SHEAR BREAKAGE OF DNA

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ABSTRACT Determinations were made of the mean length of fragments produced after shearing long (>100 *kb*) native Hela DNA in a VirTis homogenizer. (VirTis Co., Inc., Gardiner, N.Y.). The mean length (L) is a function of the speed of rotation of the homogenizer blades (ω), time of shearing (t), water concentration ($[H_2O]$), solvent viscosity (η), temperature (T), and energy of activation (E^*), but not a function of the initial length so long as the starting molecules sustain an average of three or more breaks. The relationship of the parameters is expressed by the equation $L = (b/\omega t^{1/2}\eta^{1/2}[H_2O])e^{E^*/2k_BT}$, where k_B is the Boltzmann constant and b is a constant of proportionality. The breakage rate constant k was determined to have the relationship $k = (\omega^2 L^2 \eta[H_2O]^2/2b^2)e^{-E^*/k_BT}$. These equations are valid throughout large ranges of the parameters, and a simple method is described which chooses a final mean length length between at least 0.15 and 36 *kb* by choosing the appropriate shearing conditions and initial fragment length. The heterogeneity of shearing conditions within the shearing vessel permits use of the equations at all breakage rates tested. Based on the work of others using more homogeneous shearing conditions and initial fragment lengths, more complicated forms of the equations are necessary at low breakage rates but not at high ones. A proposed model of the breakage mechanism suggests that molecules with stress-induced localized denaturations break at a rate different from that for native DNA.

INTRODUCTION

Estimates of the effect of shear force on DNA have been primarily concerned with the rate of breakage of a homogeneous DNA of known molecular weight rather than the size distribution of fragments found after shearing heterogeneous DNA of unknown but large molecular weight. In most cases where the fragment sizes were determined, it was only after the introduction of a small number of breaks (usually averaging less than three per molecule). Because the analysis of DNA sequence distribution in eukaryotes has required small fragments of particular sizes, a reproducible method for introducing a large predetermined number of breaks into a heterogeneous population of long DNA molecules is necessary. This report examines the relation between shear force and the resulting mean fragment length after shearing in a VirTis homogenizer (VirTis Co., Inc., Gardiner, N.Y.). The results permit the formulation of equations relating the breakage rate constant to the length of the DNA and the conditions of shearing. In addition, estimates of the activation energy are obtained. Finally, the results are compared to those obtained by others using homogeneous DNA.

METHODS AND MATERIALS

Cell Culture

HeLa cells were grown in suspension culture as previously described (Eagle, 1959). To label the DNA, 200 ml of a cell suspension was exposed overnight to [^{3}H]methyl-[^{3}H]thymidine (50 mC_i/μmol, 10 μC_i/ml; New England Nuclear, Boston, Mass.).

Isolation of DNA

Isolation of nuclei using Nonidet P-40 (Shell Chemical Co., New York) to lyse the cells was carried out as previously described (Penman, 1966). The nuclear pellet was washed twice in 100 vol hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.4) and resuspended in 10 vol of 1 M perchlorate buffer (sodium perchlorate at indicated concentration in 8 M urea, 100 mM EDTA, 100 mM Tris, pH 8.0) with 1% Sarkosyl (ICN. K & K Laboratories, Inc., Plainview, N.Y.). Solid pronase powder (Calbiochem, San Diego, Calif.) was added to 1 mg/ml, and the mixture incubated at 37°C with gentle rocking for at least 2 h.

DNA was purified from the pronase-digested sample by a modification of the $NaClO_4$-urea method of Wilson and Thomas (1974). Six step gradients, each containing 0.3 ml of the digested sample in the top layer, 3.5 ml of 1.5 M perchlorate buffer in the middle layer, and 1.5 ml of 5 M perchlorate buffer in the bottom layer, were centrifuged in a Beckman SW 50.1 rotor (Beckman Instruments Inc., Fullerton, Calif.) at 47,000 rpm for 2.5 h at 20°C. The centrifuge tubes were bled by puncturing the bottom with a hypodermic needle. The viscous fractions, which comprised the bottom third of the gradients, were pooled and then extracted twice at room temperature with phenol-chloroform-isoamyl alcohol (25:24:1, vol:vol:vol). The aqueous phase was extracted three times with chloroform-isoamyl alcohol (24:1, vol:vol) and then dialyzed at 4°C against 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0, until the UV absorption spectrum indicated phenol was no longer present. The DNA was stored at 4°C.

Homogenization

A 6-ml DNA solution in an 8-ml fluted microhomogenizer flask was sheared in a VirTis 45 homogenizer (VirTis Co.) fitted with a microshaft machined down to a 3.2-mm diameter supporting a single 10.9-mm long microhomogenizer blade. The blade was rotated in a horizontal plane with the cutting edge leading. The speed of homogenization was continuously monitored using a GenRad type 631-B strobe (Concord, Mass.) and manually adjusted so that it never varied more than 10% from the desired speed and then only for a few seconds. For over 90% of the shearing time, the speed was within 2% of the desired speed. Salt solutions: DNA in NaCl with 1 mM EDTA and 10 mM Tris, pH 8 was sheared at 0°C in a water-ice bath. Glycerol solutions: DNA in $\frac{2}{3}$ glycerol (vol/vol) and sodium acetate, pH 8.6, was sheared at 0°C in a water-ice bath or at −70°C in a dry ice-ethylene glycol monomethyl ether bath. After shearing, the DNA was dialyzed twice against 1 liter of 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8 at 4°C.

