VISCOELASTIC CHARACTERIZATION OF SINGLE-STRANDED DNA FROM *ESCHERICHIA COLI*

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ABSTRACT Single-stranded DNA released from *E. coli* wild type and mutant cells by alkaline-EDTA-detergent was analyzed using the recently developed biophysical technique of viscoelastometry. Under the lysis conditions used, it was possible to detect single strands of molecular weight approximately 2×10^9 daltons. Little difference was detected in the size of single-stranded DNA from log phase vs. stationary phase cultures, or from cells treated with chloramphenicol to allow completion of replicating chromosomes. The largest single strands from ligase overproducing, endonuclease$^-$, and pol A1 mutants were likewise of approximately the same size as wild type, but were present in smaller yields. The reduction in single-strand molecular weight as a result of heating intact cells was investigated as a function of time and temperature. Heating at 37°C for up to 20 min produced no additional single-strand breaks, but temperatures from 45 to 65° introduced breaks. Solutions maintained at pH 12.5 were not stable indefinitely, and the relative viscosity of such solutions was found to decrease over a period of several hours.

INTRODUCTION

During the last several years, the lysis, denaturation, and sedimentation of bacterial and eukaryotic cells on alkaline sucrose gradients has become a routine method for the analysis of single-stranded DNA. Originally introduced by McGrath and Williams (1966), the technique allows for the gentle release and denaturation of DNA directly on top of a gradient with no subsequent manipulation, transfer, or shear breakage of the DNA. The method has found widespread use for the investigation of high molecular weight bacterial and eukaryotic DNA (see, for example, Hanawalt and Setlow, 1974, and Beers et al., 1972).

Centrifugation of high molecular weight DNA, however, is subject to many difficulties. The low DNA concentrations required to avoid entanglement of large random coils necessitates the use of radioactive labels which may themselves produce breaks in DNA (Cleaver et al., 1972). Extremely low concentrations are required to avoid anomalously rapid sedimentation of clusters of high molecular weight DNA (Schumaker and Zimm, 1973). Furthermore, even low concentrations of unentangled

molecules may sediment anomalously in high centrifugal fields due to speed-dependent molecular distortions. The dependence on molecular weight of this speed-dependent sedimentation anomaly is quite marked, so that only the largest molecules in a given gradient may be affected (Rubenstein and Leighton, 1974). As a result, the trailing edge of a sedimentation profile may retain its normal Gaussian shape, while the leading edge is artificially sharpened, resulting in an overall profile shape commonly seen in the literature where the McGrath and Williams technique has been employed (Beers et al., 1972; Hanawalt and Setlow, 1974).

As an independent check of published sedimentation results which routinely obtain a molecular weight of 2–5 $\times$ 10^8 for single-stranded bacterial DNA (McBurney et al., 1972, and references therein) we employed the recently developed technique of viscoelastometry to characterize alkaline detergent lysates of *Escherichia coli*. This method is selectively sensitive to the largest molecules present, does not require radioactive labeling, and is unaffected by mild molecular distortions. Consequently, the method appears suitable for detecting molecules which are too large to sediment normally. In addition to characterizing the largest molecules present in an alkaline bacterial lysate, the method should also be useful for studying the production of small numbers of single-strand breaks which result when intact cells are heated, radioactive labels are incorporated, or high molecular weight DNA is hydrolyzed in alkali.

MATERIALS AND METHODS

Wild type *E. coli* B and K-12 cells were grown in M9 salts supplemented with 0.2% casamino acids and 0.4% glucose. A liquid overnight culture was diluted 1:100 in up to 50 ml medium and grown at 37°C with shaking to an absorbance at 450 nm of 0.4. Cells at other stages of logarithmic and stationary growth were obtained by growing to specified absorbances, as described in Results. CT 284, a dnaC mutant obtained from Dr. C. I. Davern, was grown on LB tryptone/yeast extract medium at 37°C, then shifted to 42°C, at which temperature initiation ceases and chromosome replication is completed (Schubach et al., 1973). Initiation of chromosomes in wild-type cells was inhibited by the addition of chloramphenicol to 150 μg/ml plus deoxyguanosine to 200 μg/ml (to speed replication while initiation is being inhibited), followed by incubation for 1 h at 25°C. A ligase-overproducing mutant, *E. coli* N1072, lop 8, as well as a mutant lacking endonuclease I, *E. coli* HF 560, endo I^-, were obtained from Dr. E. P. Geiduschek. *E. coli* P 3478 pol A1, a mutant lacking DNA polymerase I, was obtained from Dr. D. Smith.

