Sedimentation velocity of DNA in isokinetic sucrose gradients: calibration against molecular weight using fragments of defined length

---

Brent E. Korba, John B. Hays* and Sieghild Boehmer

Department of Chemistry, University of Maryland Baltimore County, Catonsville, MD 21228, USA

Received 30 June 1981

ABSTRACT

The relationship between sedimentation coefficient and molecular weight for DNA sedimenting in preformed alkaline and neutral sucrose gradients was determined using absolute molecular weight standards (restriction fragments of plasmid pBR322 and phage λ DNA). The range of calibration for alkaline gradients was extended to small DNA fragments (652 base-pairs) for the first time. The exponent b in the equation $S_{20,w} = aM^b$ was found to be 0.380 in neutral gradients and 0.410 in alkali. The latter value differs significantly from previous estimates. The gradients were isokinetic, and the distance sedimented was shown to be directly proportional to the sedimentation coefficient at all times.

INTRODUCTION

Accurate determination of the sizes of fragments resulting from enzymatic degradations of DNA is crucial to characterization of these processes. Often such treatments, especially those involving repair of DNA damage, result in a random distribution of molecules ranging from large to very small lengths. Although gel electrophoresis is now the preferred technique for DNA length measurements, its use in the analysis of degradation products is limited, due to lack of resolving power in the large molecular weight range, and the problem of accurately measuring relative concentrations of very small fragments in a random distribution. Sedimentation in the analytical centrifuge using optical detection of DNA offers the advantages of convenience and sector-shaped cells (no wall effects). However, detection of low concentrations of small fragments is difficult, unless total DNA concentrations are relatively high. In this case, the dependence of sedimentation coefficient upon molecular weight, itself a function of fragment size, must be taken into consideration.

Thus zonal sedimentation in preformed sucrose gradients is still the method of choice in many instances. Gradients are usually analyzed by collection and assay of fractions. If highly radioactive DNA is used, the total DNA concentration may be so low that sedimentation is effectively at

infinite dilution; nevertheless even small amounts of small DNA fragments may be detected accurately. Recently, sedimentation in alkaline sucrose gradients of DNA treated with damage-specific enzymes has been used to examine the frequency of a variety of lesions: the number of uracil residues incorporated into phage lambda DNA (1); the number of pyrimidine dimers produced by ultra-violet-light irradiation of DNA (2,3); the number of single strand breaks induced in lambda DNA by near-UV irradiation in the presence of chlorpromazine (4); the number of sites sensitive to S1 nuclease induced in DNA by *E. coli* arl mutations (5).

Accurate determination of the relationship between molecular weight and sedimentation velocity in preformed sucrose gradients has been increasingly necessary, therefore. The sedimentation coefficients of a homologous series of chain polymers can be related to their molecular weights by an expression of the form $S = aM^b$, where a and b are empirical parameters characteristic of the particular polymer (6,7). Over a very large molecular weight range the parameter values may change. This is the case for duplex DNA, which approaches random-coil behavior at very high chain lengths, and rigid-rod behavior at very low chain lengths (8,9). However, for single-stranded DNA in alkali at high ionic strengths, the same a and b values hold over a large molecular weight range (from about 10^7 to 1.2×10^8) (10). One purpose of the work described here is to extend the range of calibration to smaller chains.

If the molecular weight M of a chain with sedimentation coefficient S is to be determined by comparison with a standard (molecular weight M_0 , sedimentation coefficient S_0) then only the exponent b need be known, since $M/M_0 = (S/S_0)^{1/b}$. The value for b must be very accurate however, because S is a relatively mild function of M (b is typically about 0.4 for DNA). For an inaccuracy in b of Δb , the relative error in molecular weight $\Delta M/M$ will be given by $\Delta M/M = (M + \Delta M)/M - 1 = [M_0(S/S_0)^{1/(b + \Delta b)}]/[M_0(S/S_0)^{1/b}] - 1$, so $\Delta M/M \approx (S/S_0)^{-\Delta b/b^2}$. Thus the relative error is greater the more the unknown and standard molecular weights differ; for a polydisperse unknown, each fraction in the gradient will correspond to a different relative error. If b were in error by 10% ($b = 0.40$, $\Delta b = -0.04$), typical relative molecular weight errors would be as follows: for $S/S_0 = 3$, $\Delta M/M = 0.32$; for $S/S_0 = 1/3$, $\Delta M/M = 0.24$; for $S/S_0 = 1/10$, $\Delta M/M = 0.44$.

