Ethidium bromide-mediated renaturation of denatured closed circular DNAs. The nature of denaturation-resistant fractions of bacteriophage PM2 closed circular DNA

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ABSTRACT

Addition of the intercalating dye ethidium bromide (EtdBr) to a solution of alkali-denatured double-stranded closed circular PM2, ϕ X174, or λ b₂b₅c phage DNAs, under conditions such that the solution remains strongly alkaline, can result in the renaturation of up to 100% of the DNA upon neutralization of the solution. For a fixed time of incubation of the alkaline dye-containing solution before neutralization, there exists a minimum concentration of the dye below which no EtdBr-mediated renaturation is observed for each species of closed circular DNA examined. These minimum concentrations increase, for a given DNA, with increasing ionic strength and temperature. The kinetics of accumulation of forms renaturing upon neutralization of alkaline solutions, at fixed concentrations of dye and DNA, are dependent upon the molecular weight and superhelix density of the starting DNA. After extended periods of incubation at a fixed ionic strength and temperature, however, the profiles of percentage of DNA renatured as a function of ethidium concentration become very similar for all the closed circular DNAs tested and display a transition from an absence of dye-mediated renaturation to virtually 100% renaturation upon neutralization over a small range of dye concentration. Circular DNA containing one or more strand scissions remains strand-separated under all the conditions used to effect the renaturation of closed circular DNA. These findings indicate that configurations of closed circular DNA, in which at least some of the complementary bases are apposed, can be selectively stabilized and accumulate in the presence of ethidium in solutions containing 0.19 N hydroxide ion.

The closed circular DNA of bacteriophage PM2 has properties distinct from those of the other DNAs of this study in that it has been shown to contain fractions which exist in the base-paired duplex form after neutralization of strongly alkaline solutions of this DNA incubated at ambient temperature, while no duplex DNA is observed after exposure to alkali and neutralization of solutions of closed circular DNA from other sources.^{1,2} The fraction of denaturation-resistant PM2 DNA is shown in the present work to depend upon the temperature and time of incubation in alkali, but not upon the superhelix density of the starting DNA. PM2 closed circular DNA also behaves anomalously with respect to its kinetics of accumulation of forms renaturing upon neutralization of alkaline, EtdBr-containing solutions. Evidence is presented that the translocation of one of the strands of a closed circular molecule relative to the other, which is required for the molecule to exist in the denatured form at neutral pH, is a process to which PM2 DNA is less labile than the other closed circular DNAs of this study.

INTRODUCTION

Closed circular duplex DNA exposed to concentrations of hydroxide ion sufficient to fully titrate the molecules forms, upon neutralization, a stable denatured closed DNA species.¹⁻⁵ Such denatured closed circular DNA (denDNA I) molecules consist of two interwound strands which have been translocated relative to one another so that the complementary bases are out of register.^{3,4} The unpaired strands are interwound much as in base-paired duplex DNA, although apparently with sufficiently fewer apposed bases per turn than in duplex DNA that the structure is positively supercoiled.²

Denatured closed circular DNA does not renature under conditions which cause the renaturation of strand-separated DNA. Heating closed circular DNAs under the usual renaturation conditions of neutral pH and a temperature 25°C below that of the midpoint of the helix-coil transition (T_m) introduces strand breaks at an unacceptable rate owing to the high values of T_m characteristic of such DNAs.⁶ Pouwels *et al.*⁴ were able to renature coliphage ϕ X174 replicating form (RF) denDNA I by heating at alkaline pH (e.g., pH 12 and 50°C in 1 M NaCl) and then neutralizing the solutions. The rates of renaturation of this DNA as a function of alkaline pH, ionic strength, and temperature have also been studied by Strider and Warner.⁷

The value of T_m for a linear duplex DNA is increased in the presence of intercalating agents such as ethidium bromide,^{8,9} actinomycin, anthracyclines,¹⁰ and ellipticine.¹¹ However, the rate of renaturation of previously denatured coliphage T2 DNA was decreased drastically, to a value near zero, in solutions containing 0.4 M Na^+ and sufficient concentrations of EtdBr to render the number of moles of ethidium bound per mole of DNA nucleotide 0.06 and greater.¹²

The effects of EtdBr on the renaturation of several species of closed circular DNA under strongly alkaline conditions have been examined in the present study. The DNAs examined include supercoiled and nonsupercoiled forms of PM2 and ϕ X174 replicating form phage DNAs and the nonsupercoiled closed circular form of λ b₂b₅c phage DNA. It is shown that these DNAs, when exposed first to alkali and then to EtdBr while maintaining the alkaline conditions, can gradually accumulate in configurations from which, upon neutralization of the solutions, up to 100% of duplex (base-paired) DNA is formed. The percentage of closed circular DNA appearing as the duplex form upon neutralization has been monitored as a function of time of incubation after the addition of EtdBr to the alkaline solutions, concentration of EtdBr, ionic strength, temperature, molecular weight, and superhelix density (σ_0).

number of superhelical turns per ten base pairs¹³) of the starting DNA. The results indicate that EtdBr present in strongly alkaline solutions (0.19 N OH⁻) can cause the selective stabilization of configurations of closed circular DNA molecules in which at least some of the bases which are complementary in the native molecule are apposed, under conditions for which strand-separable DNAs exist exclusively in the strand-separated form.

Phage PM2 closed circular DNA exhibits fractions which are found in the duplex form after exposure to strongly alkaline conditions which result in the denaturation of 100% of closed circular DNA from other sources.^{1,2} The kinetics of accumulation of forms renaturing upon neutralization of EtdBr-containing alkaline solutions of this DNA are shown in this work also to be anomalous. This denaturation-resistant material is shown not to result from a fraction of PM2 DNA which is uniquely resistant to denaturation, but rather is the result of a stronger resistance in PM2 DNA, compared to that of the other closed circular DNAs examined, to translocation of the two strands relative to one another in alkaline solutions near ambient temperature.

MATERIALS AND METHODS

DNA and enzyme preparations. Supercoiled closed circular phage PM2 DNA (DNA I) was prepared as described.^{14,15} Some of the preparations used in the later stages of this work were obtained using the procedure of Richardson¹⁶ as modified by Legerski *et al.*¹⁷ Coliphage ϕ X174 RF DNA I was prepared by the procedure of Godson and Vapnek¹⁸ as modified.^{19,20} Fractions containing circular dimeric and catenated dimeric forms of this DNA,²¹ obtained from the sucrose gradient procedure,²⁰ were pooled and again centrifuged in sucrose gradients in the same manner as for the purification of the monomeric DNA. The resulting preparation contained approximately 40% (by mass) dimeric forms with the remainder consisting of monomeric RF DNA I. Coliphage λ b₂b₅c DNA was prepared as described.^{1,22}

The nicked circular forms (DNA II) of PM2 and ϕ X174 RF DNAs were generated by very limited digests of the respective closed circular forms with bovine pancreatic DNase I as suggested by Jaenisch and Levine.²³ Closed circular DNAs of varying superhelix densities were obtained from the nicked circular species through the action of *E. coli* polynucleotide ligase in the presence of various concentrations of EtdBr.²⁴⁻²⁶ λ b₂b₅c DNA was cyclized and treated with ligase to produce the nonsupercoiled closed circular form (DNA I⁰) as reported.¹ The preparation of ligase was that used in previous work from this laboratory.¹