After cells reached an absorbance of 0.4, they were harvested by centrifugation at 4,000 *g* for 5 min, resuspended in 3.0 ml cold 0.5 M glycerol, 1 mM Na_2HPO_4, and transferred immediately to the viscoelastometer chamber. Glycerol was used to stabilize the cells osmotically; it is preferable to sucrose because it does not decrease the pH of alkaline solutions as much as sucrose. Following the observation of Freifelder et al. (1971) that single-strand breaks can be minimized by lysing *E. coli* cells quickly without initial spheroplasting, the resuspended cells were lysed immediately by the addition of 1.0 ml of 2% sodium dodecyl sulfate, 0.25 N NaOH, 0.02 M Na_4EDTA ("alkaline-EDTA-detergent"). This lysing solution was added gently to the meniscus of the solution in the viscoelastometer chamber by pipet and allowed to drift slowly down through the resuspended cells. Increasing the temperature of the viscoelastometer water bath from 25 to 45°C, where it was held for 5 min, resulted in gentle convective mixing of the lysing cells, clearing of the solution within a few minutes, and de-

naturation of the DNA. After the temperature was returned to 25°C and before the viscoelastometer rotor was inserted to begin measurements, the solution was allowed to sit for 0.5 h in order to allow time for strand separation. The pH measured following the completion of each experiment was 12.5 ± 0.1; the sodium ion concentration was 0.10 M. (At this salt concentration the bacterial single strands are extended enough to make their principal retardation time measurably long.)

Heat-induced single-strand breaks were produced in one of two ways. Incubation at 52°C followed the protocol of Woodcock and Grigg (1972): the sidearm culture flask was transferred directly from a 37°C bath to a 52°C bath and shaken from 5 to 30 min. Cells were then harvested and treated as above. Incubation at temperatures from below room temperature to 65°C was accomplished by harvesting and resuspending cells as before, then heating the cells in the chamber from 2 to 20 min at the required temperature, followed by lysis and convective mixing.

DNA concentration was determined by lysing an equivalent number of cells under neutral conditions (Klotz and Zimm, 1972 *b*) and measuring the fluorescence of ethidium bromide intercalated in the native double-helical DNA.

The viscoelastic technique has been discussed in detail elsewhere (Klotz and Zimm, 1972 *a*). Basically, the instrument operates as a rotating-cylinder, Cartesian-diver viscometer equipped to record both the viscous and elastic response of DNA solutions to externally imposed stress. The experimental parameters obtained include the relative viscosity, η_{rel}, and the characteristic retardation time, τ. The method used to calculate molecular weights and solution heterogeneity has been described previously. This method makes use of all experimental parameters to establish the relative proportion of full-sized, half-sized, and sixth-sized molecules and calculates the molecular weight of the full-sized species.

RESULTS

When *E. coli* B and K-12 cells were lysed at pH 12.5 in 0.1 M Na^+, it was possible to measure principal retardation times (τ's) of approximately 80 s. Fig. 1 shows the amount of experimental error normally encountered in these measurements, as well as the very gradual dependence of τ on DNA concentration. Using the method of Uhlenhopp et al.[1] for calculating solution heterogeneity and molecular weight, an M of $2.2 \pm 0.5 \times 10^9$ daltons was obtained for the largest molecules present, which represented only 4% of the total DNA by weight. (Considering the complexities involved in these calculations and the additional uncertainties set forth below, these values should not be regarded as precise.) A vast majority of the molecules in these bacterial lysates had a considerably smaller molecular weight; approximately 75% of the DNA were estimated to have a median size roughly one-sixth that of the largest molecules, while approximately 20% of the total consisted of half-sized molecules.

