Sedimentation of Homogeneous Double-Strand DNA Molecules†

R. T. Kovacic‡ and K. E. van Holde*

ABSTRACT: Sedimentation velocity studies have been carried out with isolated double-strand DNA fragments prepared by digestion of PM2 phage with the restriction endonuclease *Hae* III. The results show that DNA molecules shorter than about 200 base pairs behave almost exactly as rigid rods with a diameter of 27 Å. The behavior of the larger fragments (up to 1735 base pairs) can be described very well by either the theory of Yamakawa and Fujii (Yamakawa, H., and Fujii, M. (1973), *Macromolecules 6*, 407) using the same diameter and a persistence length of 575 Å, or the theory of Hearst and Stockmayer (Hearst, J. E., and Stockmayer, W. H. (1962), *J. Chem. Phys. 37*, 1425) using a diameter of 20 Å and a persistence length of 525 Å.

Interest in the conformation of DNA molecules in solution has been rekindled by new questions raised in the process of extending our knowledge of the mechanisms of genetic structure and control to the molecular level. Of particular interest are the relationship between DNA conformation in solution and in chromatin and the role played by DNA tertiary structure in genetic recognition events. Unfortunately, the conformational states of DNA cannot be measured directly and so indirect measures of the conformation must be found. The simplest measure of conformational state is the translational frictional coefficient, f. From a knowledge of dependence of the frictional coefficient on molecular weight, one may easily determine if a polymer like DNA has an extended or compact conformation. With the aid of hydrodynamic models, quite detailed information about the conformation can then be obtained. In practice, however, frictional coefficients for macromolecules are difficult to measure directly in the very dilute solutions that must be used with asymmetric molecules like DNA. The usual approach, therefore, is to combine measurements of the molecular weight with the sedimentation coefficient:

$$s^0_{20,w} = \frac{M(1 - \bar{v}\rho)}{N_A f^0_{20,w}} \quad (1)$$

where M is the molecular mass in grams/mole, $\bar{v}$ is the partial specific volume of the polymer in the buffer of choice, ρ is the density of the buffer, N_A is Avogadro's number, and $f^0_{20,w}$ is the frictional coefficient at infinite dilution, corrected to the standard condition of 20 °C with water as solvent. The frictional coefficient can be interpreted in terms of hydrodynamic theories which relate the shape of a molecule in solution to its frictional properties. Such theoretical considerations are considerably simplified for double-strand DNA by the information already available about DNA structure. Double-strand DNA is extended and highly asymmetric. Base stacking and the two helically interwound backbones impart a good deal of rigidity to the structure. Therefore, a simple model for relatively short DNA might be a rigid rod. For extremely high-molecular-weight DNA, the overall conformation is thought to approach that of a random coil.

Kuhn (1934) first suggested that the random coil could be mathematically described as a series of rigid segments of uniform length separated by entirely flexible joints. The length of one such segment has been called the Kuhn statistical length (λ^{-1}) and is a measure of the flexibility of the polymer. More recent work has focused on the conformational state that is intermediate between the rigid-rod and the random-coil states. This conformational state is usually referred to as the stiff coil or wormlike coil. The Kuhn statistical length is retained as a measure of flexibility, even though the nature of the model has changed (Kratky and Porod, 1949; Hearst and Stockmayer, 1962; Yamakawa and Fujii, 1973). It should be emphasized however that these models provide a mathematical framework within which to construct a description of the behavior of DNA in solution. The physical changes at the molecular level which produce the observed conformation need not be the same as those postulated by any such model.

The principal object of this paper is to establish the empirical relationship between the $s^0_{20,w}$ and the molecular weight for double-strand DNA in the size range which covers the conformational transition from rigid rod to wormlike coil behavior and compare the data to theories that have been developed to describe the frictional behavior of such molecules. In order to do so, we have sought a source of homogeneous, unnicked, double-strand DNAs in this size range.

Size heterogeneity has always been a complication to those attempting to determine the conformation or the molecular weight of double-strand DNA in solution. Burgi and Hershey (1963) and Studier (1965) were among the first investigators to approach these problems by studying the sedimentation velocity of whole and carefully sheared bacteriophage DNAs. Crothers and Zimm (1965) and Gray and Hearst (1968) made additional measurements using phage DNAs. They compared their results with the available theories for relating molecular dimensions to the molecular weights and $s^0_{20,w}$ observed. However, bacteriophage and viral DNAs are rather large and, though useful for developing wormlike coil models, the data obtained do not reveal much about the behavior of small DNAs.

Recently, the hydrodynamic behavior of DNA of limited polydispersity has been studied to ascertain if the conformation of small DNAs in solution may be modeled by a rigid rod. Prunell and Bernardi (1973) enzymatically degraded DNA

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received November 11, 1976.* This work was supported in part by Grant PCM75-19420 from the National Science Foundation.

‡ Present address: Department of Chemistry, University of California, Berkeley, California 94720.

and then fractionated native and denatured DNA by exclusion chromatography. This material was then used to establish the relationships between $s^0_{20,w}$ and the molecular weight. Their work covered the molecular weight range of 2.9×10^4–2.9×10^5 for native DNA and 2.5×10^4–1.4×10^5 for denatured DNA. Record et al. (1975) fractionated sonicated double-strand DNA by exclusion chromatography in order to study the melting behavior of these fragments. They also determined the relationship between $s^0_{20,w}$ and molecular weight over a range similar to that studied by Prunell and Bernardi. However such studies have been complicated by residual heterogeneity and the possible presence of single-strand nicks.

In order to avoid these complications, we have isolated restriction endonuclease fragments of bacteriophage DNA by preparative electrophoresis as an alternative to fractionating heterogeneous DNA. The DNA isolated in this way is as homogeneous in both size and sequence as is undegraded bacteriophage DNA. However, the isolated restriction fragments we have used range in mass from 3.3×10^4 to 1.2×10^6 daltons, so that the relationship between $s^0_{20,w}$ and molecular weight can be studied over a much lower molecular weight range than is possible with whole bacteriophage DNAs.

The bacteriophage PM2 has been selected as a source of DNA because the DNA is small (9900 base pairs or 6.56×10^6 daltons) (Kriegstein and Hogness, 1974), the base composition is similar to calf thymus DNA (Espejo and Canelo, 1968), and the DNA can easily be isolated in 50-mg quantities. Furthermore, the bulk of the DNA isolated from the bacteriophage is circular and superhelical. Therefore, this DNA contains no single-strand breaks. Although Hays and Zimm (1970) have reported that single-strand breaks do not affect the sedimentation properties of DNA, it seemed important to minimize "nicking" as much as possible.