Length Estimates of Sheared DNA

ELECTRON MICROSCOPY. Aqueous specimen grids were prepared by the method of Davis et al. (1971). To determine the length distribution of the fragments, electron micrographs of random fields of DNA were enlarged to a final magnification of between × 20,000 and 100,000. The contour lengths of 75–200 molecules were measured with a model 240 Graphics Calculator, Numonics Corp. (Lansdale, Pa.), and the statistics of each distribution were calculated with the aid of a Control Data Corporation Cyber 174 (Minneapolis, Minn.).

VELOCITY SEDIMENTATION. A 0.1-ml aliquot of a DNA sample was layered over a 5-ml linear gradient of 5–20% neutral sucrose (sucrose in 1 M NaCl, 10 mM Tris, 1 mM EDTA,

pH 8.0) or alkaline sucrose (sucrose in 100 mM NaOH, 900 mM NaCl, 1 mM EDTA) and centrifuge at 49,000 rpm for up to 150 min at 20°C using a Beckman SW 50.1 rotor (Beckman Instruments). Each gradient was bled from the bottom and divided into approximately 35 fractions. The radioactivity in each fraction was determined using a 5-ml Triton X-100 scintillation cocktail. The molecular weight of the fragments in the peak fraction was calculated using phage T7 or λ DNA as standards and the equations of Studier (1965). The values obtained from neutral gradients were used as estimates of the number average molecular weight of double-strand DNA (Dancis and Gunn, 1977), and those obtained from alkaline sucrose gradients were used as estimates of the weight average molecular weight of single-strand DNA.

Viscosity Measurements

The viscosity of solutions at 0°C was determined with an Oswald Viscometer (Oswald Manufacturing Co., Inc., Port Washington, N.Y.). The viscosity of $\frac{2}{3}$ glycerol (vol/vol) at −70°C was determined by measuring its rate of flow through a 2-ml pipette with the constricted ends removed. The rate was compared to that for 100% glycerol (vol/vol) at 0°C in the same apparatus, which has a known viscosity of 121 (Weast, 1977). The change in diameter of the pipette was negligible (<5%) when the temperature was reduced from 0° to −70°C, and was therefore ignored in viscosity calculations.

RESULTS

When DNA is sheared, the resulting mean fragment length might be some function of the product of the blade rotation rate squared, ω^2, and the time of shearing, t. The data (Table I, Fig. 1) appear to satisfy lines drawn with arbitrary slopes of $-\frac{1}{2}$, indicating that the log of the mean fragment length is linearly related to the log of the square root of $\omega^2 t$. For DNA sheared at −70°C, fragments with a mean length longer than 1 *kb* fall on a curve (not drawn) separate from that for shorter fragments. The high viscosity at this temperature (5.7×10^4 P) requires that shearing begins before the temperature is lowered to −70°C, otherwise the liquid will form a vortex and overflow the shearing vessel. The longer fragments were sheared close to the minimal speed necessary to prevent overflowing (Davidson et al., 1973). It is likely that with the very high viscosity and the relatively low speed, shearing occurred in only a very small fraction of the solution and resulted in longer than expected fragments. These longer fragments will not be included in the analysis of shearing though they will continue to appear in the figures. The data of Harrington and Zimm (1965) and Davidson et al. (1973) who did similar experiments with the same apparatus also show a linear relation when regraphed as in Fig. 1.

Equations derived from the linear regressions for each set of data in Fig. 1 so that twice the slope of each linear regression becomes the exponent, and the antilogue of the intercept becomes the preexponent, are presented in Table II. Inasmuch as the exponents do not appear to differ significantly from −1, especially for the top and bottom curves in Fig. 1 (Eqs. II-1 and II-3, respectively, in Table II), the mean fragment length, L, is indeed inversely proportional to the square root of $\omega^2 t$ and:

$$L = B'/\omega t^{1/2}, \tag{1}$$

where B' is a constant of proportionality. Eq. 1, however, states that the final mean

