Shear degradation of DNA*

Randall E. Adam¹ and Bruno H. Zimm²

Department of Chemistry, B-017, University of California, San Diego, La Jolla, CA 92093, USA

Received 10 January 1977

ABSTRACT A concentric-cylinder flow-birefringence instrument is used to generate sufficient shear fields to break T2 DNA ($M = 1.2 \times 10^8$) and *E. coli* DNA ($M = 2.5 \times 10^9$) in dilute solution. Breakage is monitored *in situ* by measuring the change in birefringence relaxation after the flow has been stopped. The breakage of T2 DNA follows first-order kinetics. Rate constants are obtained as functions of shear rate and viscosity (varied by adding glycerol). The data are fitted by a modified Arrhenius equation, assuming that stress increases the rate by lowering the activation energy. The rate increases with temperature, pH, and water concentration, and appears to be a base-catalyzed hydrolysis of the phosphate-ester linkage. La^{3+} ions catalyze the reaction. *E. coli* DNA was reduced to half molecules at a shear stress of 0.4 dynes/cm^2 , which is about 2500 times less than that required for T2. The difference in rates is accounted for in part by the difference in size of the two, but may also reflect the presence of many single-strand nicks in the *coli* DNA.

I. INTRODUCTION

The sensitivity of DNA to degradation when its solutions are stirred is a property that must be taken into consideration in all work with the material.³ The molecular basis of this phenomenon, with DNA as well as with other chain molecules where its appearance is less dramatic, has been studied by many workers; lack of space forbids a complete survey, but an example of recent work is that of Bowman and Davidson⁴ on λ -DNA in intermittent capillary flow. In any case, the mechanism has certainly not yet been fully elucidated.

We have developed an experimental set-up which allowed for the first time studies of the degradation of DNA solutions in steady, nearly uniform, laminar flow. The results are described in this report; they are in some ways different from, and more understandable than, those found under other flow conditions.

For these measurements we employed a modified concentric-cylinder flow-birefringence machine. The DNA solution could be sheared when the outer cylinder was rotated at high speed, and the resulting effects on the molecular weight could be detected by the change in the rate of relaxation of the birefringence after the rotation was stopped, following the method of Thompson and Gill.⁵ The longest relaxation time, τ_1 , is convenient to measure, and is

theoretically⁶ related to molecular weight and viscosity by the formula

$$\tau_1 = \alpha_1 M \eta [\eta] / RT , \quad \text{I.1}$$

where α_1 is a constant, M is the molecular weight, η the solvent viscosity, $[\eta]$ the intrinsic viscosity of the DNA, and R and T have their usual meanings. Thompson and Gill⁵ showed that this relation was valid for T2 DNA. Callis and Davidson⁷ and Klotz and Zimm,⁸ using related methods, showed that equation I.1 held for DNA over a range of molecular weights. Measurement of τ_1 thus leads immediately to a molecular weight; the accompanying measurement of the amplitude of the component of the birefringence relaxing with time-constant τ_1 gives the amount of material of this size.

By alternately shearing the solution for a measured time at high speed and then measuring the amount of material of given size by birefringence relaxation after shearing at low speed, it was possible to follow the kinetics of the degradation without removing the sample from the instrument.

II. INSTRUMENTATION

The instrument, as it was modified for use in these experiments, consists essentially of three parts: (1) the mechanical rotor, stator and drive system illustrated in figure II.1, (2) the optical system used to detect the birefringence signal, also illustrated in figure II.1, and (3) the electronics used to process the signal and yield an output to the recorder. This signal processing is schematically illustrated in figure II.2.

The mechanical system is a Rao "flow birefringence viscometer."⁹ The sample is contained between an inner cylinder which is held stationary and an outer cylinder which is mounted in a thermostated bearing race and rotated by means of a variable-speed electric motor. A glass window is mounted on the bottom of the rotor through which light can pass. Another window, which has a hole through which the stator is suspended, is mounted on the top of the rotor. The cylinders themselves are machined from black Teflon or Delrin.

The annular gap is much smaller than the radius of the cylinders so that the flow approximates parallel plate flow to a very good degree (see figure II.1 for exact dimensions). The shear rate can be readily calculated as the linear velocity of the rotor divided by the annular gap width. The maximum rotor rotation rate obtainable is about 22 revolutions per second and, using the chamber with the smallest annular gap, corresponds to a shear rate of about 5000 sec⁻¹.

The optical system is a modification of that described by Zimm¹⁰ and modified by Thompson and Gill.⁵ The filament of an incandescent lamp is

Figure II.1

The Birefringence Instrument

(a) Side view of the instrument. Two sets of cylinders were used; (1) a Teflon set having an inner radius (R_1) of 17.5 mm and an outer radius (R_2) of 18.5 mm and (2) a Delrin set having an inner radius of 18.0 mm and an outer radius of 18.5 mm. (b) Top view of the instrument. P is the axis of the polarizer and A is the axis of the analyzer. The enlargement of the annulus shows the orientation of one of the principle axes of the polarizability tensor in the solution.

Figure II.2

Schematic of the Instrument Electronics

The photodetectors (PD1 and PD2) are Clairex type C1509H photoconductors. Solid state, analog circuitry was used throughout (details available from authors on request). Not shown are the filtering and attenuation sections.

focused onto a pinhole giving essentially a point source of light. The light passing through the pinhole is then collimated by another lens forming a shaft of light traveling parallel to, and coaxial with, the sample chamber. The light passing through the annulus of the chamber is first linearly polarized by placing a polarizer between the collimator lens and the sample chamber. Upon emerging from the sample chamber the light passes first through a quarter-wave plate, with its slow axis set parallel to the polarizer axis, and then through an analyzer with its axis crossed with that of the polarizer by some angle β (see figure II.1). An analysis of this optical system is given by Thompson and Gill⁵ and will be presented here in somewhat different form.