PM2 and ϕ X174 RF DNAs I were converted to the respective DNAs I^o with the DNA topoisomerase I (nicking-closing enzyme)²⁷⁻²⁹ from the nuclei of Ehrlich ascites carcinoma cells. Preparations of Ehrlich mouse ascites cells were the gifts of Mr. Normand Caron. Topoisomerase I was obtained from ascites cell nuclei³⁰ by the procedure of Germond *et al.*³¹ as modified.¹⁷ Reactions using this enzyme were carried out as reported.¹⁷ The details of separation of closed circular DNA from other forms of DNA in propidium diiodide-CsCl density gradients²⁵ have been given.¹⁷

Measurement of superhelix densities. The superhelix densities of the artificially closed circular DNAs were determined from the separations between closed and nicked circular forms after centrifugation for 48 hours in EtdBr-CsCl or propidium diiodide-CsCl buoyant density gradients according to the procedure of Gray *et al.*,²⁶ using viral PM2 DNA I as the reference closed circular DNA. The equations for the calculation of σ_0 have been modified for the use of a reference DNA other than SV40 DNA I by Upholt³² and by Burke and Bauer.³³ The details for the use of PM2 DNA I as reference are given elsewhere.³⁴ In addition, the values of σ_0 reported here are based on the redetermination³⁵⁻³⁷ of the amount of DNA helix unwinding accompanying the intercalation of a single ethidium molecule. The values of σ_0 reported refer to those in 2.83 M CsCl near neutral pH at 20°C.²⁶

Treatment of PM2 closed circular DNAs with alkali. Treatment of PM2 closed circular DNAs with alkali, in those series of experiments in which EtdBr was not involved, was performed by either of these methods: 1) 5 μ l of DNA at a concentration of 270 μ g/ml in NaCl/Tris/EDTA buffer (0.1 M NaCl, 20 mM Tris·HCl, 1 mM EDTA (pH 8.1)) were mixed with 15 μ l of 2.83 M CsCl, 0.25 M KOH, 50 mM potassium phosphate, 1 mM EDTA. The sample was incubated at 25°C for a specified time and the alkaline incubation was terminated by neutralizing the mixture with 20 μ l 2 M Tris·HCl (pH 8.1) and placing it on ice; 2) 20 μ l of DNA at a concentration of 61.3 μ g/ml in NaCl/Tris/EDTA buffer were incubated with 5 μ l 1 M NaOH at 25°C or at 70°C. Samples were neutralized with 10 μ l 1 M Tris·HCl (pH 8.1) and placed on ice after different times of incubation. A reciprocal extinction of 50 μ g/ml- $A_{260\text{ nm}}^{1\text{ cm}}$ was assumed for duplex DNA for calculating concentrations of DNA from absorbance measurements.

Treatment with ethidium bromide of closed circular DNAs in alkaline solution. A typical experiment was performed by mixing 5 μ l of 1 M NaOH with 10 μ l of duplex closed circular, duplex nicked circular, or alkali-denatured closed circular DNA at a concentration of 123 μ g/ml in

NaCl/Tris/EDTA buffer. Incubation of this mixture at a constant temperature for 5 minutes was followed by addition of 10 μ l of an appropriate solution of EtdBr in the above buffer. In experiments in which it was desired to vary the ionic strength, the addition of an appropriate amount of 5 M NaCl preceded the addition of the EtdBr solution. The combined volumes of the NaCl and EtdBr solutions was 10 μ l. Incubation (at the same temperature except where otherwise indicated) was continued for the indicated period, after which the alkaline solution was neutralized with 10 μ l of 1 M Tris·HCl (pH 8.1) and placed on ice. Concentrations of EtdBr were calculated using a reciprocal extinction of $79.2 \mu\text{g/ml} \cdot \text{A}_{485 \text{ nm}}^{1 \text{ cm}}$ in NaCl/Tris/EDTA buffer.³⁸

Cross-linking of native and denatured PM2 closed circular DNA with mitomycin C. PM2 DNA I or denDNA I was cross-linked with mitomycin C by a procedure similar to that of Pritchard and Eichinger.³⁹ A solution containing DNA in 30 mM sodium phosphate (pH 7.0) was mixed with an appropriate amount of mitomycin C (Sigma Chemical Co.) dissolved in methanol. The mitomycin C was reduced, in the presence of the DNA, by an appropriate amount of sodium dithionite while gaseous nitrogen was bubbled through the solution which was kept on ice during the reaction. Reacted samples were dialyzed into NaCl/Tris/EDTA buffer.

Under the above conditions, a substantial fraction (approximately 40%) of the closed circular DNA is converted to DNA II due to phosphodiester bond cleavage presumably caused by the reducing agents in the reaction mixture. Any PM2 DNA II present before the reaction is not cross-linked, whereas PM2 DNA I and denDNA I readily react to form interstrand cross-links (Legerski, R. J., Lau, P. P. and Gray, H. B., Jr., unpublished results). In the case of the PM2 DNA I, this is apparently another example of the preferential reactivity of negatively supercoiled DNA with reagents which do not, under the same conditions, react with nicked circular or linear duplex DNA.⁴⁰⁻⁴⁴ Thus, any cross-linked nicked circular DNA present must arise from DNA which was initially covalently closed.

The extent of cross-linking was assayed by analytical band sedimentation,⁴⁵ performed in an alkaline solvent as described.^{1,46,47} The sedimentation solvent contained 2.83 M CsCl, 0.1 M KOH, 1 mM EDTA and is denoted alkaline sedimentation solvent in the following. The nicked circular cross-linked DNA does not undergo strand separation in alkali and hence sediments much faster than non-cross-linked denatured DNA II. All of the DNA nicked during the reaction was found to contain one or more cross-links in the mitomycin C-treated preparations of this work, so that it may be assumed that

100% of the closed circular DNA was also cross-linked. Relative amounts of material in the sedimenting bands were determined by cutting and weighing bands from photoelectric scanner tracings^{48,49} in these and in numerous other experiments where relative amounts of sedimenting materials are reported.

Treatment of PM2 DNA I with Pronase, RNase, or sodium dodecyl sulfate. A solution containing 142 μ g PM2 DNA I and 500 μ g Pronase (Calbiochem) in 0.5 ml NaCl/Tris/EDTA buffer was incubated at 37°C for 1 hour before removal of protein by sequential extraction with distilled phenol and diethyl ether, followed by exhaustive dialysis into NaCl/Tris/EDTA buffer. Treatment of PM2 DNA I with bovine pancreatic RNase A was as above, except that the Pronase was replaced by 250 μ g of RNase A. Solutions of PM2 DNA I in NaCl/Tris/EDTA buffer at a concentration of 140 μ g/ml were incubated with 1% and 8% (w/v) sodium dodecyl sulfate in separate mixtures for 1 hour at 37°C. The detergent was partly removed by centrifugation in the cold before exhaustive dialysis against the above buffer.

Separation of duplex closed circular DNA from denatured closed circular DNA. Separation of the duplex closed circular and denatured closed circular forms of PM2 DNA was done by buoyant density equilibrium centrifugation in propidium diiodide-CsCl gradients at 36 Krpm at 20°C for 48 hours in a Beckman SW50.1 rotor, which conditions are similar to those of Grossman *et al.*² Rebanding of the samples obtained from puncturing of the tubes and drop collection under long-wave (365 nm) ultraviolet light was done in some cases, as the separation between DNA I and denDNA I in this procedure is sufficiently small² that cross-contamination is often significant.