Previous calibrations of S vs. M (10,11,12,13) were limited in several respects. Only a few absolute molecular weight standards (bacteriophage chromosomes) were available, and even their sizes were not known with complete accuracy. Smaller fragments were produced by physical shear of the native

chromosomes, yielding populations of fragments assumed to be, on the average, one-half the size of the starting material. Even if these assumptions were justified, the populations were polydisperse. No standards were available in the low-molecular-weight range, the region of interest in enzymatic degradation experiments. Previous calibrations thus yielded different results, even when experimental conditions were similar (for example, values for b ranged from 0.38 to 0.41 for alkaline conditions (10,12) and from 0.33 to 0.38 for neutral conditions (11,13). It is unclear whether these calibrations are valid for smaller fragments.

It is now possible, through the use of restriction endonucleases, to obtain monodisperse preparations of well-defined DNA fragments over a wide molecular weight range. Also, more accurate molecular weight measurements are now available. For example, the base sequence of plasmid pBR322 has been totally determined, so its absolute molecular weight is known exactly (14). Accurate estimates for bacteriophage λ DNA have been published, and a complete λ sequence will eventually be available (F. Sanger, personal communication). Here we use these techniques and information to calibrate sedimentation coefficients in alkaline and neutral sucrose gradients.

MATERIALS AND METHODS

DNA Samples.

Radiolabeling with [^3H]thymidine or [^{14}C]thymidine and preparation of phage and plasmid DNA was as previously described (5). Restriction endonuclease treatments were performed in accordance with suppliers instructions; completeness of digestion was verified by gel electrophoresis.

Sedimentation in Preformed Sucrose Gradients.

Alkaline sucrose solutions were made in 0.3 M NaOH ($\text{pH} \geq 12.9$) and 0.7 M NaCl. Neutral sucrose solutions were in 0.1 M Tris-HCl ($\text{pH} 8.0$), 0.001 M Na_3EDTA , 0.7 M NaCl. No special precautions were taken to exclude oxygen from the alkaline gradients. However, random degradation during sedimentation was not appreciable. In centrifuge runs lasting as long as 4.5 h., the amount of DNA (λ or pBR322) sedimenting more slowly than intact molecules was always 5% or less (data not shown).

Samples of 0.2 ml (about 0.5–1.0 μg DNA) were layered on top of preformed 4.4 ml 5–20% (w/v) sucrose gradients with a 0.4 ml 50% sucrose cushion. Centrifugation in a Spinco SW 50.1 rotor was at 45,000 RPM or 49,000 RPM for 3 to 6 hours.

Analysis of Gradient Profiles.

About 50 0.1 ml fractions were collected from the bottom of the tubes directly into vials containing 0.2 ml of 1.0 M Tris-HCl buffer (pH 7.4). Radioactivity was determined by liquid scintillation spectrometry. In some experiments linearity of the gradients was verified by measurement of refractive indices (see Fig. 1).

Molecular weights of the fragments used were based on the following information. The lambda phage DNA used here contains the al06-19 duplication, located at 2.1-20.4 λ^+ map units (15) and the b538 deletion, located at 43.0-60.1 λ^+ map units (8). Based on the estimate of 49,502 bp. for λ^+ DNA (Blattner, personal communication) the phage DNA used here contains approximately 50,096 bp. (1.012 λ^+ lengths). The DNA of plasmid pBR322 contains 4,362 bp. (14). The locations of restriction enzyme sites are those given by Szybalski and Szybalski (16) for lambda DNA and Sutcliffe (19) for pBR322.

