SEDIMENTATION COEFFICIENT AND FRAGILITY UNDER HYDRODYNAMIC SHEAR AS MEASURES OF MOLECULAR WEIGHT OF THE DNA OF PHAGE T5

A. D. HERSHEY, ELIZABETH BURGI, *and* LAURA INGRAHAM

From the Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, Long Island

ABSTRACT T5 DNA molecules resemble fragments of T2 DNA of molecular weight 84×10^6 with respect to sedimentation coefficient and susceptibility to breakage under hydrodynamic shear. The sedimentation coefficient falls by the same factor when either T2 or T5 DNA is broken at its characteristic critical shear rate. At a given high rate of shear, both DNA's are broken into fragments exhibiting the same sedimentation coefficient. It follows that 84×10^6 is a proper estimate of the molecular weight of T5 DNA, and that particles of phage T5, like those of T2, contain a single DNA molecule.

INTRODUCTION

Study of the breakage of T2 DNA by stirring made available a series of products of known molecular weights and physical properties (Burgi and Hershey, 1961; Rubenstein *et al.*, 1961). These materials should serve as standards of reference for other DNA's in the range of molecular weights between 25 and 130 million.

Matching another DNA to the appropriate T2 fragments by measuring any molecular weight–dependent property gives an estimate of the unknown molecular weight that is valid if structural differences between the two DNA's can be neglected. The latter condition is satisfied when matching by two or more dissimilar properties yields mutually consistent results.

In this paper we compare the DNA's of T2 and T5 and their fragmentation products with respect to sedimentation coefficient and fragility under hydrodynamic shear. The results show that the molecular weight of T5 DNA is 84×10^6 (relative to 130×10^6 for T2 DNA), and that the difference in molecular weight is sufficient to account for the differences in physical properties.

METHODS

DNA was prepared by shaking phage suspensions of optical density 15 cm^{-1}, measured at 260 mμ wave length, with aqueous phenol as described by Mandell and Hershey (1960). A heat stable mutant, T5 *st* (Adams and Lark, 1950), obtained from Dr. Y. T. Lanni, provided the source of T5 DNA.

For present purposes, DNA from T2 and T5 labeled with P^{32} at a specific activity of 1.0 c/gm of phosphorus was required. At this level radiation damage, recognizable by abnormal retention of DNA by the fractionating columns and failure of the eluted band to match that of admixed carrier, was encountered several times. The radioactive DNA was stable when diluted to 10 μg/ml or less (often 0.05 μg/ml) immediately after extraction. DNA purified by ion exchange chromatography proved especially prone to radiochemical damage, presumably because of removal of protective substances. To avoid this effect we often added 0.1 per cent ethanol to the solvents used for chromatography.

A model GT21 stirrer and thyratron controller (G. T. Heller Co., Las Vegas), with its flat, steel, clover-leaf stirring blade, 3.7 cm in diameter, attached to the faster motor shaft, was used to stir 50 ml of DNA solution in a fluted, conical flask. The controller was fed through a constant voltage transformer and modified by the addition of a fine adjustment potentiometer and a larger (0.025 μf) phase-shifting capacitor to improve the performance at low speeds. An electromechanical revolution counter, attached to the slower shaft of the motor, permitted continuous monitoring of stirring speeds. Drift in speed during a 30 minute period did not exceed 4 per cent at 300 RPM or 1 per cent at higher speeds. Reproducible geometry was achieved by means of a flask-holding clamp and platform.

Our experience with several stirrers suggests that a thin, flat blade produces a shear rate little affected by variations in the shape or size of the stirring vessel, presumably because the maximum shear is produced at the surface of the blade. With a propeller type of stirrer, on the other hand, minor changes in geometry greatly affect breakage of DNA. A related effect is brought out by the data presented in Fig. 1, which show that the dependence of effective shear rate on stirring speed is more critical when the solution is stirred by a glass rod than when it is stirred by a razor blade. For these practical reasons a flat stirring blade is to be preferred.

Since the two curves shown in Fig. 1 are not parallel, effective shear stress is not proportional to speed of stirring in one or both stirrers. For this reason the data are of doubtful theoretical interest (Levinthal and Davison, 1961). In any case they are pertinent to what follows chiefly because they illustrate the precision of the analytical methods.

Applications of chromatography to the measurement of breakage of DNA have been described by Hershey and Burgi (1960) and Burgi and Hershey (1961).

Sedimentation coefficients were measured at 35,600 RPM and 10 μg DNA/ml in 0.7 M NaCl as described by the authors last mentioned.