Assay of percentage of duplex closed circular DNA after exposure to alkali. After neutralization in the procedures involving incubation of the DNA in alkaline solution, the samples were placed on ice and centrifuged within 1 hour in the neutral sedimentation solvent (2.83 M CsCl, 20 mM Tris·HCl (pH 8.1), 1 mM EDTA) using the band sedimentation method as described.¹ Fully denatured closed circular DNA and fully renatured closed circular DNA separate readily.^{1,4,5} Contaminating nicked circular DNA remains completely denatured and sediments at a rate between that of duplex closed circular DNA and denDNA I (ref. 1). The percentage of duplex closed circular DNA was calculated as that of the total amount of closed circular DNA.

RESULTS

Ethidium bromide-mediated renaturation of denatured closed circular DNA. Samples of closed circular DNA were exposed to alkali for 5 minutes at 25°C and then mixed with an aliquot of dye solution (or buffer alone) such that the concentration of hydroxide ion was 0.19 N. Incubation at 25°C was continued for the indicated times after which the solutions were neutralized. The neutralized sample was sedimented in the neutral solvent in order to monitor the percentages of denatured and duplex forms. In Figure 1(a), which represents experiments with viral closed circular PM2 DNA, the band corresponding to the renatured closed circular DNA (22 S) has the same sedimentation coefficient as that of viral PM2 DNA I. The 42 S band represents the denatured closed circular material. When an aliquot of the same sample was centrifuged in the alkaline solvent, nearly all the DNA sedimented at the rate (65 S) characteristic of closed circular viral PM2 DNA in this solvent (Figure 1(b)). The denatured nicked circular DNA, which is barely evident as a 28 S band in Figure 1(a), sediments as an 18 S species in

Figure 1. Photoelectric scanner tracings from band sedimentation experiments with samples, treated as indicated, of PM2 phage DNA containing predominantly the closed circular form. Sedimentation was at 25°C with the monochromator adjusted to 265 nm and the scanner sensitivity adjusted to 100 mV/cm. Direction of sedimentation is from left to right. (a) PM2 DNA I was exposed to alkali for 5 minutes at 25°C, after which EtdBr was added to the alkaline solution to a final concentration of 100 µg/ml. Incubation at 25°C was continued for 5 minutes before neutralization and sedimentation in the neutral solvent (Materials and Methods). Numbers above the sedimenting bands represent sedimentation coefficients (s) in Svedberg units (S) to the nearest integer. Species represented by each band are, in order of increasing s, PM2 DNA I, denatured PM2 DNA II, and PM2 denDNA I. (b) Aliquot of sample in (a) sedimented in the alkaline solvent (Materials and Methods). The only band detectable in the tracing represents denatured closed circular PM2 DNA. The number above the band has the same meaning as those in (a) above. Arrow indicates the position expected for denatured DNA II in this solvent (18 S). The concentration of DNA after addition of EtdBr was 49 µg/ml in these and in all other experiments except where otherwise indicated.

the alkaline solvent but is not detectable in Figure 1(b), largely due to the strong ultraviolet absorption by the EtdBr near the meniscus. Figure 1(b) indicates that the 22 S material in the neutral solvent corresponds to closed circular DNA and not to another species such as partially renatured nicked circular DNA.

(a) Fraction of DNA renaturing as a function of ethidium bromide concentration. Below about 35 $\mu\text{g/ml}$ of EtdBr at the temperature and DNA concentration of these experiments, viral PM2 closed circular DNA displays a fraction (approximately 10%) which exists in the duplex form upon neutralization, while the ϕX174 RF and $\lambda\text{b}_2\text{b}_{5\text{c}}$ closed circular DNAs are fully denatured under these conditions (Figure 2). This denaturation-resistant fraction of PM2 closed circular DNA is discussed more fully in a later section. Above about 35 $\mu\text{g/ml}$ of dye, the three species of closed circular DNA examined display dye-mediated renaturation. The fraction of fully renatured DNA depends strongly upon the dye concentration at a given time of incubation and upon the time of incubation at a fixed concentration of EtdBr (Figure 2).

The percentage of renatured DNA, at a fixed time of incubation in alkaline solution after addition of the dye, is observed in at least one instance for each DNA to display a maximum when plotted as a function of dye concentration. The dye concentration corresponding to such maxima is about the same (near 70 $\mu\text{g/ml}$) for all the DNAs. Although different extents of renaturation are observed for these DNAs at each dye concentration above 35 $\mu\text{g/ml}$ and each time of incubation, there is a tendency of the profiles to converge with increasing incubation time. After 120 minutes of exposure, 100% of ϕX174 RF DNA I is renatured at all concentrations of EtdBr above approximately 35 $\mu\text{g/ml}$, and there are sharp transitions, over the same range of dye concentration as for the ϕX174 RF DNA, from zero percent renaturation (except for PM2 DNA I, which displays approximately 10% in the duplex form independently of the presence of EtdBr) to high percentages of renaturing DNA for the PM2 and $\lambda\text{b}_2\text{b}_{5\text{c}}$ closed circular DNAs (Figure 2(c)). When the PM2 and $\lambda\text{b}_2\text{b}_{5\text{c}}$ closed circular DNAs were incubated for 19 hours in alkaline EtdBr solutions, no dye-mediated renaturation was observed at dye concentrations of 0 and 30 $\mu\text{g/ml}$ (3 to 4% of the PM2 DNA renatured), but 100% of renaturing closed circular DNA was observed at 50 and 70 $\mu\text{g/ml}$ of EtdBr (data not shown). At 40 $\mu\text{g/ml}$ of dye, 17% and 100% of renatured DNA was observed for the PM2 and $\lambda\text{b}_2\text{b}_{5\text{c}}$ closed circular DNAs, respectively. The difference in the percentages of renaturing DNA between these two closed

Figure 2. Percentage of closed circular DNA renaturing upon neutralization of the solutions after exposure first to alkali alone, then to alkali in the presence of various concentrations of EtdBr. Solutions of DNA were mixed with NaOH solution as described in Materials and Methods, incubated for 5 minutes at 25°C, subjected to the addition of EtdBr at the indicated concentrations, incubated at 25°C for the times given below, and neutralized. Solutions were analyzed for the relative amounts of denatured and renatured closed circular DNA in these and in all other experiments by band sedimentation (Materials and Methods). The time of incubation in the presence of dye in alkaline solution was 5, 15, and 120 minutes in (a), (b), and (c), respectively. Δ , PM2 DNA I; $\square$, ϕ X174 RF DNA I; $\bullet$, λ b₂b₅c DNA I°; $\circ$, PM2 DNA II. Note changes in abscissa scale in (a) and (b). Point immediately after scale change in (a) corresponds to 120 μ g/ml EtdBr.

circular species at 40 μ g/ml of EtdBr cannot be taken as indicative of any difference in the renaturation profiles for these two DNAs at long times of incubation, owing to the small range of dye concentration over which the transition from no dye-mediated renaturation to very significant dye-mediated renaturation takes place.

However, even after 19 hours of incubation, only 53% and 32% of the PM2 and λ b₂b₅c species, respectively, are found in the duplex form after neutralization of alkaline solutions of these DNAs containing 200 μ g/ml of EtdBr. While it is shown that the profiles from experiments such as shown in Figure 2 become indistinguishable at long times of incubation for the DNAs of this study at dye concentrations up to at least 70 μ g/ml, establishing that the larger two DNAs can accumulate 100% of renaturing closed circular material at dye concentrations as high as 200 μ g/ml would apparently require times of incubation which would be prohibitively long due to such problems as alkaline hydrolysis of the DNA. It was also found that perturbation of the alkaline

EtdBr solutions by vigorous mixing (as with a vortexing mixer operated at top speed) during incubation reduced or rendered non-existent the renatured DNA found upon neutralization. The factors which preferentially stabilize renaturable forms of the closed circular DNA are thus sensitive to hydrodynamic shear forces and/or heating which occur during vigorous stirring.

