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The Isolation of Terminally Cross-Linked DNA and Kinetics of Venom Phosphodiesterase†

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ABSTRACT: The action of venom phosphodiesterase (exonuclease) on non-cross-linked and mitomycin C cross-linked *Escherichia coli* DNA fragments has been investigated. Kinetic studies reveal that the rate of phosphodiesterase catalyzed hydrolysis is primarily controlled by the cross-link density, and that added alkaline phosphatase and Mg^{2+} in the digest have a slight stimulatory effect on the reaction rate. Cross-links are shown to inhibit exonuclease hydrolysis, and limited digestion can be used to decrease the concentration of non-cross-linked fragments in randomly cross-linked DNA. Thermal denaturation profiles of partially digested DNA show that end effects in the helix-coil transition of low molecular weight cross-linked DNA are slight compared to the effects of cross-link density and nucleotide composition heterogeneity.

The thermal denaturation of duplex DNA has been the subject of much experimental and theoretical study. From this work, many features of DNA molecular structure have come to be recognized as determinants of the denaturation behavior. Specifically, melting is dependent upon (1) the nucleation of helical regions, (2) the differing thermal stability of A-T and G-C base pairs, (3) molecular weight, (4) the loop entropy, (5) slip degeneracy, (6) base sequence, (7) electrostatic effects, and (8) specific interactions with solvent and/or other solutes. Several of these factors have been investigated in detail from both theoretical and experimental sides. For instance, the molecular weight dependence of transition midpoints and breadths seems well understood (Crothers *et al.,* 1965); the linear dependence of midpoint temperatures on gross A-T composition is well known; the difference in breadths of transitions for homopolymers and heteropolymers is rationally ascribed to slip degeneracy. Other factors, such as (7) and (8) above, are less well understood, but at least are experimentally controlled to be constant.

From the biological standpoint, the ultimate aim of investigations on denaturation behavior is the deduction of base sequence information, of whatever kind, from melting profiles. However, this goal cannot be achieved unless and until the several influential aspects of DNA structure listed above have been quantified.

It is most probable that the major current difficulty confronting this interpretation results from the interplay between (4) loop entropy, (5) slip degeneracy, and (6) base sequence. This paper describes the procedures we have used to produce modified DNA of such structure that at least partial separation of these effects is achieved. The aim of the work is to produce relatively short and fractionated DNA fragments that are cross-linked at both ends, and which therefore cannot melt by unzipping. The sequence of operations, up to the fractionation, is depicted schematically in Figure 1. In a subsequent paper we will present the denaturation behavior of the fractions.

The procedure makes use of the known (Iyer and Szybalski, 1963, 1964; Szybalski and Iyer, 1964, 1967; Weissbach and Lisio, 1965) capability of the reduced form of mitomycin C to cross-link duplex DNA. Subsequently, the 3′ chain ends may be removed with venom phosphodiesterase (Laskowski, 1966; Razzell and Khorana, 1959; Richards and Laskowski, 1969) in the expectation that cross-links effectively impede more complete hydrolysis of the chains (Pricer and Weissbach, 1965; Sarkar, 1967). The data presented here further characterizes this inhibitory action of cross-links. In addition, we have investigated the stimulatory effect of phosphatase and Mg^{2+} on the exonuclease digestion. The kinetics of the digestions lead to a plausible conjecture concerning the mode of action of venom phosphodiesterase on duplex DNA.

Experimental Section

Materials. The DNA was isolated from strain B *Escherichia coli* cells by a modification of the method of Marmur (Marmur, 1961); the 2-propanol precipitation was omitted and a deproteinization by phenol extraction was added. The venom phosphodiesterase from *Crotalus adamanteus* and bacterial alkaline phosphatase were purchased from Worthington Biochemical Corp. The exonuclease assays were done by the method of Koerner and Sinsheimer (Koerner and Sinsheimer, 1957) using calcium bis(*p*-nitrophenyl) phosphate (0.12 mg/ml) as a substrate. Concentrations are expressed in units/ml where 1 unit is arbitrarily defined as the activity of enzyme in a 3-ml assay volume which produces a total increase of 0.30 absorbance unit in 30 min.