Salt solutions mentioned in this paper contain, in addition to the stated concentration of NaCl, 0.05 M sodium and potassium phosphates giving a final pH of 6.7.

RESULTS

Chromatography of T5 DNA. T5 DNA elutes from our fractionating column at a lower salt concentration than does either T2 DNA or its half length fragments (Fig. 2). The separation therefore depends on properties other than

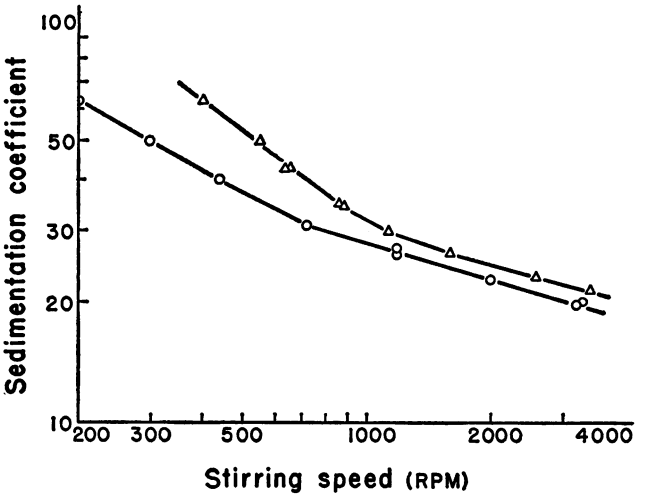

FIGURE 1 Relation between speed of stirring of T2 DNA and sedimentation coefficient of the products. Triangles, the glass rod stirrer and circles, the razor blade stirrer of Burgi and Hershey (1961). A single point on either curve was determined as follows. A 50 ml sample of P^{32}-labeled DNA at 0.05 μg/ml in 0.6 M NaCl was stirred at the indicated speed for 30 minutes at room temperature. The stirred sample was mixed with about 0.4 mg of appropriately broken unlabeled DNA, and the mixture was fractionated chromatographically. The sedimentation coefficient of the DNA eluting with the median weight fraction of the labeled fragments was then measured. Where two nearly identical results are shown on the curves, a second sample was analyzed after stirring for 60 minutes. Sedimentation coefficients measured in this way refer to DNA fragments of about 0.9 of the greatest unbreakable length characteristic of the stated speed of stirring (Burgi and Hershey, 1961).

The above description refers to fragments of sedimentation coefficient less than 50S. In addition, we have entered on each curve the sedimentation coefficient (63S) of unbroken DNA plotted against the highest speed of stirring that caused no breakage, and the sedimentation coefficient (50S) of the longest fragments unbreakable at the critical speed of stirring plotted against the speed giving 90 per cent breakage.

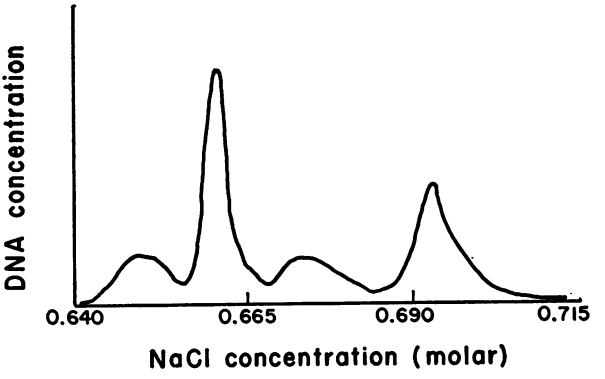

FIGURE 2 Chromatographic separation (left to right) of half length fragments and unbroken T5 DNA and of half length fragments and unbroken T2 DNA. Tracer amounts of a P^{32}-labeled mixture.

molecular weight, presumably the substitution in T2 of glucosylated hydroxymethylcytosine for cytosine and the somewhat greater content in adenine and thymine of that DNA (Wyatt and Cohen, 1953). Breakage of T5 DNA further lowers the salt concentration at which it elutes. The initial breakage products, which prove to be half length fragments, form a single band well separated from the unbroken DNA.

In the course of the present work we discovered that stirring at room temperature causes local denaturation of T5 DNA. Details concerning this phenomenon will be published separately. The effect pertinent here is to cause about half of the DNA that has been stirred at its critical speed to fail to elute from a fractionating column. However, the denaturation proves to be independent of the breakage, and measurement of breakage in the fraction of the DNA eluting from the column is not appreciably biased by selective retention of broken or unbroken molecules. Moreover, by stirring at high salt concentration or at low temperature, breakage can be accomplished without concomitant denaturation. In the more critical experiments described below denaturation was avoided by stirring at 5°C or in 2.6 M NaCl. When necessary, the solutions were diluted with buffered water after stirring to obtain the proper salt concentration for application to the columns.