Referring to Thompson and Gill,⁵ the transmission of light per unit area of the annulus is given by

$$T = \cos^2\beta + \frac{1}{2}(\sin\delta)(\sin 2\beta)(\sin 2\gamma) - \frac{1}{2}(\sin^2\delta/2)(\cos 2\beta)(1 - \cos 4\gamma) \quad (\text{II.1})$$

where δ is the retardation angle in radians of the birefringent solution contained in the annulus of the sample chamber, β is the angle between the axis of the polarizer and that of the analyzer, and γ is the angle that the slow axis of the solution makes with the polarizer axis. Since the retardation angle δ is always small in our experiments, the last term of the above expression is negligible and can be ignored in the ensuing analysis. Referring to figure II.1, it can be seen that

$$\gamma = \theta - \chi + \pi/2 \quad (\text{II.2})$$

where θ is the angular position along the annulus and χ is the extinction angle which is the smaller angle that the principle optic axes make with the stream lines. Substituting this into the transmission expression yields

$$T = \cos^2\beta - \frac{1}{2}(\sin\delta)(\sin 2\beta)(\sin 2[\theta - \chi]) \quad (\text{II.3})$$

It can be seen that the transmission is made up of a term which is constant around the annulus and a second sinusoidal term with an amplitude proportional to the birefringence of the sample and a periodicity of 180° about the annulus. Therefore with the onset of birefringence the intensity of light transmitted through the analyzer increases over two opposing 90° sectors of the annulus and decreases over the other two.

Since the change in transmission corresponding to a change in δ is essentially zero when averaged over the whole annulus (being the integral of a sine function over two periods) it is necessary to separate the signal of the two positive-going sectors from that of the two negative-going sectors. To achieve this a lens (the proximal lens) with two opposite 90° sectors cut away is placed above the analyzer so that the light from two of the sectors--say

the positive-going two--are intercepted by it and focused onto a photodetector while the light from the other two negative-going sectors passes by. The light from these latter two impinges on a second (distal) lens, placed above the first, which in turn focuses it onto a second photodetector. As is evident from equation II.3, the extinction angle χ , which is characteristic of the particular system being investigated, must first be determined in order to place the analyzing lens in the appropriate angular position.

Suppose that, in the absence of birefringence, the intensity of light from the positive-going quadrants is I_+^0 and that from the negative-going quadrants is I_-^0 . When the solution becomes birefringent the corresponding intensities become

$$I_+ = I_+^0 + kI_+^0 \quad (\text{II.4a})$$

$$I_- = I_-^0 - kI_-^0 \quad (\text{II.4b})$$

where k is proportional to the integral of equation II.3 over the two 90° sectors appropriate to each quantity. These two quantities appear as analog voltages at the input amplifiers of the electronics.

By means of operational amplifiers the two voltage signals are then both added and subtracted and the sum is then divided into the difference. The yields

$$V_{\text{out}} \propto \frac{I_+^0 + kI_+^0 - I_-^0 + kI_-^0}{I_+^0 + kI_+^0 + I_-^0 - kI_-^0} \quad (\text{II.5})$$

which, after regrouping terms, becomes

$$V_{\text{out}} \propto \frac{(I_+^0 - I_-^0) + k(I_+^0 + I_-^0)}{(I_+^0 + I_-^0) + k(I_+^0 - I_-^0)} \quad (\text{II.6})$$

Due to imperfections in the optics and windows and slight misalignments in the system it is generally the case that I_+^0 and I_-^0 are of slightly different magnitudes. Still, for $k \ll 1$ (which it is in these experiments) and I_+^0 nearly equal to I_-^0 , equation (II.6) simplifies to

$$V_{\text{out}} \propto \frac{(I_+^0 - I_-^0)}{(I_+^0 + I_-^0)} + k \quad (\text{II.7})$$

Here it can be seen that the divider output is a sum of a constant term and a term proportional to the birefringence. The salient feature here is that a change in k (such as an exponential decay of the birefringence) will be reflected in a corresponding change in V_{out} . This output, after suitable atten-

uation and filtering, is then fed to a strip-chart recorder.

Another notable feature of equation II.6 is that while the intensity of the lamp enters each factor I_+^0 and I_-^0 , it divides out at the complete expression. This lack of dependence on lamp stability improves the signal to noise ratio, a condition which becomes important at low shear rates and concentrations.

III. METHODS AND MATERIALS

A. *Preparation of T2 phage DNA samples.* *E. coli* B was grown in M9S medium¹¹ with shaking. At an absorbance of 0.5 at 650 nm the culture was infected with T2 phage at a multiplicity of 0.1 phage particles per cell. After four hours a few ml of chloroform were added and shaking was continued for ten minutes. The phage was purified using differential centrifugation. This involved a ten minute spin at 5000 rpm to bring down cellular debris whereupon the supernatant was transferred and centrifuged at 10,000 rpm for two hours to pellet the phage. This procedure was repeated until the phage centrifuged out as a clear, translucent pellet. It was then resuspended and stored at 4°C over chloroform. This procedure is detailed by Adams.¹²

Samples of phage were withdrawn and the DNA was released and purified by phenol extraction according to the method of Massie and Zimm.¹³ The purified DNA was then dialyzed against pH 7 phosphate buffer containing 0.008 M phosphate, 0.001 M EDTA and 0.016 M Na⁺. The intrinsic viscosity of these samples ranged from 310 to 325 dl/gm. Concentrations were determined by spectroscopy at 260 nm assuming that 55.2 µg/ml would give an absorbance of one absorbance unit. An independent check of molecular weight was made by M. Weissman using a new method of fluctuation spectroscopy.¹⁴ His results ranged from 1.2×10^8 to 1.3×10^8 on various samples. One sample was analyzed for single-strand breaks by denaturing in 0.2 M alkali and then dialyzing against a buffer containing 0.005 M NaOH and 0.0001 M EDTA. A melting curve showed this sample to be completely melted at room temperature. This sample had an intrinsic viscosity of 480 dl/gm. According to Rosenberg and Studier (1969), single-stranded T7 phage DNA, under similar low salt conditions and having 30 to 40% single-strand breaks, has an intrinsic viscosity 1.5 times that of the double-stranded DNA in a neutral buffer containing 0.2 M Na⁺. Since we find the same ratio, we feel that our single-strand breakage was also in the range of about 30 to 40%.