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The assays of alkaline phosphatase were performed according to Garen and Levinthal (Garen and Levinthal, 1960) using *p*-nitrophenyl phosphate as a substrate. Concentrations are expressed in units/ml where 1 unit is that activity which liberates 1 μmol of *p*-nitrophenol/min. The DNA concentration was determined from absorbance measurements at 259 nm using E_{1cm}(mg/ml) 20. The acid-soluble nucleotide concentration was determined from the absorbance at 260 nm. A mean nucleotide molar extinction coefficient of 1.0×10^4 was determined from the absorbance of acid-soluble nucleotides after complete digestion of a non-cross-linked DNA sample (defined by the plateau absorbance attained as a function of incubation time); the concentration of nucleotides in the sample was obtained from the DNA absorbance before digestion.

The desired DNA molecular weight was obtained by shearing in a French pressure cell. Molecular weights were determined by viscosity (Eigner and Doty, 1965) and band sedimentation velocity (Studier, 1965) methods.

Cross-Linking. The sheared *E. coli* fragments were cross-linked using a modification of a previously published method (Iyer and Szybalski, 1964). Mitomycin C, dissolved in a small amount of methanol, was added to a large volume of 0.03 M PO_4[1] containing sheared *E. coli* DNA such that the final concentration of the cross-linking agent was 0.05–0.15 mg/ml while the DNA was 0.3–0.4 mg/ml. Then, sodium dithionite dissolved in water was added to a final concentration of 0.2–0.4 mg/ml. Nitrogen was bubbled through the DNA and sodium dithionite solutions prior to and during the cross-linking reaction. The buffer concentration was adjusted to 0.3 M PO_4 prior to precipitation of DNA by addition of two volumes of 95% ethanol. The DNA was pelleted by centrifugation, after which the pellet was dissolved in the desired buffer and dialyzed against the same. The fraction of DNA that was cross-linked, f_L, was calculated from the measured hyperchromic shift after thermal denaturation, quenching in an ice bath, and equilibration to room temperature by

$$f_L = \frac{(A_q(\text{not linked})/A_n) - (A_q(\text{linked})/A_n)}{(A_q(\text{not linked})/A_n) - 1} \quad (1)$$

where A_q is the absorbance after denaturation and quenching and A_n is the absorbance before denaturation measured at room temperature. This method of calculating the fraction of cross-linked molecules has been used previously (Kohn *et al.*, 1966; Cohen and Crothers, 1970). The cross-link distribution is calculated from this value of f_L by assuming a Poisson distribution of cross-links throughout a uniform molecular weight population. The cross-link density is expressed as the average number of cross-links per molecule, $\bar{X}$.

Exonuclease digestions were followed by spectrophotometric determination of acid-soluble nucleotides. The venom phosphodiesterase was incubated with DNA in 0.1 M Tris (pH 9.1) at 37°. Alkaline phosphatase, when used, was added 1 hr prior to the addition of the exonuclease. The reaction with venom phosphodiesterase was terminated at a specified time by the addition of an equal volume of chilled 2.5% perchloric acid containing 0.25% uranyl acetate. The mixture was then centrifuged at 4° for 15 min at 14,000*g*, and the absorbance of the supernatant at 260 nm was determined along with that of a blank that lacked only the exonuclease.

Denaturation Curves. The exonuclease digests were deproteinized prior to the determination of thermal denaturation profiles by one of two methods. (1) Twice the volume of 95% ethanol containing one-tenth a volume each of 10 × SSC and 0.5 N HCl was added to one volume of the digestion mixture and the DNA precipitate was collected by centrifugation. After dissolving the pellet in 0.3 M PO_4 buffer, the DNA was precipitated again by adding two volumes of ethanol; the precipitate was recovered by centrifugation. (2) Protein was removed by three successive phenol extractions consisting of the addition of freshly prepared liquified phenol (saturated with water, pH 8.0), shaking or stirring, and centrifugation to separate layers. All samples were exhaustively dialyzed against either 0.03 M PO_4 or 0.1 × SSC prior to the determination of melting curves. The first procedure, employed only for samples whose denaturation curves were determined in 0.03 M PO_4 buffer, was an unusual but effective means of deproteinization as judged by the ratio of absorbances of the resulting DNA solutions at 280 and 259 nm (0.52–0.53). The observed effect of the exonuclease digestion on the denaturation curves was the same regardless of which deproteinization procedure was employed.