Nicked circular forms of DNA remained denatured under all the conditions of dye concentration and incubation time represented in Figure 2. This was established by noting the sedimentation behavior of the low percentages of DNA II present in some of the samples. It was also found that PM2 denDNA I, produced by alkaline denaturation and purified as described in Materials and Methods, renatures upon neutralization after exposure to alkaline solutions containing 100 $\mu\text{g/ml}$ EtdBr under the conditions of solvent and temperature represented by the data of Figure 2.

(b) Kinetics of the dye-mediated renaturation: dependence upon molecular weight and superhelix density of the DNA. The kinetics of accumulation of closed circular DNA renaturing upon neutralization, after alkaline denaturation and incubation in alkaline solution containing 100 $\mu\text{g/ml}$ of EtdBr, are shown in Figure 3. The points at zero time of incubation represent

Figure 3. Kinetics of the dye-mediated renaturation of alkali-denatured closed circular DNAs. Samples of DNA were denatured, incubated in the presence of EtdBr (100 $\mu\text{g/ml}$) in the alkaline solution at 25°C for the times indicated, and neutralized as described in Materials and Methods. $\square$, ϕX174 RF DNA I; $\blacksquare$, ϕX174 RF DNA I $^\circ$; Δ , PM2 DNA I; $\blacktriangle$, PM2 DNA I $^\circ$; $\circ$, dimeric forms of ϕX174 RF closed circular DNA; $\bullet$, $\lambda\text{b}_2\text{b}_{5c}$ DNA I $^\circ$. Note change in abscissa scale in (b).

experiments in which the alkaline solution was simply neutralized, without addition of EtdBr, after 5 minutes of incubation.

The data represented in Figure 3(a) suggest that, for a given species of DNA, the rate at which forms accumulate which renature upon neutralization increases with increasing number of negative superhelical turns (increasing values of $-\sigma_0$) in the DNA prior to its exposure to alkali. The rates of renaturation increase with increasing values of $-\sigma_0$ in the case of the ϕ X174 closed circular DNAs and apparently also in the case of the PM2 closed circular DNAs. At times of exposure greater than one minute, at which the curves for the two forms of PM2 closed circular DNA level off somewhat, the more highly supercoiled species definitely renatures to a greater extent after a given time of incubation in alkali in the presence of dye.

The effect of molecular weight at constant superhelix density upon the kinetics of renaturation is not monotonic in that the PM2 closed circular forms initially accumulate renaturable DNA at a much faster rate than either of the ϕ X174 RF closed circular DNAs or λ b₂b₅c DNA I° (Figure 3(a)), while at longer times of incubation, the rate of accumulation of renaturing forms of PM2 DNA I can become slower than that of either the smaller ϕ X174 RF DNA I or the larger λ b₂b₅c DNA I° (Figure 3(b)). This biphasic character of the renaturation kinetics of the PM2 closed circular species is not observed in any of the other DNAs examined. A series of experiments very similar to those of Figure 3(b) was done at an EtdBr concentration of 70 μ g/ml (data not shown). The rate of accumulation of renaturing forms of DNA was generally more rapid as might be expected from Figure 2. The profile for PM2 DNA I was again biphasic and the initial renaturation rate again more rapid than that of the ϕ X174 RF I and λ b₂b₅c I° DNAs.

The dimeric forms of ϕ X174 RF closed circular DNA display kinetics of accumulation of renaturing forms which are comparable to those of the monomeric RF DNA I (Figure 3(a)). Although the sample used contained approximately 60% of monomeric RF DNA I, the sedimentation coefficients of the denatured and duplex monomeric and dimeric closed circular forms differ sufficiently that it was possible to monitor the relative amounts of the duplex and denatured dimeric forms. The dimeric forms of this DNA should have a molecular weight of 7.10×10^6 (ref. 50) which is comparable to the value of 6.5×10^6 for PM2 DNA.⁵¹ Any comparison between the kinetics of renaturation of the PM2 and dimeric ϕ X174 RF DNAs is complicated by the fact that an appreciable fraction of the ϕ X174 dimeric DNA (roughly 40% in the absence of chloramphenicol in the cultures) exists as catenated dimeric species

instead of cyclic dimeric forms, and that the fraction of catenated species depends upon the concentration of chloramphenicol in the infected cultures;⁵² chloramphenicol was used in this work at a concentration of 30 $\mu\text{g/ml}$. Moreover, the cyclic dimeric $\phi\text{X174 RF}$ DNA is a head-to-tail tandem duplication of the monomer genome, whereas PM2 DNA represents a single genome. However, these data can be taken to indicate that the behavior observed for PM2 closed circular DNAs in these experiments is not a general characteristic of closed circular DNAs of molecular weight near that of PM2 DNA.

It proved possible to alter the behavior of PM2 DNA I and PM2 DNA I^o in the above kind of experiment so that both features which were peculiar to these DNAs, i.e., the biphasic renaturation profiles and the existence of duplex forms after incubation with alkali and neutralization in the absence of dye (Figure 3), were no longer present. Dye-mediated renaturation experiments were carried out as above except that the initial denaturation in alkali took place at 70°C, with the samples cooled to 25°C for 5 minutes before addition of EtdBr and further incubation at 25°C. This procedure had no great effect upon the renaturation profiles for $\phi\text{X174 RF}$ DNA I, $\phi\text{X174 RF}$ DNA I^o, and $\lambda\text{b}_2\text{b}_{5c}$ DNA I^o, but dramatically altered the behavior of both the closed circular PM2 DNAs (Figure 4). These last curves no longer display biphasic behavior; moreover, comparison of the curves of Figure 4 for the experiments starting with $\phi\text{X174 RF}$ I^o, PM2 I^o, and $\lambda\text{b}_2\text{b}_{5c}$ I^o DNAs, for which the value

Figure 4. Kinetics of dye-mediated renaturation of closed circular DNAs which were exposed to alkali at 70°C. Solutions of DNA were mixed with NaOH solution, heated for 5 minutes at 70°C, and then incubated for another 5 minutes at 25°C before the addition of EtdBr to the alkaline solution to a concentration of 100 $\mu\text{g/ml}$. After incubation for the indicated times at 25°C, solutions were neutralized and placed on ice. There was no significant loss of closed circular DNA caused by the heating. The symbols represent the same starting DNAs as in the legend to Figure 2.

of σ_0 for the starting DNAs is the same, reveals that the percentage of DNA accumulating in renaturable forms, at a fixed time of alkaline incubation, now increases monotonically with decreasing molecular weight. The qualitative relationship between percentage of renaturing DNA and superhelix density, noted in connection with Figure 3, is also observed for the samples of PM2 DNA heated at 70°C in alkali.

Scanner tracings from experiments in which PM2 DNA I was mixed with NaOH solution and incubated (in the absence of EtdBr) for 5 minutes at 25°C or 70°C before neutralization are shown in Figure 5(a) and 5(b), respectively. The sample heated at 70°C was cooled to 25°C before neutralization. Figure 5 demonstrates the virtual disappearance of the 22 S₂ species, which represents duplex DNA I, upon heating in alkali at 70°C.