To prepare solutions for the breakage experiment, an aliquot was withdrawn from the stock solution and added to a solvent mixture of appropriate composition for the experiment being run. The total sample, which usually had a volume of 25 to 30 ml, was contained in a 400 ml beaker, thereby spreading

the sample into a relatively thin layer to enhance the rate of diffusion. The sample was tilted and gently rotated at about .3 rev/sec followed by a period of a few hours in which diffusion was allowed to take place. This was repeated several times until the sample was totally mixed. Alternatively, it was found that gentle swirling of the sample enhanced mixing in a shorter time without causing any apparent damage, even at the highest viscosity and pH conditions. The sample was swirled at about one revolution per second for about three to four seconds. The solution was then drawn into a disposable, polypropylene syringe which had been drilled out and fitted with a length of Teflon tubing (i.d. = 2.5 mm). The solution was then pumped into the instrument at a rate such that the maximum shear rate was less than 10 sec^{-1} . The sample was allowed to equilibrate thermally for a number of hours, and the experiment was then run.

B. *Preparation of E. coli DNA samples.* *E. coli* was grown two to four hours into stationary phase and the cells were harvested by centrifugation. An appropriate number of cells were suspended in a boric acid buffer of pH 8.3 containing 0.01 M pyroborate, 0.001 M EDTA and 0.195 M Na^+ , incubated at 65°C for ten minutes, cooled in an ice bath, and placed in a dialysis bag. Boric acid buffer solutions of Pronase, Brij 58 and lysozyme were then added in that order and the sample quickly mixed before lysis had begun. The bag was then immersed in a test tube filled with boric acid buffer, and this test tube was then placed in a 50°C water bath for 16 to 20 hours. This is essentially the lysis procedure used by Klotz and Zimm.⁸

DNA concentrations were estimated by measuring the absorbance of the cell suspension at 500 nm and using an empirical relationship between the absorbance and the DNA concentration as measured by the method of Klotz and Zimm.⁸

To alter the pH of the lysate the dialysis bag was removed from the 50° water bath at the end of the digestion period and, after cooling, was immersed in an equivalent buffer of the desired pH for about 24 hours. The external buffer was changed several times during this period, at the end of which the sample was pumped into the instrument. The actual pH of the solution was always measured at the conclusion of the experiment.

The dialysis bag was attached to a piece of Teflon drilled out and machined to fit the filling hole of the instrument. The Teflon piece was fitted into a rubber stopper so that the dialysis bag could be sealed into a test tube designed for pumping the solution. This tube was fitted with a piece of glass tubing such that buffer could be pumped in or out of the interstitial space between the wall of the tube and the dialysis bag, thus col-

lapsing or inflating it. In this way, by pumping buffer in while the device was inserted into the filling hole of the sample chamber, the DNA solution could be forced out of the bag and into the instrument at a controlled pumping rate.

An alternate device was made of a 20 cc disposable polypropylene syringe drilled out and fitted with a piece of 2.5 mm i.d. Teflon tubing. The lysis components were combined in the syringe, and, with the tubing plugged, the syringe barrel was immersed in the 50° water bath to allow digestion to take place. This apparatus was used in the experiments where the viscosity was increased by adding glycerol. The glycerol could be drawn into the syringe at the end of the digestion period and would underlie the DNA solution. The syringe would then be laid on its side and diffusion allowed to take place at room temperature (a process that took eight to ten days to complete!). Finally, the syringe was placed in a syringe pump, and, inserting the tubing into the filling hole of the chamber, the solution was pumped in.

The pumping rate for all *E. coli* solutions was such that the maximum shear rate was kept below 1 sec^{-1} .

C. *Molecular Weight Determination by Relaxation Techniques.* In an experiment to determine molecular weight, the rotor was turned at a low shear rate (5 sec^{-1} for T2 DNA and 1 sec^{-1} for *E. coli* DNA) until a steady-state signal was reached. The shear was then abruptly halted, and the birefringence signal was observed to relax back to zero in an exponential fashion. The experiment would be allowed to continue until a linear baseline had been well established on the strip chart. The baseline was then extrapolated back to the time of shear stoppage ($t=0$) and the height of points along the curve was measured from the extrapolated baseline and replotted on semilogarithmic graph paper. There was ordinarily a fast-relaxing initial component corresponding to break-age products in the solution and faster relaxation modes of the whole molecules, but these died out much more rapidly than the principle relaxation (τ_1), so that the tails of the curves appeared as pure, single-component exponential decays. On a semilogarithmic plot this portion appeared linear with a slope equal to $-1/\tau_1$. Thus the time constant was obtained by taking the negative reciprocal of the terminal slope. The linear portion of the semilogarithmic plot was then extrapolated back to $t=0$ and the initial amplitude, Γ_1 , of the longest relaxation mode was so obtained. This value is proportional to the number of molecules in the solution relaxing with that characteristic time constant; i.e., the number of whole molecules. Typical semilogarithmic plots are pictured in figure III.I.

D. *Breakage Rate Determination.* For breakage experiments the solution, after equilibrating thermally, was sheared at the low speed setting used for relaxation experiments, as described above, and the initial values for Γ_1 and the relaxation time, τ_1 , were obtained. The sample was next sheared for a measured time interval at a rate sufficient to cause breakage. After the birefringence had disappeared, a low-speed run was made to again determine the concentration of whole molecules. This process was repeated until the whole-molecule concentration had been reduced sufficiently to obtain a satisfactory curve of concentration versus time of shear. The relaxation data were then plotted as described above, and the value of Γ_1 was obtained for each run.