RESULTS AND DISCUSSION

DNA samples were digested with restriction enzymes AvaI, EcoRI, HindII, PstI or combinations of these enzymes to produce fragments spanning a wide range of sizes (see Table 1). Mixtures of these fragments were subjected to zonal sedimentation in preformed sucrose gradients. Each sample was centrifuged for a time and speed calculated to move all the fragments away from the meniscus regions, where accurate analysis of sedimentation velocity is least certain, and to spread the fragments over as much of the gradient as possible for optimal resolution of peaks. A representative profile is shown in Figure 1.

If it can be assumed that the sedimentation coefficient of linear DNA is the same function of molecular weight, $S^{\circ}_{20,w} = aM^b$, over a wide range of lengths (10,13,17), then the relative positions of two DNA molecules sedimenting in the same gradient will be related to the molecular weights by $M_1/M_2 = (D_1/D_2)^{1/b}$ (12,18), where D_1 is the position in the gradient. This is strictly true if the gradients are isokinetic, that is, the velocity V_1 of a given molecule is the same in all regions of the gradient, and if the distance D_1 moved by that molecule is directly proportional to the time of sedimentation, $D_1 = V_1 t$. The constant b is then determined by plotting $\log M$ vs. $\log D$ for each peak (molecular weight species) in the gradient.

Plots for several alkaline and neutral sucrose gradients, derived from profiles similar to Figure 1, are shown in Figure 2. Slopes were calculated by linear regression analysis. The plotted straight lines are shown; the

Table 1. Exponents (values of b) for functions relating sedimentation coefficient to molecular weight.

Fragment Sizes (bases or base-pairs)	Fragment Sources *	Exponent (b) values [#]	
		Alkaline gradients	Neutral gradients
652 to 4362	EAH4/E1/H2 H2/E1/PE2	0.4052 0.4110	0.3801 0.3771
652 to 15445	E5/E1/H2 A8/E1/H2	0.4113 0.4073	ND ND
1682 to 50096	E5/L1,A8 A8/L1	0.4114 0.4126	0.3794 0.3812
Average (+ standard deviation)		0.4098 ± 0.0015	0.3795 ± 0.0009

* EAH4: EcoRI, AvaI, HindII triple digest of [³H]pBR322 (4 fragments). E1: EcoRI digest of [³H] or [¹⁴C]pBR322 (1 fragment). H2: HindII digest of [¹⁴C]pBR322 (2 fragments). PE2: PstI, EcoRI double digest of [³H]pBR322 (2 fragments). E5: EcoRI digest of [³H] λ al06-19 b538 (5 fragments). A8: AvaI digest of [³H] λ al06-19 b538 (8 fragments). L1: undigested [³H] λ al06-19 b538.

[#] Slopes (least-squares fits) of plots of log D vs. log M (see Fig. 2); linear regression coefficients were 0.995 to 0.998. ND, not determined.

absence of detectable curvature justifies the assumption of constant b, and results of these determinations of b are summarized in Table 1. The value calculated for neutral gradients (0.38) is in good agreement with earlier values. In alkaline conditions the value for b (0.41) differs significantly from previous published estimates for sedimentation in sucrose gradients. It is slightly closer to the ideal value of 0.50 expected for a random coil (13), and is in good agreement with the value for sedimentation in the analytical ultracentrifuge (10). It is also in excellent agreement with a value of 0.406 determined elsewhere using DNA of length 6000 bp and higher, in alkaline sucrose gradients containing 0.5 M NaCl plus 0.2 M NaOH (R. Setlow, personal communication).

The values obtained here are essentially constant over the entire size range from full-sized lambda DNA (50,000 bp.) to the smallest pBR322 fragment (652 bp.). Thus profiles corresponding to heterogeneous distributions of DNA fragments falling within the size range used here can be analyzed using a single exponent. Kovacic and van Holde (17), using restriction fragments of phage PM2 DNA (200 to 1700 bp.) in the analytical ultracentrifuge under

Figure 1. DNA sedimentation in alkaline sucrose after restriction enzyme digestion. Samples were centrifuged for six hours at 49,000 RPM. Sedimentation direction is right to left as shown. Fragment sources (see Table 1 legend for abbreviations): (●—●), E1/PE2; (○---○), H₂.

neutral conditions, found a constant function relating molecular weight and sedimentation coefficient ($b = 0.37$). Under similar conditions, Purnell and Bernardi (20) and Record, et al. (21), using duplex DNA fragments in the molecular weight range of 50 - 500 bp. (isolated by exclusion chromatography) found $b = 0.32$.

