Circular, but Not Circularly Permuted, Deoxyribonucleic Acid Reacts Slower Than Linear Deoxyribonucleic Acid with Complementary Linear Deoxyribonucleic Acid†

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ABSTRACT: The electron microscope was used to count the number of double-stranded linear and circular renaturation products of a 2:1 by weight mixture of restricted double-stranded linear ϕX174 RFI and single-stranded circular ϕX174 viral deoxyribonucleic acid (DNA). More linear molecules than circular molecules are observed. However, when a 1:1 mixture of aliquots of either ϕX174 RFI or SV40 DNA, each previously cleaved with a different single-site restriction enzyme, is denatured and renatured under conditions which assure circularization with out of phase molecules, an equal number of linear and circular molecules is observed. These experiments indicate that the nucleation rate is not affected by circular permutation of linear strands but is decreased ~3-fold when one of the reacting strands is circular. An excluded volume theory is developed which is consistent with these as well as previous results concerning the effects of DNA strand lengths on renaturation rates.

Deoxyribonucleic acid (DNA) renaturation (reassociation) is a second-order reaction characterized by a rate constant, k_2. This rate constant depends upon the length of the reacting single strands (L) and the complexity of the DNA (N) as well as upon various environmental factors such as temperature, viscosity, and ionic strength. For a detailed discussion of how these variables are related to the mechanism of DNA renaturation, see Wetmur (1976). The DNA renaturation reaction may be separated into a rate-determining nucleation event, involving the formation of the first few base pairs, and a faster propagation reaction involving base pair formation to the end of the molecule. The greater the DNA complexity, the lower the concentration of a particular nucleation site at constant DNA nucleotide concentration. All other variables being constant, k_2 should be inversely proportional to N.

Because propagation proceeds to the maximum extent possible, k_2 should be directly proportional to L_s, the length of the shorter of two reacting complementary DNA strands. The only exception should be a decrease in base pair formation of 33% when molecules of equal length but undetermined origin are allowed to react (Wetmur & Davidson, 1968). Thus, one expects

$$k_2 = k_N L_s / N \quad (1)$$

where k_N, the nucleation rate constant, should depend only on environmental factors. Wetmur & Davidson (1968) and Wetmur (1971) observed, however, that

$$k_2 = k_N' L_s^{0.5} / N \quad (2)$$

where k_N' is a length-independent nucleation rate constant. The unexpected nature of the dependence of k_2 on L was proposed to be a manifestation of an excluded volume effect. Although no experimental evidence has been found which contradicts the excluded volume hypothesis, neither does there exist any evidence requiring an excluded volume effect on DNA renaturation. In this paper we compare DNA reassociation reactions which differ only in the topology of the reacting strands. We also present a quantitative excluded volume theory consistent with both the observations relating k_2 to strand topology and previous observations relating k_2 to strand lengths. Taken together, these experimental and theoretical results greatly strengthen the argument for an excluded volume effect on DNA renaturation reactions.

Materials and Methods

Nucleic Acids and Restriction Endonucleases. Bacteriophage ϕX174 viral DNA was obtained from Miles Laboratories. Bacteriophage ϕX174 RFI DNA and ^{3}H-labeled SV40 DNA component I were obtained from Bethesda Research Laboratories. Restriction endonucleases *Ava*I and *Ava*II were obtained from New England Biolabs. All other restriction endonucleases were obtained from Bethesda Research Laboratories.

Nucleic Acid Concentration. Double-stranded DNA concentrations were determined spectrophotometrically by using a Beckman Model 25 recording spectrophotometer. Relative concentrations of various circular permutations of SV40 DNA produced by restriction endonuclease digestion were determined by liquid scintillation counting in a Beckman LS 9000. Single-stranded ϕX174 DNA concentrations were determined by melting the DNA in a Gilford Model 2400 spectrophotometer equipped with a linear temperature programmer from Neslab Inc. It was assumed that denatured ϕX174 single-stranded DNA and linearized ϕX174 RF DNA had the same absorbance at temperatures above the melting temperature.

Single-Strand Breaks. The extent of single-stranded breakage of ϕX174 viral DNA was determined by alkaline band velocity sedimentation in a Beckman Model E analytical ultracentrifuge. Photographs showing linear and circular DNA bands, as well as standards, were traced with a Canalco Model J densitometer. The areas under the peaks for the two bands were determined. The double-stranded starting DNAs were determined to be primarily covalently closed circular DNA by gel electrophoresis. After linearization with a restriction endonuclease these DNAs were denatured and renatured as described below, mounted for electron microscopy by using the aqueous Kleinschmidt technique described by Davis et al. (1971), and observed in an AEI Model EM 801 electron microscope. Histograms were prepared. Only enzymes leaving DNA in good condition were used in subsequent experiments.