The restriction nuclease chosen to digest the PM2 DNA was endonuclease R *Hae* III, which was isolated from *Haemophilus aegyptius* bacteria. There are several properties of endonuclease R *Hae* III which make it particularly suited to the requirements of this study. The nuclease has been reported to cleave each strand of a double-strand DNA between the guanine (G) and cytosine (C) residues of the sequence 5′-GGCC-3′ (Smith and Nathans, 1973). The result is a double-strand cleavage with no single-strand character because G and C form complementary pairs. In contrast, both shearing and random nuclease digestion will likely produce some single-strand ends. If the *Hae* III enzyme preparation is completely free of contaminating endo- and exonucleases, the PM2 *Hae* III digest should be completely double strand. Preliminary experiments with the *Hae* III preparation revealed it to be remarkably free of other nuclease activity; large excesses of the enzyme incubated for long time periods with small amounts of DNA revealed no apparent degradation of the double-strand material. The small size of the restriction site recognized is also particularly useful in this study because more small fragments are produced. The broad range of sizes produced also make the PM2 *Hae* III digest particularly useful in this study.

Materials and Methods

Preparation and Purification of PM2 Hae III Fragments. Preparation of PM2 Lysates. PM2 bacteriophage was grown on the marine pseudomonad BAL 31 in BAL broth as described by Espejo and Canelo (1968). Both the phage and the bacterial stocks were the generous gifts of Dr. Stuart Linn, University of California, Berkeley.

BAL 31 was preserved by growing the cells to early midlog phase in BAL broth, making the suspension 20% in glycerol, and freezing in liquid nitrogen. PM2 lysate was preserved by preparing a fresh lysate as described below, adding glycerol to 7.5%, and freezing. Freezing immediately reduces the titer by two orders of magnitude, but the remaining viable phages are stable indefinitely at −70 °C.

The procedures of Espejo and Canelo (1968), as modified by Le Pecq (1971), formed the basis for the large scale preparation of PM2 outlined here. High-titer lysates (1×10^{11} plaque-forming units (pfu)/mL) were prepared 1 to 2 days in advance of each large-scale preparation by inoculating 50 mL of a culture of BAL 31 in early log phase with 0.1 mL of low titer (10^7–10^9 pfu/mL) PM2 stock and incubating overnight at 25 °C. Growth medium was BAL broth, vigorously shaken during incubation. Titers were assayed as outlined by Le Pecq (1971) using BAL top and bottom agars.

Large-scale lysates were prepared in a 100-L fermentor (Stainless Steel Products Co.) by adding the salts and nutrient broth (Difco) required to 50 L of distilled water. The medium was sterilized 15–45 min and then cooled to 25 °C. The fermentor was then inoculated with 50 mL of an overnight culture of BAL 31 diluted with 500 mL of sterile BAL broth. Aeration was maintained at 20 L/min with the slowest stirring rate available. After 4 h of continuous growth, aliquots were withdrawn at 30-min intervals and air bubbles trapped in the aliquot were allowed to disperse; the absorbance at 550 nm (A_{550}) was then measured on a Bausch and Lomb 340 Spectrometer. When the A_{550} reached 0.15, the input port was sterilized and an aliquot of fresh lysate equivalent to 3.5×10^{10} pfu of PM2 lysate was diluted with 500 mL of sterile BAL broth and added to the contents of the fermentor. As a result of the sterilization procedures, the infection is delayed approximately 20 min from the initial reading; by 30 min, the A_{550} was usually equal to 0.2. Lysis is generally complete 4–6 h after infection.

To avoid wasting dextran sulfate (used in the concentration step following), titers were taken before harvesting the contents of the fermentor. Because 8–12 h are required for plaques to develop on the plates, aeration was stopped and cold tap water was circulated through the water jacket, effectively halting all growth.

If the titer was greater than 1×10^{11} pfu/mL, cell debris was removed by passing the lysate through a Super Sharples continuous-flow centrifuge and collecting the lysate in a polyethylene garbage can. Alternatively, the cell debris was removed and the lysate was placed in a cold room so that the concentration step could be begun as soon as the results of the titer were obtained. All subsquent operations were carried out in a cold room (4 °C) or on ice.

The polyethylene glycol-dextran sulfate method of phase separation as described by Espejo and Canelo (1968) was used with slight modification for concentration of PM2 lysates. Final concentrations of the polymers on a weight/water, respectively, and the polymers were dissolved in the lysate.

The dextran sulfate rich phase, which appeared after 20 h of settling, was pooled with the phase appearing after a further 48 h of settling. The interphase was collected as described by Espejo and Canelo (1968) and the dextran sulfate was then removed as described by Le Pecq (1971). The virus suspension was dialyzed overnight against 1.0 M NaCl, 0.01 M $CaCl_2$, 0.02 M Tris,[1] pH 7.1 (NTC), at 25 °C. After dialysis 0.359 g of optical grade CsCl per gram of dialyzed phage solution

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; SSC, standard saline citrate.

was dissolved in the pooled dialysate and the phage–CsCl mixture was then banded to equilibrium by centrifuging for 24 h at 45 000 rpm in the Spinco 50Ti rotor. The phage band from each tube was collected using a Pasteur pipet.

The bacteriophage may be stored indefinitely in CsCl. However, we have found the DNA in some preparations to become rapidly nicked, so that the DNA should be prepared from the phage as soon as possible.

PM2 DNA Isolation. In a typical preparation, a suspension of PM2 equivalent to 50 A_{260}/mL is dialyzed against two changes of 0.1 M NaCl, 0.01 M Na_2EDTA, 0.1 M Tris, pH 8.0, at 25 °C (SCTE). After dialysis, the suspension is made 2% in Sarkosyl by adding 1 volume of 20% Sarkosyl–SCTE for each 9 volumes of suspension. The mixture is then heated at 60 °C for 10 min and cooled to 37 °C, and a 1% pronase stock added to a final concentration of 1 mg/ml. The Pronase stock is made by dissolving Pronase in 0.1 M NaCl, 0.01 M Na_2EDTA, 0.1% Sarkosyl, 0.1 M Tris, pH 8.0, heating at 80 °C for 10 min, and predigesting for 2 h at 37 °C. The DNA mixture containing Pronase is incubated at 37 °C for 4 h to complete deproteinization. After the incubation, 6.7 g of CsCl is combined in a polyallomer tube for the Spinco 50Ti rotor with (a) 1.25 mL of digestion mixture, (b) 1 mL of ethidium bromide (2.8 mg/mL of 2% Sarkosyl–SCTE), and (c) 2% Sarkosyl–SCTE to bring the weight of liquid to 7.25 g. The CsCl is dissolved by sealing the tube with Parafilm and inverting; the tube is then capped, filled completely with mineral oil, and sealed. The filled tubes are then centrifuged at 37 000 rpm for the 36–48 h required to form the CsCl gradient. The double-strand covalently closed DNA is then collected and the ethidium bromide is removed from the DNA by repeated extraction with water-saturated isoamyl alcohol. After all visible color disappears, the DNA solutions are extracted six more times and then dialyzed against at least three changes of 0.15 M NaCl, 0.015 M trisodium citrate (SSC) to remove CsCl and isoamyl alcohol. The DNA is routinely stored in SSC at 4 °C.