(c) Dependence of the dye-mediated renaturation of closed circular DNAs upon ionic strength and temperature. Alkaline solutions of PM2 DNA I and λ b₂b₅c DNA I^o were incubated in the presence of dye in media of different ionic strengths, under the same conditions of temperature and DNA concentration as for the data of Figure 2, and then neutralized to obtain the renaturation profiles shown in Figure 6. The data at the lowest ionic strength for PM2 DNA I (open upright triangular plot symbols) is the same as shown in Figure 2(a). The concentrations of EtdBr required for significant dye-mediated renaturation of each DNA increases markedly upon increasing

Figure 5. Photoelectric scanner tracings from band sedimentation experiments, in the neutral solvent with the scanner sensitivity adjusted to 100 mV/cm, with samples treated as indicated, of predominantly closed circular phage PM2 DNA. Numbers above bands have the same meanings and represent the same forms of PM2 DNA as in the legend to Figure 1(a). (a) Sample was exposed to NaOH solution for 5 minutes at 25°C, after which the solution was neutralized and placed on ice as described in Materials and Methods. (b) Aliquot of DNA solution in (a) treated as in (a) except that the solution was heated at 70°C for 5 minutes in the alkaline solution before the solution was cooled and neutralized. Arrow indicates the position expected for duplex DNA I (22 S₂). The slight increase in percentage of denatured DNA II (28 S₂) is the result of hydrolysis in the alkaline solution at 70°C.

Figure 6. The effect of ionic strength upon the percentage of renaturing closed circular DNA as a function of EtdBr concentration. After 5 minutes of incubation in alkaline solution at 25°C (Materials and Methods), aliquots of 5 M NaCl and EtdBr stock solution were added, in that order, to give the indicated ionic strengths and dye concentrations and the solutions were incubated at 25°C for an additional 5 minutes (PM2 closed circular DNA) or 25 minutes ($\lambda b_2 b_{5c}$ closed circular DNA). These solutions were then neutralized, placed on ice, and examined by band sedimentation. Open symbols and filled symbols represent experiments starting with PM2 DNA I and $\lambda b_2 b_{5c}$ DNA I°, respectively. Ionic strengths of reaction mixtures before neutralization were: ●, Δ, 0.28 M; ▲, ○, 0.38 M; ■, ▽, 0.48 M.

the ionic strength from 0.28 M to 0.48 M in the alkaline solutions. The fraction of PM2 DNA which is in the duplex form at EtdBr concentrations below those for which dye-mediated renaturation is observed decreases with increasing ionic strength, as seen from the data represented by open plot symbols in Figure 6.

The minimal requirement of EtdBr for appreciable dye-mediated renaturation increases with temperature for each species of closed circular DNA (Figure 7). The curves for the incubations at 25°C are the same in Figure 7 as for the corresponding species of DNA in Figure 6. No fraction of either species of DNA was observed to renature at 70°C at EtdBr concentrations as high as 300 μg/ml. The fraction of PM2 DNA existing in the duplex form at concentrations of dye too low to affect dye-mediated renaturation is highest at the lowest temperature of incubation (6°C) and is no longer present at the highest temperature (40°C) of these experiments (see curves defined by open plot symbols in Figure 7).

The $\lambda b_2 b_{5c}$ closed circular DNA displayed a high percentage of molecules renaturing upon neutralization after the DNA was exposed to 0.19 M OH⁻ at 6°C in the absence of dye (Figure 7). When the neutralized solutions of $\lambda b_2 b_{5c}$ DNA treated at 6°C were sedimented in order to determine the percentage of denatured and renatured species, the band corresponding to

Figure 7. The effect of temperature upon the percentage of renaturing closed circular DNA as a function of EtdBr concentration. After 5 minutes of alkaline incubation (Materials and Methods) at the indicated temperature, EtdBr was added to the indicated concentration and incubation at the same temperature was continued for an additional 5 minutes (PM2 closed circular DNA) or 25 minutes (λ b₂b₅c closed circular DNA). These solutions were then neutralized, placed on ice, and examined by band sedimentation. It was necessary to heat the sample of λ b₂b₅c DNA treated at 6°C after its neutralization as noted in the text. Open symbols and filled symbols represent experiments starting with PM2 DNA I and λ b₂b₅c DNA I°, respectively. Temperatures of incubation were: ■, O, 6°C, ●, Δ, 25°C, ▲, □, 40°C. Note change in abscissa scale at 80 μg/ml.

denatured DNA was present, but there was a considerable portion of the material sedimenting at rates intermediate between those of the relatively small amount of denatured DNA II and duplex DNA I° (Figure 8(a)). When aliquots of these neutralized samples were heated at 40°C for 15 minutes, stirred vigorously, placed on ice, and subjected to band sedimentation, the material sedimenting near 48 S had disappeared and the amount of material in the band corresponding to duplex DNA I° had concomitantly increased (Figure 8(b)). Figure 8(c) shows that the bulk of the material of Figures 8(a) and (b) sediments as closed circular DNA in the alkaline solvent; the 48 S material thus does not represent species arising from nicking of closed circular DNA. The data corresponding to λ b₂b₅c DNA incubated at 6°C in Figure 7 was obtained after heating the neutralized solutions as above.

The 48 S species of Figure 8(a) is attributed to partially renatured closed circular DNA. DNA in this form clearly has some base pairs in register (since mild heating effected its renaturation), but does not have sufficient thermal energy to overcome topological "blocks" to renaturation presumably present in the collapsed structure even during sedimentation at 25°C in 2.83 M CsCl. These partially renatured forms were not observed at any temperature for the smaller PM2 and ϕ X174 RF closed circular species and

Figure 8. Photoelectric scanner tracings from band sedimentation experiments at 25°C with samples, treated as indicated, of λ b₂b₅c DNA initially consisting of approximately 80% DNA I°. Scanner sensitivity was adjusted to 100 mV/cm. (a) Alkali-denatured sample incubated in the presence of 10 μ g/ml EtdBr for 15 minutes at 6°C, neutralized, and placed on ice before sedimentation in the neutral solvent. Numbers above bands have the meanings given in the legend to Figure 1(a). Bands represent, in order of increasing *s*, renatured DNA I°, an apparently partially renatured form of this closed circular DNA, denatured nicked circular DNA, and denatured closed circular DNA. (b) Aliquot of sample treated as in (a) except that it was heated at 40°C for 20 minutes and stirred vigorously before sedimentation in the neutral solvent. Numbers above bands correspond to the same species of DNA as in (a). (c) Sample treated similarly to that in (a) sedimented in the alkaline solvent. Faster band (134 S) represents denatured closed circular DNA; slower band (37 S) represents denatured nicked circular DNA.

not at the higher temperatures for the λ b₂b₅c closed circular DNA.

The denaturation-resistant fractions of PM2 closed circular DNA. In reporting that a fraction (7 to 8%) of the closed circular DNA from PM2 phage exists in the duplex form after its exposure to alkali (pH 12.2 to 14) in 2.1 M CsCl solution at 25°C for 10 to 15 minutes, followed by rapid neutralization and cooling of the solution on ice, Ostrander and Gray¹ ruled out several possible explanations, but were otherwise unable to account for the existence of this denaturation-resistant DNA. Grossman *et al.*² also reported 7 to 27% of duplex PM2 DNA I upon neutralization after exposure to 0.1 M KOH at ambient temperature; they did not attempt to explain these observations. Fractions resistant to alkaline denaturation were also noted in closed circular chloroplast DNA.⁵³ The existence of duplex fractions of PM2 closed circular DNA at neutral pH after exposure to strongly alkaline solutions (in the absence of dye) has been further documented in the present study (Figures 2, 3, 5-7) and the presence of such fractions demonstrated also in experi-

ments starting with PM2 DNA I° (Figure 3). The fraction of duplex DNA found upon neutralization was shown above not to depend upon the presence of EtdBr in the alkaline mixtures until a rather well-defined dye concentration is reached at which dye-mediated renaturation becomes significant.