Molecular Weights from Sedimentation Coefficients. Twelve measurements utilizing three preparations of T5 DNA, tested both before and after purification by chromatography, yielded sedimentation coefficients of 48.5 ± 1.3S (expressed as mean and standard deviation of the several measurements). According to the empirical relation proposed by Rubenstein *et al.* (1961), the stated sedimentation coefficient calls for a molecular weight of 82 ± 5 million.

Ten measurements of half length fragments, identified chromatographically among the products of initial breakage of T5 DNA by stirring (Burgi and Hershey, 1961), yielded sedimentation coefficients of 32.8 ± 1.4S. These data were obtained with two preparations stirred at room temperature in 2.6 M NaCl. and one stirred in 0.6 M NaCl. There was no sign that the excessive retention of DNA by the column in the latter case biased the result. The half molecular weight indicated by these measurements is 40 ± 3 million.

Since the molecular weights cited conform to the expectation that initial breaks should produce half length fragments, one can infer that they are correct at least in relation to each other, and that the ratio between sedimentation coefficients for whole molecules and half length fragments is the same whether measured for DNA's from T2 or T5.

Critical Stirring Speed. The DNA of T5 is considerably more resistant to breakage by stirring than is the DNA of T2. At effectively infinite dilution, stirring for 30 minutes at 640 RPM is required for 50 per cent breakage of T5 DNA, whereas 430 RPM suffices for T2 DNA under the same conditions (Fig. 3). These results show that the sedimentation coefficients are correlated with susceptibility to breakage under shear, as expected if both are molecular weight-dependent properties.

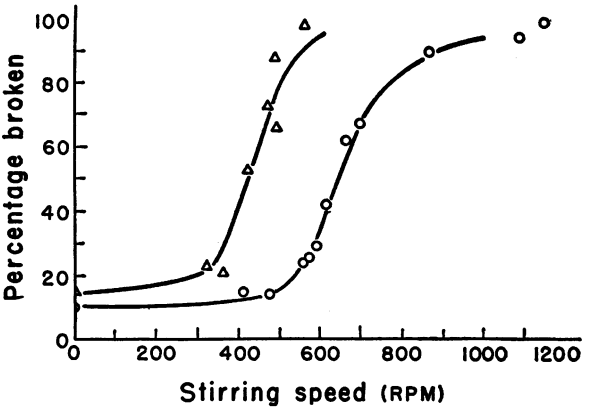

FIGURE 3 Critical stirring speeds. Triangles, T2 DNA. Circles, T5 DNA. DNA concentration, 0.05 μg/ml; stirring time 30 minutes; solvent, 0.6 M NaCl at room temperature; stirrer described in this paper; breakage measured chromatographically.

Relation between Speed of Stirring and Sedimentation Coefficient of the Resulting Molecular Fragments. If solutions of T2 DNA and T5 DNA are separately stirred at a given speed sufficient to break either into fragments, one expects in general that the sedimentation coefficients of the two products will match only if two propositions are valid; namely, that the fragility per unit length of the two DNA's is the same, and that the relation between sedimentation coefficient and molecular weight is the same for both. The appropriate experiments, described in Fig. 4, show that the two DNA's yield similar products when stirred at any of several speeds. We conclude that the DNA's of T2 and T5 exhibit similar intrinsic fragilities and that the molecular weights of T5 DNA and its longer fragments can be measured without large errors from their sedimentation coefficients and from the calibration established with T2 DNA.

The data of Fig. 4 also reinforce an earlier conclusion (Burgi and Hershey, 1961) that the DNA of T2 does not show signs of a few weak linkages, which might be expected to reveal themselves as a discontinuity in the plot. Rather, it seems likely that similar bonds are being broken both in T2 and T5 DNA's over the range shown, which represents about 7 breaks per molecule of T2 DNA and 4 per molecule of T5 DNA. One should not look for significance in the linearity of the plot, however. Other stirrers yield somewhat different relationships (Fig. 1).