Renaturation with Single-Stranded Circular Molecules. ϕX174 RFI DNA was digested with 1.5 units of *Ava*I/μg of

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DNA at 37 °C for 60 min. Ethylenediaminetetraacetic acid (EDTA) and NaCl were added to produce final concentrations of 0.02 M and 0.2 M, respectively. The DNA was extracted with phenol and dialyzed at 4 °C against 0.15 M NaCl, 0.01 M 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl), and 0.001 M EDTA, pH 7.8. An aliquot of this DNA was mounted for electron microscopy to determine the extent of restriction enzyme digestion. The remaining DNA was divided into two aliquots. ϕX174 viral DNA was added to one aliquot of double-stranded ϕX174 DNA in a weight ratio of 1:2. Both aliquots were denatured with alkali, final concentration 0.1 M, with 0.01 M EDTA, at 37 °C for 5 min. A total of 0.1 volume of 2 M Tris-HCl was then added together with NaCl to produce 0.4 M NaCl. The DNA, with a plus strand concentration of $\sim$2 μg/mL, was renatured at 70 °C for 2–10 min. The number and length of circular and linear molecules were determined by electron microscopy as described above.

Renaturation of Circularly Permuted Molecules. ϕX174 RFI DNA was digested with either *Ava*I or *Ava*II. The restriction endonuclease digestion, extraction, dialysis, and sample preparation for electron microscopy were as described above. Samples analyzed included *Ava*I- or *Ava*II-cut DNA, *Ava*I- or *Ava*II-cut, denatured and renatured DNA, and *Ava*I-cut DNA plus *Ava*II-cut DNA mixed 1:1, denatured and renatured. Similar experiments were performed with SV40 DNA cut with *Eco*RI, *Bam*HI, or *Hpa*II. Mixtures (1:1) were assured by monitoring the ^{3}H label in the SV40 DNA. Experiments were performed in a number of aqueous solvents as well as in formamide. The exact renaturation conditions are given below under Results for each experiment. The number and length of circular and linear molecules were determined by electron microscopy as described above.

Theory

A number of attempts have been made to develop theoretical models of various aspects of the kinetic excluded volume effect on reactions between polymer residues (Wetmur, 1971; Morawetz et al., 1973; Cho & Morawetz, 1973; Horie & Mita, 1978). None of these attempts has been wholely successful. The model presented below is also incomplete and contains many simplifying assumptions which cannot be fully justified. Nevertheless, the model is in remarkably good agreement, both qualitatively and quantitatively, with the DNA renaturation kinetic data presented in this paper as well as with previously published data.

General Aspects of the Theory. The random flight treatment of a polymer (Tanford, 1961) leads to a distribution function for the end to end distance

$$W(h)\,dh = 4\pi[3/(2\pi\langle h^2\rangle)]^{1.5}e^{-3h^2/(2\langle h^2\rangle)}h^2\,dh \qquad (3)$$

where $\langle h^2\rangle = Ll_e = \sigma l_e^2$ is the mean square end to end distance. L is the contour length and σ and l_e are the equivalent segment number and length, respectively, for the polymer chain. Consider two polymer chains (1 and 2) which meet at an origin 0. The probability of overlap of two segments L_1 and L_2 bases distant from 0 on the respective chains is given by

$$\theta_{12} = V^*\int_0^\infty [W_1(h)W_2(h)/4\pi h^2]\,dh \qquad (4a)$$

and

$$\theta_{12} = V^*[3/(2\pi l_e)]^{1.5}[1/(L_1 + L_2)]^{1.5}$$

or

$$\theta_{12} = V^*[3/(2\pi l_e^2)]^{1.5}[1/(\sigma_1 + \sigma_2)]^{1.5} \qquad (4b)$$

If eq 4b is true in general, it must also be true for the special case where L_1 and L_2 add up to a single polymer chain ($L_1 + L_2 = L$). Equation 4b may then be simplified to

$$\theta_{ends} = V^*[3/(2\pi Ll_e)]^{1.5} = V^*j \qquad (5)$$

where V^* is the excluded volume of an end segment and j is the Jacobson–Stockmayer factor. Wang & Davidson (1966b) experimentally verified the Jacobson–Stockmayer factor for the interaction of the ends of a λ DNA molecule. We are aware that the intramolecular excluded volume will affect chain statistics, but for simplicity we continue without taking this factor into account. In fact, the magnitude of the intramolecular excluded volume for single-stranded DNA under renaturation conditions is unknown.