Denaturation curves were obtained by measuring the DNA absorbance at 259 nm at various temperatures. Thermal control was achieved by circulating water from a constant-temperature bath through a modified cell compartment of a Beckman DU-2 spectrophotometer.

The thermally induced changes in DNA absorbance were found to be influenced by the presence of single-stranded DNA which exhibits an hyperchromic shift over a wide temperature range below the duplex DNA denaturation. Single-stranded DNA results from exonuclease hydrolysis of 5′-mononucleotides from 3′ termini; the 5′-polynucleotide chain ends which remain are single stranded. To eliminate the influence of this base-line drift, the degree of denaturation was defined as the normalized increase in absorbance above a somewhat arbitrary temperature (70° for 0.03 M PO_4 denaturations and 63° for 0.1 × SSC denaturations), chosen to lie just below the double-strand helix-coil transition. The increase in native DNA absorbance between room temperature and the temperature defined above was always less than 5%. All absorbances were corrected for the thermal expansion of water. The midpoint and breadth of the denaturation curve for a particular sample were reproducible to within at least ±0.3 and ±0.5°, respectively.

Hydroxylapatite Chromatography. Double- and single-stranded DNA were separated by hydroxylapatite chromatography. The DNA was denatured in 0.03 M PO_4 buffer by immersing a screwcap vial containing the DNA in boiling water for 10–20 min. The hot solution was then passed through a hydroxylapatite (Bio-Rad Laboratories) column maintained at 60°. The column was eluted stepwise with 0.03 M PO_4, 0.12 M PO_4, and 0.3 M PO_4. Single-stranded DNA is eluted at 0.12 M PO_4, while native DNA elutes at 0.3 M PO_4 (Britten and Kohne, 1968). Under these conditions, using non-cross-linked DNA, we found that after denaturation only 80% of the DNA was eluted with 0.12 M PO_4. The fraction eluted at 0.3 M PO_4 was identified as DNA from spectra and it possesed a hyperchromic shift

[1] Abbreviations: A 2*X* M PO_4 solution is *X* M NaH_2PO_4 and *X* M Na_2HPO_4, pH 7.0; SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0. The temperature at 50% denaturation is T_m; T_n is the temperature at 100*n* % denaturation; $\bar{X}$ is the average number of mitomycin C cross links per molecule.

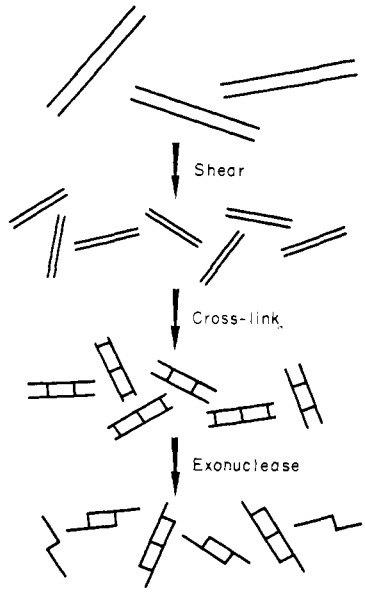

FIGURE 1: Schematic of the isolation of DNA with cross-links at duplex termini.

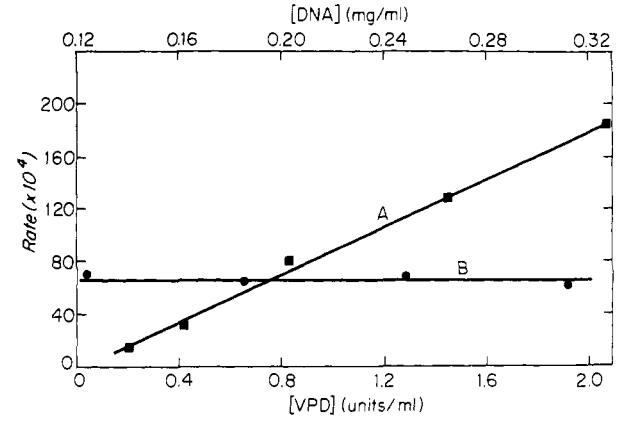

FIGURE 2: Rates of exonuclease digestion of sheared (5×10^5) non-cross-linked *E. coli* DNA in 0.1 M Tris (pH 9.1) at 37°. The DNA was incubated for 1 hr with the alkaline phosphatase (AP) prior to the addition of the venom phosphodiesterase (VPD). (A) [DNA] = 0.298 mg/ml, [AP] = 0.30 units/ml, $[Mg^{2+}] = 0$. (B) [AP] = 0.30 unit/ml, $[Mg^{2+}] = 0$, [VPD] = 1.0 unit/ml. The rate of formation of acid-soluble nucleotides is expressed in mM min^{-1}.