The size of the largest single strands did not appear to depend upon stages in the growth cycle of the culture, as shown in Fig. 2. The τ obtained at equivalent concentrations of early, mid-, and late exponential as well as stationary cells was in all cases 80 ± 25 s. Furthermore, when chloramphenicol was added to inhibit initiation of DNA synthesis while allowing completion of replicating chromosomes, the retardation time remained about the same. Similar results were obtained with the temperature-sensitive dnaC mutant CT 284; when shifted to 42°C to inhibit initiation and allow chromosome replication to finish, no significant change in τ was observed. Any

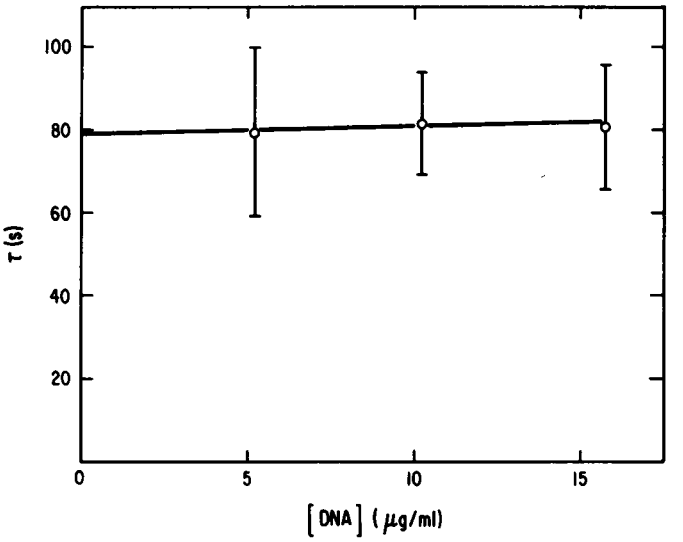

FIGURE 1 Retardation time of *E. coli* B as a function of concentration. Cells were grown, harvested, and lysed as described in Materials and Methods. Error bars indicate the range of τ values obtained for an average of eight relaxations at each concentration.

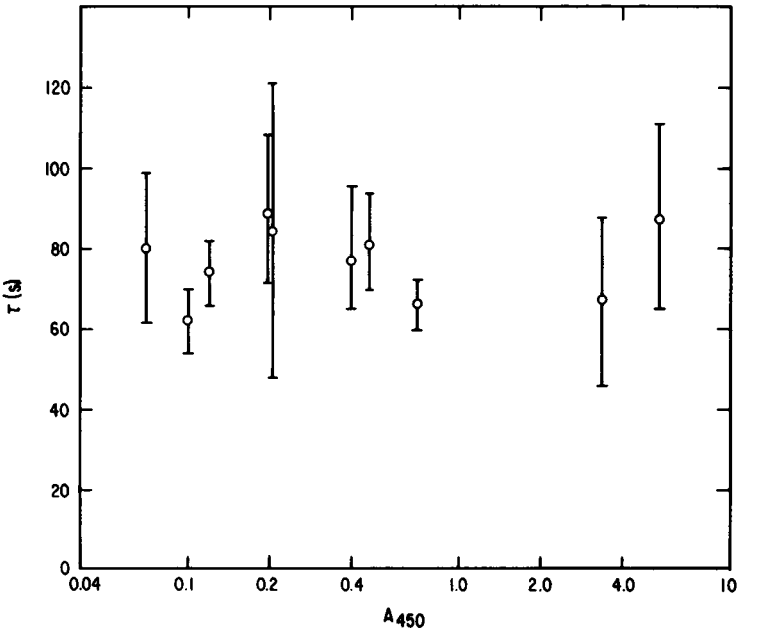

FIGURE 2 Retardation time of *E. coli* W3110 single-stranded DNA as a function of stage in the growth of the culture. Cells were grown to the indicated A_{450} and a sufficient quantity harvested to give a final DNA concentration of approximately 10 μg/ml. Cells harvested at an $A_{450} \leq 0.2$ had been inoculated at a sufficiently small density to allow at least three doublings before centrifugation.

significant difference in molecular weight between replicating and completed single strands should have been detected by a noticeable change in τ, since τ depends approximately on $M^{5/3}$ (Klotz and Zimm, 1972 *b*; see also the following paper).

Mutant strains of *E. coli* K-12 were examined to determine whether an overproduction of ligase (using *E. coli* N 1072, lop 8), or a lack of DNA endonuclease I (*E. coli* Hg 560, endo I^-) or DNA polymerase I (*E. coli* P 3478 pol A1) had any effect on the size or yield of the largest single-stranded DNA molecules observed. Although the molecular weight of the mutant single strands remained the same as the wild type, the yield decreased to 60% of the wild-type level for N 1072, 40% for HG 560, and 35% for P 3478.