Interactions between Two Linear Molecules. Now consider four chains of a, b, c, and d segments, respectively, meeting at 0. This point of intersection is going to be the nucleation site for DNA renaturation. Let two chains be connected to produce $\sigma_1 = a + b$. Let the other two chains be connected to produce $\sigma_2 = c + d$. The total probability of new overlaps resulting from this requirement for intersection of the two polymers at 0 may be determined by integrating eq 4b over all of the possible overlaps. Most of the new overlaps will involve noncomplementary DNA sequences. These overlaps contribute to the excluded volume effect but do not enhance nucleation rates significantly.

$$\theta = V^*[3/(2l_e^2)]^{1.5}\left(\int_0^a d\sigma_1 + \int_0^b d\sigma_1\right)\times \left(\int_0^c d\sigma_2 + \int_0^d d\sigma_2\right)[1/(\sigma_2 + \sigma_1)]^{1.5} \qquad (6)$$

Evaluation of eq 6 gives

$$\theta = 4V^*[3/(2\pi l_e^2)]^{1.5}[2(a^{0.5} + b^{0.5} + c^{0.5} + d^{0.5}) - (a + c)^{0.5} - (a + d)^{0.5} - (b + c)^{0.5} - (b + d)^{0.5}] \qquad (7)$$

Equation 7 will be used for all θ calculations involving linear DNA chains.

Effect of Length on DNA Renaturation. Let us consider the evaluation of the overlap function (eq 7) for two DNA chains of identical number of segments (σ). As extreme cases we will consider the nucleation event for renaturation (the origin) to occur at the end of both molecules ($a = c = \sigma$ and $b = d = 0$) and the nucleation event for renaturation to occur at the middle of both molecules ($a = b = c = d = \sigma/2$). The results of the overlap calculations are

$$\theta_{center} = 4V^*[3/(2\pi l_e^2)]^{1.5}1.7L^{0.5} \qquad (8a)$$

$$\theta_{ends} = 4V^*[3/(2\pi l_e^2)]^{1.5}0.58L^{0.5} \qquad (8b)$$

The total number of overlaps possible in solution cannot be increased by connecting the segments of a polymer together to form chains. Therefore, let the probability of finding any conformation with a particular origin configuration be proportional to $1/(1 + \theta)$. The probability of reaction at the origin is then given by $\theta_1/(1 + \theta)$ where θ_1 is $4V^*[3/(2\pi l_e^2)]^{1.5}$. Referring back to eq 1 and 2, we find that

$$k_N = k_N'\theta_1/[s(\theta + 1)] \qquad (9)$$

where s is the number of bases in a segment. For the renaturation reactions with nucleation events at the centers or ends of the molecules, we find that if $\theta \gg 1$

$$k_N \approx k_N'/(1.7L^{0.5}) \qquad (10a)$$

for center reactions, and

$$k_N \approx k_N'/(0.58L^{0.5}) \qquad (10b)$$

for end reactions. The predicted dependence of k_N on L is precisely that seen experimentally by Wetmur & Davidson (1968) and scales the same as previous excluded volume calculations by Wetmur (1971) for DNA renaturation kinetics. The difference between reaction rates for nucleation at the end or center of DNA molecules has not been observed but will be discussed below.

Reactions between Short and Long DNA Strands. Evaluation of eq 7 with long and short (L_s) strands gives, for the same cases of reaction at the end and center

$$k_N \approx k_N'/(2.8L_s^{0.5}) \quad (11a)$$

for center reactions and

$$k_N \approx k_N'/(1.0L_s^{0.5}) \quad (11b)$$

for end reactions. This dependence on L_s is exactly that seen experimentally by Wetmur (1971) and similar to that seen experimentally by Hinnebusch et al. (1978). This result will be considered again in the discussion below.