Preparation of Endonuclease R *Hae* III from *Haemophilus aegyptius*. *Haemophilus aegyptius* ATCC # 11116 is readily cultured in well-aerated brain–heart infusion broth (BHI, Difco) supplemented with 2 μg of NAD/mL and 10 μg of hemin/mL. Our stock culture and detailed instructions for growing the bacteria were the generous gift of Dr. Marshall H. Edgell and Dr. Clyde W. Hutchinson, III, University of North Carolina. The required cells were grown and harvested according to the following protocol: A 50-mL stock culture is maintained by transferring 0.5 mL of growing cells to 50 mL of fresh medium every 8 h. At midnight of the day before the large scale preparation, 1 L of supplemented BHI is innoculated with 10 mL of the stock culture and incubated at 37 °C with vigorous shaking. The following morning the 1-L stock is checked for contamination by examination with a phase contrast microscope and a rabbit blood agar plate streaked to serve as a further check. The entire 1 L volume is then emptied into 10 L of prewarmed medium in a Fermentation Design, Inc., fermentor. The medium is maintained at 37 °C and aerated at 12 L/min with Dow Corning Antifoam Y-30 added to control foaming. When the cells have grown to midlog, 1 L of the suspension is transferred to 10 L of fresh prewarmed medium, which is then maintained at 37 °C and aerated as above. The remaining suspension is immediately harvested by passing the uncooled medium through a Sharples continuous-flow centrifuge; the freshly innoculated fermentor is harvested when the cells have again grown to midlog. After each harvest, the cells were scraped from the centrifuge bowl, immediately frozen in liquid nitrogen, and stored at −70 °C. The average yield for 21 L under these conditions was 28 g of cells. The above process was repeated until approximately 115 g of cells (wet weight) was accumulated to be carried through the following purification procedure.

Isolation of Endonuclease R *Hae* III. The procedure used was kindly provided by Dr. John Newbold and is a modification of that described by Huang et al. to prepare "endonuclease Z", the same activity now called *Hae* III (Huang et al., 1973).

The isolated *Haemophilus aegyptius* cells were thawed and then suspended in 200 mL of 0.001 M dithiothreitol, 0.025 M Tris, pH 7.9, at 25 °C. The cells were disrupted by sonicating in the cold at full power for 5 min, turning off the power and monitoring the A_{650} of a 1:200 dilution of the cells, and then resuming sonication after 5 min. As the sonication proceeds, the A_{650} drops to a plateau in about 30 min, indicating that all of the cells have been disrupted. The sonicated suspension was then centrifuged for 2 h at 10 000 rpm in the Sorval GSA rotor to remove the cell debris. The supernatant was then decanted and the absorbance of diluted aliquots was read at 230, 260, and 280 nm. This procedure provides a crude estimate of the protein and nucleic acid in the solution. A fresh solution of streptomycin sulfate (10%) was then added slowly with stirring to precipitate the DNA, which interferes with the enzyme isolation; about 0.2 mL/mL of supernatant was required. The solution was allowed to stir overnight in the cold. The precipitate was removed by centrifuging for 10 min at 15 000 rpm in the SS34 rotor and decanting the supernatant. The supernatant was then made 50% saturated in ammonium sulfate by adding 31.3 g of solid $(NH_4)_2SO_4$/100 mL of supernatant to the solution with stirring in the cold room. Stirring was continued for 15 min and the precipitate was collected by centrifuging for 15 min at 15 000 rpm. The supernatant was decanted and then made to 70% saturation in ammonium sulfate by adding 15.9 g of $(NH_4)_2SO_4$ per 100 mL of the decanted supernatant. The supernatant was decanted and discarded. The pellet was then redissolved in cold 0.01 M phosphate buffer, pH 7.2, containing 0.001 M Na_2EDTA, 0.001 M β-mercaptoethanol, 5% glycerol (GPB). The extract was then desalted by exclusion chromatography on a 2.5 × 50 cm Sephadex G75 column previously equilibrated with GPB. The brown salt-free extract emerges from the column at the void volume: all fractions containing color and free of ammonium sulfate were pooled and protein was determined by the Lowry method (Lowry et al., 1951). A column of freshly prepared phosphocellulose was then packed so that there was 1 mL of phosphocellulose for each 100 mg of protein. The packing was done in 0.05 M Tris, pH 7.9, and the column then washed with 20 bed volumes of GPB. The pooled extract was then passed through the column at 10–15 mL/h. Four bed volumes of GPB following the extract removed the colored material and unbound protein; the bound protein was then eluted stepwise with 2 bed-volume portions of GPB, which were successively 0.1, 0.2, 0.3, 0.5, and 1.0 M in NaCl. Fractions corresponding to 0.5 bed volume were collected and assayed for *Hae* III activity by incubating 5 μL of each fraction with 2 μg of PM2 DNA (see below). The restriction fragments were detected by electrophoresis in 1.5% agarose–ethidium bromide gels (see below). Aliquots taken from the fractions containing the bulk of the restriction activity completely digested the PM2 DNA in 30 min at 37 °C. A complete digest yielded a set of 17 fragments (A-P, Figure 1), with no material remaining at the origin. The active fractions were preserved by adding 1 mL of sterile glycerol to 1-mL aliquots of the fractions, mixing and storing at −20 °C.

Digestion of PM2 DNA with *Hae* III. PM2 DNA was precipitated from SSC by adding 2 volumes of cold absolute ethanol, mixing, and chilling at −20 °C for 4 h or overnight. The DNA was pelleted by centrifugation and the drained pellet was redissolved in 0.15 M NaCl, 0.05 M Tris, pH 7.4, at 25 °C (TBS).

Assays for *Hae* III activity were carried out with a solution which was 40 μg of DNA/mL of TBS. To each 50-μL aliquot was added 5 μL of 0.5 M β-mercaptoethanol and 5 μL of 0.13 M $MgCl_2$. Five microliters of the fraction to be assayed was added to initiate the reaction and each reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 50 μL of 0.08 M Na_2EDTA, 20% glycerol. The samples may then be layered directly onto the gel.

Large-scale digestions were carried out with a solution of 1 mg of DNA/mL of TBS. To each mL of DNA solution, 0.1 mL of 0.5 M β-mercaptoethanol and 0.2 mL of 0.13 M $MgCl_2$ was added. The reaction was initiated by adding 0.05 mL of enzyme for each mg of DNA and incubating at 37 °C. Two more 0.05-mL portions of enzyme were added at 2 and 4 h from the beginning of the digestion. At 6 h, the digestion was stopped by adding Na_2EDTA to 0.05 M. The solution was then extracted three times with 24:1 chloroform–isoamyl alcohol (v/v) and the DNA either then precipitated with 2 volumes of ethanol or dialyzed against 0.02 M NaOAc, 0.001 M Na_2EDTA, 0.04 M Tris, pH 7.2, at 25 °C (buffer E) and precipitated. The DNA pellets were redissolved in 1/10 buffer E plus 10% glycerol at concentrations of 1–5 mg of DNA/mL prior to layering onto the preparative gel.