TABLE I

SHEARING CONDITIONS

Solution*	Speed	Time	Viscosity	$[H_2O]$	[DNA]	$[Na^+]$	Temperature	Length‡
	rpm	*min*	*P*	*mol/liter*	*μg/ml*	*mol/liter*	*°K*	
Salt	2,000	20	0.0224	52.75	5.0	2.5	273	36,720
	3,000	20	0.0224	52.75	5.0	2.5	273	22,927
	3,000	20	0.0224	52.75	5.0	2.5	273	20,000
	5,000	20	0.0224	52.75	5.0	2.5	273	14,497
	5,000	20	0.0209	53.34	5.0	2.0	273	13,365
	5,000	20	0.0224	52.75	5.0	2.5	273	14,500
	7,500	20	0.0224	52.75	5.0	2.5	273	8,652
	7,500	20	0.0224	52.75	5.0	2.5	273	10,750
	7,500	20	0.0224	52.75	5.0	2.5	273	9,200
	9,000	20	0.0224	52.75	5.0	2.5	273	7,190
	9,000	20	0.0224	52.75	5.0	2.5	273	6,331
	9,500	30	0.0224	52.75	5.6	2.5	273	4,310
	10,000	20	0.0209	53.34	5.0	2.0	273	6,369
	20,000	20	0.0209	53.34	5.0	2.0	273	4,549
Glycerol	10,000	10	1.072	18.02	5.1	0.6	273	3,597
	10,000	20	0.926	18.26	12.0	0.3	273	3,012
	10,000	30	0.926	18.26	12.0	0.3	273	2,304
	10,000	30	1.072	18.02	5.1	0.6	273	2,431
	10,000	30	1.072	18.02	5.1	0.6	273	2,747
	20,000	10	1.072	18.02	5.1	0.6	273	1,555
	20,000	20	1.072	18.02	5.1	0.6	273	1,400
	20,000	20	1.072	18.02	5.1	0.6	273	1,568
	20,000	30	1.072	18.02	5.1	0.6	273	1,198
	30,000	10	1.072	18.02	5.1	0.6	273	1,348
	30,000	20	1.072	18.02	5.1	0.6	273	1,086
	30,000	30	1.072	18.02	5.1	0.6	273	1,146
	15,000	30	5.65×10^4	18.26	12.0	0.3	203	3,188
	20,000	30	5.65×10^4	18.26	12.0	0.3	203	496
	25,000	20	5.65×10^4	18.42	5.0	0.2	203	2,317
	25,000	20	5.65×10^4	18.26	12.0	0.3	203	485
	30,000	32	5.65×10^4	18.26	12.0	0.3	203	326
	40,000	40	5.65×10^4	18.26	12.0	0.3	203	183
	45,000	52	5.65×10^4	18.26	12.0	0.3	203	159

‡Mean length after shearing in base pairs.

*Shearing solution contains either high salt or ⅔ glycerol.

fragment length, L, is independent of the initial length, L_1, a relation that cannot be true unless t_L, the time necessary to shear an infinitely long molecule to length L, is not, under the shearing conditions, significantly different from the actual shearing time. If t_{L_1} is the time that would be necessary to shear an infinitely long molecule to the actual initial length, then $t_L = t + t_{L_1}$. Thus, the relation of fragment length to time should be:

$$L = B'/\omega t_L^{1/2} = B'/\omega(t_{L_1} + t)^{1/2}. \quad (2)$$

Consequently, whenever two or more breaks are introduced into molecules of finite

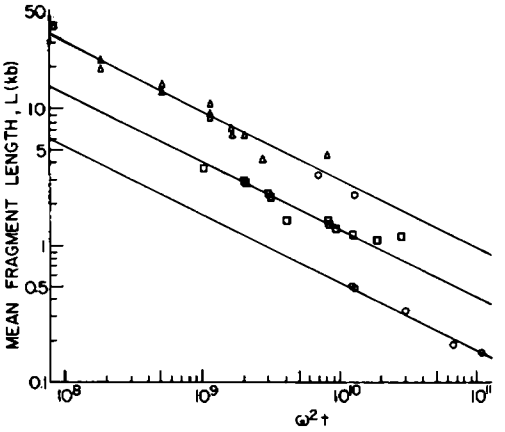

FIGURE 1 Effect of $\omega^2 t$ on mean fragment length. Long native HeLa DNA (single-strand mean length > 100 kb) was sheared in a VirTis homogenizer. Shearing at 0°C in NaCl with 10 mM Tris, 1 mM EDTA pH 8, (Δ), at 0°C (□) or at −70°C (○) in ⅔ glycerol and sodium acetate. For all DNA sheared in NaCl, the mean size (number average length) was determined by neutral sucrose sedimentation and for half of the samples it was also determined from electron micrographs. The two methods gave essentially the same results. Estimates of the weight average length from these micrographs also agreed with those based on alkaline sucrose sedimentation, indicating that the single- and double-strand lengths were the same and that shearing breaks but does not nick; for all DNA sheared in ⅔ glycerol, the mean size of the resulting fragments was determined by measurements of electron micrographs. Diagonal lines satisfy the equation $L = B'/\omega t^{1/2}$ where B' is a constant of proportionality chosen to give the best fit for each set of data. For DNA sheared at −70°C, the values of $L > 1$ *kb* are not included in the estimate of B'. Conditions and results of shearing given in Table I.

initial length (L_1), t_{L_1} is at most 12.5% of t and Eqs. 1 and 2 are equivalent. Inasmuch as the data in Fig. 1 appear to satisfy Eq. 1, the mean size of the fragments before shearing should have been over 100 *kb*, a prediction verified by sedimentation through a linear alkaline sucrose gradient.