In a preliminary experiment designed to confirm the hypothesis that the material giving the long retardation time was actually DNA, we searched for the heat-induced breakage of DNA described by Woodcock and Grigg. In fact, the molecular weight of single-stranded DNA obtained from wild-type K-12 *E. coli* was found to decrease when intact cells were heated before lysis. The effect on retardation time of heating at 52°C is shown in Fig. 3. The time required to reduce by one-half the molecular weight of the largest single-stranded molecules, corresponding to a reduction of τ by a factor of about 3.2, was 12–13 min. Thereafter, molecular weight appeared to continue drifting downward, but there was no indication of an exponential increase of breaks with time, as was observed by Woodcock and Grigg (1972).

Since heating at 52°C produced breaks in the DNA, further experiments were conducted to determine the temperature dependence of this heat-induced breakage. In

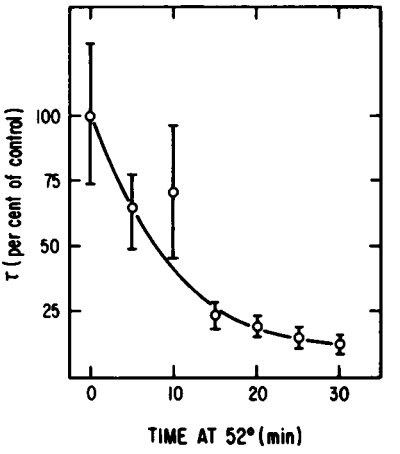

FIGURE 3 Dependence of the principal retardation time of *E. coli* W3110 single-stranded DNA on time of heating at 52°C. Cells growing in mid log-phase at 37°C were transferred to a second shaker bath at 52°C and incubated for the indicated times. The cells were then cooled, centrifuged, resuspended, lysed, and mixed by convection. Because the extensively heated samples had smaller single-strand molecular weights, higher DNA concentrations were required to observe relaxation curves (4.5 times as many cells were required for the sample which was heated for 30 min compared with the unheated control).

TABLE I

HIGH TEMPERATURE INCUBATION OF *E. COLI*

Temperature	Time	η_{rel}	τ	$L_1 \times 10^7$	k
C°	*min*				*breaks/10⁹ daltons-min*
65	3	1.39	79 ± 18	0.8	0.32
55	2.5	1.82	77 ± 22	2	0.22
45	7	1.95	105 ± 27	1.8	0.09
37	20	2.0	79 ± 12	7	—
35	10	2.02	76 ± 19	7	—
<25	<1	1.82	69 ± 12	6	—

Effect of heating on the viscoelastic properties of single-stranded DNA obtained from *E. coli* W3110. Cells grown to an A_{450} of 0.40 were centrifuged, resuspended in cold 0.5 M glycerol, 10 mM Na_2HPO_4, and heated in the viscoelastometer chamber at the indicated temperature for the specified time. Cells were then lysed with alkaline-EDTA-detergent and mixed by convection. The lowest temperature, <25°C for < 1 min, involved resuspending cells in cold glycerol/phosphate, transferring them immediately to the viscoelastometer chamber with the circulating bath off to avoid warming to room temperature, lysing quickly, and mixing by convection. Relative viscosity values have all been extrapolated to conditions of zero shear, but τ values are uncorrected for DNA concentration, which in all cases was 10 μg/ml ± 5%. The number of molecules per milliliter of largest molecules, L_1, was calculated as described by Kavenoff and Zimm (1973). The breakage rate, k, was calculated for the three highest temperatures from $L_1 = L_{1,0}e^{-kt}$, where L_1 is the number of largest molecules surviving, after a given heat treatment, $L_{1,0}$ is the number of largest molecules for the unheated control (averaged over the lowest three temperatures), and t is the time of incubation at a given temperature.

these experiments, more complete data accumulation allowed calculation of the number of largest molecules present in each lysate, L_1, using the method of Kavenoff and Zimm (1973). Table I shows that only temperatures of 45°C and above were successful in reducing L_1. No temperature produced a noticeable decrease in τ, indicating that even after incubation at the higher temperatures, a sufficient number of full-sized molecules remained to yield a principal retardation time of 80 ± 20 s. Only the highest temperature, 65°C, produced a significant decrease in viscosity, which is consistent with the results of Dugle and Dugle (1971), who observed that only temperatures of 65°C and above were effective in reducing the molecular weight of single-stranded *B. subtilis* DNA as measured by alkaline sucrose-gradient centrifugation.

