in which every other glycosidic torsion angle and phosphodiester linkage differ from the standard B-DNA conformation observed in 1 M NaCl where the symmetry repeat occurs every base pair. The calorimetric data indicate that these two conformations do not manifest significantly different enthalpies of transition. A reasonable candidate for the 1 M Me_4NCl dinucleotide repeat conformation is a right-handed, alternating B-DNA structure.

Acknowledgments

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Alkaline Gel Electrophoresis of Deoxyribonucleic Acid Photoreacted with Trimethylpsoralen: Rapid and Sensitive Detection of Interstrand Cross-Links†

Thomas R. Cech

ABSTRACT: Restriction fragments of phage λ and φX174 deoxyribonucleic acid (DNA) were photoreacted with 4,5′,8-trimethylpsoralen to various extents, and the amount of covalent cross-linking was determined by electron microscopy of the DNA under totally denaturing conditions. The DNA was then analyzed by electrophoresis in alkaline agarose gels. A single cross-link in a DNA molecule produced a large decrease in its electrophoretic mobility. With DNA fragments 0.3–4 kilobase pairs in size, the apparent M_r (molecular weight) of the cross-linked DNA was 2.0 ± 0.1 times the M_r of the unreacted, single-stranded DNA. A single cross-link in a larger DNA molecule resulted in an even greater increase in apparent M_r. Further cross-linking produced a decrease in the apparent M_r of the DNA, reaching a plateau at a value of 1.4 ± 0.1 times the M_r of the unreacted, single-stranded DNA over a large range of fragment sizes (0.6–10 kilobase pairs). The apparent M_r of the cross-linked DNA was weakly dependent on the percentage of agarose in the gel. Although highly sensitive to interstrand cross-links, the electrophoretic mobilities appeared to be unaffected by low levels of monoadducts (trimethylpsoralen covalently bound to one strand of the DNA). The DNA bandwidths increased by as much as 4-fold at low extents of cross-linking, presumably due to heterogeneity in the locations of the cross-links in the DNA molecules. The bands became sharp again at high levels of reaction. These observations form the basis of a new assay for interstrand DNA cross-links that is both more sensitive and more convenient than previous methods.

Psoralen and its derivatives undergo photochemical reactions with deoxyribonucleic acid (DNA).[1] The psoralen can react with a pyrimidine base on one strand of the DNA to form a covalent monoadduct. Subsequent photoreaction with a pyrimidine on the opposite strand of the DNA can form a stable

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[1] Abbreviations used: DNA, deoxyribonucleic acid; Me_3psoralen, 4,5′,8-trimethylpsoralen; EDTA, (ethylenedinitrilo)tetraacetic acid; φX174 RFII, nicked double-stranded replicative form of bacteriophage φX174 DNA; bp, base pair(s); kbp, kilobase pair(s); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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interstrand cross-link. The photochemistry and photobiology of the psoralens have been recently reviewed (Song & Tapley, 1979).

Photochemical cross-linking of DNA with psoralens has become a useful tool for nucleic acid chemistry. The cross-linking reaction has been used to probe the arrangement of chromosomal proteins with DNA in chromatin (Hanson et al., 1976; Weisehahn et al., 1977; Cech & Pardue, 1977; Hallick et al., 1978; Cech & Karrer, 1980) and to identify DNA–protein interactions in the *Drosophila* mitochondrial genome (Potter et al., 1980). Psoralen cross-linking acts as a fixative for DNA secondary structure (Cech & Pardue, 1976; Shen & Hearst, 1976) and has also been used to hold the two strands of DNA in register during R-loop hybridization (Kaback et al., 1979). Because psoralen derivatives plus irradiation are used to treat various skin diseases, the psoralen–DNA interaction is also of clinical interest (Scott et al., 1976).