The rate constant of the breakage could next be calculated. If the reaction is assumed to be first order, then $c(t)$, the whole-molecule concentration at time t , is given by

$$c(t) = c_0 e^{-kt} \quad (\text{III.1})$$

where c_0 is the whole-molecule concentration at $t=0$, k is the rate constant, and t is the time of shearing. Since c is proportional to Γ_1 in the relaxation experiments, the values for Γ_1 can be plotted versus time of shear on a semilogarithmic plot. This plot will be linear for a first order reaction and the negative of the slope will be equal to k . Figure III.1 illustrates treatment of the data from a typical experiment in this manner. For experiments using T2 DNA, it was found that the data fit a curve of this type very well. Experiments using *E. coli* DNA were fraught with more complications, and the breakage data obtained from them was treated in a somewhat different fashion.

In an experiment using *E. coli* DNA, the sample would be sheared at a low rate to obtain an initial relaxation time. It would then be sheared at a relatively high rate for a fixed increment of time--usually one minute. A slow run would again be made and the relaxation time noted, followed by a high-speed run using a shear rate which was increased over that of the last high-speed run by some increment. The process was repeated a number of times--each time increasing the shear rate of the high speed run. The high speed shear rate which first caused the relaxation time of the solution to significantly decrease, as measured by the following low-speed run, was taken to be the "critical shear," G_{crit} . A breakage rate, as such, was therefore not obtained. The results of an experiment of this type are illustrated in figure III.2. The problems encountered in the *E. coli* experiments will be detailed in the sections on results and discussion.

Figure III.1

Replotted Data of a Typical Breakage Experiment

This is the data obtained on a sample containing 5.7 $\mu\text{g/ml}$ T2 DNA in 60% glycerol ($\eta = 8.82 \text{ cp}$) at 25.0°C . Na^+ concentration is 0.2 M and the pH is 6.3. The shear rate used for breakage is 3380 sec^{-1} . (a) Relaxation from steady state of the unsheared sample. (b) Relaxation after shearing for 90 seconds. (c) Relaxation following additional shearing for 90 seconds. (d) Γ_1 values of all three relaxations plotted versus time of shear.

FIGURE III.2

Breakage Experiment on *E. coli* DNA

These data were obtained from a sample containing 4 $\mu\text{g/ml}$ DNA. The temperature is 20°C and the pH is 10.7

IV. RESULTS.

A. *T2 DNA. Birefringence relaxation.* In figure IV.1 is a double-logarithmic plot of the relaxation time versus solvent viscosity for T2 DNA, samples having the same concentration of DNA and salt and identical pH. The solvent viscosity was changed by either changing the concentration of glycerol or changing the temperature. The data are fit by a straight line with a slope very close to unit in good agreement with theory. This result implies that the conformation of the molecule is not affected by glycerol concentration or temperature over the ranges studied. We therefore define a solvent-viscosity-independent relaxation time, τ^0 , such that

$$\tau^0 \equiv \tau_{\text{obs}} / \eta_{\text{solv}}, \quad (\text{IV.1})$$

where τ_{obs} is the experimental relaxation time and η_{solv} is the relative viscosity of solvent to that of water at 20°C. Figure IV.2 shows a plot of τ^0 versus DNA concentration. The relaxation time decreases with decreasing concentration, as is expected.⁵ A new, concentration-independent variable, τ_0^0 , is introduced which is defined as follows:

$$\tau_0^0 \equiv \tau^0 - 0.0144c. \quad (\text{IV.2})$$

Here c is the DNA concentration in $\mu\text{g/ml}$ and the factor 0.0144 is the slope of the line in figure IV.2.

FIGURE IV.1

Relaxation Time Versus Solvent Viscosity for T2 DNA

The graph is a double-logarithmic plot of τ versus solvent viscosity. DNA concentration is 5 $\mu\text{g/ml}$, $[\text{Na}^+] = 0.2\text{M}$ and $\text{pH} = 10.6$. The slope of the straight line is 0.96 and the τ -intercept is 0.68 sec.

FIGURE IV.2

Concentration Dependence of Relaxation Time

τ_0^0 is plotted vs. concentration for T2 DNA. Data points are averages of all data collected at those concentrations adjusted to a solvent viscosity of one centipoise.

There is a possibility that τ_0^0 might be affected by pH, but a plot of τ_0^0 versus pH for all experiments run in this study on T2 DNA showed that the pH had no perceptible effect on the relaxation time.

As can be seen in figure IV.2, the value arrived at for τ_0^0 is 0.61 seconds. This value is in good agreement with the accepted value for the relaxation time of T2 DNA as obtained both theoretically and experimentally by the investigators cited in the introduction.

The ionic strength of the DNA solution has a well-established effect on the intrinsic viscosity.¹⁵ Some data are shown in Table IV.1. Rosenberg and Studier¹⁵ reported that the intrinsic viscosity of T7 DNA increases by a factor of about 1.5 when the salt concentration is decreased from 0.2 M to 0.005 M, and so, from equation I.2, we might expect τ_0^0 to likewise increase by this factor in our experiments---assuming T2 DNA behavior is about the same as that of T7 DNA. The measured value of 0.94 for τ_0^0 in 0.005 M Na^+ compared with the value of 0.61 obtained in 0.2 M Na^+ gives the expected ratio almost exactly.