Preparative Gel Electrophoresis. The PM2 *Hae* III fragments were separated by electrophoresis in acrylamide gels. The preparative gel apparatus described by Hagen and Young (1974), which is currently available from Savant Instruments, Inc., Hicksville, N.Y. 11801, was used for all preparative electrophoresis. The effluent from the apparatus was continuously monitored at 254 nm with an Altex, Model 153, column monitor equipped with the 100-μL biochemical flow cell.

In order to obtain the most efficient separation, several combinations of gel, elution flow rates, and DNA loadings were tried; details are given elsewhere (Kovacic, 1976).

A typical preparative electrophoresis run was carried out as follows: About 250 μg of PM2 *Hae* III digest in 200 μL of 1/10 E buffer is applied to the top of a 4-cm 4% acrylamide (40:1 ratio of acrylamide–biscrylamide) gel (see below). Electrophoresis is carried out at 16 mA and ~40 V, and is complete in 20 h; fragments A, B, and C are well resolved; the groups of fragments D–H, I–K, and L–P are well resolved from each other; and H, K, and P may be recovered in a pure state if the fractions are well resolved. For high resolution electrophoresis, a 12 × 1.6 cm 3.5% acrylamide gel (20:1) is used. Pooled fragments from several low resolution runs are concentrated on hydroxylapatite (Bio-Rad, DNA grade) and eluted with 0.4 M phosphate buffer (Britten et al., 1974). The eluate is dialyzed against E buffer and the DNA is collected by mixing with 3 volumes of cold ethanol, precipitating at −20 °C overnight, and centrifuging at 12 500 rpm in the Sorval SS34 rotor for 0.5 h. The pellet is redissolved in 200 μL of 0.1 buffer E, 10% glycerol, and layered on top of the gel. Electrophoresis is initiated at 50–75 V and 15 mA, and is continued for 24–48 h. Yields are 80–90% of the applied absorbance but may vary depending on the exact loading. The limiting factor seems to be the amount of DNA per peak rather than the size of the DNA in the peak. If the DNA concentration is too high, the peak broadens and trails so that both the yield and resolution are reduced.

Preparation of Other DNA Restriction Fragments. Restriction endonuclease fragments of SV-40 DNA and λ DNA were also prepared for use in calibrating the size of PM2 *Hae* III fragments. The SV-40 DNA samples were gifts from Drs. Gary Ketner and John Newbold. The λ DNA was kindly provided by Dr. Lyle Brown. *Hind* endonuclease was purchased from New England Biochemical. Conditions for *Hae* III digestion of SV-40 and λ DNA were as described for PM2 DNA. Conditions for *Hind* digests were as given by Fiers et al. (1975).

Sedimentation Analysis. Conditions for Runs. All measurements were made on the Model E ultracentrifuge using scanner optics. The accuracy of the scanner response was checked by centrifuging nucleotide solutions of known absorbance. Sedimentation velocity measurements were obtained from conventional boundary sedimentation experiments in 12- or 30-mm double-sector cells at DNA concentrations of 5–25 μg/mL. In most cases, 12 or more scans were used for each determination. All sedimentation velocity measurements were carried out in BPES buffer (see below) at 48 000 rpm. In this buffer (ionic strength 0.202), DNA concentration had no effect on the sedimentation coefficient values obtained for fragment H (498 bp) over a concentration range from 5 to 25 μg/mL. Because concentration effects are more pronounced for large DNAs, further tests were made with fragment A (1735 bp), which was sedimented at concentrations of 7.5 and 15 μg/mL. No significant effect (>1% difference) was found. It was not necessary to exceed 15 μg/mL because 30-mm double-sector cells and the scanner were available. Therefore, the remaining velocity measurements were performed at or below 15 μg/mL in 30-mm cells and no extrapolation to zero DNA concentration was made.

To test for the speed dependence of the sedimentation coefficient, fragment D (854 bp) at a concentration of 5 μg/mL was sedimented at 48 000 rpm and at 34 000 rpm. There was no significant difference between the sedimentation coefficient obtained from these two runs (difference on the order of 1%). Because the speed dependence of the sedimentation coefficient increases as a function of both DNA size and concentration, fragment A was also tested as above at a concentration of 15 μg/mL. The two values obtained deviated from each other by less than 1%; they were also the extremes of the deviations observed. In any event, the speed dependence for the sedimentation coefficient of the DNA samples studied here at concentrations below 15 μg/mL seems negligible.

Corrections to Standard Conditions. All sedimentation velocity measurements were made at 20 °C in order to eliminate the temperature correction in subsequent calculations. The RTIC of the ultracentrifuge was checked by observation of the melting of diphenyl ether, according to the method of Gropper and Boyd (1965). All of the velocity measurements were made in 0.179 M NaCl, 0.001 M Na_2EDTA, 0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4 (BPES), which has a sodium ion concentration of 0.195 M and an ionic strength of 0.202. We have used the Cohen and Eisenberg (1968) values of $(\partial\rho/\partial C_2)^0_\mu$ to define the quantity $1 - \bar{v}\rho$ in eq 1. For Na DNA in dialysis equilibrium with 0.2 M NaCl, $(\partial\rho/\partial C_2)^0_\mu = 0.457$. According to Svedberg and Pederson (1940), $\eta_{20,b}/\eta_{20,w} = 1.017$, $\Delta\rho = \rho_{20,b} - \rho_0 = 0.0083$ g/mL and $\rho_0 = 0.9982$ g/mL for this solution. The correction of the s value is therefore easily made:

$$s_{20,w} = (\eta_{20,b}/\eta_{20,w})\{(1 - \bar{v}\rho)_{20,w}/(1 - \bar{v}\rho)_{20,b}\}s_{20,b} \quad (2)$$

$$s_{20,w} = 1.027\, s_{20,b} \quad (3)$$

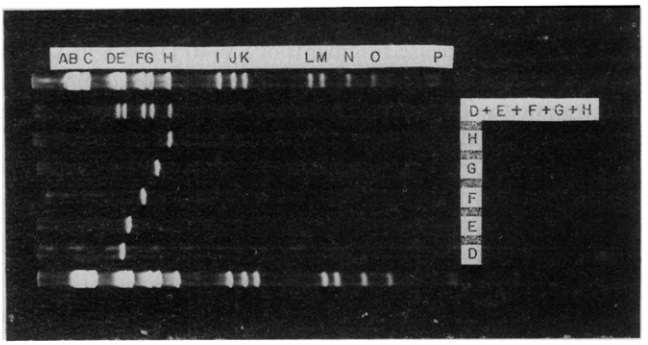

FIGURE 1: Gel electrophoresis of whole *Hae* III digests of PM2 DNA and some isolated fragments. The top and bottom gels represent complete digests, with individual fragments indicated by letters. The five gels with individual bands represent individual isolated fragments. The remaining gel contained a mixture of five isolated fragments. Electrophoresis is in 3.5% polyacrylamide (20:1) and the gel has been stained with ethidium bromide.