Quantification of psoralen cross-links in DNA is essential to all the lines of research outlined above. The methods that have been most useful for determining frequencies of cross-links in DNA fall into three categories. First, there are techniques that make use of the reversibility of the denaturation of cross-linked DNA (Geiduschek, 1961; Cole, 1970; Dall'Acqua et al., 1970). Because one cross-link is sufficient to hold the complementary DNA strands in register during denaturation, these techniques do not differentiate between singly cross-linked and multiply cross-linked DNA molecules. Furthermore, many naturally occurring DNAs contain inverted repeat sequences; these produce a background level of rapid intramolecular renaturation which makes it difficult to detect a low frequency of cross-links in the DNA. Sedimentation of DNA in alkaline sucrose gradients provides a second method for identifying interstrand cross-links. When used to analyze a uniform-size population of DNA molecules, this method allows one to distinguish between un-cross-linked, singly cross-linked, and multiply cross-linked molecules (Cole & Zusman, 1970). The third method involves the preparation of the DNA for electron microscopy under totally denaturing conditions. The cross-links are visualized as points of contact of the single strands of DNA (Hanson et al., 1976; Cech et al., 1977). This method is especially powerful because it gives the locations of the cross-links as well as the number of cross-links. It is not a reliable method for quantifying low extents of cross-linking (<1 cross-link/molecule).

In the present study, alkaline gel electrophoresis is used to analyze restriction fragments of DNA photoreacted with Me_3psoralen.[1] These observations form the basis of a new assay for covalent cross-links in DNA that is highly sensitive to low levels of reaction, that gives an estimate of the number of cross-links per molecule, and that is much more rapid and facile than previous methods.

Materials and Methods

Materials. Me_3psoralen was supplied by the Paul B. Elder Co., Bryan, OH. [^{3}H]Me_3psoralen (5.4×10^5 cpm/μg) was a gift of S. T. Isaacs and J. E. Hearst. Bacteriophage λ DNA cleaved with *Hin*dIII restriction nuclease and ϕX174 RFII DNA cleaved with *Hae*III restriction nuclease were purchased from New England Biolabs. The λ DNA restriction fragments have the following sizes (kbp): A, 23.5; B, 9.59; C, 6.76; D, 4.45; E, 2.29; F, 1.94 (Daniels et al., 1980). The ϕX174 DNA fragments have the following sizes (kbp): A, 1.35; B, 1.08; C, 0.87; D, 0.60; E, 0.31; F, 0.28; G, 0.27; H, 0.23 (Sanger et al., 1978).

Photoreaction of DNA with Me_3psoralen. Solutions contained 80 μg/mL DNA in 0.15 M NaCl, 0.01 M Tris-HCl, and 0.001 M EDTA, pH 8.4. Me_3psoralen [300 μg/mL in $(CH_3)_2SO$] was added to a final concentration of 3 μg/mL, resulting in a $(CH_3)_2SO$ concentration of 1%. Aliquots (20 μL) in siliconized-glass 100-μL micropipets (Corning) were irradiated for various times at 25 °C. The irradiation source (320–400 nm) consisted of two 40 W fluorescent lamps (GTE Sylvania FR40T12/PUVA). The samples were placed 1.9 cm above the lamps on a sheet of Plexiglass (0.6 cm thick), which served both as a support and as a filter for radiation with a wavelength below 340 nm. The fluence was $\sim$4 mW/cm^2. Circulation of air around the lamps and samples served to provide constant temperature. Halfway through its irradiation period, each sample was mixed by repetitive pipetting. Samples irradiated for more than 6 min received additional 3 μg/mL portions of Me_3psoralen after each 6-min period. After irradiation with unlabeled Me_3psoralen, samples were ethanol precipitated to remove the majority of the unreacted drug. For determination of the amount of [^{3}H]Me_3psoralen bound to the DNA, unreacted drug was completely removed by chloroform extractions as described previously (Cech & Pardue, 1977).