At a constant rotation rate, ω, the rate of change of the mean fragment length with time is the derivative of Eq. 2 and is equal to:

$$dL/dt = -L/2t_L. \tag{3}$$

Substituting $\omega^2 L^2/(B')^2$ for $(1/t_L)$ gives:

$$dL/dt = -\omega^2 L^3/2(B')^2. \tag{4}$$

Thus, the rate at which the length decreases is proportional to the cube of the length. Because the product of the number of fragments (n) and the mean fragment length is constant throughout the course of the shearing (i.e., $nL = n_1 L_1$), the fraction of molecules breaking at any instant in a fragment population of mean length, L, will be:

$$dn/n dt = -dL/L dt. \tag{5}$$

Combining Eqs. 4 and 5 yields

$$dn/ndt = \omega^2 L^2/2(B')^2. \tag{6}$$

The value (dn/ndt), however, is by definition equal to the rate constant of breakage, k, so that:

$$k = \omega^2 L^2/2(B')^2 = 1/2t_L. \tag{7}$$

Notice that for a given rate constant, the mean fragment length, L, and the rotation rate, ω, are inversely related. A similar relation has been found between shear rate and length for homogeneous DNA (Adam and Zimm, 1977; Bowman and Davidson, 1972; Levinthal and Davison, 1961).

The requirement for two equations for DNA sheared at 0°C (Eq. II-1, II-2, and Table II) (Δ, □ Fig. 1) may be due to the effect of glycerol on both the viscosity and the water concentration. If the breakage of DNA is an hydrolysis reaction, then a decrease in the water concentration should decrease the breakage rate. Adam and Zimm (1977) have shown that the breakage rate is proportional to the square of the water concentration. Thus, one effect of increasing the glycerol concentration should be to decrease the water concentration and thereby decrease the breakage rate. Conversely, an increase in viscosity, η, due to added glycerol should cause an increase in the shear stress and therefore an increase in the breakage rate. Thus, the net effect of added glycerol on the breakage rate resulting from changes in viscosity and water concentration may be:

$$k = \eta\omega^2[H_2O]^2L^2/2B^2, \tag{8}$$

where $B^2 = (B')^2\eta[H_2O]^2$.

TABLE II

RELATION OF MEAN FRAGMENT LENGTH TO SHEARING CONDITIONS

	Symbol	Shearing conditions	Equation based on linear regression*
Fig. 1	Δ	0°C, salt	1. $L = 2.82 \times 10^8 (\omega t^{1/2})^{-0.994}$
	□	0°C, $\frac{2}{3}$ glycerol	2. $L = 1.40 \times 10^7 (\omega t^{1/2})^{-0.800}$
	o‡	−70°C, $\frac{2}{3}$ glycerol	3. $L = 1.65 \times 10^8 (\omega t^{1/2})^{-1.095}$
Fig. 2	Δ, □	0°C	4. $L = 1.57 \times 10^9 (\omega t^{1/2}\eta^{1/2}[H_2O])^{-0.976}$
	o‡	−70°C	5. $L = 1.62 \times 10^{12} (\omega t^{1/2}\eta^{1/2}[H_2O])^{-1.095}$
			Equation assuming slope of −1§
	Δ, □	0°C	6. $L = 2.44 \times 10^9 (\omega t^{1/2}\eta^{1/2}[H_2O])^{-1}$
	o‡	−70°C	7. $L = 2.27 \times 10^{11} (\omega t^{1/2}\eta^{1/2}[H_2O])^{-1}$
		all	8. $L = 4.75 \times 10^3 (\omega t^{1/2}\eta^{1/2}[H_2O])^{-1} e^{3589/T}$

*The slope of the linear regression for the graph of $\log L$ vs. $\log(\omega^2 t)$ has been doubled so that $\omega t^{1/2}$ can be used instead of $\omega^2 t$.

‡Does not include data when $L > 1$ *kb*.

§Numerical coefficients in Eqs. 6 and 7 are average values of $L\omega t^{1/2}\eta^{1/2}[H_2O]$ for all data points at a given set of shearing conditions. The numerical preexponent and exponent in Eq. 8 were derived from the simultaneous solution of Eqs. 6 and 7 after inclusion of a temperature-dependent exponent.

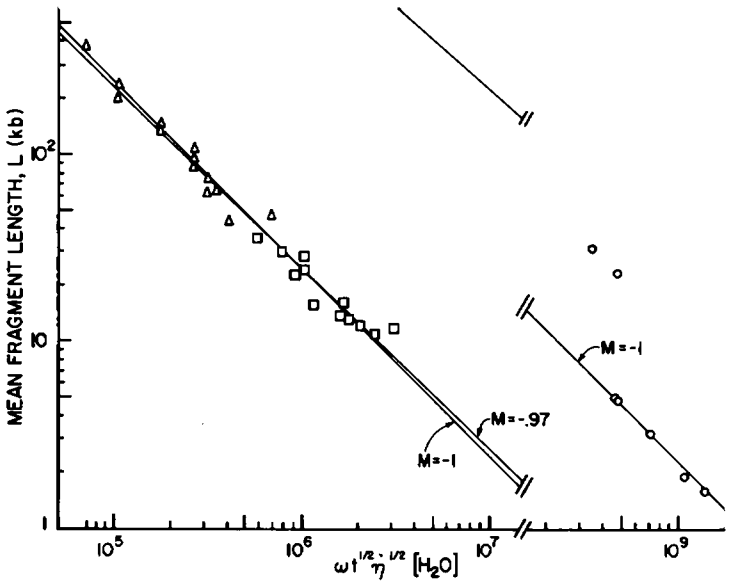

FIGURE 2 Effect of the square root of $\omega^2 t$, after correction for water concentration and viscosity, on mean fragment length. Data, after correction for water concentration ($[H_2O]$ in moles per liter) and viscosity (η in poise), and symbols same as in Fig. 1. Diagonal lines are either the best fit assuming slope of -1 or linear regression for shearing at 0°C.