To investigate the stability in alkali of bacterial lysates, *E. coli* W3110 cells were lysed at pH 12.5 and their viscoelastic properties monitored over a period of several days. Fig. 4 illustrates the change in relative viscosity observed for lysates obtained from cells which had been heated for 3 min at 55°C (curve *A*) or 20 min at 37°C (curve *B*) before lysis and prolonged incubation at 25°C and pH 12.5. After a rapid initial rise, the viscosity dropped gradually over several hours. Experimental error tended to obscure similar trends in curves of τ vs. time, but an initial rise in τ followed by a subsequent decline to a value which persisted after overnight incubation at 25°C could still be detected (Fig. 5).

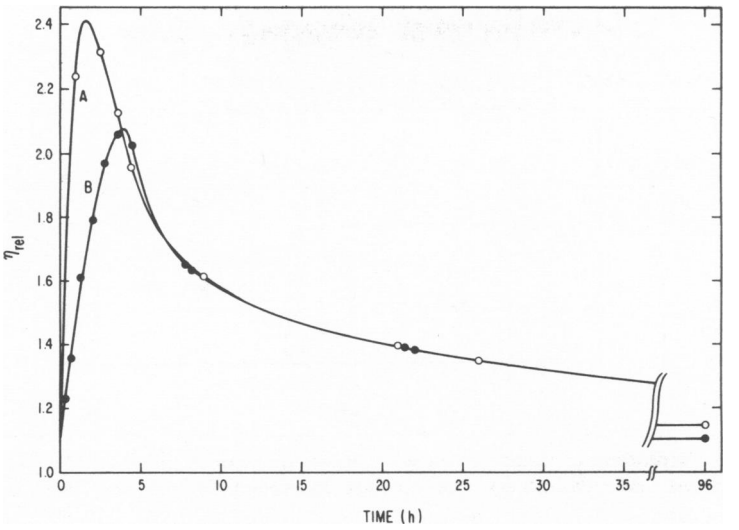

FIGURE 4 Dependence of viscosity of denatured *E. coli* DNA on time of incubation at pH 12.5, 25°C. *E. coli* W3110 cells were harvested, resuspended in 3 ml 0.5 M glycerol, and transferred to the viscoelastometer chamber for an incubation of 3 min at 55°C (curve *A*) or 20 min at 37°C (curve *B*). Cells were lysed by the addition of 1.0 ml alkaline-EDTA-detergent and mixed gently by dropping the temperature to 25°C. Each viscosity measurement has been extrapolated to zero shear. DNA concentrations: 15.5 μg/ml (*A*) and 10.5 μg/ml (*B*).

DISCUSSION

Although analyses employing the lysis and sedimentation technique of McGrath and Williams (1966) routinely yield average-size estimates of 2–5 × 10^8 daltons for single-stranded DNA released from either bacterial and eukaryotic cell (McBurney et al., 1972), the viscoelastic results reported here reveal a small amount of material in bacterial lysates with a calculated molecular weight in excess of 10^9 daltons. Such molecules could account for the entire, intact bacterial genome (Klotz and Zimm, 1972 *a*). The following report (Uhlenhopp, 1975.) suggests that lysates of mammalian cells may contain single-stranded molecules of even greater size, perhaps in excess of 10^{10} daltons. It must be emphasized in the present case that very few of the very large molecules were observed: only 4% of the total DNA population, by weight, had M = 2.2 × 10^9 and only about 20% were present as half-molecules. This small percentage of large molecules would very easily have escaped detection in a conventional sedimentation study. Viscoelastic measurements emphasize the largest molecules present, while alkaline sucrose-gradient centrifugation counts all species equally by weight. The lower sedimentation estimate could also be influenced by the anomalous sedimentation exhibited by molecules of molecular weight greater than 10^8 daltons in high centrifugal fields (Rubenstein and Leighton, 1974).