Circular Permutations. One of the experiments described below deals with reactions between linear single-stranded DNAs which are circularly permuted. Integration of eq 7 over all possible nucleation sites and division by the number of nucleation sites gives the average value of θ, $\langle\theta\rangle$. $\langle\theta\rangle$ depends on L and P where P is the number of bases by which the reacting strands are out of phase. We find

$$\langle\theta\rangle = c[8L^{1.5} - (2L - P)^{1.5} - 0.5(L - P)^{1.5} - (L + P)^{1.5} - 1.5(L - P)(L + P)^{0.5} - 1.5P(2L - P)^{0.5} - 0.5P^{1.5}]/L \quad (12)$$

where c is a constant. Evaluation of $\langle\theta\rangle$ for $P = 0$ and $P = L/2$ shows that $\langle\theta\rangle$ for $P = L/2$ is within 3% of $\langle\theta\rangle$ for $P = 0$. This small difference would be impossible to determine experimentally. Thus, we predict that DNA renaturation rates between circularly permuted molecules will not depend on the extent of the circular permutation (phase). Results presented in this section are in agreement with this prediction.

Circular Molecules. We have reevaluated θ for the case where one molecule is linear and one molecule is circular. The circular molecule is described by the same distribution function $W(h)$ but with altered $\langle h^2\rangle$ (Sharp & Bloomfield, 1968). Let i and o be arbitrary positions on a molecule. The replacement of

$$\langle h_{io}^2\rangle = L_{io}l_e \quad (13a)$$

for linears with

$$\langle h_{io}^2\rangle = [(L - L_{io})/L]L_{io}l_e \quad (13b)$$

for circles results in an overlap function:

$$\theta = 4V^*[3/(2\pi l_e^2)]^{1.5}L^{0.5}\{\pi - \sin^{-1}(1/[1 + 4(a/\sigma)]^{0.5}) - \sin^{-1}(1/[1 + 4(b/\sigma)]^{0.5})\} \quad (14)$$

where the linear strand has σ segments, the same as the circular strand, and $\sigma = a + b$. Again, considering end and center nucleations for the linear strands we find

$$k_N \approx k_N'/(1.9L^{0.5}) \quad (15a)$$

for center reactions, and

$$k_N \approx k_N'/(1.11L^{0.5}) \quad (15b)$$

for end reactions. Thus the rate of renaturation of circular or linear molecules with linear molecules may be compared by looking at eq 10a,b and 15a,b. For experiment to agree with theory, circular–linear reactions must be slower than linear–linear reactions. We shall see that this is indeed the case.

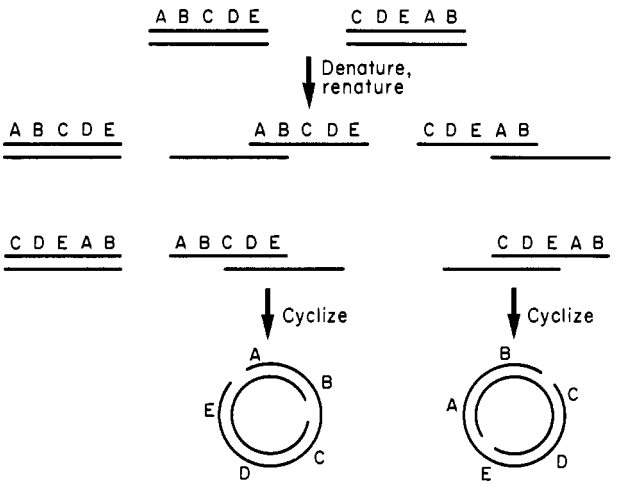

FIGURE 1: Protocol for testing effect of circular permutation on DNA renaturation rates. The products of the renaturation reaction are shown. The double-stranded linear starting molecules were generated from the same pool of ϕX174 RFI molecules but were cleaved with different single-site restriction enzymes.

The theory above deals with various effects of strand length and strand topology on DNA renaturation rates. Applicability of this theory to other polymer systems will be considered under Discussion.

Results

DNA renaturation rates are not affected by circular permutation. The products of denaturing and renaturing a mixture of circularly permuted DNA molecules are illustrated in Figure 1. Renaturation between complementary DNA strands which are in phase results in the formation of fully duplex linear molecules. When two complementary, but out of phase, DNA strands renature with one another, the initial product is a partially duplex molecule with complementary single-stranded tails; the final product is a double-stranded circle with two staggered nicks. The experiments to be described here used dilute DNA solutions to promote cyclization of partially duplex molecules before either of the tails could react with a third strand or with another partially duplex molecule. The requirements for such conditions may be calculated from the results of Wang & Davidson (1966a,b).