When the data in Fig. 1 are regraphed based on Eq. 8, they appear as in Fig. 2 and satisfy the following equation:

$$L = (\eta^{1/2}\omega t)^{1/2}[H_2O])^{-1}B. \tag{9}$$

The actual equations for the data in Fig. 2 based on either a linear regression or the assumption that the exponent is -1 are shown in Table II.

It appears that the two sets of data for shearing at 0°C satisfy the same curve and that the deviation from a slope of -1 observed in Fig. 1 for shearing in $\frac{2}{3}$ glycerol at 0°C was due to experimental variation rather than to an error in Eq. 2.

The energy of activation (E^*) for shearing can be calculated from the two curves in Fig. 2 if it has the following relation to k:

$$k = (\eta\omega^2[H_2O]^2L^2/2b^2)e^{-E^*/k_BT} \tag{10}$$

where k_B is the Boltzmann constant, T the absolute temperature, and $2b^2$ a preexponent constant. Because, for a given temperature $2B^2 = 2b^2e^{E^*/k_BT}$, and because the numerical coefficients of Eqs. 6 and 7 in Table II are values of B at two different temperatures, the values of b and E^* can be determined. Thus, the breakage rate is

$$k = (\eta\omega^2[H_2O]^2L^2/4.52 \times 10^7)e^{-7179/T}, \tag{11}$$

with an activation energy of (7179 × 1.98=) 14.2 kcal/mol and the relation of mean

fragment length to temperature is:

$$L = b(\omega t_L^{1/2} \eta^{1/2} [H_2O])^{-1} e^{E^*/2k_B T}. \quad (12)$$

DISCUSSION

The linear relation found between log L and log $\omega t^{1/2}$ (Fig. 1) permitted for the first time the derivation of one equation (Eq. 10) which simultaneously relates the breakage rate constant to fragment length, viscosity, rotation speed, water concentration, energy of activation, and temperature. This equation has only one ad hoc constant, b, which probably contains corrections for at least pH, ionic strength, divalent cation concentration, and possibly DNA concentration as well. Adam and Zimm (1977) showed that breakage rates are affected by changes in both pH and divalent cation concentration, but the observation of such effects in the present experiments were prevented by the narrow pH range (8–8.6) and the absence of added divalent cations.

DNA Concentration and Self-Protection during Shearing

Hershey and Burgi (1960) observed self-protective effects when shearing DNA in the same apparatus used in the present experiments as did Yew and Davidson (1968) and Bowman and Davidson (1972) when shearing DNA by repeated passage through a narrow capillary. At DNA concentrations that affected the breakage rate, the effect decreased with shearing time (Hershey and Burgi, 1960) and therefore longer DNA had a greater self-protective effect than shorter DNA. Under such conditions, the breakage rate constant for molecules of a given length increased as the average fragment size decreased (see Fig. 3 in Hershey and Burgi, 1960). In Fig. 1, however, if the individual breakage rates had continually increased with increasing time or decreasing mean fragment size, then the linear relation between log mean fragment length and log $\omega^2 t$ would not have been observed for the range of shearing times and fragment lengths used. Thus, DNA breakage in the present experiments does not show self-protective effects and therefore between 5 and 12 μg/ml is not concentration dependent. A similar conclusion was reached by Adam and Zimm (1977) who sheared DNA at 5 μg/ml between concentric rotating cylinders.

Breakage Rates for Homogeneous vs. Heterogeneous Fragment Populations

All the equations in this paper have been for heterogeneous fragment populations where changes in, and relations to, the mean fragment length are descriptive of the population as a whole. If the equations are also applicable to homogeneous fragment populations, they will have a greater utility. Taking the log of Eq. 10 and rearranging terms gives:

$$T \log (k/\eta G^2 L^2 [H_2O]^2) = T \log 2b_1^2 + E^*/k_B, \quad (13)$$

where b_1 is a constant similar to b but corrected for the difference between shear rate, G, and rotation rate, ω. Consequently, a graph of $T \log (k/\eta G^2 L^2 [H_2O]^2)$ vs. ηGL should have a slope of zero and an intercept equal to $T \log 2b_1^2 + E^*/k_B$. When the data for the homogeneous T2 DNA sheared under conditions approaching laminar

flow (from Adam and Zimm, 1977) are graphed in this way, the result appears as in Fig. 3. With the exception of the extrapolated point in parentheses, the data appears to satisfy a single complicated function (curve 1). At high values of ηGL ($>9 \times 10^7$), the slope of the line connecting the data is, as predicted by Eq. 10, not significantly different from zero. At low values of ηGL ($<4 \times 10^7$), the ordinate is a linear function of ηGL and the transformation used in Fig. 3 appears to have reconciled the differences between data obtained at 20°C (●, ○) and 30°C (Δ).