Direct Matching of T5 Molecules and T2 Fragments. The principles just discussed can be exploited more precisely by isolating a series of fragments of different lengths from T2 DNA, determining which of them matches T5 DNA with respect to fragility under hydrodynamic shear, and then comparing the sedimentation coefficients of the pair. This experiment, described in Table I, shows that T5 DNA, with a sedimentation coefficient of 48.5S is equally as fragile as T2 fragments with sedimentation coefficients lying between 48.5 and 49.5S.

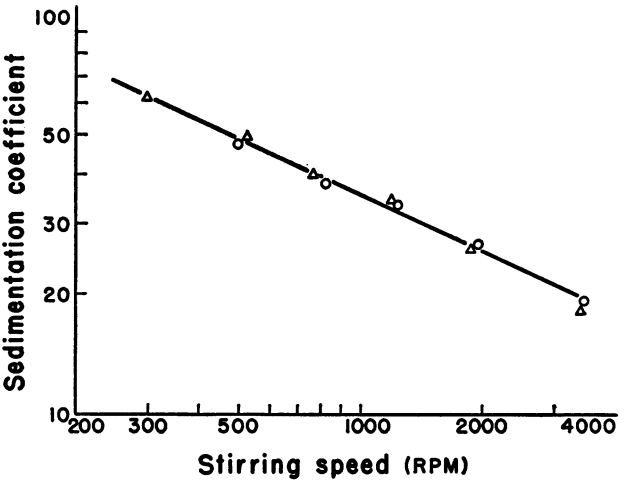

FIGURE 4 Relation between speed of stirring of DNA and sedimentation coefficient of the products. Triangles, T2 DNA; circles, T5 DNA. 50 ml samples were stirred for 30 minutes at 0.05 μg P^{32}-labeled DNA/ml in 0.6 M NaCl at room temperature in the stirrer described in this paper. Procedure as in legend of Fig. 1. Only the 800 RPM fragments of T5 DNA were appreciably denatured under these conditions.

TABLE I

EQUAL FRAGILITY UNDER HYDRODYNAMIC SHEAR OF T5 DNA AND T2 DNA FRAGMENTS OF SIMILAR SEDIMENTATION COEFFICIENT

Sample	Chromatographic interval	Sedimentation coefficient	Molecular weight ($\times 10^{-6}$)	Breakage at 630 RPM
	per cent	*s*		*per cent*
T2 fragments, tube 30	10.6	31.8	37	—
" " " 31	23.4	36.2	47	—
" " " 32	36.6	38.6	55	—
" " " 33	49.4	42.4	64	0
" " " 34	60.7	44.1	71	15
" " " 35	69.8	44.9	73	27
" " " 36	77.9	47.2	79	51
" " " 37	83.8	48.5	82	60
" " " 38	88.5	49.5	86	73
" " " 39	92.3	51.0	89	95
T5 DNA	—	48.5	82	65

P32-labeled T2 DNA was stirred to produce 90 per cent breakage and passed through a fractionating column. The resulting fractions, eluting at successively higher salt concentrations, are described above in terms of the percentage distribution of DNA among the fractions and the sedimentation coefficient of the DNA in each fraction. Molecular weights were calculated from sedimentation coefficients according to Rubenstein *et al.*, 1961. Breakage at 630 RPM was measured chromatographically after stirring 50 ml samples at DNA concentration 0.05 μg/ml for 30 minutes in 0.6 M NaCl at 5°C. Chromatograms of all samples showed single bands before, and two bands after, partial breakage by stirring. The DNA in tube 33 was half broken when stirred at 800 RPM.

Thus from the stirring tests alone we arrive at a molecular weight for T5 DNA lying between 82 and 86 $\times 10^6$, as compared to 82 $\pm$ 5 $\times 10^6$ from the sedimentation coefficient alone. Expressed as a fraction (84/130) of the molecular weight of T2 DNA, this result is probably reliable within a few per cent. The absolute molecular weights are subject, of course, to the errors of the measurements for T2 DNA (Rubenstein *et al.*, 1961).

The results displayed in Table I, like similar data given earlier (Burgi and Hershey, 1961), show that sedimentation velocity measurements permit recognition of molecular weight differences of about 5 per cent. The breakage tests show that molecular fragility is an equally sensitive index of molecular weight.

A Test of the Chromatographic Resolution of Molecular Weight Classes from Broken T2 DNA. Application of our "mixed carrier" method of analysis exemplified in Fig. 4 depends on the sorting out of DNA fragments of different lengths by chromatography. Evidence cited by Burgi and Hershey (1961) shows that the resolution of the longer fragments derived from T2 DNA is quite good. The following experiment extends the tests to lower molecular weight classes.