TABLE IV.1 Effect of Na^+ Concentration on Relaxation Time and Breakage Rate

$[\text{Na}^+]$ (moles/liter)	T (°C)	pH	η (poise)	G (sec^{-1})	τ_0^0 (sec)	$k \times 10^3$ (sec^{-1})
0.005	20	7.0	0.0605	3394	0.94	6.9
0.20	20	7.0	0.0605	3394	0.61	5.5

That the relaxation time does give a measure of the molecular weight can be demonstrated by comparing the value of τ_1 for whole molecules of T2 DNA with that obtained from a solution of once-broken, half molecules (Table IV.2). The theoretical relationship between molecular weight and relaxation time is given in equation I.2. According to Crothers and Zimm,¹⁶ the empirical relationship between intrinsic viscosity and molecular weight is given by

$$[\eta] + 5 = KM^{.665}, \quad (\text{IV.3})$$

where $[\eta]$ is given in dl/gm and K is a constant. For T2 DNA whole and half molecules the intrinsic viscosity is large enough so that the relationship simplifies to

$$[\eta] = KM^{.665}, \quad (\text{IV.4})$$

Combining this relationship with equation I.2 leads to

$$\tau \propto M^{1.665}. \quad (\text{IV.5})$$

The ratio of the relaxation times of whole molecules to that of half molecules would therefore be

$$\frac{\tau_{\text{whole}}}{\tau_{\text{half}}} = 2^{1.665} = 3.17. \quad (\text{IV.6})$$

The experimentally determined ratio from the data in Table IV.2 is 2.5; the agreement seems reasonable, considering that the broken sample probably is not perfectly homogeneous.

B. *T2 DNA. Shear Degradation.* If the DNA breakage mechanism is a thermal reaction, we would expect the rate constant to be related to the shear stress in a logarithmic fashion such that

$$\ln k \propto \eta G \quad (\text{IV.7})$$

since the activation energy E^* would be reduced by an amount $f\eta G$ where f is a

TABLE IV.2 *Relaxation and Breakage Data
for T2 DNA Whole and Half Molecules*

The solvent contained 60% glycerol and 0.2 M Na^+ at a pH of 10.6. The temperature was 20°C. DNA concentration was 4.3 $\mu\text{g/ml}$.

Molecular Size	ηG (dynes/cm ²)	$k(\text{sec}^{-1})$	τ (sec)
wholes	537	117 ^a	6.8
halves	537	9.8	2.7

^aThis figure is derived from the breakage rate curve in figure IV.6

constant dependent on molecular dimensions, conformation, etc., and r is the distance along the reaction coordinate that the tensile force is exerted over. Further details and discussion of this proposed mechanism are given in the next section.

The results of breakage experiments at various shear rates and viscosities are given in Table IV.3. To check the relationship between k and shear stress we first plot G versus $\ln k$ from these data. The viscosity η , as well as all other variables, is held constant for each plot, and the results are illustrated in figure IV.3. It can be seen that, while the rate is proportional to G , as suggested by equation IV.7, it increases much more slowly with η than would be expected. It is however true that when the viscosity is changed by adding glycerol, the water concentration also varies. It occurred to us that the water activity may affect the reaction rate, which is not unreasonable, since the hydrolysis of an ester involves the uptake of a molecule of water.

Therefore, an experiment was run where the glycerol concentration was kept at 50%, but the water was partly replaced by ethanol. Since ethanol has a viscosity very close to that of water, its effect is to decrease the water activity without significantly increasing the solvent viscosity. The results are also shown in Table IV.3 along with the control experiment which contained no ethanol. The relaxation time seems to indicate that the DNA conformation has not been grossly affected, and a melting curve showed the DNA to still be double-stranded at the temperature and pH used in the breakage experiments.

TABLE IV.3 Breakage Rate of T2 DNA as a Function of η , G , and Solvent Composition

Solvent Composition	T (°C)	η (poise)	pH	G (sec ⁻¹)	$k \times 10^3$ (sec ⁻¹)	$k_{50} \times 10^3$ (sec ⁻¹)
50% Glycerol	20	0.0605	10.6	2425	1.9	1.9
50% Glycerol	20	0.0605	10.6	3394	8.6	8.6
50% Glycerol	20	0.0605	10.6	4364	21.5	21.5
60% Glycerol	20	0.1096	10.6	2425	8.6	13.4
60% Glycerol	20	0.1096	10.6	3394	22	34
70% Glycerol	20	0.2294	10.6	2425	10	40
70% Glycerol	20	0.2294	10.6	3394	19	76
50% Glycerol	20	0.0605	7.0	4364	15	—
50% Glycerol* 25% Ethanol	20	0.0605	7.0	4364	4.4	—

*Average of two experiments agreeing within 10%

FIGURE IV.3

Breakage Rate Versus Shear Rate for T2 DNA

In all samples the temperature is 20°C and the pH is 10.6. The solvent viscosity is indicated on the figure for each sample.

(Higher ethanol concentration caused denaturation and salt-solubility problems.) The result shows the breakage rate to vary approximately with the square of the water concentration. We can then define an adjusted rate constant k_{50} such that

$$k_{50} \equiv (k_x) (50/x)^2 \quad (\text{IV.8})$$

where x is the water concentration in per cent and k_x is the observed breakage rate. The quantity k_{50} is the breakage rate that would be observed at the same viscosity but at a water concentration of 50%. These values are also given in Table IV.3 for each experiment.

Even the values of k_{50} when plotted versus ηG , the shear stress, did not fall on one curve. However, when each value of k was multiplied by the associated viscosity, a single curve could be fitted to the data (figure IV.4).

FIGURE IV.4

Product of the Viscosity and Breakage Rate Versus Shear Stress

The breakage rate adjusted for water concentration, k_{50} , multiplied by the corresponding solvent viscosity and plotted versus ηG . For all samples the temperature is 20°, and the pH is 10.6; (o) η is 0.0605, (Δ) η is 0.1096, ($\square$) η is 0.2294.

This implies an equation of the type

$$\ln(\eta k) = F(\eta G), \quad (\text{IV.9})$$

where $F(\eta G)$ is some mildly non-linear function. The significance of this will be discussed in the next section.

In these experiments, data has been compared for solutions covering a concentration ranging from 1 to 15 $\mu\text{g/ml}$. In contrast to the previous work, where a marked reciprocal relationship between concentration and breakage was reported, we observed no such correlation. In comparing data in these experiments, therefore, the concentration is not considered, although in experiments where DNA concentration was not being purposely varied, the concentration was usually between 5 and 8 $\mu\text{g/ml}$, since a birefringence signal of convenient amplitude was obtained in this range.