The electron microscope was used to quantitate the products of various denaturation–renaturation mixtures. The following control was performed to be certain that electron microscopy was free from bias and accurately reflected the number of linear and circular molecules present in a solution. An equimolar mixture of *Eco*RI-cut ^{3}H-labeled SV40 linear DNA and DNase I cut ^{3}H-labeled open circular SV40 DNA was prepared. The 1:1 mixture was assured by liquid scintillation counting of aliquots of the samples. The mixture was examined in the electron microscope. To prevent operator bias, we made adjustments at one grid square before translation to an adjacent square where a photograph would be taken before observing the field. The result shown in line 1 of Table I confirms that neither linear nor circular DNA is preferentially detected.

Samples of linear ϕX174 RF DNA with different beginning sequences are generated by cleaving one aliquot of ϕX174 RFI DNA with restriction enzyme *Ava*I and another aliquot with restriction enzyme *Ava*II. Samples of ^{3}H-labeled SV40 linear DNA are generated in exactly the same way by using restriction enzymes *Eco*RI, *Hpa*II, or *Bam*HI. In ϕX174 the 5′ terminus of the recognition sequence for *Ava*I is at nucleotide 162; for *Ava*II it is at nucleotide 5042. The zero

Table I: Renaturation of Circularly Permuted DNA[a]

DNA source		renaturation conditions of 2 μg of DNA/mL: salt	T (°C)	t (min)	f_c
SV40					
*Eco*RI, DNase I	(1)	not denatured–renatured			0.490 ± 0.020
SV40					
*Hpa*II, *Bam*HI	(2)	1 M NaCl	65	5	0.500 ± 0.030
	(3)	0.15 M NaCl	39	60	0.440 ± 0.030
	(4)	50% $HCONH_2$	25	60	0.460 ± 0.030
SV40					
*Eco*RI, *Hpa*II	(5)	1 M NaCl	65	5	0.490 ± 0.020
	(6)	0.15 M NaCl	39	60	0.480 ± 0.020
	(7)	50% $HCONH_2$	25	60	0.520 ± 0.050
φX174					
*Ava*I	(8)	0.4 M NaCl	70	2	0.013 ± 0.007
*Ava*II	(9)	0.4 M NaCl	70	2	0.010 ± 0.006
*Ava*I and *Ava*II	(10)	0.4 M NaCl	70	2	0.440 ± 0.025

[a] Measure N molecules. f_c^m = fraction found circular; $f_c = f_c^m \pm (f_c^m(1 - f_c^m)/N)^{0.5}$.

position is the *Pst*I cleavage site, and the total number of nucleotides in the DNA is 5386 (Fuchs et al., 1978). In SV40 the 5′ terminus of the recognition sequence for *Eco*RI is at nucleotide 1700, for *Hpa*II it is at 264, and for *Bam*HI it is at 2451. The zero position is near the origin of DNA replication, and the total number of nucleotide pairs in the DNA is 5226 (Fuchs et al., 1978). The *Eco*RI-cut and the *Hpa*II-cut SV40 DNAs are 27% out of phase with one another; the *Hpa*II-cut and the *Bam*HI-cut SV40 DNAs are 42% out of phase with each other; the *Ava*I- and *Ava*II-cut φX174 DNAs are 9% out of phase with one another. These three combinations span most of the possible range of phases which would be found in a sample with random circular permutation. The φX174 DNA concentration was determined spectrophotometrically. The ^{3}H-labeled SV40 DNA concentration was determined by liquid scintillation counting. The integrity of restriction enzyme cut SV40 or φX174 RF DNA is verified by observing that histograms of denatured and self-reannealed DNA were the same as those for undenatured DNA (data not shown). For a few restriction enzymes the histograms indicated that degradation had accompanied digestion. These enzymes were not used any further.

For determination of the effect of circular permutation on DNA renaturation rates, equimolar amounts of circularly permuted φX174 linear DNAs were mixed together and denatured, or two of the circularly permuted SV40 DNA samples were mixed together and denatured. Renaturation conditions for each trial are given in Table I along with the fraction of the molecules that were observed in the electron microscope to be circles.