Gel Electrophoresis. Alkaline agarose gels were prepared according to the method of McDonell et al. (1977). Both the gel and the running buffer contained 33 mM NaOH and 2 mM EDTA, pH 12.4. Gels (22 cm long, 14.7 cm wide, 0.46 cm thick) were poured on a horizontal bed apparatus and submerged under 0.2 cm of running buffer. Samples contained 5 μL of DNA solution (0.2–0.4 μg of DNA in 0.1 mM EDTA, pH 8.4) plus 1 μL of 60% sucrose and 0.05% bromophenol blue. Electrophoresis was carried out for 12–14 h at 100-mA constant current (50 V) with continuous circulation of the buffer. Following electrophoresis, gels were neutralized by soaking for 5 h in 3 changes of 30 mM Tris-HCl, pH 7.5, and then stained for 30 min in 2 μg/mL ethidium bromide in the same buffer. Gels were illuminated by a 300-nm radiation source (Ultra-Violet Products model C-63) and photographed on Kodak Tri-X 4 × 5 in. sheet film through an orange filter. A fluorescent density wedge, constructed to meet the specifications given by Pulleyblank et al. (1977), was photographed along with the gel to allow determination of the film-response curve. Tri-X negatives were scanned by using the gel scanner attachment of the Cary 219 spectrophotometer at 480 nm, where the background absorbance of the film is minimal. Measurement of peak widths was carried out on negatives in which the film densities were within the linear region of the film-response curve. For nondenaturing agarose gel electrophoresis, the buffer system contained 40 mM Tris-HCl, 5 mM sodium acetate, and 1 mM EDTA and was adjusted to pH 7.9 with glacial acetic acid.

Electron Microscopy. DNA was denatured in the presence of glyoxal and spread for electron microscopy as described previously (Cech & Pardue, 1976; Cech et al., 1977). Double-stranded ϕX174 RFII DNA, cross-linked for 80 s as described above, was added to the samples before glyoxal denaturation. It served as a molecular weight standard (5386 bp; Sanger et al., 1978) and also as an internal control for the completeness of denaturation. DNA contour lengths (L) were measured on photographic prints with the aid of a Numonics electronic graphics calculator interfaced to a Nova 2 computer. Apparent molecular weights (bp) were calculated as $1.09L/[5386/L(\text{RFII})]$, where L(RFII) is the average contour length of the cross-linked ϕX174 RFII DNA standards and 1.09 is the length of cross-linked ϕX174 RFII DNA relative to the length of single-stranded ϕX174 DNA (Cech et al., 1977).

