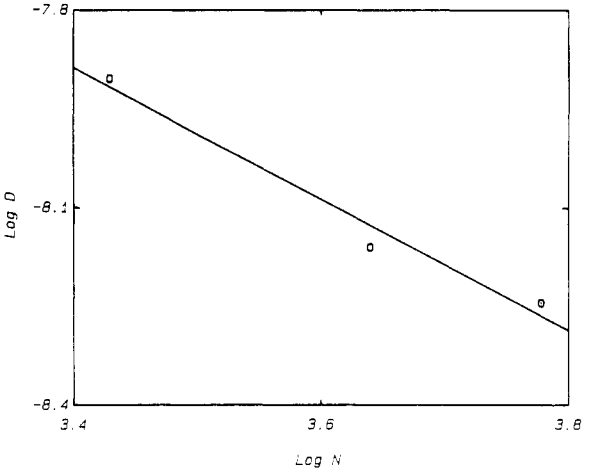

Figure 3. Logarithm of the diffusion coefficient versus the logarithm of the molecular length (in base pairs) of the linearized plasmids pUC19, pBR322, and pBR325 in 0.5% agarose. The slope of the least-squares best-fit line is −1.000 and the intercept is −4.488.

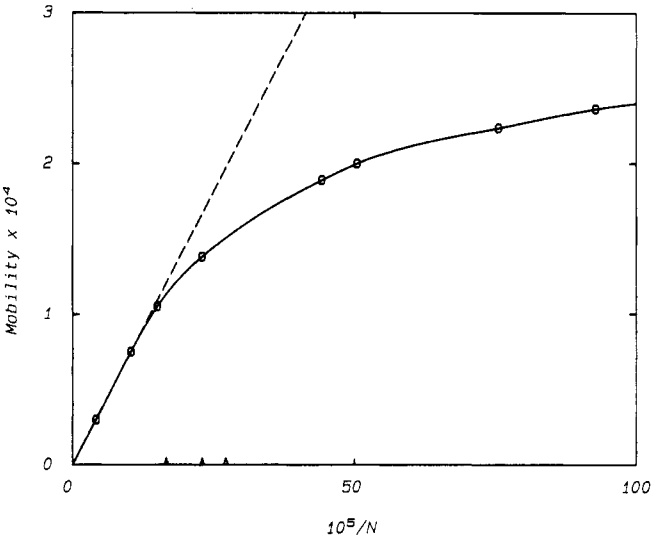

Figure 4. Electrophoretic mobility (cm²/(Vs)) versus the inverse of the DNA length, $1/N$; data from ref 40. N is in base pairs. The arrrowheads on the abscissa mark $1/N$ for our three samples.

length for a large range of DNA lengths. Electrophoretic mobility should scale as D times length because the driving force is proportional to the total charge, which is proportional to the length. Some of the data of Hervet and Bean for 0.6% agarose, 0.2 V/cm, are replotted in Figure 4. For the smallest fragments the slope approaches the free-solution value of zero, where the mobility of DNA is independent of the molecular length,[40] and it approaches the reptation[3,4] value, −1 in electrophoresis, for the largest fragments. The sizes of the fragments used in this study are marked by arrowheads on the abscissa of Figure 4, and the Hervet–Bean fragments in this size range are seen to lie in the transition region between free-solution conditions and reptation. Thus the scaling behavior of diffusion and electrophoresis are consistent.

There should be something close to a direct proportionality between our measured values of D and the electrophoretic mobility in the same gel. We define a frictional resistance coefficient, ζ, by the Einstein relation,

$$\zeta = k_B T/D \quad (17)$$

where k_B is the Boltzmann constant and T the absolute temperature. Then we would expect that the electrophoretic mobility, μ, under the same conditions would be

the ratio of the electric driving force to ζ. This driving force is $2Nqe$, where N is the number of base pairs, e is the charge on the electron, and q is the effective fraction of this charge on each phosphate group. Thus we get

$$\mu = 2Nqe/\zeta \tag{18}$$

The effective charge q is expected to be considerably less than unity because (1) some counterions are bound to the DNA and (2) part of the electric force on the counterions reaches the DNA through friction and electrostatic forces between the moving counterions and the DNA (see discussion by Stigter[41]). By combining eqs 17 and 18, we get an expression for q:

$$q = \frac{k_B T \mu}{2eND} \tag{19}$$

We can estimate values of μ from the Hervet–Bean curve of Figure 4; we then get values for q of 0.065, 0.058, and 0.041 for pUC19, pBR322, and pBR325, respectively. These are similar to the values of 0.03 to 0.05 found by Smith and Bendich[42] from measuring the extension in an electric field of DNA tethered at one end. Stigter[41] has developed a theory for q and compared his results to those of Smith and Bendrich, finding his to be higher by about a factor of 2, which he suggested might be within the uncertainties of his approximate treatment of hydrodynamic interaction. The hydrodynamic interaction probably is also somewhat dependent on the molecular size. In our case an additional uncertainty arises from unknown differences between the gels used by Hervet and Bean and ourselves. Taking account of these problems, we feel that there is reasonable agreement between the diffusion and electrophoretic measurements.

Comparison with Other Diffusion Measurements

Guo et al.[17] measured the diffusion of polystyrene solutions in porous glasses. They found that samples with R/P_E ratios between 0.2 and 1.0 had D scaling with the −1 power of M, similar to our results. Rotstein and Lodge[13] measured diffusion of polystyrene in poly(vinyl methyl ether) gels and found D scaling as about the −2.7 power of M, but their gels were about 30 times more concentrated than ours, so the results are perhaps not comparable. Bansil et al.,[15] measuring diffusion of polystyrene in 12% poly(methyl methacrylate) gels, found the scaling exponent to increase from −0.6 at low molecular weights to −1.8 at high molecular weights; they considered the transition to be abrupt, but the precision of the data would also allow an intermediate region of −1 exponent. Thus our results on DNA are similar to those of other systems.

Conclusion

The scaling behavior of our DNA samples with molecular length shows them to lie in the transition region between the Ogston and reptation regimes, in agreement with the electrophoresis behavior of samples of similar size. This is consistent with the difference between the gel diffusion coefficients and the estimated free-solution values. It was not possible for us to measure larger samples that would lie fully in the reptation regime because the diffusion coefficients would have been too small for our technique.

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