Several putative explanations for the denaturation-resistant fractions of PM2 closed circular DNAs can be convincingly ruled out. It is not possible, for example, that the duplex DNA arises from fractions which remain substantially duplex in strongly alkaline solvents. PM2 closed circular DNA sediments entirely as the fully denatured 65 S species of Figure 1(b) in the alkaline solvent as routinely observed in this laboratory.

Another possible cause for the denaturation-resistant fractions is that a fraction of the molecules contain covalent cross-links between the complementary strands. This was considered to be an unlikely explanation on the grounds that nicked circular PM2 DNA, produced directly from samples of DNA I for which fractions existing as the duplex form after neutralization of strongly alkaline solutions had been shown to exist, contained only material sedimenting as the strand-separated form upon incubation in alkali, neutralization, and sedimentation in a neutral solvent.¹ If covalent cross-links were present, some of this material should have been present as duplex DNA II, unless the cross-links are cleaved upon the introduction of one or more scissions into a closed circular molecule. All of the nicked circular PM2 DNA present in the samples of the present study was similarly found to be denatured after exposure to NaOH solution and neutralization as described in Materials and Methods.

The above explanation is further shown to be untenable in the present study. If cross-links present in viral PM2 DNA I were removed upon its conversion to DNA II, then the supercoiled DNAs produced from the action of ligase upon the nicked circular form should not contain duplex forms upon alkaline incubation and neutralization. Such DNAs do contain denaturation-resistant fractions as shown below. The PM2 DNA I°, produced from the alternate nicking and closing action of the mammalian nicking-closing enzyme⁵⁴⁻⁵⁷ should likewise display no duplex forms upon neutralization of alkaline solutions were the above explanation valid. However, such duplex forms were shown to be present (Figure 3).

The duplex DNA remaining after exposure of viral PM2 DNA to NaOH solution for 5 minutes at 25°C as described in Materials and Methods (approximately 10% of the closed circular DNA; see Figure 5(a)) was separated from the denatured closed circular DNA. When either this purified duplex DNA I

or the purified denDNA I was subjected to alkaline incubation and neutralization as before, the percentage of duplex DNA was approximately that observed in the original sample from which the duplex and denatured closed circular forms had been separated (Figure 9). These observations indicate that the fraction of PM2 closed circular DNA present as the duplex form upon neutralization of alkaline solutions does not represent a unique species within the population of PM2 DNA molecules. If this duplex DNA were due to such a unique species, it would be expected that a solution of this DNA would give rise to predominantly duplex DNA upon a second exposure to alkali and neutralization, which is not the case (Figure 9(a)). The purified denDNA I, on the other hand, should not display a duplex fraction after the second exposure to alkali and neutralization; however, such a fraction is observed (Figure 9(b)). The findings above also serve as additional evidence against the existence of interstrand cross-links in a fraction of the DNA, as this fraction would clearly comprise a unique species which, when isolated, should display 100% renaturation upon a second incubation with alkali and neutralization.

Solutions of PM2 DNA I which had been cross-linked with mitomycin C so that the complementary strands were held in register at one or more positions

Figure 9. Photoelectric scanner tracings from band sedimentation experiments in the neutral solvent with samples, treated as indicated, of predominantly closed circular phage PM2 DNA. Scanner sensitivity was adjusted to 50 mV/cm in (a) and to 100 mV/cm in (b). (a) Sample was the duplex DNA I remaining after exposure of DNA I to NaOH solution (5 minutes at 25°C) and neutralization (Materials and Methods). This duplex DNA was separated from the denDNA I (Materials and Methods) and again exposed to alkali followed by neutralization of the solution as above. The numbers above the sedimenting bands have the same meanings and represent the same forms of PM2 DNA as in Figure 1(a). (b) Sample was the denDNA I from exposure to alkali and neutralization of a solution of PM2 DNA I as in (a). This DNA was separated from the duplex material remaining after the above procedure and a solution subjected to a second alkaline incubation-neutralization treatment. The three sedimenting bands correspond to the same species of DNA as in (a).

in each molecule were mixed with NaOH solution as above, incubated for 5 minutes at 25°C, and neutralized. Sedimentation of these mixtures showed that 100% of the closed circular DNA was present as the 22 S duplex form. It is thus highly unlikely that the PM2 denDNA I present after alkaline incubation of solutions of PM2 DNA I and neutralization represents DNA with some complementary bases still apposed, but which fails to renature due to, e.g., topological constraints. Conversely, PM2 denDNA I, cross-linked after denaturation and purification, behaved as expected in that these solutions contained no material sedimenting as duplex DNA I after alkaline incubation and neutralization. These results show, in accord with the results of Pouwels *et al.*⁴ in their studies of cross-linked ϕ X174 RF closed circular DNA, that translocation relative to one another of the two strands of PM2 DNA is necessary for this DNA to exist in the denatured form in the absence of denaturing agents.

Exposure of PM2 DNA I to alkaline CsCl solution at 25°C as described in Materials and Methods, which represents conditions very similar to those used in the earlier work,¹ for 5 minutes consistently gave rise to 3 to 4% duplex DNA I upon neutralization of the solution. This percentage of renaturing DNA was not detectably affected with samples of PM2 DNA I which had been extensively treated with pronase, pancreatic RNase, or sodium dodecyl sulfate as described in Materials and Methods.

At times of exposure to alkali shorter than 5 minutes, a dependence of the percentage of duplex DNA I upon time of incubation in alkaline solution was observed, for both the sets of conditions of alkaline denaturation (NaOH-NaCl or the much higher ionic strength KOH-CsCl solutions) used in these studies. The kinetics of disappearance of the closed circular DNA renaturing upon neutralization were examined as a function of the superhelix density of the starting DNA in experiments employing alkaline CsCl solution as the denaturant (Figure 10). It is evident that the initial rate of loss of the renaturing DNA is not strongly dependent upon superhelix density over a range of this property from near zero ($\sigma_0 = -0.011$) to a value corresponding to a starting DNA which contains substantially more superhelical turns ($\sigma_0 = -0.154$) than viral PM2 DNA I ($\sigma_0 = -0.117$). The percentage of duplex closed circular DNA found in the neutralized mixtures tends to become independent of the time of exposure to alkali after about 6 minutes, with an average of near 3% of the renaturing species present (Figure 10). This last finding is consistent with the general lack of dependence of the fraction of DNA I present upon time of alkaline incubation, at greater than 5 minutes of

Figure 10. Kinetics of disappearance of renaturing PM2 closed circular DNA. The percentage of duplex closed circular DNA is plotted logarithmically as a function of time of exposure to alkaline CsCl solutions at 25°C (Materials and Methods) before neutralization and band sedimentation in the neutral solvent. Superhelix densities of the starting closed circular DNAs and corresponding plot symbols are: ▲, -0.011; □, -0.054; △, -0.117; ■, -0.154. Note changes in abscissa scale.

such incubation, noted earlier in connection with the experiments in which NaOH solution was used as the denaturant.