Adam and Zimm (1977) said an inverse relation exists between the breakage rate corrected for water concentration and viscosity because a graph of log $\eta k/[H_2O]^2$ vs. ηG gave a single nonlinear curve for $T2$ DNA sheared at 20°C. Such a relation was consistent with their modification of the Yew and Davidson (1968) model of DNA breakage represented by the following equation:

$$k = (A[H_2O]^2/\eta)e^{-(E^* - f\eta Gr)/k_B T}. \tag{14}$$

Here, A is a constant of proportionality, f a friction factor depending upon the fragment length, conformation, and segmental frictional coefficient, and r is the increase in bond length due to shear stress. However, because $k/[H_2O]^2$ is proportional to ηG^2 in Eq. 10, $k\eta/[H_2O]^2$ is proportional to $\eta^2 G^2$ and a graph of log $\eta k/[H_2O]^2$ vs. ηG should give the single curve observed by Adam and Zimm (1977). Consequently, Eq. 10, which has a direct relation between $k/[H_2O]^2$ and η, surprisingly predicts the inverse relation found by Adam and Zimm (1977). Thus, Eq. 10, after correcting rotation rates for shear rates, appears to satisfy homogeneous as well as heterogeneous fragments at higher values of ηGL and requires some modification at lower values.

Equations for the Breakage Rate Constant

The requirement for modifying Eq. 10 suggests the possibility that there may actually be two breakage mechanisms. The mechanism predominating at high values of ηGL could satisfy the equation:

$$k = (\eta G^2 L^2 [H_2O]^2/2b_1^2)e^{-E^*/k_B T}, \tag{15}$$

and is represented by line 2 (Fig. 3), whereas the mechanism predominating at low values of ηGL could satisfy:

$$k = (\eta G^2 L^2 [H_2O]^2/2b_2^2)e^{-(E^* - C\eta GL)/k_B T}, \tag{16}$$

and is represented by line 3 (Fig. 3) where C is the slope and T log $2b_2^2 - E^*/k_B$ is the intercept of that line. Thus, the observed breakage rate, k_{obs}, would be the weighted average of Eqs. 15 and 16 and would be equal to:

$$k_{obs} = (\eta G^2 L^2 [H_2O]^2/2)e^{-E^*/k_B T}\{(d/b_1^2 + s/b_2^2)e^{-C\eta GL/k_B T}\}, \tag{17}$$

with d and s the fraction of molecules breaking according to Eqs. 15 and 16, respectively. A possible model for the two mechanisms is suggested by three observations: (*a*) shear stress can sometimes induce localized denaturations in the DNA without breakage (Hershey et al., 1963), (*b*) shearing sometimes produces double-strand DNA

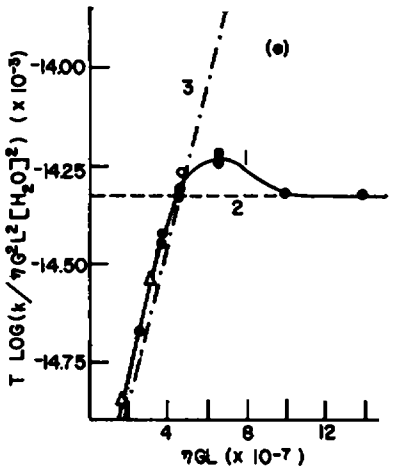

FIGURE 3 Breakage of homogeneous DNA. The data in Tables IV.2–IV.4 from Adam and Zimm (1977) for DNA sheared at pH 10.6 have been used to calculate the ordinate and abscissa values of the points in the figure. With the exception of one point using half molecules (○), all data is for whole phage T2 DNA. Their value of k for the point in parentheses is based on a linear extrapolation beyond the range of two of the other data points. DNA sheared at 293°K (●, ○), 303°K (Δ). The choice of the ordinate is based on Eq. 10 in the text and is a function of the energy of activation. The choice of the abscissa is based on the postulated effect of η, G, and L on the activation energy (Yew and Davidson, 1968). Curve 1 is an empirical fit to the data and line 2 is the best fit to the data of Eq. 15 when $\eta GL > 9 \times 10^7$. Line 3 represents one of an infinite number of lines that satisfies both Eq. 16 and, when weighted with the solution to Eq. 15, can approximate the empirical data. All such lines must pass through the intersection of curve 1 and line 2 and have slopes greater than or equal to that for line 3 which has the same ordinate intercept as curve 1. The range of the ordinate intercepts giving values of breakage rate constants between zero and one inclusive is about 0.1 kcal/mol or about 1.2% of the value of the ordinate intercept. Breakage rate estimates uncorrected for temperature obtained by Adam and Zimm (1977) at 25°C (not shown) are the same as, or lower than, those obtained at 20°C under otherwise similar conditions even though the higher temperature should have resulted in higher values. This lack of consistency has resulted in omitting the 25°C estimates from inclusion in the figure and discussion in the text.

with single-strand tails depending upon the salt concentration and temperature of the shearing solution (Pyeritz et al., 1972), and (*c*) DNA breakage under conditions producing no single-strand tails satisfies Eq. 10 which is similar to Eq. 15 (this report). The model proposes that shear stress either denatures DNA in the region of maximal stress before breakage and thereby produces single-strand tails upon breakage (Eq. 16) or it breaks the DNA before denaturing it without producing the tails (Eq. 15). At the moment of breakage, the fraction of molecules with (*s*) and without (*d*) stress-induced denatured regions would determine the observed breakage rate (Eq. 17). Further tests are needed to determine the validity of this model.