Mixtures of SV40 DNA molecules that are either 42% (Table I, line 2) or 27% (Table I, line 5) out of register produced essentially the same number of linear and circular molecules when renatured in 1 M NaCl for sufficient time (~5 half-times) to assure renaturation and almost complete cyclization. φX174 molecules 9% out of register (Table I, line 10) produced 44% circles. The decrease in the fraction of φX174 circular molecules compared to the fraction of SV40 molecules may reflect the somewhat less favored cyclization of a molecule that has single-strand tails at each end amounting to only 9% of the total length of the molecule and the fact that more stringent renaturation conditions were used. A total of 2 min for renaturation in 0.4 M NaCl is only one half-time for φX174. In light of the qualifications, these three

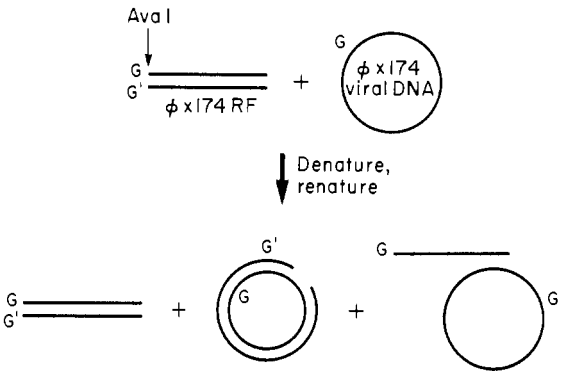

FIGURE 2: Protocol for examining rate of renaturation of a linear molecule with its linear and circular complements.

data points are in good agreement with one another. In another trial with SV40 DNA, conditions for renaturation were chosen that might be expected to enhance any excluded volume effect (Table I, lines 3 and 6). Lower salt (0.15 M) has the effect of permitting the negatively charged DNA strands to repel each other more, and the lower temperature of 39 °C slows down renaturation. The lower rate of DNA renaturation at temperatures which are more than 25 °C below the melting temperature may itself reflect an excluded volume phenomenon. Even with these renaturation conditions the relative populations of linear and circular molecules are unaffected by circular permutation of 27% or 42%. Finally, the results of renaturing SV40 DNA in 50% formamide, 0.2 M Tris-HCl, and 0.01 M EDTA, pH 8 (Table I, lines 4 and 7) are consistent with little or no effect of 27% or 42% circular permutation on DNA renaturation rates. Taken together, the SV40 and φX174 data indicate that circular permutation due to phase shifts ranging from 9% to 42% (out of a possible 50%) does not affect the rate of DNA renaturation. Furthermore, neither salt concentration, temperature, nor use of organic solvents alters this conclusion. These results are in complete accord with the excluded volume theory resulting in eq 12 which indicates that the average overlap of two reacting DNA strands is essentially independent of the percent out of phase, $100P/L$. A method that does measure an excluded volume effect on DNA renaturation rates is presented below.

DNA renaturation rates are decreased if one of the complementary strands is circular. In order to study the effect of circular vs. linear DNA structures on the rate of renaturation of DNA, φX174 RFI DNA was cut with *Ava*I to produce linear molecules. Two parts of these molecules were mixed with one part of φX174 viral DNA, and then the mixture was denatured and renatured. The reactions will lead to the formation of linear and circular duplex molecules as outlined in Figure 2. With the 2:1 ratio of *Ava*I-cut φX174 RF DNA to viral DNA, the negative DNA strand from the restricted φX174 RF DNA would be as likely to encounter the positive linear complementary strand as to encounter the positive circular complementary strand of viral φX174 DNA. If this ratio is not achieved, then the distribution of double-stranded linear and circular molecules obtained after renaturation could be attributed to the presence of unequal concentrations of reacting species in the mixture rather than to a difference in their abilities to renature with each other. DNA concentrations were measured spectrophotometrically by using a correction factor which was determined for single-stranded DNA hypochromicity by equating the absorbance of native and denatured single-stranded DNA at temperatures above the melting temperature. An aliquot of the same preparation of *Ava*I-cut φX174 RFI DNA was denatured and renatured

Table II: Linear and Circular Molecules Counted after Denaturation and Renaturation of a Mixture of φX174 RFI and Viral DNA[a]

DNA source	renatured	time (min)	no. of molecules linear	no. of molecules circular	$f_{circles}$	k_L/k_c
φX174 RFI						
*Ava*I	no		484	13	0.026 ± 0.007	
*Ava*I	yes	2	410	9	0.021 ± 0.007	
*Ava*I, φX174 viral DNA	yes	2	231	123	0.350 ± 0.030	3.8
φX174 RFI						
*Ava*I	no		407	8	0.019 ± 0.007	
*Ava*I	yes	2	398	8	0.020 ± 0.007	
*Ava*I, φX174 viral DNA	yes	2	172	103	0.370 ± 0.030	3.2
*Ava*I, φX174 viral DNA	yes	10	161	99	0.380 ± 0.030	2.9

[a] f = fraction of broken φX174 viral DNA; f_c, corrected for uncut DNA; $k_L/k_c = \log f_c/\{\log [(1-f_c-ff_c)/(1-f)]\}$.

in order to determine the extent of single-stranded breaks in the molecule. The results are given in Table II.