(25%) between that of denatured DNA (11%) and native DNA (38%).

This chromatographic procedure yielded a reproducible measure of the amount of denatured, and therefore non-cross-linked, molecules in our samples of sheared and mitomycin alkylated DNA. Based on the results of the chromatography of denatured nonalkylated DNA, we assume that amount of DNA eluted with 0.12 M PO_4 in the chromatography of alkylated DNA to be 80% of the total amount of denatured DNA. The accuracy of this procedure was confirmed by calculating the fraction of nonrenaturing DNA in the mitomycin C treated sample from the hyperchromic shift as discussed above; the same result was obtained by both methods.

Results

The concentration dependence of the rate of enzymatic hydrolysis of non-cross-linked DNA is shown in Figure 2. The reaction rate was found to be constant if no more than about 75% of the DNA becomes acid soluble (Figure 4); rates shown in Figure 2 were determined in this range. The results show that the rate is linearly dependent on exonuclease concentration but is independent of the amount of DNA in the specified concentration range. The substrate is therefore in excess compared to the amount of active enzyme.

We have also investigated the effect of 3′-phosphoryl groups on the exonuclease digestion rates. It is known that terminal 3′-phosphates inhibit the rate of venom phosphodiesterase hydrolysis of oligonucleotides (Koerner and Sinsheimer, 1957; Richards and Laskowski, 1969). Since the isolation scheme depicted in Figure 1 requires the removal of molecules with no or one cross-link, and since shear produces molecules with various end groups, we sought to remove remaining terminal phosphates that might inhibit the rate of hydrolysis of these sparsely linked fragments. Cross-linked DNA ($\bar{X} = 2.1$) was first treated with an excess of alkaline phosphatase to remove all terminal phosphates. The phosphatase was then removed by two phenol extractions and the remaining DNA was dialyzed against 0.1 M Tris (pH 9.1) buffer. Digestion of this sample with exonuclease in the presence and in the absence of alkaline phosphatase ($[Mg^{2+}] = 0$) was observed (Figure 3). The digest containing alkaline phosphatase exhibits a greater initial

TABLE I: Solubilization of Nucleotides by Exonuclease Digestion.[a]

Run	[AP][b]	$[Mg^{2+}]$[c]	% Acid Soluble	
			60 min	540 min
1	0.0	0.0	7	35
2	0.0	10^{-3}	14	39
3	0.447	0.0	17	43
4	0.447	10^{-3}	25	68

[a] DNA of 5×10^5 daltons, $\bar{X} = 1.6$ cross-links/molecule. The concentration of DNA is 0.251 mg/ml, and venom phosphodiestrase is present at 0.58 unit/ml for all runs. [b] Concentration of alkaline phosphatase (AP) in units/ml. [c] Molar concentration of Mg^{2+}.

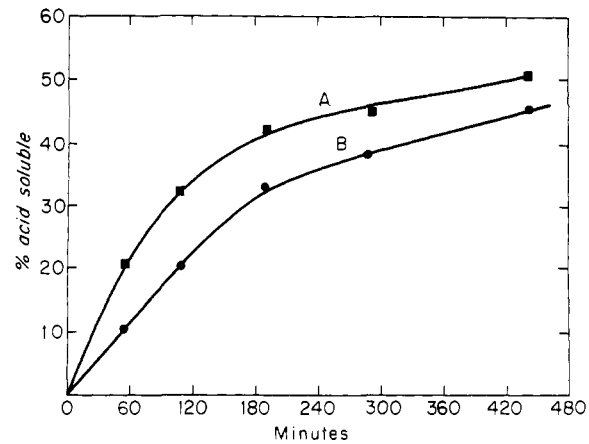

FIGURE 3: The formation of acid soluble nucleotides by exonuclease digestion. The *E. coli* DNA is sheared (5×10^5) and cross-linked ($\bar{X} = 2.1$). The concentrations are [DNA] = 0.199 mg/ml, [VPD] = 0.6 unit/ml, and $[Mg^{2+}] = 0$, and for A, [AP] = 0.364 unit/ml, while for B, [AP] = 0. Minutes refers to time of digestion.