Results

Quantification of Extent of DNA Cross-Linking by Electron

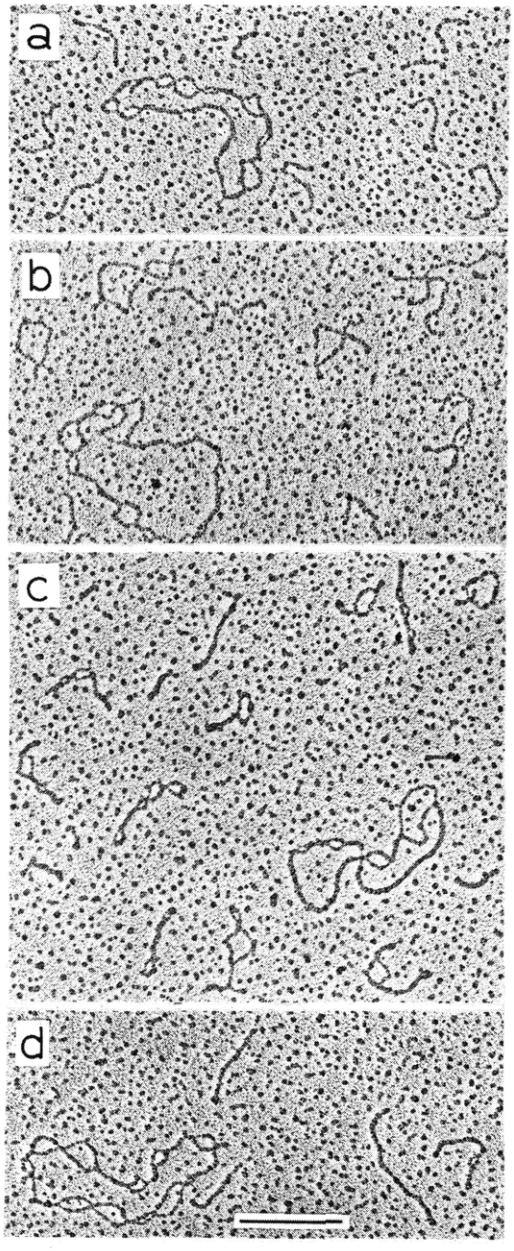

FIGURE 1: *Hae*III restriction fragments of ϕX174 RFII DNA, photoreacted with Me_3psoralen and spread for electron microscopy under totally denaturing conditions. (a) DNA fragments irradiated for 1.5 min in the absence of Me_3psoralen. The other photographs show DNA irradiated in the presence of Me_3psoralen for (b) 0.5 min, (c) 1.5 min, and (d) 24 min. The circular ϕX174 RFII DNA, photoreacted for 80 s in a separate experiment, was included in each preparation as a molecular weight standard. The length of the bar corresponds to 1.00 kbp of cross-linked DNA or 1.09 kb of single-stranded DNA.

Microscopy. ϕX174 *Hae*III fragments that had been photoreacted with Me_3psoralen were totally denatured in the presence of glyoxal and examined in the electron microscope. As shown in Figure 1, the control DNA sample contained only single strands of DNA. The sample photoreacted for 0.5 min contained single strands of DNA as well as molecules with two strands joined by an interstrand Me_3psoralen cross-link ("X" forms with two pairs of equal length arms). The molecules in the 1.5-min sample were heterogeneous in shape. They contained denatured "bubble" regions of varying size, as well as regions that had the appearence of double-stranded DNA. In accordance with earlier studies (Cech et al., 1977), the apparently double-stranded DNA was interpreted to contain denatured regions that were too small to be observed by

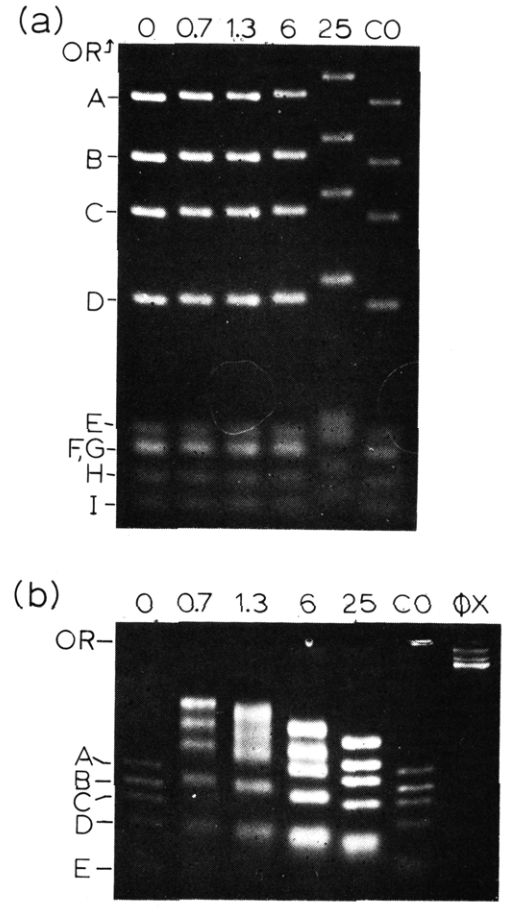

FIGURE 2: Electrophoresis of cross-linked DNA in neutral and alkaline agarose gels. *Hae*III fragments of ϕX174 DNA, designated by the capital letters, were mixed with Me_3psoralen and irradiated for the times (minutes) given. (CO) DNA exposed to neither Me_3psoralen nor radiation. (ϕX) Unrestricted ϕX174 DNA; the prominent band is the 5386 base linear DNA. The same samples were electrophoresed on (a) a neutral 1.4% agarose gel and (b) an alkaline 1.4% agarose gel. (OR) Origin. The origin of the neutral gel was above fragment A by a distance equivalent to that separating (A) and (I). The gels were stained with ethidium bromide.

electron microscopy. The DNA photoreacted for 24 min was almost entirely double-stranded in appearance; visible dentaured regions were rare.