In this and all other examinations of DNA in the electron microscope, the same random sampling procedure described above was used. The extent of digestion of φX174 RFI DNA by *Ava*I was determined by electron microscopy and is reported in Table II. This result was unaffected by denaturation and renaturation for slightly greater than one half-time. Digestion was from 97 to 98% complete. Analysis of histograms of the *Ava*I-cut φX174 RFI DNA which had been denatured and renatured indicated few single-strand breaks in the restricted DNA (data not shown). The single-stranded φX174 viral DNA used in these experiments was determined by analytical band velocity sedimentation to be 81% intact. The remaining 19% of the DNA, containing one break at a random location, would be expected to renature with the rate of a linear DNA with complementary in phase linear DNA but to cyclize rapidly to a circular form. These kinetics are the same as those of the circularly permuted DNAs described above.

The results of the two separate experiments with linear and circular DNA are given in Table II. In one trial 123 out of 354 molecules were circles; in another 103 out of 275 molecules were circles. The technique used to mount the DNA on the grids does not spread single-stranded DNA, so all the molecules seen are duplexes. The renaturation reactions were done in 0.4 M NaCl at 70 °C for the indicated times. Incubation for slightly greater than one half-time (2 min) resulted in the same fraction of circular molecules as incubation for more than five half-times (10 min). The results clearly indicated a preference for reaction between linear molecules. The relative renaturation rate constants, k_L/k_C, for renaturation of a linear molecule with complementary linear or circular molecules, are also given in Table II. These rate constants are calculated by taking into account the fraction of RFI DNA uncut by AvaI, the fraction of viral DNA which is broken to yield linear molecules, and the amounts of each strand remaining at all stages of the renaturation reaction. This calculation indicates that reactions between linear molecules proceed ~3 times faster than reactions between linear and circular molecules. This experimental result agrees qualitatively as well as semiquantitatively with the excluded volume theory. Analyses of eq 10a,b and 15a,b indicate that linear–linear reactions might be expected to be up to 2-fold faster than linear–circular reactions. This result showing an effect of DNA strand topology on DNA renaturation rates is another indication, along with the form of the effect of DNA strand length on renaturation rates, that an excluded volume effect is involved in the mechanism of DNA renaturation.

Discussion

An excluded volume theory for DNA renaturation kinetics is proposed above. In order to be complete, such a theory needs to account for both the previously published data concerning the effects of strand lengths on renaturation rates and the new data concerning the effects of DNA strand topology on renaturation rates.

The theory predicts that the rate of renaturation of DNA will depend on the square root of the length of the reacting single strands ($L^{0.5}$). This prediction is clearly in agreement with the experimental results of Wetmur & Davidson (1968) and subsequent investigators [for a review, see Wetmur (1976)]. The theory is not unique in making the $L^{0.5}$ prediction. Other theories based on hard-sphere or random-coil models of DNA (Wetmur & Davidson, 1968; Wetmur, 1971) have also led to the same conclusion.

The theory also predicts that renaturation will depend on the square root of the length of the shorter of two reacting DNA single strands ($L_s^{0.5}$). This prediction is clearly in agreement with the experimental results of Wetmur (1971) and subsequent investigators [for a review, see Wetmur (1976) and Hinnebusch et al. (1978)]. In this case, the simple hard-sphere model of Wetmur & Davidson (1968) failed to agree with experiment and the more complex random-coil model based theory of Wetmur (1971) agreed over a limited range of ratios of long to short reacting DNA strands. One reason that the new theory presented above might be expected to be more correct than the theory of Wetmur (1971) is that Wetmur's theory involves interactions at the limit of the distribution functions [$W(h)$] where these functions are expected to be the least reliable as models of real DNA configurations. The new theory uses distribution function data from the entire range of the functions. Whatever the reason, the new theory is in better agreement with experiments relating DNA renaturation rates to strand length than any previously published theory.