If the activation energy is a constant and the same for both homogeneous and heterogeneous DNA, then the value of b_1 in Eq. 15 is 9×10^4. The estimate of 14 kcal/mol for the activation energy obtained from Fig. 3 and Table II is low compared

with 20–40 kcal/mol based on the graphs of Adam and Zimm (1977) and lower still compared with 37–120 kcal/mol found by Davidson (Yew and Davidson, 1968; Bowman and Davidson, 1972) where both groups based their estimates on the slopes of graphs of log k vs. $1/T$. When the preexponential variables are not properly used to correct the rate constant before graphing, the estimate of the activation energy will be incorrect and could lead to the different estimates obtained in different laboratories.

The model represented by Eq. 17 suggests that the effective activation energy (the factor of $1/k_BT$ in the exponent of equations such as Eqs. 10 and 14–17) for Eq. 16 (partially denatured DNA) is affected by shearing conditions whereas that for Eq. 15 (native DNA) is not. Thus, at lower values of ηGL, increases in the breakage rate are due at least in part to a decrease in the effective activation energy, whereas at high values it is due only to increases in the preexponent.

The preexponent, also called the frequency factor, is a measure of the fraction of reactants which are in the proper state for the reaction to occur. If that state is a function of the solvent velocity gradient spanned by the molecule, then conditions which extend the molecule should increase its breakage rate. Thus, increasing viscosity or rotation speed may act by extending the molecule. Increasing the length of the molecule should have a similar effect.

Effect of Shearing Apparatus

After correcting for experimental error, all the data at a given temperature in Fig. 2 fall on a single line with zero slope when graphed as in Fig. 3 (not shown). This is true even for DNA sheared at values of $\eta\omega L$ much less than 10^7 where based on Fig. 3 the data should have a positive slope. This apparent discrepancy is probably due to differences in shearing apparatuses rather than to differences between ω and G. Adam and Zimm (1977) used an apparatus where turbulence, if any, was minimal resulting in laminar flow and uniform shearing. The VirTis homogenizer used in the present experiments had glass projections in the shearing vessel and thereby created a great deal of turbulence during shearing. In addition, the rotating blade of the homogenizer was in contact with only a very small fraction of the shearing solution at any given time. Consequently, in some parts of the solution, shear rates were very high and in other parts, they were much lower. Inasmuch as the breakage rate is proportional to the square of the shear rate, the vast majority of breaks occurred in those regions of maximal shear rate and also constant effective activation energy (Eq. 15). The long shearing times used in Fig. 1 ensured that all molecules were subjected to the maximal rate for comparable times. Consequently, at any given moment, actual breakage occurred at high values of $\eta\omega L$, but only involved a small fraction of the total fragment population. Thus, the observed breakage rate was linearly related to η, ω^2 and $[H_2O]^2$, even at low average values of $\eta\omega L$, because the actual breakage occurred at high values.

Effect of Fragment Length on Shearing

The inverse relation between shear rate and mean fragment length implied by Eq. 10 was also found previously for homogeneous DNAs. The only major discrepancy

between the present and earlier work is the relation between fragment size and breakage rate. Eq. 10 predicts that the breakage rate is proportional to L^2 so that fragments twice as large should break four times faster. Adam and Zimm (1977), however, calculate that it is 10 times faster. Their calculation, however, is based on a linear extrapolation of a nonlinear function (see Fig. IV.4 in Adam and Zimm, 1977) to a point almost twice the data range beyond the data. As can be seen in Fig. 3, their extrapolated value (in parentheses) is not consistent with their other data, whereas the measured value of half molecules (o) is. It is not surprising that they overestimated the relative breakage rate of the larger molecules. At high values of ηGL, the breakage rate is proportional to length squared (Eq. 15) and at lower values to more than the length squared (Eq. 17).

The threshold effect for breakage of phage T2 DNA reported by Hershey and Burgi (1960) agrees neither with my observation of a continuous relation between fragment length and rotation rate nor with Eq. 17 which satisfies the data of Adam and Zimm (1977). The observation of a threshold may actually have been an artifact due to the lack of sensitivity of their methods and the presence of L^2 in the preexponent of Eq. 10, the operative equation in their shearing apparatus.