The high-temperature and high-pH incubation experiments demonstrate that the

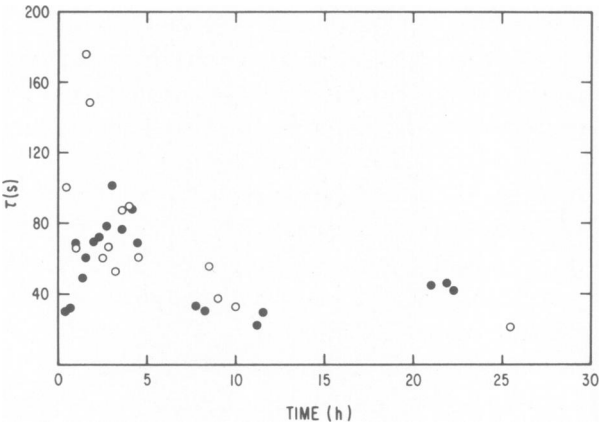

FIGURE 5 Dependence of retardation time on time of incubation at pH 12.5, 25°C for the same two experiments described in Fig. 4. Open circles correspond to curve *A*, closed circles to curve *B*.

material with long retardation times behaves as single-stranded DNA would be expected to behave, and also that the viscoelastic technique is suitable for the detection of small numbers of single-strand breaks. The data for the highest temperature, 65°C, are of particular interest. Previous studies on native bacterial DNA relied on a preliminary 70°C heat shock of intact cells to denature nucleases (Klotz and Zimm, 1972 *a*). Table I illustrates that for single-stranded work this incubation is not only unnecessary, but undesirable if the yield of high molecular weight single-stranded DNA is to be maximized. When our protocol is used, nuclease action does not appear to be a significant problem at normal temperatures since even prolonged incubation of intact cells at 37°C following a change from growth medium to buffered glycerol does not decrease the yield of high molecular weight single strands (Table I).

The problem of DNA instability during prolonged storage at high pH has been described only recently (Hill and Fangman, 1973; Grossman et al., 1973; Ormerod, 1973). This instability must be at least in part responsible for the gradual decline in viscoelastic parameters with time at pH 12.5 (Figs. 4 and 5).

The change of viscoelastic properties with time at high pH can be interpreted as follows. The initial rise occurs during the finite time required to detach proteins, lipids, RNA, etc., from the DNA and to complete chromosome decondensation, denaturation, and unwinding. Preliminary heating at 55°C introduces a sufficient number of single-strand breaks (Table I) to allow more rapid unwinding and hence an earlier peaking of the viscosity vs. time curve (Fig. 4, curve *A*). (This curve also peaks higher because the DNA concentration is 50% greater than in the 37°C experiment.) The subsequent decrease in viscoelastic properties can be explained, at least in part, by alkali-catalyzed scission of the phosphodiester backbone of single-stranded DNA, as has been observed previously by Hill and Fangman (1973).

It is also possible that a slow strand-separation process contributes to the decrease

of viscosity and τ with time. If this process is involved, however, it must become insignificant after about 10 h, since τ appears to reach a plateau after that time. If this is the correct description of the situation, then the τ corresponding to isolated single strands is the plateau value of 30–40 s, and the corresponding molecular weight is about 1.3×10^9. It is not possible definitely to rule out this possibility with the present data alone. (The existence of the plateau does not deny the possibility of the backbone-scission process, since the number of molecules with large τ still decreases with time, as indicated by the decrease in viscosity and in the amount of recoil.)

Another observation eliminates the possibility that disaggregation and untangling of strands is the *only* cause of the slow decline in properties. In the following paper we show that mammalian DNA of still higher molecular weight degrades faster than the bacterial lysates, no viscoelasticity being observable after overnight incubation at pH 12.4. This behavior is expected if hydrolysis of the backbone is the dominant process, since the rate per molecule should increase as the molecular weight, and hence the target size, goes up. However, larger molecules would surely be slower in unwinding and diffusing apart, so that the observed behavior is inconsistent with a purely diffusion-limited process.

Consequently, we favor the former explanation, namely, that the rise in η_{rel} is due to unfolding and unwinding while the decline in η_{rel} is mostly due to alkali-catalyzed scission of the phosphodiester backbone.

Finally, one comment should be made regarding the exact configuration of the DNA being examined in this and related studies. We have assumed throughout that the viscoelastic material being analyzed in bacterial lysates is unaggregated, single-stranded DNA. The response of retardation time and η_{rel} to high-temperature and high-pH incubation supports that assumption. Related experiments analyzing bacterial lysates by alkaline sucrose-gradient centrifugation make a similar assumption. However, in light of the complexities encountered when lysing mammalian cells in alkaline detergent (Uhlenhopp, 1975), additional proof will be required before the existence of partially double-stranded or non-specifically aggregated species can be conclusively excluded.

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