DISCUSSION

Accumulation of closed circular DNA in renaturable configurations in alkaline solutions containing ethidium bromide. The EtdBr-mediated accumulation of closed circular DNA in renaturable configurations in the presence of 0.19 N hydroxide ion described in this study, as well as the earlier work indicating that the renaturation of closed circular ϕ X174 RF DNA requires rather stringent conditions such as heating at pH near 12 followed by cooling and neutralization⁴ demonstrate that the renaturation of closed circular DNA differs qualitatively from that of strand-separable DNAs in that it can be promoted effectively by conditions under which strand-separable DNAs are unstable with respect to denaturation with strand separation. The optimal renaturation of strand-separated DNAs, however, takes place under conditions ($T_m - 25^\circ\text{C}$) for which the duplex form is clearly stable. The presence of EtdBr definitely does not permit the existence of stable duplexes of strand-separable DNA in the strongly alkaline solutions of this study, as was

clearly demonstrated by adding EtdBr to the DNA solutions before these were made strongly alkaline. In such experiments it was observed that any DNA II in the mixture remains strand-separated independently of dye concentration or time of incubation before neutralization. The failure of strand-separable DNAs to undergo EtdBr-mediated renaturation in the present work is thus not a result of the drastic reduction of renaturation rates for strand-separated DNA which can be effected in the presence of EtdBr under conditions for which the DNA duplex is stable.¹²

Since translocation of the two strands relative to each other is necessary for molecules of closed circular DNA incubated in alkaline solution to exist in the denatured form upon neutralization, the conditions for renaturation of denatured closed circular DNAs must be such that: 1) a dynamic process can occur in which the molecules of closed circular DNA undergo interstrand translocation; 2) when a given molecule attains a configuration from which renaturation to the duplex form will occur immediately upon neutralization of the solution, it tends to remain in that configuration. These renaturable configurations presumably have some bases which are complementary in the native molecule apposed or nearly enough apposed so that nucleation sites for the rapid renaturation reaction upon neutralization are present. Molecules existing in such "in-register" configurations in the alkaline dye solutions must tend to remain in these configurations in order to account for the observation that up to 100% of each of the closed circular DNAs of this study, at least at some of the dye concentrations used, are found to exist in the duplex form if incubation is continued for sufficiently long periods before neutralization. An observation that 100% of the DNA exists in the duplex form simply means that a band corresponding to the denatured closed circular form is not detectable in the band sedimentation assay, and does not preclude the presence of a very low percentage (less than 2%) of the denatured form.

The relative stability of the "in-register" configurations means that the rate of translocation of molecules into such configurations is much greater than the rate of translocation out of these renaturable configurations. It seems most likely that the gradual accumulation of 100% of closed circular DNA into renaturable configurations in alkaline dye solutions is a result of the near-cessation of translocation among molecules which have attained these "in-register" configurations. This would imply a high average free energy of activation for translocation away from these configurations compared to the average value for translocation out of configurations from which renaturation does not take place upon neutralization. An alternative explanation, requir-

ing a rapid equilibrium between the "in-register" configurations and a very small fraction of material in "out-of-register" configurations, is not consistent with all the data of this study.

The enhanced stability of "in-register" configurations must be a feature of closed circular DNAs denatured in alkali independently of the presence of EtdBr, since denatured closed circular DNA can be renatured by heating at strongly alkaline pH followed by cooling and neutralization of the solutions.^{4,7} However, if interstrand translocation can take place in the presence of 0.19 N OH⁻ but in the absence of EtdBr, then it is apparent that the stability of "in-register" configurations compared to other configurations is not sufficient for the accumulation of molecules in renaturable configurations in the absence of EtdBr over the range of temperatures and ionic strengths represented in this study. Only when well-defined dye concentrations, which depend upon ionic strength and temperature, are reached can the accumulation of forms renaturing upon neutralization be observed. In this view, the accumulation of renaturable closed circular molecules results from the fact that the binding of EtdBr above a certain ratio of moles of dye bound per mole of DNA nucleotide is sufficient to block further translocation of the more stable renaturable configurations, but not that of the non-renaturable configurations. There could be an enhanced binding of the dye to molecules in "in-register" configurations to aid in their selective stabilization, although this is not a required feature of the model due to the greater inherent stability of renaturable configurations. The possibility that denatured molecules in 0.19 N OH⁻ do not continually undergo interstrand translocation, and that this process is accordingly facilitated by the addition of the dye, has been considered and can be rejected on the basis of work to be described elsewhere.

It is apparent that in closed circular DNAs, in which the strands cannot separate, there is some form of recognition in strongly alkaline solutions of configurations in which at least some bases which are complementary in the native molecule are apposed. In the presence of EtdBr, such recognition is possible at values of pH for which, according to tabulated pK_a values for deoxyribonucleotides, there should be at least partial titration of the hydrogens associated with the exocyclic nitrogens of the deoxyadenosine and deoxyguanosine residues, in addition to the complete titration of the protons of the ring nitrogens of the deoxyguanosine and thymidine residues. The positive charge associated with the quaternary nitrogen of the ethidium moiety could have the general effect of decreasing the repulsions of the negative

charges on the phosphate groups and the DNA bases, and this could help to selectively stabilize the inherently more stable "in-register" configurations. It is by no means certain that such an effect exists, however, as titration of the exocyclic amino groups of the ethidium ion should take place which could give rise to a net negative charge on this species. Such an explanation cannot in any event account for the greater stability of renaturable configurations in strongly alkaline solutions in the absence of EtdBr.

A plausible explanation for the greater stability of "in-register" configurations (independently of the presence of EtdBr) is that, since the majority of the charge repulsion which is apparently the single most important factor causing denaturation of strand-separable DNAs in alkali is due to the full titration of ring protons of deoxyguanosine and thymidine residues, the minimal charge repulsion in a closed circular molecule in alkaline solution should occur when the bases are all in register. This is because there would be no deoxyguanosine residues directly apposed to thymidine residues in such a configuration. The apposed charge repulsions would rather consist of presumably partially titrated residues opposite fully charged residues, a situation which is expected, in this view, to give rise to a lower electrostatic free energy than that associated with configurations in which a substantial fraction of fully titrated residues (and partially titrated residues) are directly apposed. Hence, nucleation in a relatively small region of the molecule could rapidly be propagated in a process completely analogous to the process of duplex formation which takes place when two separated, complementary strands of DNA undergo nucleation under conditions for which the Watson-Crick helix is stable.

Dependence of the ethidium bromide-mediated renaturation of closed circular DNA in alkaline solution upon factors affecting interstrand translocation. Any factors increasing the lability of the denatured closed circular DNAs with respect to interstrand translocation should, at a fixed concentration of DNA, either increase the minimum concentration of EtdBr required for dye-mediated renaturation at a fixed time of incubation or increase the rate of accumulation of renaturing molecules at a fixed dye concentration. These expected effects were observed in this study, both for the parameters corresponding to the DNA itself which might be expected to affect the kinetics of interstrand translocation (molecular weight and initial superhelix density) and for the solvent parameters of ionic strength, temperature, and dye concentration.

Increasing the molecular weight of the closed circular DNA, at a fixed

superhelix density of the duplex form, should create a progressively more formidable kinetic barrier to the translocation process. When the non-supercoiled closed circular forms of ϕ X174 RF, PM2, and λ b₂b₅c DNAs are allowed to undergo dye-mediated renaturation under the same conditions, the rate of accumulation of closed circular molecules renaturing upon neutralization of the alkaline EtdBr solutions decreases with increasing molecular weight, provided that the PM2 species has first been heated in alkali (Figure 4). Heating the samples did not substantially affect the renaturation kinetics of the ϕ X174 RF and λ b₂b₅c DNAs but removed anomalies observed in the case of PM2 DNA as noted earlier.