If the breakage mechanism is a hydrolysis reaction, it is likely to be catalyzed by acid or base. To test this possibility, we performed breakage experiments on samples of varied pH but of otherwise identical viscosity, salt concentration, and temperature (Table IV.4). Although there was no evidence that the molecular conformation had changed in any way, as was pointed out in the previous section, the breakage rate increased as the pH was raised from 7.0 to 11.5. Under the experimental conditions used the DNA appeared to either melt or precipitate at a pH of about 4.5; therefore we did not study the breakage rate below a pH of 5.3.

TABLE IV.4 Breakage Rate as a Function of Temperature and pH

pH	η (poise)	$[H_2O]$ (X)	T ($^{\circ}C$)	G (sec^{-1})	$k \times 10^3$ (sec^{-1})
5.3	0.0882	40	25	2425	4.3
6.3	0.0882	40	25	2425	3.4
10.6	0.0882	40	25	2425	5.7
10.6	0.0882	40	25	3394	21
11.5	0.0882	40	25	2425	11
5.3	0.0882	40	25	3394	9.1
6.3	0.0882	40	25	3394	8.3
6.6	0.1096	40	20	3394	7.7
8.3	0.1096	40	20	3394	10
10.6	0.1096	40	20	3394	21
7.0	0.0605	50	20	3394	5.5
10.6	0.0605	50	20	3394	9.4
10.6	0.0731	40	30	2425	13
10.6	0.0425	50	30	2425	4.2

Another test of the reaction mechanism is the breakage behavior of the molecules in the presence of metal ions known to catalyze the hydrolysis of phosphate esters. The effects of both lanthanum III and calcium II were studied, using a Tris buffer and no EDTA, and the results are shown in Table IV.5 along with those from the control experiments with the same buffer but no added metal ions. The presence of 50 μM La^{3+} ion increased the rate of breakage dramatically, while the same concentration of Ca^{2+} ion had no discernible effect at a pH of 7.5.

TABLE IV.5 *Effect of Multivalent Cations on Breakage Rate*

The Tris buffer described in the text was used in all samples. In each case the temperature was 20°C, the viscosity 0.1096, and shear rate was 2425 sec⁻¹.

Cation concentration	pH	k x 10 ³ (sec ⁻¹)
50 μM La ³⁺	7.5	36
—	7.5	3.6
50 μM La ³⁺	10	18
—	10.6	7.1
50 μM La ³⁺	5.5	36
—	5.3	4.3
50 μM Ca ²⁺	7.5	4.0

The data in Table IV.4 also show the effect of temperature on the breakage rate. In every case the rate increases as the temperature is raised.

C. *E. coli* DNA. The results of the relaxation experiments are given in Table IV.6. There appeared to be much more polydispersity of molecular size than with T2 DNA, in agreement with the observations of Klotz and Zimm;⁸ we therefore used only the long-time part of the relaxation curve. This tail of the curve still replotted as a straight line in most cases, however, allowing us usually to obtain a value for τ . The data reported are the average of several experiments in most cases. The scatter in the results is considerably greater than in the T2 experiments.

TABLE IV.6 *Relaxation and Breakage Data for E. coli DNA Experiments*

T (°C)	η (poise)	pH	C (μg/ml)	τ (sec)	G _{crit} ^a (sec ⁻¹) ± 15%
20	0.010	9.6	4	210±30	40
20	0.010	5.8	4	190±20	45
20	0.010	10.7	4	220±30	40
20	0.010	8.3	4	180±20	40
20	0.017 ^b	8.3	4	240±30	25
20	0.028 ^b	8.3	4	410±40	8
20	0.010	8.3	2	170±10	40

^a Shear rate at which degradation became apparent.

^b The viscosity was raised by adding glycerol to the digested lysate.

From Table IV.6 it can be seen that the relaxation time tends to increase with solvent viscosity as is predicted by equation I.2. The concentration dependence of τ is also shown in Table IV.6. The apparent decrease in relaxation time when the concentration is reduced is expected.

The breakage data are also given in Table IV.6. The DNA begins to degrade in the neighborhood of a critical shear, G_{crit} . The dependence of G_{crit} on the solvent viscosity, as shown in Table IV.6, seems to indicate that there is a value of the shear stress above which the DNA will degrade substantially during the one minute time period in which the shear was applied in these experiments. This is in qualitative agreement with the situation with T2 DNA.

V. DISCUSSION

A. *The Model.* Two ways in which the rate can be accelerated in a reaction are to increase the temperature or to decrease the activation energy. In many reactions the activation energy can be decreased by the addition of a catalyst. In the case of high-molecular-weight linear polymers, the activation energy for bond breaking can be altered significantly by mechanical stress. We follow here the model proposed by Yew and Davidson.¹⁷ Consider the tensile force exerted on the central bond of the chain to be $f\eta G$, where f is a friction factor which depends on the segmental friction coefficient of the molecule, molecular conformation, molecular length, etc. When the reactant molecule goes to the activated state, the force $f\eta G$ would be exerted along the reaction coordinate over a distance r . The result would be an energy $f\eta Gr$, which would reduce the activation energy by the same amount. The breakage rate constant for a DNA molecule in a shear field, according to this model, would then be

$$k = Ae^{-(E^* - f\eta Gr)/k_B T} \quad (V.1)$$

where k_B is the Boltzmann constant. It is from this equation that equation IV.7 was derived.