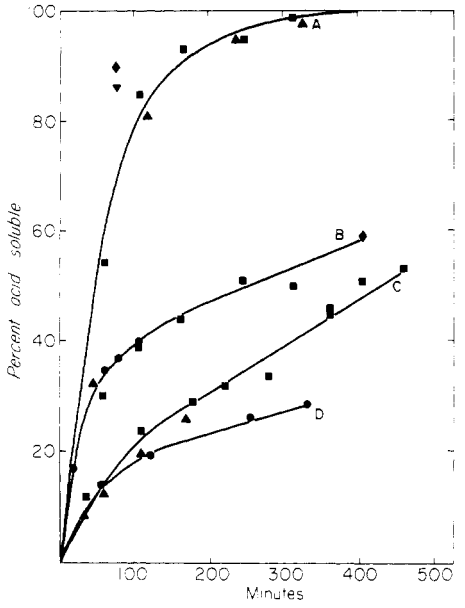

FIGURE 4: Exonuclease digestion of sheared (5×10^5) non-cross-linked and cross-linked DNA. Points are from a number of runs under slightly different experimental conditions encompassing variations in the enzyme and magnesium chloride concentrations as indicated below. The average number of cross-links per molecule, $\bar{X}$, was calculated from the number of molecules that have no cross-links assuming a Poisson distribution. Concentrations given for components in the incubation reaction are in mg/ml for DNA, units/ml for the enzymes, and molarity for magnesium ion. Minutes refer to time of VPD digestion. (A) $\bar{X} = 0$: (■) [DNA] = 0.27, [VPD] = 0.57, [AP] = 0.06, $[Mg^{2+}] = 10^{-3}$; (▲) [DNA] = 0.30, [VPD] = 1.0, [AP] = 0.36, $[Mg^{2+}] = 0$. (B) $\bar{X} = 1.4$: (●) [DNA] = 0.33, [VPD] = 2.7, [AP] = 0, $[Mg^{2+}] = 10^{-3}$; (■) [DNA] = 0.27, [VPD] = 0.47, [AP] = 0.064, $[Mg^{2+}] = 10^{-3}$; (♦) [DNA] = 0.29, [VPD] = 0.57, [AP] = 0, $[Mg^{2+}] = 10^{-3}$. (C) $\bar{X} = 2.3$: (■) [DNA] = 0.28, [VPD] = 0.60, [AP] = 0.34, $[Mg^{2+}] = 10^{-3}$; (▲) [DNA] = 0.29, [VPD] = 0.61, [AP] = 0.35, $[Mg^{2+}] = 0$. (D) $\bar{X} = 3.2$: (●) [DNA] = 0.30, [VPD] = 1.0, [AP] = 0.36, $[Mg^{2+}] = 0$. Points are also shown above (A) for the digestion of denatured DNA, (♦) [DNA] = 0.25, [VPD] = 2.0, [AP] = 0, $[Mg^{2+}] = 10^{-3}$. For DNA which was denatured and then alkylated with mitomycin, under conditions which produced an average cross-link density of 1.39 for a sample of native DNA, the digestion is given by (▼) [DNA] = 0.30, [VPD] = 2.4, [AP] = 0, $[Mg^{2+}] = 10^{-3}$.

rate of hydrolysis, but the rates with and without phosphatase are comparable at long times. The same trend was observed with a DNA substrate of low cross-link density ($\bar{X} = 1.6$), results for which will be found in Table I.

The stimulatory effect of alkaline phosphatase is very different in the presence of Mg^{2+}. We have observed the rate of digestion to increase markedly with Mg^{2+}, especially at long times, as inspection of run 4 in Table I will reveal.

The substrates in both of these experiments were dephosphorylated prior to venom phosphodiesterase digestion as described above. The results therefore suggest that this hydrolytic enzyme is inhibited by the product nucleotide monophosphate, the concentration of which may be reduced by the action of phosphatase present in the venom phosphodiesterase digest. This phenomenon was not investigated further.