Four samples of T2 DNA were stirred at different speeds, and the median sedimentation coefficients measured for each proved to be 18.8, 26.2, 33.6, and 44.0S, respectively. An equal mixture of the four preparations was then passed through a column, and samples corresponding to the ⅛, ⅜, and ⅝ elution points, measured as cumulative fractions of the total DNA eluting from the column, were analyzed in the centrifuge. (The concentration of DNA eluting at the ⅞ point was too low for analysis.) The sedimentation coefficients proved to be 19.5, 25.4, and 34.1S, respectively. It appears, therefore, that the chromatographic separation of molecular weight classes is satisfactory independently of the complexity and molecular weight of the mixture analyzed. This would not be true, of course, if the mixture were heterogeneous in two or more independently varying characteristics recognizable by the column.

DISCUSSION

The principal results presented in this paper show that molecules of T5 DNA (sedimentation coefficient 48.5 $\pm$ 1.3S) are broken at the same rate of hydrodynamic shear as fragments of T2 DNA having the same sedimentation coefficient (48.5 to 49.5S). These and other findings already discussed can only mean that the two materials mentioned have the same molecular weight, 84 million for T2 fragments of 49S according to Rubenstein *et al.* (1961).

The particle of phage T2 contains a single DNA molecule of molecular weight about 130 $\times 10^6$ (Rubenstein *et al.*, 1961; Davison *et al.*, 1961). Particles of phage T5 are somewhat smaller than those of T2 (Williams and Fraser, 1953). Since we now find that the molecular weight of T5 DNA is 84 $\times 10^6$ measured relative to that of T2, particles of phage T5 must also yield single DNA molecules.

The molecular weights cited above disagree directly or indirectly with other commonly employed measurements (Rolfe, 1962). It may be appropriate to summarize reasons for believing that the figures given here are correct.

Relative molecular weights for T2 DNA and its larger fragments were measured independently by autoradiographic counts of the number of radiophosphorus atoms per particle of DNA (Rubenstein *et al.*, 1961) and by direct cleavage into halves and quarters (Burgi and Hershey, 1961) with excellent agreement. The relationship between relative molecular weight and sedimentation coefficient arrived at in these ways is shown in this paper to apply also to T5 DNA.

The absolute molecular weight for T2 DNA, measured autoradiographically both for the isolated DNA and in the phage particle, fell between 123 million and 140 million in three independent measurements. Independently of the reliability of these numbers, the conclusion follows that a single phage particle yields a single DNA molecule (Rubenstein *et al.*, 1961; Davison *et al.*, 1961).

The fact last stated permits an estimate of molecular weight based on more direct measurements of DNA content per phage particle. Rubenstein *et al.* (1961) found by phosphorus analysis a molecular weight of $160 \times 10^6 E$ for T2 DNA, where E is the efficiency of counting the phage particles by the plaque method. The efficiency E almost certainly lies between 0.5 and 1.0 (Luria *et al.*, 1951; Kellenberger and Arber, 1957). It may be added that the development of precise methods for counting phage particles as a means of measuring molecular weights for phage and other DNA's is now called for.

The length (Cairns, 1961) and diameter (Beer and Zobell, 1961) of the molecule of T2 DNA are about 50 μ and 20 A, respectively. Together with the molecular weight, these measurements seem to show that the molecule is two strands thick. The twofold reduction in apparent molecular weight on denaturation in the presence of formaldehyde is also consistent with this interpretation (Berns and Thomas, 1961). Levinthal and Davison (1961) showed that the shearing force required to break T2 DNA calls for a long, thin molecule, indeed one having approximately the dimensions cited above. Our present finding that a single relation between sedimentation coefficient and susceptibility to hydrodynamic shear applies to both T2 DNA and T5 DNA places them in the same structural category. We conclude that both are high molecular weight DNA's whose mass to length ratio is consistent with an extended double helical structure.

Our results also show that the bonds cleaved by stirring are of equal strength and similar distribution in the DNA's of T2 and T5, and are therefore likely to prove to be bonds common to all DNA's. This should be so if, as indicated by estimates of the bond strength in T2 DNA (Levinthal and Davison, 1961), the cleavage points are covalent bonds. The measurement of molecular weight of DNA in terms of fragility under shear thus recommends itself as a general method, subject only to some check that the molecules are of conventional structure and extendable under

shear. The results presented in this paper demonstrate that matching against T2 DNA fragments with respect to sedimentation coefficient and susceptibility to breakage furnishes a convenient and adequate check.

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