Since, as shown above, no significant dependence of $s_{20,w}$ on DNA concentration or rotor speed was observed, the values for the separated PM2 *Hae* III fragments are therefore reported as $s^0_{20,w}$ values.

Computer-Aided Calculations. Analysis of sedimentation velocity and sedimentation equilibrium data was greatly aided by the Fortran program SVEDCOF written by Dr. Robert D. Dyson, Oregon State University, for the CDC 3300 computer in the OS-3 time-sharing system. The program accounts for the difference in time at which the scanner encounters the boundary in successive scans, plots the relationship between log r and t, calculates the least-squares fit for the points, and calculates the sedimentation coefficient from the slope. Viscosity and temperature corrections are then applied to yield the $s_{20,w}$ value. A version of this program allows the insertion of the measured values for the distance from the center of the rotor to the outer reference hole and the distance from the inner hole to the outer reference hole, rather than the nominal values incorporated into the original version. All plots were performed on a Hewlett Packard 7200 Graphic plotter when using OS-3 to analyze data.

Extensive use was also made of a Hewlett Packard 9821A programmable calculator equipped with the following peripherals: the 9864A digitizer, the 2748 B paper-tape reader, a 9864A plotter, and a standard Teletype. Several programs were written to plot and analyze gel electrophoresis data. Separate programs allowed us to calculate and plot the theoretical relationship between $s^0_{20,w}$ and M predicted by the Hearst–Stockmayer and the Yamakawa–Fujii theories for any choice of λ^{-1} and the helix diameter.

Analytical Gel Electrophoresis. Acrylamide Gels. The methods of Loening (1967) were followed, except that the pH of buffer E was 7.2 at 25 °C. A detailed description is given elsewhere (Kovacic, 1976). The percentage of acrylamide was varied over a range of 3.0–6.0% by increasing or decreasing the amount of a stock solution of acrylamide monomer and bisacrylamide, which was added to the other constituents. In some cases, the bisacrylamide–acrylamide ratio was lowered from 1:20 to 1:40, and 4–6% gels were cast. Gels were routinely formed in 0.7 × 30 cm Plexiglas tubes sealed at the bottom with several layers of Parafilm. After at least 1 h of polymerization time, the Parafilm was replaced with nylon mesh secured with a rubber band and the gels preelectrophoresed at 80 V for a minimum of 2 h. Samples in 25–100 μL of buffer made 10% in glycerol were layered onto the gel top and electrophoresed for 4–8 h at 125 V. Following electrophoresis, the gels were extruded from the tubes with compressed air and stained in 1.0 μg of ethidium bromide/mL of buffer E. After staining for 1–3 h, the DNA could be visualized and photographed by the orange fluorescence of the DNA–ethidium bromide complex under ultraviolet light. Gels were usually photographed through both an ultraviolet haze filter (Tiffen Photar Haze 2-A) and an orange filter (Tiffen Photar Orange) with a Polaroid MP-3 Land camera using Type 55 or 107 film.

Agarose Gels. Electrophoresis grade agarose (Bio-Rad or Sigma) was dissolved in buffer E by autoclaving for 10 min. After autoclaving, the solution was mixed and then allowed to solidify. For casting analytical gels, this solidified agarose was remelted by autoclaving for 10 min, and the liquid allowed to cool to 50–60 °C. The warm agarose solution was pipetted into prepared tubes and allowed to solidify at room temperature for 30 min. To obtain a flat surface to "stack" the sample, the top of the tube was netted, the tube inverted, and the gel seated firmly against the netting. No preelectrophoresis was needed; the samples were applied as above and electrophoresed for 2–6 h at 125 V and ~5 mA/tube. The gels could then be extruded, stained, and photographed as above.

Agarose–Ethidium Bromide Gels. Alternatively, 0.5 μg of ethidium bromide/mL of agarose solution was added when the melted gel reached 60 °C (Sharp et al., 1973); the gels were then cast as described for agarose gels. Running buffer was 0.4 μg of ethidium bromide/mL of buffer E.

Results

PM2-Hae III Fragments. Figure 1 shows the PM2 *Hae* III fragments A–P separated by analytical electrophoresis in 3.5% acrylamide gels (20:1). Figure 1 also illustrates the resolution of bands D through H by preparative electrophoresis. In this experiment, the separated bands were run in individual tubes to determine if the separation is complete. To further verify the identity of the fragments, aliquots of each of the five separated bands were mixed together. From these gels, it can be seen that there is no visible sign of cross-contamination and that the fragments have been properly identified.

Figure 2A is a scan of a gel containing the complete PM2 *Hae* III digest which has been stained with Toluidine Blue 0 and scanned at 546 nm using a Gilson gel scanner. The stain is nearly quantitative; increasing the loading of DNA proportionately increases the area under each peak. From this scan, it can be seen that peaks A through O represent unique fragments, since the relative area under each peak is proportional to the estimated size of each fragment (see calibration of molecular weights by gel electrophoresis). Peak P contains about twice the area expected and therefore must consist of at least two unique fragments. Electrophoresis of the mixture in 6% acrylamide gels (40:1) resolves P into two distinct bands (not shown). It has not been possible to resolve P into two components by preparative gel electrophoresis, although all the other bands may be resolved from each other. Therefore, P is the only "fragment" which is not unique in both size and base sequence.

Figure 2B is a scan of a formamide gel containing the complete PM2 *Hae* III digest stained with Toluidine Blue. The procedures of Staynov et al. (1972), as modified by Boedtker et al. (1973) were used in preparing this gel. The DNA has been denatured by boiling, and runs as single-strand fragments. Therefore, this procedure should detect single-stranded nicks in the double-strand fragments if they are present. The result (Figure 2B) shows that the PM2 *Hae* III digest must be largely

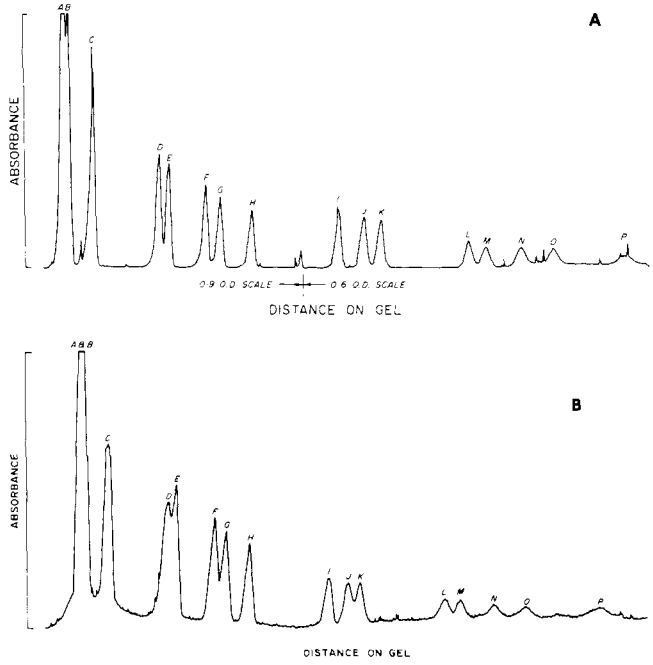

FIGURE 2: (A) A scan of a polyacrylamide gel containing the complete PM2 *Hae* III digest which has been stained with Toluidine Blue. It was scanned at 546 nm. In this gel, the fragments are double strand. (B) A scan of the whole PM2 digest electrophoresed under denaturing conditions, according to the method of Staynov et al. (1972), as modified by Boedtker et al. (1973). The fragments here are single strand.