Rate data for the exonuclease digestion of cross-linked DNA is shown in Figure 4. The data encompass varying concentrations of enzymes and Mg^{2+}. A comparison of data determined with different cross-link densities but similar reaction conditions reveals a pronounced decrease in the rate of hydrolysis due to an increase in the cross-link density. The figure also contains comparative data for the digestion of nonalkylated, denatured DNA as well as that for

TABLE II: Determination of Non-Cross-Linked Species.[a]

Min	h	% Acid Soluble	% Non-Linked Species[b]	
			Method 1	Method 2
0	0.39	0	17.5	17.5
31	0.35	32	14.3	13.7
89		45		2.2
154	0.36	52	9.0	8.4

[a] The DNA was digested for the indicated number of minutes, after which the sample was extracted three times with phenol and was then dialyzed against 0.1 × SSC or 0.03 M PO_4. [b] Method 1: The ratio, A_q(not linked)/A_n, required in eq 1 properly depends on the total hyperchromicity which is due in part to single stranded chain ends. We therefore use for this case A_q(not linked)/$A_n = 0.606h + 1$ in 0.1 × SSC buffer, where the fractional hyperchromicity, h, is defined by the highest absorbance recorded in a thermal denaturation of a partially digested cross-linked sample. The factor 0.606 was empirically derived from data for non-cross-linked DNA without VPD digestion. Method 2: Concentrations were determined from the absorbance at 259 nm of hydroxylapatite chromatography fractions. The extinction coefficients E_{1cm}(mg/ml) 20.0 for double-stranded fractions and E_{1cm}(mg/ml) 24.4 for single strands were used for determination of concentrations at 25°. The values given in the third line are for a sample that was not thermally denatured prior to chromatography.

DNA that was denatured and subsequently alkylated with mitomycin C. [Labeled mitomycin has been shown to alkylate denatured DNA as easily as native DNA (Weissbach and Lisio, 1965).] As seen from data presented in Figure 4, the denatured samples are hydrolyzed rapidly; incorporated, but non-cross-linked mitomycin has only a slight effect on the rate. Therefore, the much slower rates of digestion of cross-linked duplex DNA must result from interstrand linkages and not from simple alkylation of nucleotides.

Inspection of the data in Figure 4 also shows that variations in the concentration of enzymes or divalent cation have only a slight effect on the rate of digestion when compared with the influence of cross-link density. Data for curve B were obtained for a wide range of venom phosphodiesterase concentrations, yet the overlapping families of points describe a single curve. Exonuclease digestion of the highest cross-link density sample was slower than that of samples with fewer cross-links, despite a relatively high venom phosphodiesterase concentration for the former. In contrast, the digestion of non-cross-linked DNA is more sensitive to enzyme concentration as was shown above (see Figure 2).

Since the low cross-link density DNA sample is hydrolyzed at a much faster rate than samples with high cross-link density, exonuclease digestion of randomly linked DNA will certainly reduce the population of sparsely linked molecules faster than that of densely linked molecules. To verify this hypothesis, we measured directly the change in the fraction of non-cross-linked molecules after limited digestion. Prior to analysis the solutions were deproteinized by phenol extraction and were then dialyzed into an appropriate buffer. Two methods of analysis were employed: (1) the fraction of nucleotides in double-stranded regions of

TABLE III: Denaturation Curves in 0.03 M PO_4 Buffer.

Sample	Mol wt[a]	$\bar{X}$[a]	% Acid Soluble	T_m	$T_{0.8} - T_{0.2}$	$T_{0.9} - T_{0.1}$
	9×10^7	0	0	81.2	4.2	7.1
	5×10^5	0	0	80.6	4.3	7.4
(6–1)LX	5×10^5	1.4	0	81.4	5.4	8.8
			35	81.9	5.7	9.5
			37	82.1	5.5	8.9
$(6–1)LX_2$[b]			41	82.2	5.6	9.3
(6–23)LX	5×10^5	1.4	0	81.6	5.7	9.6
			35	82.0	5.8	9.7

[a] Molecular weight and average number of cross-links per molecule, $\bar{X}$, is given for the DNA sample before exonuclease digestion. [b] The exonuclease digestion for this sample was done in two steps, 60 min apiece, separated by a deproteinization procedure, by precipitation as described in the text, and dialysis. The degree of digestion is an estimate corresponding to one 120-min digestion.

TABLE IV: Denaturation Curves in 0.1 × SSC.