The factor A is often referred to as the frequency factor, since it may be thought of as the frequency with which the reactants happen into a configuration in which reaction can occur. If this frequency is diffusion-dependent, and therefore inversely proportional to η , the existence of such dependence as we found in these experiments would be explained. Alternatively, we must consider that the DNA molecules tumble in the flow at a rate which might contribute to the frequency factor, since the molecules may rupture only when tumbling brings them into the right configuration. The rate of tumbling of macroscopic fibers in laminar flow was studied by Forgacs and Mason.¹⁸

They found that the rate of tumbling, as measured by the reciprocal of the time needed to revolve 360° , was nearly proportional to the shear rate, G . Assuming that A is proportional to this rate, we would then have

$$A = A'G = A'S/\eta,$$

where A' is a proportionality factor. (This factor A' was found by Forgacs and Mason to decline with increasing S , so that the apparent proportionality of A to S is in part removed; the effect varied with the nature of the particle, however, so no quantitative statement can be made.) Thus the observed reciprocal dependence of A on η might be accounted for.

Focusing next on the exponential term in equation V.1, we see, as was noted in chapter IV, that the dependence of $\ln(\eta k)$ on ηG is not exactly linear. This non-linearity can be accounted for if we assume f , the friction factor, in equation V.1 depends on S , which is likely, since f is dependent on the molecular conformation.

Since the pre-exponential factor A in equation V.1 has an inverse viscosity dependence, we see that equation V.1 predicts that the logarithm of ηk should be a linear function of $1/T$ with a slope equal to $-(E^* - f\eta Gr)/k_B$. To measure the temperature dependence, the values of k were experimentally obtained for several temperatures above 20°C . These values were adjusted to 50% water concentration according to equation IV.4 and were multiplied by the viscosity of the solvent at that temperature. The values of ηk expected at 20° for the same shear stress were obtained from the plot in figure IV.4. Finally, these values of ηk were plotted against $1/T$, and the results are shown in figure V.1. The slope of these plots should be equal to $(E^* - f\eta Gr)/k_B$. After these many interpolation steps the results are not highly precise, however. (Also, the apparatus was not designed for precise temperature control; temperature variations of about 1° might have occurred.) The average value for $(E^* - f\eta Gr)/k_B$ is about 25 kcal/mole which is reasonable for a chemical reaction. It does not agree at all with the value of 130 kcal/mole reported by Yew and Davidson,¹⁵ however.

B. The Mechanism. We assume that the chemical mechanism of breakage is a phosphate-ester hydrolysis, (Richards and Boyer¹⁹). The experiments on water activity, pH and multivalent cations bear directly on this question.

The dependence of rate on pH is shown in figure V.2; it is apparent that the reaction rate is enhanced by hydroxyl ions and possibly by hydronium ions as well. This is reasonably compelling evidence for a base-catalyzed (and perhaps acid-catalyzed) ester hydrolysis.

FIGURE V.1

Temperature Dependence of the Breakage Rate

The values at 20°C were calculated as described in the text. (Δ) G is 3394 sec $^{-1}$ and η is 0.0882 poise. (□) G is 2425 sec $^{-1}$ and η is 0.0882 poise. (○) G is 2425 sec $^{-1}$ and η is 0.0731 poise. (■) G is 2425 sec $^{-1}$ and η is 0.0425 poise. The pH of all samples is 10.6 and breakage rates have been adjusted to a water concentration of 50% in all cases.

The water-activity experiment also seems to support this type of mechanism, although the results are somewhat tentative since the experiment was run only at one ethanol concentration and only at neutral pH. The participation of water and hydroxyl ions suggests to us a mechanism wherein a hydroxyl ion attacks the phosphorus, forming a pentacoordinate intermediate. A nearby water molecule transfers a proton to the leaving group in a concerted manner as it comes off the phosphorus. (Glycerol or ethanol might function as proton donors in such a mechanism, but the concentration of available protons in glycerol is low compared to water, while ethanol is a much weaker acid.) It can be seen that the above reaction scheme involves acyl cleavage, however, which, according to Richards and Boyer,¹⁹ occurs about 10% of the time in a sonic degradation experiment. They do report that this fraction increases for alkaline degradation, but it is still not the major reaction, which is alkyl cleavage.

FIGURE V.2

Breakage Rate Versus pH

Data is presented for samples of varied pH but of otherwise identical composition and conditions: (○) G is 3394 sec⁻¹, η is 0.1096 poise, and T is 20°C; (□) G is 3394 sec⁻¹, η is 0.0605 poise, and T is 20°C; (△) G is 2425 sec⁻¹, η is 0.0889 poise, and T is 25°C; (●) G is 3394 sec⁻¹, η is 0.0882 poise, and T is 25°C.

A mechanism for alkyl cleavage would probably involve an initial, spontaneous scission of the C-O bond. The carbonium ion would then react with a molecule of water which would, in turn, lose a proton to a nearby hydroxyl ion.

The multivalent cation experiments also lend credence to the proposed mechanism. As was pointed out in chapter I, La³⁺ ions are known to catalyze hydrolysis of polynucleotides. The effect is greatest on RNA, and a mechanism has been proposed by Butcher and Westheimer²⁰ involving coordination of the 2' hydroxy group. This coordination is of course not possible in the case of DNA. Nonetheless, the catalytic effect observed in our experiments was considerable—the addition of 50 μ M/ml La³⁺ to a neutral solution being more effective than a change of pH to 11.5. The lanthanum ion probably coordinates a hydroxyl ion and a water molecule as well as the phosphate ester. This might hold two or three of the necessary reactants in close proximity. At higher pH the rate enhancement is not as pronounced. This can possibly be explained by assuming that La(OH)²⁺ is the predominant species at neutral pH,

and, furthermore, is the reactive form. At high pH, the predominant species would most likely be $\text{La}(\text{OH})_2^+$, and the concentration of effective catalyst would thereby be reduced. Ca^{2+} is reported to have a pronounced effect on the hydrolysis of RNA as evidence by loss of biological activity.²¹ There has been no detailed mechanism proposed for its interaction with RNA, but our result showing no Ca^{2+} activity in shear-assisted DNA hydrolysis implies that here the 2' hydroxy group may play a crucial role.