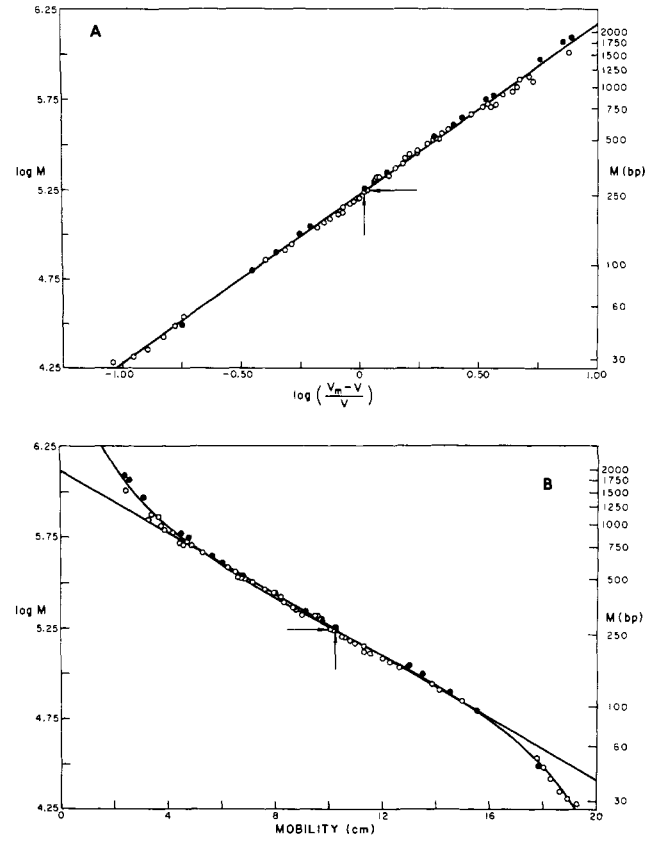

FIGURE 3: Electrophoresis calibration data used to establish the sizes of the PM2 *Hae* III fragments. Electrophoresis was on a 3% polyacrylamide (20:1) slab gel. All of the sets of DNA fragments listed in Table I are shown here. The PM2 *Hae* III fragment sizes given by Noll (1976) are shown as filled circles. The arrows point to the position of SV40 *Hind* H, a sequenced fragment. (A) Data graphed according to eq 4. (B) Data graphed in the conventional manner. The sigmoidal curve corresponds to the straight line in A; the straight line in B is the best "linear" fit. The sizes we have adopted are taken from migration of PM2 *Hae* III fragments on the same gel, using the sigmoidal fit to the whole data set.

unnicked, since the same apparent size distribution is seen. The erratic baseline, which is a usual problem with formamide gels, prevents one from detecting small amounts of interband DNA which would represent nicked strands. Baseline difficulties also make it difficult to precisely compare the area under the peaks with the amount of DNA applied. The areas usually check within 10% of that expected from the applied amounts, so that a conservative estimate would be that at least 90% of the DNA fragments are wholly unnicked.

Calibration of Molecular Weights by Gel Electrophoresis. The molecular weights of the PM2 *Hae* III fragments were determined by comparison, in gel electrophoresis, with other DNA fragments of known size. With the availability of a number of sets of restriction endonuclease fragments, including some of known sequence, this is becoming a highly accurate method for the measurement of DNA molecular weight.

The principal danger in this technique appears to be the occasional observation of anomalous mobilities (Maniatis et al., 1975), which may be related to base-compositional effects (Zeiger et al., 1972). However, in the most careful study of this kind (Maniatis et al., 1975), only a few samples behaved anomalously, and even then the maximum error in molecular weight was about 10%. Since several gel systems have been used in our experiments, with consistent results, and the sedimentation coefficient data show no anomalous points (see below), we feel that such artifacts are unlikely in these calibrations.

A second problem in the application of gel electrophoresis to such a calibration is selection of the best representation for the relationship between mobility and molecular weight. It has become traditional to utilize graphs of log M vs. mobility (V), with the understanding that there usually will be found significant deviations from this linear relationship at high and low molecular weights. We have found an empirical relationship that extends the range of linearity of the M–V relationship:

$$\log M = m \log \left\{ \frac{V_m - V}{V} \right\} + b \qquad (4)$$

where V_m is a "maximum mobility" chosen to yield the best fit to the data. This representation (which we call the sigmoidal fit) will be discussed in detail elsewhere (Kovacic, Lohr, and Van Holde, in preparation). At the present, we use it only as an empirical aid to calibration of the molecular weight set.

Figure 3A,B graphically display the results of the calibration of the PM2 *Hae* III digest by comparison with four other restriction digests in the 3.0% acrylamide slab gel. The sigmoid fit includes equally weighted data from SV40 *Hae* III, SV40 *Hind*, SV40 *Hind–Hae* III, λ *Hind*, and PM2 *Hae* III digests. The molecular weights of the PM2 *Hae* III fragments are from an independent calibration carried out by Noll (1976). For identification, the PM2 *Hae* III data with the Noll molecular weights are indicated by closed circles. The data for the SV40 *Hae* III, SV40 *Hind*, and the SV40 *Hind–Hae* III restriction fragments are those reported by Yang et al. (1976), assuming the size of SV40 DNA to be 5000 bp. SV40 *Hind* H has been sequenced by Fiers et al. (1975). Although originally reported to be 277 bp plus a four-base single-stranded end, the size was recently revised to be 266 bp plus the four-base "tail". The mobility and molecular weight of this sequenced fragment are indicated in Figure 3. The sizes of the λ *Hind* fragments are from Maniatis et al. (1975). The straight line in Figure 3B is

TABLE I: Sizes of PM2 *Hae* III Fragments from Sigmoidal and Linear Fits to the Data for Five Restriction Digests Coelectrophoresed in a 3.0% Acrylamide (20:1) Slab Gel.