The effect of increasing the value of $-\sigma_0$ of the duplex form of the DNA, at a fixed molecular weight, is to increase the rate of accumulation of renaturing forms (Figures 3(a), 4). The difference in rates of accumulation of renaturing species is noted between PM2 DNAs I and I° both in the absence of heating (Figure 3a) and after heating in alkali (Figure 4). This effect is in the direction expected since denatured closed circular DNA in strongly alkaline solutions is highly positively supercoiled.⁵⁸ Hence, molecules with higher values of $-\sigma_0$, which contain more negative superhelical turns in the duplex form before exposure to alkali, should contain fewer positive superhelical turns upon denaturation in alkaline solution. It is assumed that the molecules with fewer positive superhelical turns in alkali should display more rapid rates of interstrand translocation than molecules with more such turns, which should be more entangled and thus more resistant to a process requiring the relative motion of one circular strand to the other. In the presence of dye, the effect may be magnified, since such binding will presumably increase the superhelix density of these denatured structures even further.

The number of moles of ethidium bound per mole of DNA nucleotide is known to decrease with increasing ionic strength or temperature for duplex DNA near neutral pH (ref. 8). Such a decrease can reasonably be assumed to accompany increases in ionic strength or temperature for the binding of EtdBr to closed circular DNA in alkaline solution, and must partly account for the increase in the threshold concentration of dye required for renaturation, at a fixed time of incubation in alkaline dye solution, when either of these solvent parameters is increased (Figures 6,7). It is reasonable to expect that an increase in the ionic strength of the medium should increase the lability of interstrand translocation owing to the decrease in the effective charge repulsion between the two strands, and it is fully expected that the increased

thermal motion of the DNA chains associated with an increase in temperature would facilitate interstrand translocation. The above factors thus all favor an increase in the amount of EtdBr required, at the fixed DNA concentration of these studies, to maintain DNA molecules which have translocated into renaturable configurations in these configurations when the ionic strength and/or temperature of the solvent is increased.

At times of incubation sufficiently short that 100% renaturation is not observed at all dye concentrations above the lowest value for which renaturation takes place, all of the DNAs of this study can exhibit an optimal dye concentration of near 70 $\mu\text{g/ml}$ for the accumulation of renaturing forms (Figure 2). In the case of the two larger DNAs, 100% renaturation could be achieved when these were incubated for 19 hours at EtdBr concentrations between 50 and 70 $\mu\text{g/ml}$, but not when they were incubated for the same period at a dye concentration of 200 $\mu\text{g/ml}$. While the decrease in the rate of accumulation of renaturing forms at dye concentrations above 70 $\mu\text{g/ml}$ is attributable to a decrease in the lability of the DNAs to translocation with increasing ratio of moles dye bound per DNA nucleotide, the existence of an optimal dye concentration for such accumulation, which is well above the lowest concentration for which accumulation of 100% of the DNA in renaturing forms can take place, is not yet readily explained. However, these findings do suggest that the most reliable conditions for renaturation of closed circular DNAs in general are to incubate for some hours at a dye concentration slightly above the lowest value for which renaturation takes place.

The convergence, at long incubation times, of the minimal concentration of EtdBr required for renaturation of ϕX174 RF DNA I, PM2 DNA I, and $\lambda\text{b}_2\text{b}_5\text{c}$ DNA I^o (Figure 2) indicates that the amount of dye bound per DNA nucleotide which is required to maintain the in-register configurations depends upon neither the superhelix density of the duplex form nor the molecular weight, as the above DNAs vary widely in these properties. This contrasts with the findings that the rate at which these DNAs accumulate in renaturable configurations is markedly dependent upon these parameters.

Denaturation-resistant fractions and anomalous kinetics of dye-mediated renaturation in PM2 closed circular DNA. Previous work from this laboratory¹ and the results of the present study have demonstrated that the fraction of PM2 closed circular DNA which is present in the duplex form after solutions of this DNA are exposed to alkali and neutralized is not the result of inter-strand cross-linking and does not otherwise correspond to a unique subpopulation within the collection of PM2 DNA molecules. The behavior of PM2 DNA

can, however, be explained if the circular strands of this DNA, relative to those of the other DNAs of this study, are less labile to the translocation process. The presence of the duplex fraction upon treatment with alkali and neutralization at 25°C (in the absence of dye) under the conditions of this study, and the biphasic kinetics of dye-mediated renaturation, suggest that PM2 closed circular DNA in alkali contains, at any instant, three general populations: 1) molecules which have at least some base pairs in register; 2) molecules which are not in configurations from which renaturation can occur upon neutralization, but which have undergone translocation to a relatively limited extent (presumably due to factors which result in an "energy barrier" to extensive translocation beyond the fully in-register configuration); 3) molecules which have overcome the putative barrier above and thus can be found translocated out of register to arbitrary extents. Since the rationale for the existence of these three populations requires the presentation of additional data, further discussion of some observations, such as the biphasic kinetics of dye-mediated renaturation for PM2 DNA, will not be given here.

The absence of denaturation-resistant fractions for PM2 closed circular DNA after heating in alkali at 70°C is interpreted to mean that translocation can be driven to any extent at the elevated temperature. The presence of the above anomaly for both PM2 DNAs I and I° and its disappearance upon heating for both DNAs indicates that this property is not unique to the viral form of PM2 DNA. In addition, the decrease in the fraction of PM2 DNA I renaturing upon neutralization with increasing ionic strength (Figure 6) or temperature (Figure 7), at dye concentrations insufficient for dye-mediated renaturation, is consistent with restricted interstrand translocation in PM2 DNA which can be facilitated by increasing either of the above parameters.

The PM2 DNA I which is present in the duplex form after exposure to alkali and neutralization (in the absence of EtdBr) appears to be in equilibrium with at least a fraction of the molecules existing as denDNA I after such treatment, as suggested by the independence of the percentage present in the duplex form from the time of exposure to alkali and by the presence of roughly this same percentage of duplex material independently of whether exposure to alkali and neutralization is done starting with purified denDNA I or with the DNA I purified from a mixture resulting from a previous exposure to alkali and neutralization of a sample of DNA I. The equilibrium between renaturable and non-renaturable forms of viral closed circular PM2 DNA at 25°C requires several minutes to become established upon exposure of the DNA to alkali, since the percentage of this DNA present as the duplex form upon neutralization decreases for approximately the first 5 minutes after such

exposure (Figure 10). The kinetics of disappearance of the duplex species is not detectably dependent upon the superhelix density of the starting DNA (Figure 10), which observation may be contrasted with the dependence of the kinetics of dye-mediated renaturation upon σ_0 for all the closed circular DNAs of this study. This could be partly due to the fact that the closed circular DNAs are more positively supercoiled in alkaline EtdBr solutions than in the presence of alkali alone, which could result in a significant difference in the relative lability of molecules of different superhelix densities to translocation in the presence of dye owing to the greater degree of chain entanglement at the higher superhelix densities. Also, the bound ethidium clearly retards translocation in alkali; this could enhance slight differences between DNAs having different values of σ_0 in the duplex form with respect to the relative rates of translocation in alkali.

The reason for the decreased lability of PM2 DNA, compared to the other DNAs of this study, with respect to strand translocation in alkali is unknown. No "odd" bases were detected in PM2 DNA¹⁴ and this DNA is similar in G + C content to the other DNAs of this study.^{14,59} The effect is clearly not one of decreasing lability to translocation with increasing molecular weight since λ b₂b₅c DNA I° does not display a duplex fraction upon neutralization of its alkaline solutions. Hence, the structural properties which give rise to the apparent barrier to extensive translocation in PM2 DNA clearly merit further investigation.

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