It should be mentioned that the control experiment, containing DNA, Tris buffer, but no multivalent cations, gave results identical to those obtained using the phosphate-EDTA buffer at the same pH. This indicates that at least this change in negative ions does not seem to affect the breakage rate and, perhaps more importantly, that the absence of EDTA, which is used to chelate metal ions and thus prevent nuclease activity, had no detectable detrimental effects on the DNA preparations.

It is interesting that, in the study of chemical or thermal degradation of double-stranded DNA, the shear stress may be thought of as acting as a mechanical catalyst which speeds the reaction rate to a convenient level.

C. *General*. As was previously stated, the molecular length and conformation affect the breakage rate through the friction factor f . Although we have no adequate theory quantitatively relating f to these quantities, we still expect f to increase with increase of molecular length or molecular expansion. The results in Table IV.2 show the breakage rate to decrease by a factor of about 10 when the molecular weight is reduced by a factor of 2. For a rigid-rod molecule, Frenkel²² showed that the tensile force is proportional to the square of the length. Breaking a half molecule, therefore, might be expected to require four times the shear rate needed to break whole molecules. Referring to Table IV.3, we see that the shear rates required to produce the same breakage rate for whole and half molecules under identical conditions are 2500 sec^{-1} and 4900 sec^{-1} respectively, the ratio being 1/2. This indicates the relationship between force and the length is linear rather than quadratic. This was also the result of Levinthal and Davison,²⁰ as well as Bowman and Davidson.⁴ *E. coli* DNA, which has a length some 20 times that of T2, is found to break at a shear stress about 2000 times lower than T2 DNA; however the differences in water concentration, viscosity, pH, etc., as well as the probability of extensive nicking in the *coli* DNA, make a quantitative comparison meaningless.

The DNA conformation can be varied by lowering the Na^+ concentration which causes the coiled molecule to expand, as is evidenced by an increase in

the intrinsic viscosity.¹⁵ This might be thought of as an effective increase in the molecular length, thus increasing the molecules sensitivity to shear. The results given in Table IV.1 show that such a decrease in Na^+ ion concentration does indeed lead to an increase in the breakage rate k , a result also found by Yew and Davidson.¹⁷

It is unclear why we did not observe the concentration dependence of k that was reported by Burgi and Hershey,²⁴ Levinthal and Davison,²³ and Bowman and Davidson.⁴ According to their results we should have observed a variation in breakage rate of about 20% over our concentration range. Such a variation is somewhat larger than the error in our T2 experiments, so we might have expected to detect it. Two differences in our setup compared with the others are (1) in our case the shear rate was constant throughout the sample, while in the others it was highly variable both in space and in time, and (2) in our case we used glycerol-water-salt mixtures rather than plain water plus salt as the solvent. It is not clear why either of these differences should have reduced or eliminated the concentration dependence. It is also worth mentioning that Burgi and Hershey as well as Bowman and Davidson looked for and did not find a viscosity dependence of the breakage rate such as we found. Possibly both of these discrepancies have a common origin.

D. *Conclusions.* In conclusion, we find that breakage of T2 DNA in a shear field behaves as a shear-assisted hydrolysis. The breakage rate exhibits the pH and temperature dependence expected of such a reaction. In addition, the reaction rate is increased by lanthanum ions, which are known to catalyze phosphate-ester hydrolysis. To what extent these results depend on our special conditions--constant shear field and use of glycerol-water solvent mixtures--remains to be determined in further work.

References

- * Dedicated to Jerome Vinograd
- 1. Present address: IBM Research Laboratories, Monterey & Cottle Roads, San Jose, California 95193.
- 2. To whom correspondence should be addressed.
- 3. Davison, P. F. (1959). *Proc. Nat. Acad. Sci. U.S.* 45, 1560.
- 4. Bowman, R. D. and Davidson, N. (1972). *Biopolymers* 11, 2601.
- 5. Thompson, D. S. and Gill, S. J. (1967). *J. Chem. Phys.* 47, 5008.
- 6. Zimm, B. H. (1956). *J. Chem. Phys.* 24, 269.
- 7. Callis, P. R. and Davidson, N. (1969). *Biopolymers* 8, 379.
- 8. Klotz, L. C. and Zimm, B. H. (1972a). *J. Mol. Biol.* 72, 779.
- 9. Rao Instrument Company, 245 Washington Avenue, Brooklyn, New York 11205.
- 10. Zimm, B. H. (1958). *Rev. Sci. Instr.* 29, 360.
- 11. Bolle, A., Epstein, R. H., Salser, W., and Geiduschek, E. P. (1968). *J. Mol. Biol.* 31, 325.

12. Adams, M. (1959). Bacteriophages, Wiley (Interscience), New York, New York.
13. Massie, H. R. and Zimm, B. H. (1965). Proc. Nat. Acad. Sci. U.S. 54, 1641.
14. Weissman, M., Schindler, H., and Feher, G. (1976). Proc. Nat. Acad. Sci. U.S. 73, 2776.
15. Rosenberg, A. H. and Studier, F. W. (1969). Biopolymers 7, 765.
16. Crothers, D. M. and Zimm, B. H. (1965). J. Mol. Biol. 12, 525.
17. Yew, F. F. H. and Davidson N. (1968). Biopolymers 6, 629.
18. Forgacs, O. L. and Mason S. G. (1959). J. Colloid Sci. 14, 473.
19. Richards, O. and Boyer, P. D. (1965). J. Mol. Biol. 11, 327.
20. Butcher, W. W. and Westheimer, F. H. (1955). J. Amer. Chem. Soc. 77, 2420.
21. Huff, J. W., Shastry, K. S., Gordon, M. P., and Wacker, W. E. C. (1964). Biochemistry 3, 501.
22. Frenkel, J. (1944). Acta Physicochim. U.R.S.S. 19, 51.
23. Levinthal, C. and Davison, P. F. (1961). J. Mol. Biol. 3, 674.
24. Burgi, E. and Hershey, A. D. (1962). J. Mol. Biol. 4, 313.