Sample	Mol wt	$\bar{X}$	% Acid Soluble	T_m	$T_{0.8} - T_{0.2}$	$T_{0.9} - T_{0.1}$
	9×10^7	0	0	75.1	4.2	7.2
	1×10^6	0	0	74.5	4.6	7.2
	5×10^5	0	0	74.3	4.7	8.0
SX	5×10^5	0	16	74.3	5.1	8.6
		0	34	74.4	5.0	9.1
		0	42	74.1	5.0	8.3
(7–16)LX	1×10^6	1.6	32	75.5	5.6	9.3
			52	75.9	5.0	8.4
(2)LX	5×10^5	3.2	0	75.9	6.4	10.4
			28	75.7	5.9	9.3
(5)LX	1×10^6	3.2	0	75.7	6.0	9.8
			26	75.5	6.7	10.6

non-cross-linked, and therefore nonrenaturing, molecules was determined from the relative hyperchromicity in accordance with eq 1; and (2) single strands were isolated by hydroxylapatite chromatography, and relative amounts of DNA in eluted fractions were determined by absorbance measurements at 259 nm.

The measured fractions of nucleotides in non-cross-linked molecules as obtained by these methods are shown in Table II. The results demonstrate that there is indeed a decrease in the fraction of non-cross-linked molecules as a result of exonuclease digestion. This experiment and the kinetic experiments together provide evidence that the venom phosphodiesterase digestion procedure can be used to isolate DNA fragments in which cross-links occur predominantly at the duplex termini. The thermal denaturation of these fragments is affected neither by strand separation nor by unzipping from the ends. Additional support for this conclusion is provided by an analysis of denaturation curves of this partially digested DNA.

Denaturation Curves. Melting profiles of DNA samples characterized by different cross-link densities and degrees of exonuclease hydrolysis are summarized in Tables III and IV. The reaction conditions for the VPD digestion of each sample is given in Table V. From inspection of the data we note the following observations which are important to the discussion. (1) The denaturation curves of non-cross-linked DNA reveal the expected molecular weight dependence of the T_m and breadth: the melting temperature decreases and the breadth increases with decreasing molecular length. However, the increase in breadth associated with the cross-links is greater than that arising from molecular weight dependence in non-cross-linked DNA. (2) The exonuclease digestion of sheared, non-cross-linked DNA (sample SX, Table IV) produces a surprisingly small change in the helix-coil transition; even after 42% of the nucleotides have become acid soluble, the T_m is depressed only slightly and there is no substantial increase in breadth. (3) Denaturation curves of sheared and low cross-link density DNA [(6–1)LX and (6–23)LX in 0.03 M PO_4, Table III, and (7–16)LX in 0.1 X SSC, Table IV] show increases in T_m with exonuclease treatment, but there are virtually no changes in breadths. (4) The higher cross-link density samples, (2)LX and (5)LX, show both slight increases and decreases in transition breadths after digestion but there are no substantial shifts in the mean melting temperatures.

TABLE V: Reaction Conditions.[a]

Sample	DNA	VPD	AP	Mg^{2+} (M)
(6–1)LX	0.37	7.5	0	10^{-3}
$(6–1)LX_2$	0.27	7.5	0	10^{-3}
(6–23)LX	0.305	6.2	0	10^{-3}
(2)LX	0.30	1.0	0.358	0
(5)LX	0.30	1.0	0.361	0
SX	0.41	0.98	0.380	0
(7–16)LX	0.183	1.2	0.328	0

[a] Concentrations are given in mg/ml for DNA, and units/ml for the venom phosphodiesterase (VPD) and alkaline phosphatase (AP).

Discussion

Before interpreting the transition breadths for cross-linked and venom phosphodiesterase digested DNA, we must further characterize the products of limited venom phosphodiesterase action. The denaturation curves of nuclease-treated non-cross-linked DNA are revealing in this respect. In particular, if reaction with venom phosphodiesterase produced a significant concentration of duplex regions which were much shorter than the initial substrate, the denaturation curves of the partially digested DNA would be broadened and shifted to lower temperatures (Crothers *et al.*, 1965). Such low molecular weight species could be produced, for example, if there were contaminating endonuclease activity or if the exonuclease produced an accumulation of partially digested intermediates of low molecular weight. The presence of such molecules is excluded on consideration of the denaturation profiles of the non-cross-linked venom phosphodiesterase treated DNA (sample SX, Table IV). The venom phosphodiesterase digestion of sheared, non-cross-linked DNA results in only small changes in the midpoints and breadths of the denaturation curves (observation 2). The curves do not reflect the presence of large amounts of very short molecules, even though

as much as 42% of the nucleotides become acid soluble. The denaturation profile is that which would be expected for double-stranded molecules with the same molecular weight as the undigested material.