Cross-linked *Hin*dIII fragments of λ DNA were also analyzed by electron microscopy. Measurement of the average distance between points of contact of the complementary DNA strands (the "average bubble size") and measurement of [^{3}H]Me_3psoralen covalently bound to the DNA were used to estimate the cross-link density of the three largest fragments (Table I).

The measured molecular weights of the lightly cross-linked molecules (0.5-min irradiation) corresponded closely to the published values (Table I). The contour lengths of all the DNA fragments increased with increasing photoreaction time. The data are consistent with apparent double-stranded regions having a length per bp 1.10 times that of single-stranded DNA, in agreement with the results of Cech et al. (1977).

Electrophoretic Mobility of Cross-Linked DNA in Neutral and Alkaline Agarose Gels. Electrophoresis of cross-linked ϕX174 *Hae*III fragments in a neutral agarose gel is shown in Figure 2a. The electrophoretic mobility of the DNA fragments decreased slowly with increased photoreaction. The only DNA substantially shifted in mobility was that irradiated for 24 min, which contained ~22 cross-links/kbp (calculated from radioactivity measurements as in Table I).

Table I: Electron Microscopic Analysis of Cross-Linked Restriction Nuclease Fragments of λ DNA

irradiation time (min)	fragment M_r (kbp)[a]	n[b]	M_r^{app} (kbp)[c]	wt fraction bubbles[d]	av bubble size (bp)[e]	no. of cross-links/kbp
0.5	6.76	10	6.71 ± 0.25	0.96 ± 0.03	1220 ± 690	1.0[f]
	9.59	29	9.80 ± 0.43	0.96 ± 0.03	920 ± 190	1.3[f]
	23.5	20	23.77 ± 1.00	0.98 ± 0.02	1260 ± 240	0.9[f]
1.5	6.76	9	6.90 ± 0.13	0.76 ± 0.08	390 ± 80	3.5[f]
	9.59	22	10.07 ± 0.31	0.65 ± 0.07	360 ± 40	4.2[f]
	23.5	32	24.68 ± 0.70	0.81 ± 0.05	450 ± 50	3.1[f]
4.5	6.76	29	7.31 ± 0.37	0.32 ± 0.11	220 ± 50	7.0[g]
	9.59	24	10.39 ± 0.55	0.23 ± 0.12	190 ± 50	8.4[g]
	23.5	25	25.10 ± 1.26	0.44 ± 0.07	260 ± 30	6.2[g]
12	6.76	37	7.41 ± 0.29	0.03 ± 0.03	180 ± 80	14[g]
	9.59	31	10.64 ± 0.48	0.01 ± 0.01	160 ± 40	17[g]
	23.5	26	25.94 ± 0.96	0.08 ± 0.02	230 ± 30	12[g]