	Standard Sizes[a]					Predicted PM2 *Hae* III Sizes	
Fragment	SV40 *Hae* III[b]	SV40 *Hind*[b]	SV40 *Hind*–*Hae* III[b]	λ *Hind*[c]	PM2 *Hae* III[d]	Sigmoidal[e]	Linear[f]
A	1535	1057	770	1125	1860	1735	1236
B	787	768	420	1090	1760	1606	1193
C	512	508	350	980	1410	1310	1075
D	350	505	314	930	890	854	817
E	335	425	305	900	845	794	773
F	315	420	265	800	672	642	650
G	305	350	260	700	615	592	606
H	305	260	240	580	525	498	516
I	215	240	215	550	333	322	330
J	164	223	184	520	295	288	292
K		195	176	500	272	263	265
L			164	480	167	160	155
M			133	440	152	145	141
N			123	400	120	117	114
O			108	375	95	94	94
P			52	340	47	50	60
Q			46	320	10 058	9 470	8 317
R			40	230			
S			34	200			
T			31				
U			29				

[a] Sizes are in bp; 1 bp = 663 daltons. [b] Sizes reported by Yang et al. (1976) for SV40 DNA of 5000 bp. [c] Sizes reported by Maniatis et al. (1975). [d] Sizes reported by Noll (1976). [e] Calculated from the mobility, V, and the Vm from the equation $\log M = m \log (Vm - V/V) + b$. Vm is 21.02 cm, m is 0.94412, and b is 5.2208. M is in daltons. [f] Calculated from the mobility, V, and the equation $\log M = mV + b$. V is in cm and M is in daltons. The slope, m, is −0.08526 and b is 6.1181.

a least-squares linear fit to the data for which the mobility ranges between 4.5 and 16 cm.

Table I lists the molecular weights of all the fragments used in the calibration graphed in Figure 3. In addition, the PM2 *Hae* III sizes calculated from both the sigmoidal and the linear fits are listed. The equations for each curve are also given. Both fits predict a size of 263 bp for SV40 *Hind* H, which is in very good agreement with the sequenced size. The PM2 DNA size predicted by the sigmoid fit is also in quite good agreement with the reported molecular weight for PM2 DNA (Kriegstein and Hogness, 1974). The linear fit gives substantially the same sizes over the range of mobility for which the log M vs. mobility plot seems linear, but the sigmoidal fit performs better in both the high- and low-molecular-weight range. Therefore, the PM2 *Hae* III sizes predicted by the sigmoidal fit will be used in the examination of the relationship between the $s^0_{20,w}$ and M which follows.

Sedimentation Velocity Results. The average $s^0_{20,w}$ for each of the PM2 *Hae* III fragments is given in Table II. In addition, the individual $s^0_{20,w}$ values which make up the average for each fragment are listed. In many cases, a number of repeat determinations have been made. The scatter of the data seems independent of the concentration or rotor speed. The maximum deviation from the mean for any sample was 2.0%. The standard deviations average about 1%, which seems to be of the order of precision expected with scanner optics. The molecular weight of each fragment, as determined in the manner described above, is also included in Table II. This table summarizes the empirical relationship between the $s^0_{20,w}$ and molecular weight over the transition from rigid rod to stiff coil behavior for double-strand DNA molecules. The data in Table II will be used in all the discussion which follows.

There have been a number of theoretical analyses of the frictional coefficient (and hence sedimentation coefficient) for rodlike molecules. In general, for a rod of length L and a diameter d, the results take the form:

$$\frac{1}{f^0_{20,w}} = \left(\frac{1}{3\pi\eta_0 L}\right)\left(\ln\frac{L}{d} + \gamma\right) \tag{5}$$

or using eq 1

$$s^0_{20,w} = \left(\frac{M(1-\bar{v}\rho)}{N_A 3\pi\eta_0 L}\right)\left(\ln\frac{L}{d} + \gamma\right)$$

$$= \left(\frac{M_L(1-\bar{v}\rho)}{N_A 3\pi\eta_0}\right)\left(\ln\frac{L}{d} + \gamma\right) \tag{6}$$

where M_L is molecular mass per unit length. While most calculations of the frictional coefficient for rodlike molecules yield this form, the value of γ differs from one theory to another. Values given in the literature include 0.577 (Kirkwood, 1954; Yamakawa and Tanaka, 1972) for a linear string of beads, and values of 0.58 (Burgers, 1938), 0.38 (Broersma, 1960), 0.392 (Bloomfield et al., 1967), and 0.3863 (Yamakawa and Fujii, 1973) for right circular cylinders. The most accurate computations are probably the last three, all of which give values of $\gamma \simeq 0.39$.

Since, for a series of rodlike DNA molecules L should be proportional to M, eq 6 predicts a linear relationship between $s^0_{20,w}$ and ln M. In Figure 4 are shown the experimental data from Table II, graphed vs. log M. As can be seen, the data asymptotically approach a straight line at low M. This straight line corresponds to a value of d = 27 Å. In fact, DNA molecules smaller than about 200 base pairs appear to behave as rigid rods in solution. As will be seen below, the parameters

TABLE II: Molecular Weight and Average $s^0_{20,w}$ for PM2 *Hae* III Fragments.

Fragment	Mol Wt (bp)	Av $s^0_{20,w}$	Individual
A	1735	10.78	10.81, 10.78, 10.75, 10.82, 10.73, 10.74, 10.79, 10.78
B	1606	10.67	10.66, 10.66, 10.63, 10.84, 10.76, 10.56, 10.63, 10.63
C	1310	9.99	9.97, 9.96, 9.99, 10.06, 10.05, 10.09, 9.87, 9.90
D	854	8.72	8.75, 8.69
E	794	8.59	8.52, 8.65
F	642	8.03	7.97, 8.09
G	592	7.89	7.87, 7.88, 7.93
H	498	7.62	7.63, 7.60, 7.63, 7.61
I	322	6.70	6.70
J	288	6.37	6.37
K	263	6.24	6.24
L	160	5.41	5.38, 5.44
M	145	5.20	5.20
N	117	4.92	4.92
O	94	4.58	4.58
P	50	3.51	3.58, 3.44