It was important for these calibrations to show that the gradients used were in fact isokinetic (7). To demonstrate this, undigested lambda DNA and linear pBR322 (cut once by restriction endonuclease *EcoRI*) were centrifuged for various times at the same speed (49,000 RPM). The distance traveled by each of these molecules through alkaline sucrose in a given time appeared to be the same throughout the experiment (Fig. 3). The migration of lambda DNA at the longest time point was somewhat retarded; these molecules are probably beginning to enter the 50% sucrose cushion. The plots do not intersect the ordinate at zero, presumably because of acceleration and deceleration times and any initial diffusion that occurs during loading. However, both lines appear to converge at the abscissa (within experimental error); the intercept (about -25 min.) corresponds to the effective time involved in acceleration-deceleration. A similar demonstration of isokineticity was

Figure 2. Plots of distance traveled vs. length for DNA fragments after zonal sedimentation in alkaline (panel A) or neutral (panel B) sucrose. Some plots lack one point or contain an additional point due to nearly identical lengths of two fragments or partial digestion by one enzyme. Fragment sources (see Table 1 legend for abbreviations) were as follows. Panel A: (●—●) E1/H2/PE2; (○—○) E1/EAH4; (▲—▲) E1/E5; (△—△) E1/A8; (■—■) L1/E5; (□—□) L1/A8. Panel B: (●—●) E1/H2/PE2; (○—○) E1/EAH4; (▲—▲) L1/E5; (△—△) A8.

obtained when the samples were run together in the same rotor, one tube being removed at each time point (data not shown).

The non-zero ordinate intercepts appear to violate the assumed proportionality between distance moved and time of sedimentation, $D_i = V_i t$, and this would seem to invalidate molecular weight calibrations in experiments where acceleration-deceleration time is a significant fraction of running time. This need not be the case however, as long as the distance moved by each molecule during acceleration-deceleration is the same function of molecular weight as is the distance moved at the steady-state speed. This can be seen in the following way. Let $D_i = V_i t + D_i^0$, where D_i^0 is the distance moved during

Figure 3. Plots of sedimentation distance vs. time at speed in alkaline sucrose. Samples of lambda DNA (o—o) and pBR322 DNA (digested with EcoRI) (●—●) were centrifuged for various times at 49,000 RPM.

acceleration-deceleration by the i 'th molecule. If $D_i^\circ = CM_i^b$, where C is a constant and b has the same value as it does in the relation $S_i = aM_i^b$, then $D_i = V_i t + CM_i^b$. Since by definition the velocity V_i is proportional to the sedimentation coefficient $(S_{20,w}^\circ)_i$, then $D_i = (\text{const}) M_i^b = (\text{const}) S_{20,w}^\circ$ and $M_1/M_2 = (D_1/D_2)^{1/b}$, as assumed originally. What is the relationship between D_i° and M_i in these experiments? From the two limiting ordinates in Fig. 3 we calculate $D_i^\circ = (\text{const}) M_i^{0.44}$, i.e. the value of the exponent is so close to 0.41 that sedimentation during acceleration-deceleration will not significantly affect the calibration.

Three conclusions here are particularly relevant to the determination of molecular weight distributions of partially degraded DNA by sedimentation in alkaline sucrose gradients. First, under conditions of high ionic strength sedimentation coefficients of fragments at least as large as 50,000 nucleotides and at least as small as 652 nucleotides conform to the equation $S_{20,w}^\circ = (\text{const}) M^{0.41}$. The calibration rests on standard fragments whose molecular weight is known absolutely, or nearly so. It seems reasonable to assume that this equation holds accurately for larger fragments and at least approximately

for smaller ones. Whether or not it is accurate for those very small fragments that remain near the meniscus when degraded DNA is analyzed is not important; determination of the true distance moved for these meniscus fractions is inaccurate anyway (1).