If F is the fraction of unbroken molecules, then

$$F = e^{-kt} \tag{18}$$

and based on Eq. 10, L^2k' can be substituted for k to give

$$F = e^{-L^2k't}. \tag{19}$$

If we assume that molecules break exactly in half, then based on Eq. 19, by the time 80% of the T2 molecules were once-broken, only 17% would have been twice broken. Thus, the shearing solution would contain by weight 20% whole, 63% half, and 17% quarter molecules. Because Hershey and Burgi (1960) sized their molecules by a crude chromatographic method, the twice-broken fragments might be difficult to detect in the larger population of once-broken molecules and cause them to conclude that all or almost all of the molecules were once-broken before any were twice-broken. Because, as Hershey and Burgi (1960) first observed, molecules are not broken exactly in half but form a distribution with a coefficient of variation (σ/L) of about 20–30%, there should be considerable overlap of fragment sizes produced from once-broken and twice-broken molecules and thereby further decrease the ability to observe a small population of twice-broken molecules in a much larger population of once-broken ones. Hershey and Burgi (1960) also observed that T2 DNA was 60% broken after 30 min shearing at 8,000 rpm but not broken after 30 min at 6,000 rpm. Modifying Eq. 18 to explicitly express the effect of rotation rate gives:

$$F = e^{-\omega^2k''t}, \tag{20}$$

where k'' is the proportionality constant relating ω^2 and the breakage rate constant. From Eq. 20, conditions which give 60% breakage at 8,000 rpm should give 40% break-

age at 6,000 rpm. Hershey and Burgi (1960), however, performed this experiment at a high concentration of DNA (0.4 mg/ml) where they also observed significant self-protective effects with 100% intact DNA but not with 50% intact DNA. Based on their graph of log F vs. t demonstrating the self-protective effects (Fig. 3 in Hershey and Burgi, 1960), conditions causing 60% breakage at 8,000 rpm should cause at most 10% at 6,000 rpm, an amount that might be overlooked with their methods. Thus, a threshold for breakage based on fragment length and/or shear rate may exist, but it has yet to be demonstrated.

Choosing a Final Mean Fragment Length

The results suggest a simple procedure for predicting sheared fragment lengths. A sample of DNA is sheared in high salt (> 1.0 M) at a low temperature (0°C) preventing stress-induced localized denaturations and then the mean length of the fragments is determined by sedimentation or some other suitable method. Using log-log paper, the length is plotted against $\omega^2 t$ similar to Fig. 1 and a line with a slope of one-half is drawn through the plotted point. This line then determines the shearing time and rotation speed necessary to produce fragments of the desired mean length as long as the other shearing conditions are unchanged. Within a sheared population, the lengths of the individual fragments will be normally distributed and quite heterogeneous. The coefficient of variation (δ/L) will average 0.45 with a range from 0.35 to 0.6 that is independent of mean lengths between at least 0.5 and 10 *kb* (B. M. Dancis, unpublished results). This procedure is independent of initial fragment size and amount of turbulence and requires neither a replica of the shearing apparatus used in this report nor an estimate of the shear rate, G, which is often impossible to obtain. The procedure is useful over at least the broad range of conditions studied here where the fragment size varied by a factor of 200 (0.15–36 *kb*), the viscosity by 2 million (0.02 to 5×10^4 P), the ionic strength by 8 (0.3–2.5), the water concentration by 3 (18.3–52.7 M), the rotation rates by 20 (2,000–45,000 rpm), the shearing time by 5 (10–52 min), and the temperature by 70°C (−70°–0°C). Including the results of others, increases the utility of this simple procedure even more.

Methodology

Previous attempts to relate the breakage rate constant to the parameters of shearing (Yew and Davidson, 1968; Adam and Zimm, 1977) began with a theoretical model modified to correct for discrepancies with the data. The corrections were able to produce a single curve but not a linear one and therefore the corrected model could only demonstrate the existence but not the form of the relationships.

This paper, however, started only with the then naive idea that the log of the final fragment length was related to the log of $\omega^2 t$. The results confirmed the validity of this idea at low temperatures and high breakage rates. The relationship does not appear to be affected by the heterogeneity of the fragment population or shear stress, it permits the breakage rate constant to be determined indirectly, and it is valid over broad ranges of shearing conditions and mean fragment lengths. At low breakage

rates, the requirement for corrections resulted in a new model suggesting that DNA containing stress-induced localized denaturations breaks at a different rate from native DNA. Further experiments are needed to determine the validity of the model, but the results presented here about the nature of the exponent and preexponent define more clearly the conditions under which these experiments must be conducted.

The equations for homogeneous and heterogeneous DNA populations appear to be the same, but applying Eq. 10 to the individual molecules of a theoretical, normally distributed heterogeneous population and then calculating the changes in the whole population (B. M. Dancis and F. C. Hsuan, unpublished results) gives:

$$k = \omega^2 L^2(1 + R)/2(B')^2, \tag{21}$$

where R is the square of the coefficient of variation (σ^2/L^2). As long as R is constant, Eqs. 7 and 21 are equivalent and the individual molecules are being sheared in the same way as the population as a whole. Preliminary results (B. M. Dancis and F. C. Hsuan, unpublished results) indicate that the changes in R are small after an average of 5–10 breaks have been introduced into the original molecules.

The author thanks Doctors T. Punnett, R. Thompson, F. C. Hsuan, and G. Holmquist for critical comments and useful suggestions, and C. Anderson, F. Doganiero, J. Gunn, S. Pervaiz, A. Salzman, and K. Will for technical assistance.

The work was supported by National Institutes of Health, grant GM 21671.

Received for publication 26 June 1978.

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