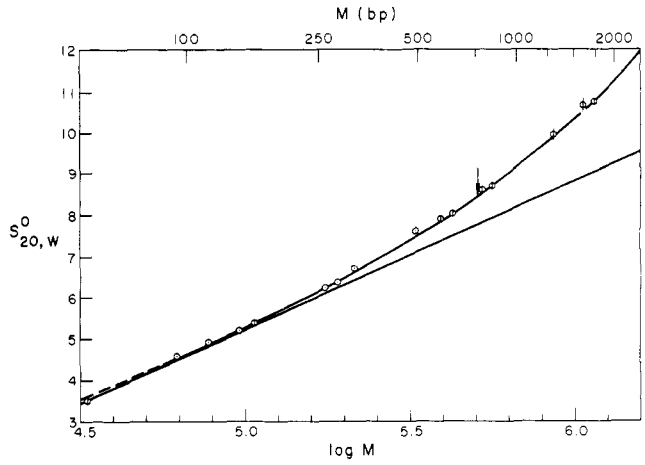

FIGURE 4: A graph of $s^0_{20,w}$ vs. log M for the PM2 *Hae* III fragments. Points for which multiple determinations of sedimentation coefficient have been carried out have vertical bars. The length of the bar represents the extreme range of results. The straight line corresponds to the sedimentation coefficients predicted for 27 Å diameter rigid rods (see text). The solid curve represents the best fit from the Yamakawa-Fujii theory. The equation for this curve for $M < 5.11 \times 10^5$ (arrow) is: $s^0_{20,w} = 3.620$ (log $M - 3.552 + 3.23 \times 10^{-7}$ M $+ 1.63 \times 10^{-13}$ M$^2 - 7.85 \times 10^{-20}$ M^3), where the sedimentation coefficient is in svedbergs. Above that molecular weight, the appropriate expression is obtained from the high-molecular-weight form of the Yamakawa-Fujii expression. With the parameters used here, it is: $s^0_{20,w} = 6.117 \times 10^{-3}$ M$^{1/2} + 4.232 + 1.029 \times 10^2$ M$^{-1/2} - 1.12 \times 10^5$ M$^{-1} - 5.0 \times 10^6$ M$^{-3/2}$. The broken curve (which is indistinguishable from the solid curve except at low M) represents the best fit from the Hearst-Stockmayer theory.

used to generate this asymptotic relationship appear reasonable.

It is quite clear from Figure 4 that deviations from rigid-rod behavior are observable at fairly low molecular weights. In order to analyze the behavior in this transition region, in which neither rigid rod nor random coil models will suffice, we must turn to "wormlike chain" models for the stiff polymers. Such models are formulated in terms of a stiffness parameter, the Kuhn statistical length (λ^{-1}), which is related to the "persistence length" of Kratky and Pörod (1949), (the persistence length = $1/2\lambda$). For example, the "beaded string" theory of Hearst and Stockmayer (1962) yields, for polymers of contour length L less than 2.2, the result

$$s^0_{20,w} = \left(\frac{M_L(1-\bar{v}\rho)}{N_A\, 3\pi\eta_0}\right)\left(\ln\frac{L}{d} + 0.166\,L + 0.02\,L^2 - 0.002\,L^3 - 0.332\,d - 0.060\,d^2 + 0.008\,d^3 + d/L \cdots\right) \quad (7)$$

where L and d are in units of λ^{-1}. Another expression is used when $L > 2.2$; see Hearst and Stockmayer (1962).

The somewhat more realistic theory of Yamakawa and Fujii (1973), which treats the polymer as a smooth, stiff, wormlike chain, yields a quite similar result in the same size range:

$$s^0_{20,w} = \frac{M_L(1-\bar{v}\rho)}{N_A 3\pi\eta_0}\left(C_1 \ln\frac{L}{d} + C_2 + C_3L + C_4L^2 + C_5L^3\right) \quad (8)$$

where the quantities C_1–C_5 are very slightly dependent upon powers of d. (For the detailed expression and the expression applicable at larger L, see the original paper). While both represent $s^0_{20,w}$ in terms of ln L/d plus a power series, eq 7 and 8 differ principally in the absence of a constant term in eq 7 corresponding to the γ term in eq 6 and in the existence of a (generally small) d/L term in eq 7. Otherwise, the coefficients of corresponding powers of L are virtually identical in the two expressions. The coefficient C_1 in the Yamakawa-Fujii paper will be very close to unity in most cases, and the terms in powers of d in eq 7, as well as contributions of powers of d to the Yamakawa-Fujii coefficients, will usually be negligible. The absence of the C_2 term in the Hearst-Stockmayer theory can be traced to a replacement of summation by integration, which is inappropriate in the low-molecular-weight limit. As a consequence, while the Yamakawa-Fujii theory gives a limiting rigid-rod result identical with eq 6 with $\gamma = 0.39$, the Hearst-Stockmayer result gives $\gamma = 0$. The practical consequence of this is that the Hearst-Stockmayer equation will give too low a value for d, if d is treated as an adjustable parameter.

The fitting of the Hearst-Stockmayer and Yamakawa-Fujii theories to the experimental data is shown in Figure 4. In each case, we have chosen a value of $1 - \bar{v}\rho$ corresponding to the value of $(\partial\rho/\partial C)^0_\mu$ obtained by Cohen and Eisenberg (1968) under similar salt conditions. The mass per unit length of DNA (M_L) has been taken to be 195 daltons/Å (Yamakawa and Fujii, 1973). The two variable parameters (d and λ^{-1}) were adjusted until a best fit was obtained. The values for these parameters are: using the Yamakawa-Fujii theory, $d = 27$ Å, $\lambda^{-1} = 1150$ Å; using the Hearst-Stockmayer theory, $d = 20$ Å, $\lambda^{-1} = 1050$ Å. As Figure 4 shows, the "best fit" curves for the two theories are almost indistinguishable over the range covered, however, the parameters required are significantly different.

Discussion

Our measurements show that very small double-strand DNA molecules (<200 base pairs) behave almost exactly as predicted for rigid rods in sedimentation transport. The only adjustable parameter in eq 6 is the rod diameter d; the value obtained from the data is 27 Å, a reasonable result for hydrated

DNA. This same value for the rod diameter, together with a value of 1150 Å for λ^{-1} (a persistance length of 575 Å) allows us to fit the data by the Yamakawa–Fujii theory over the whole molecular-weight range we have studied. The whole data set can be fitted equally well by the Hearst–Stockmayer theory, but, in this case, the diameter is required to be 20 Å and the value of λ^{-1} is 1050 Å. We feel that this diameter value is unreasonably low for hydrated B-form DNA in solution. According to Arnott and Hukins (1972), the *center* of oxygen O_2 lies at a radius of 10.2 Å. Taking into account the van der Waals radii of the atoms involved and the presence of an appreciable hydration shell, a diameter of 20 Å seems low. As indicated in the preceding section, we would expect the Hearst–Stockmayer theory to yield too low a value for *d*. On the other hand, the value of 27 Å obtained from either rigid rod or Yamakawa–Fujii theory seems a bit high. In any event, to attach a *precise* meaning to the diameter calculated from such analyses is probably unjustified. It is satisfying, however, that the data are reproduced with *reasonable* physical parameters.

Our results are in good agreement with the data obtained by Prunell and Bernardi (1973) and Record et al. (1975) over the more limited molecular-weight ranges covered by these workers. The agreement of these three independent studies gives confidence in the use of $s^0_{20,w}$ to measure molecular weight for small DNA molecules.

The rigidity of low-molecular-weight DNA that this study establishes clearly indicates that kinking of DNA, in the manner proposed by Crick and Klug (1975), or Sobell et al. (1976), must be a rare event in aqueous salt solutions. This would indicate that if such kinking does occur frequently in structures, such as nucleosomes, it must occur primarily as a result of energetically favorable interaction with other substances (such as histones). Similarly, uniform bending of DNA fragments smaller than 200 base pairs (an alternative hypothesis for nucleosome structures) seems, likewise, very unfavorable for free DNA. As had been suspected, the compact forms of DNA found in structures like chromatin must be formed only at a considerable price in energy.

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