Similar conclusions may be drawn from the melting behavior of cross-linked and exonuclease digested samples. With the low cross-link density samples, exonuclease digestion produces an increase in the midpoints of the denaturation curves (observation 3). This fact is further evidence that the fraction of non-cross-linked molecules (approximately 25% in the undigested sample) is decreasing with digestion time. It has been established that low molecular weight non-cross-linked DNA melts at a lower temperature than very long molecules due to the entropy gain associated with the separation of strands (Crothers *et al.*, 1965). Conversely, cross-linked DNA which cannot undergo strand separation possesses an increased T_m compared to non-cross-linked DNA of similar molecular weight (Cohen and Crothers, 1970). We therefore conclude that the increase in T_m after exonuclease digestion is the result of the diminished concentration of non-linked molecules (which melt with concomitant strand separation). The high cross-link density DNA does not exhibit a change in T_m with exonuclease digestion (observation 4) because the fraction of non-cross-linked molecules in the undigested sample is less than 5%.

Thus, based on a consideration of the behavior of the product denaturation curves after exonuclease hydrolysis, we conclude that the fraction of non-cross-linked molecules decreases as a result of nuclease action. Phosphodiesterase treatment does not produce a significant number of short, non-cross-linked double-stranded molecular fragments by, for example, contaminating endonuclease activity.

Finally, we draw attention to substantiating results obtained by chromatography of hydrolyzed, cross-linked DNA. The third line of Table II contains data for an aliquot removed during the run which was not denatured prior to loading it on the hydroxylapatite column. Even though 45% of the nucleotides have become acid soluble, only 2% of the remaining nucleotides are in single-stranded chains. As was the case with digestion of non-cross-linked DNA, significant concentrations of single-stranded segments are not produced by exonuclease activity or a combination of endonuclease and exonuclease action. [The endonuclease from snake venom, a possible contaminant of the venom phosphodiesterase preparation, has optimal activity at pH 5.0 and requires no Mg^{2+} (Georgatsos and Laskowski, 1962).]

The analysis of the denaturation curves discussed above suggests that venom phosphodiesterase releases nucleotides from the same molecule before attacking another polynucleotide chain and, therefore, since the substrate is in excess compared to the venom phosphodiesterase, the concentration of single-stranded chains and short duplex regions resulting from partial digestion of molecules in the incubation mixture remains low. If only a small fraction of the molecules are hydrolyzed at a time, the average molecular weight of the sample does not vary appreciably during most of the digestion; denaturation curves do not change since the average duplex length is constant. However, the evidence presented here is not definitive, and these contentions should be regarded as suggestive. Evidence controverting these results has been obtained for the action of venom phosphodiesterase on poly(A) (Nossal and Singer, 1968). Many of the results discussed here, on the other hand, were obtained with cross-linked DNA, and we have shown that the cross-links affect the course of the reaction; it is possible that they also alter the hydrolysis mechanism. It is significant, however, that (1) the observed helix-coil transitions of the partially digested samples do not reflect the effects of molecular ends which might have been introduced during the venom phosphodiesterase digestion, and (2) that the enzyme preferentially hydrolyzes molecules with relatively few cross-links.

We can now offer an interpretation of the fourth observation on the denaturation curves. First note that the transition curve for non-cross-linked DNA of 5×10^5 is slightly broader than the melting curves of longer molecules (observation 1). The denaturation profile of the smaller molecule reflects melting from the molecular ends. The curves for the cross-linked, venom phosphodiesterase treated samples show variations in transition breadths, but there is no consistent trend with increasing time of hydrolysis (observation 4). The breadths do not consistently increase or decrease even though the concentration of molecules which are subject to end effects is constantly decreasing. Evidently end effects perturb the melting behavior of these cross-linked fragments less than that due to cross-link density and compositional heterogeneity. It is a combination of these more important, but variable, factors that produces the observed variations in breadths.

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