Second, under at least these conditions (5 to 20% linear gradients in 1/2 x 2-inch tubes), the distance sedimented by any given molecule is directly proportional to its sedimentation coefficient, $D_1 = (\text{const}) S_1$. This is true at all times, even those early times for which D_1 is not directly proportional to time because of the acceleration-deceleration "dead time". The constant of proportionality for any given molecule is different at different times, but the same for all molecules at any given time. Thus species at all positions in these gradients (except very near the meniscus) may be characterized accurately. It has been brought to our attention that for a uniformly accelerating rotor, timing should begin when the rotor reaches 2/3 final speed. Under these circumstances, time-course curves (Fig. 3) should more nearly extrapolate to the meniscus at zero time. However, as the analysis here demonstrates, these considerations do not affect the relationship between molecular weight and distance sedimented.

Finally, a trade-off between precision and accuracy should be noted. The closer the molecular weights of unknown and standard, the more accurate the measurement, since errors in the calibration (determination of the exponent b) have the least effect. On the other hand, the closer the two molecular weights, the less precisely the difference can be determined by analysis of gradient fractions. Molecules whose length disparity corresponds to less than one fraction cannot be distinguished at all.

ACKNOWLEDGEMENTS

We thank Dr. Daniel Yarosh, National Cancer Institute, and Dr. Richard Setlow, Brookhaven National Laboratory, for critical reading of this manuscript and Alice Combs for preparation of the manuscript. The work was supported by American Cancer Society grant NP-293 and by a grant from the National Institutes of Health (GM 22586).

*To whom correspondence should be addressed.

REFERENCES

1. Hays, J.B., Duncan, B.K. and Boehmer, S. (1981) *J. Bacteriol.* **145**: 306-320.
2. Paterson, M.C. (1978). *Adv. Radiat. Biology* **7**: 1-53.
3. Ahmed, F., and Setlow, R.G. (1977). *Cancer Research* **37**: 3414-3419.

4. Fujita, H., Endo, A. and Suzuki, K. (1981) *Photochem. and Photobiol.* 33: 215-222.
5. Hays, J.B. and Korba, B.E. (1979) *Proc. Natl. Acad. Sci. USA.* 76: 6066-6070.
6. Tanford, C. (1961) *In Physical Chemistry of Macromolecules*, John Wiley and Sons, New York, p. 382.
7. Freifelder, D.M. (1976) *In Physical Biochemistry*, W.H. Freeman and Sons, San Francisco, p. 302.
8. Eigner, J. and Doty, P. (1965) *J. Mol. Biol.* 12: 549-580.
9. Crothers, D. and Zimm, B. (1964) *J. Mol. Biol.* 9: 525-536.
10. Studier, F.W. (1965) *J. Mol. Biol.* 11: 373-390.
11. Burgi, E. and Hershey, A.D. (1963) *Biophys. J.* 3: 309-321.
12. Abelson, J. and Thomas, C.A., Jr. (1968) *J. Mol. Biol.* 18: 262-291.
13. Doty, P., McGill, B.B. and Rice, S.A. (1958) *Proc. Natl. Acad. Sci. USA.* 44: 432-438.
14. Sutcliffe, J.G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* 42: 77-90.
15. Emmons, S.W. and Thomas, J.O. (1975) *J. Mol. Biol.* 91: 147-152.
16. Szybalski, E. and Szybalski, W. (1979) *Gene* 7: 217-270.
17. Kovacic, R.T. and van Holde, K.E. (1977) *Biochem.* 16: 1490-1498.
18. Burgi, E. (1963) *Proc. Natl. Acad. Sci. USA.* 49: 151-155.
19. Sutcliffe, J.G. (1978) *Nucleic Acids Res.* 5: 2721-2728.
20. Prunell, A. and Bernardi, G. (1973) *J. Biol. Chem.* 248: 3433-3440.
21. Record, M.T. Jr., Woodbury, C.P. and Inman, R.B. (1975) *Biopolymers* 14: 393-408.