Morawetz et al. (1973) published an excluded volume theory for the interactions of polymers in solution. Two versions of the theory involved a hard-sphere model and a random-coil model for the polymers. Cho & Morawetz (1973) then made two types of polymers to test the theory. One contained an ester to be hydrolyzed and the second contained a catalyst. Three different lengths of catalyst-containing polymer were prepared. When mixed with the ester-containing polymer, all three catalytic polymers were about equally effective in inducing the hydrolysis of the ester. Furthermore, the hydrolysis rates with the polymers were not substantially different from those found with monomers of ester and catalyst. Because this result did not agree with their theory, Cho and Morawetz concluded that excluded volume did not play a part in this type of polymer reaction. The lack of length dependence in the experiments of Cho and Morawetz could easily be attributed to the fact that the length of the ester-containing polymer was in every case equal to or shorter than the length of the catalytic polymer and that the length of the ester-containing polymer

(L_s) was invariant. However, the relatively high rates of reaction of the polymer residues are best explained if there were little or no excluded volume in this system. This would occur if V^* is small enough so that θ is <1 (eq 7) leading to no length dependence for k_N (eq 9). DNA molecules are polyelectrolytes. It would be interesting to reinvestigate a system of polymers similar to those studied by Cho and Morawetz under conditions where the lengths of both polymers as well as their charge densities could be varied in an attempt to bridge the gap between the experimental results with acrylamide polymers in solvents close to the ideal solvent and DNA renaturation for which no ideal solvent is known.

In 1970, Lee and co-workers found that coliphage 15 DNA was circularly permuted and terminally redundant. Examination of the end to end spacings of the terminal single strands in circular molecules generated after denaturation and renaturation of the DNAs revealed a nonrandom distribution. Such a nonrandom distribution of end to end spacing could have been the result of a nonrandom distribution of circular permutations in the original coliphage 15 DNA population. As an alternative explanation, Lee et al. (1970) suggested that nearly in phase molecules renatured preferentially and advanced an excluded volume argument to explain this preference. The ends of DNA are, on the average, further from the center of mass than are the centers of the molecules. Reactions involving nucleations between complementary ends of molecules with similar phase which are far from the excluded center of mass might be expected to provide sufficient advantage over nucleation events between out of phase molecules to account for the observed nonrandom distribution of end to end spacings. More recently, Tye et al. (1974), using the circularly permuted terminally redundant *Salmonella* phage P22 DNA, showed that nonrandom distribution of the end to end spacings reflected a nonrandom set of circular permutations. The DNA replication begins at a unique origin. A headful packaging mechanism produces the permutations. Only for deletion mutants does the replication and packaging lead to a population of random circular permutation. When the circular permutations are random so are the end to end spacings found in the renaturation products of the DNA. This result does not eliminate the possibility of an excluded volume effect on DNA renaturation. Rather, the theory above predicts that circular permutation of DNA will have no effect on DNA renaturation rates. The prediction, albeit negative, is borne out by the data in this paper as well as the data of Tye et al. (1974).

This agreement between experiment and theory complements the positive evidence related to the effects of length on DNA renaturation rates. Also, the theory makes a new and testable prediction, namely, that circular single-stranded DNA will renature slower than linear single-stranded DNA. The results above are in agreement with this prediction.

Any DNA renaturation mechanism must involve either a preequilibrium plus a rate-determining step or a diffusion-controlled rate-determining step. Excluded volume is the only way in which length may be involved in the preequilibrium step. No reasonable model exists for a length dependence at the level of the rate-determining step following a preequilibrium. It is hard to imagine the remainder of a freely jointed molecule affecting the formation of the second (or third) base pair during a nucleation event, and there is theoretical argument to the contrary (Wetmur & Davidson, 1968). This leaves models involving diffusion control as the only alternatives to an excluded volume model. A DNA renaturation mechanism which involves translational diffusion as a rate-limiting step might be expected to lead to a rate constant inversely proportional to solvent viscosity, proportional to the square root of the length of the shorter of two reacting complementary DNA strands, and independent of circular permutation. All of these predictions agree with experiment. Wetmur & Davidson (1968) dismissed this mechanism because the absolute rate of renaturation was too slow to be consistant with translational diffusion and especially because the temperature dependence of DNA renaturation is totally inconsistent with a translational diffusion mechanism. The temperature profile for DNA renaturation implies a nucleation step with a preequilibrium followed by the rate-determining step. Segmental diffusion control could only make the expected absolute rate of renaturation increase and be less in agreement with experiment and could not resolve the inconsistency between a diffusion-controlled mechanism and the observed temperature profile. A final prediction of a translational diffusion model would be more rapid renaturation rates for more compact circular DNA, a result opposite to that observed in the experiments described in this work. The existence of a single excluded volume theory which explains all the known data concerning DNA strand length and topology effects on DNA renaturation rates does not prove that the theory is correct. However, until contradictory data are obtained or another equally satisfactory theory is derived, an excluded volume effect remains the best explanation for these various phenomena.

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