[a] Daniels et al., 1980. [b] Number of molecules measured. [c] The contour length of the λ DNA fragment relative to that of cross-linked φX174 RFII DNA, calculated as described under Materials and Methods. Each value is the average molecular weight of n molecules ± standard deviation. [d] The remainder of the DNA occurred as apparent double-stranded regions. The weight fraction was determined for each molecule; the value reported is the mean for the n molecules ± standard deviation. [e] A bubble with two halves each 200 bases in length is said to have a size of 200 bp. Average bubble size was determined for each molecule; the mean ± standard deviation is reported. [f] Calculated from the electron microscopy data as $1000[F/B + (1 - F)/150]$, where F = weight fraction bubbles and B = average bubble size (bp), and apparent double-stranded regions are taken to have 1 cross-link/150 bp (Cech et al., 1977). This method is accurate at low cross-linking densities but underestimates the number of cross-links in more heavily reacted DNA, where a large fraction of each molecule is involved in apparently double-stranded regions of unknown cross-link content. [g] The number of cross-links in the more heavily reacted samples is estimated from radioactivity measurements of DNA photoreacted with [^{3}H] Me_3psoralen. It is assumed that the number of cross-links is proportional to the number of covalently bound Me_3psoralen molecules. Sample calculation: [^{3}H] Me_3psoralen bound after 4.5 min/amount bound after 1.5 min = 2.0, so the number of cross-links after 4.5 min is taken to be twice the number seen after 1.5 min of irradiation. Note that, at all levels of photoreaction, the 9.6 kbp fragment was the most heavily cross-linked and the 23 kbp fragment the least heavily cross-linked. The degree of cross-linking increased with increasing A + T content of the λ DNA (Davidson & Szybalski, 1971), as noted previously for other naturally occurring DNAs (Dall'Acqua et al., 1978; Cech & Karrer, 1980). Despite these sequence-specific effects, the four DNA samples analyzed here were nonoverlapping with respect to their cross-linking densities.

When analyzed by alkaline gel electrophoresis, even the samples that had been photoreacted for a short time with Me_3psoralen contained DNA molecules with a substantially decreased mobility (Figure 2b). In contrast to the results obtained with neutral gel electrophoresis, under alkaline conditions the largest shift in mobility occurred with the most lightly photoreacted DNA. The shift in mobility occurred only upon treatment with both Me_3psoralen and radiation. DNA irradiated in the absence of the drug and DNA–Me_3psoralen mixtures that were kept in the dark had the same electrophoretic mobilities as untreated DNA.

For evaluation of the effect of a single Me_3psoralen cross-link on the electrophoretic mobility of DNA in an alkaline gel, shorter irradiation times were used. With DNA irradiated for 10 s, 23% of the strands (φX fragments A–D) were involved in singly cross-linked molecules and 77% were single-stranded, as judged by electron microscopy. With DNA irradiated for 30 s, the same type of analysis showed 24% of the strands in multiply cross-linked structures, 38% in singly cross-linked structures, and 38% un-cross-linked. Alkaline gel electrophoresis of these samples showed superpositions of two DNA fragment patterns (Figure 3). One set consisted of bands that had the same mobilities as the un-cross-linked DNA fragments; they decreased in intensity as irradiation proceeded. The other set of bands formed a similar pattern displaced to lower mobilities; these bands increased in intensity as irradiation proceeded. The first set is interpreted to be composed of un-cross-linked DNA fragments, including DNA containing Me_3psoralen monoadducts. The low mobility set is interpreted to contain DNA molecules with one or two cross-links.

The largest DNA fragments were the first to disappear from the band pattern of un-cross-linked DNA. For example, from the data in Figure 3 it was determined that only 29% of the fluorescence of the 1342 bp DNA remained in the single-stranded DNA band after 0.5 min of irradiation, while 43% of the 1078 bp band, 65% of the 872 and 606 bp bands, and almost all of the 271–310 bp bands remained. With the assumption that all fragments have about the same frequency of Me_3psoralen cross-linkable sites, the larger DNA fragments are expected to be preferentially cross-linked because they

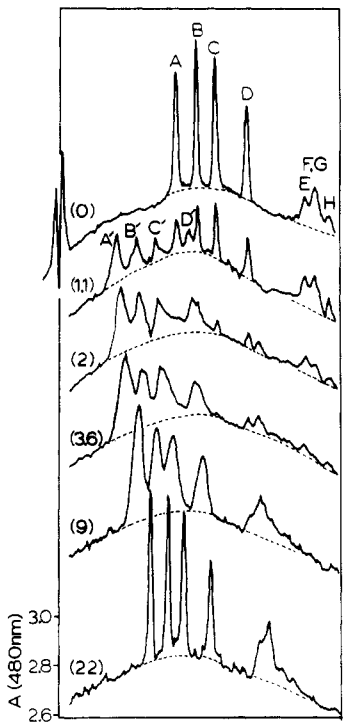

FIGURE 3: Alkaline gel electrophoresis of *Hae*III fragments of φX174 RFII DNA photoreacted with Me_3psoralen. A photographic negative of the ethidium-stained 1.4% agarose gel was scanned in a spectrophotometer. Electrophoresis was from left to right. The deflection in absorbance at the sample well, which is shown only in the first tracing, looked the same in all the scans. The numbers in parentheses give the average number of cross-links per kbp (Table I). The irradiation times were 0, 0.5, 1.0, 1.5, 6, and 24 min. The capital letters refer to the un-cross-linked fragments and the primed letters to the corresponding cross-linked fragments.

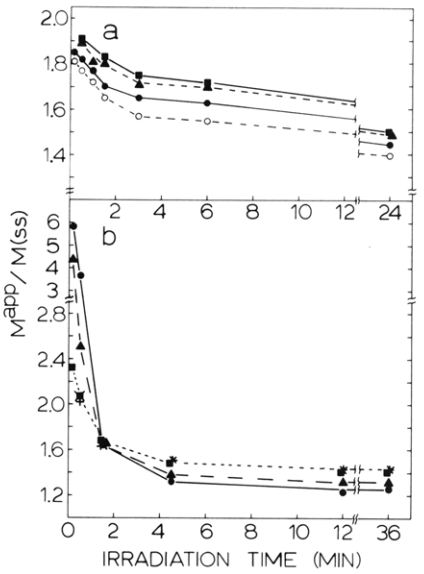

FIGURE 4: Apparent molecular weight of DNA in alkaline gels as a function of extent of cross-linking. (a) Alkaline 1.0% agarose gel electrophoresis of *Hae*III fragments of ϕX174 DNA: (○) fragment A; (●) fragment B; (▲) fragment C; (■) fragment D. (b) Alkaline 0.7% agarose gel electrophoresis of *Hin*dIII fragments of λ DNA: (●) fragment B; (▲) fragment C; (■) fragment D; (+) fragment E; (×) fragment F. Fragment A is omitted, because its mobility after cross-linking was well outside the range of the marker DNAs. For the other fragments, all values of $M_r^{app}/M(ss) > 3$ are minimum estimates based on a linear extrapolation of the log M_r vs. mobility curve beyond the 23 kb fragment. In both parts a and b, the zero time and short irradiation time samples contained un-cross-linked DNA fragments with $M_r^{app}/M(ss) = 1.0$; these points are not included.

Table II: Apparent Molecular Weight of Cross-Linked DNA in Alkaline Gels: Dependence on DNA Fragment Size

DNA fragments	$M(ss)^a$ (kb)	% agarose	$M_r^{app}/M(ss)$ (mean ± range)[b] 1 cross-link	heavily cross-linked
λ B–D	4.4–9.6	0.4	*c*	1.47 ± 0.09
		0.5	*c*	1.44 ± 0.06
		0.7	≥2.3	1.36 ± 0.05
			c	1.39 ± 0.07
		0.9	≥2.2	1.34 ± 0.02
λ E–F	1.9–2.3	0.7	2.04 ± 0.03	1.44 ± 0.02
		0.9	2.05 ± 0.02	1.42 ± 0.02
φX A–D	0.6–1.3	0.8	*c*	1.49 ± 0.06
		1.0	1.87 ± 0.06	1.47 ± 0.07
			1.84 ± 0.09	1.39 ± 0.01
			c	1.51 ± 0.07
		1.4	2.12 ± 0.30	1.37 ± 0.08
			1.93 ± 0.05	1.53 ± 0.04
			c	1.47 ± 0.07
φX E–F	0.3	1.0	2.00	1.50
		1.4	2.00	1.72
			1.98	1.70
			c	1.73

[a] The molecular weight of the single-stranded DNA in kilobases. [b] Each value is the mean for all the fragments in that group. Where multiple values are given for a single percent agarose, each line of data was derived from a separate electrophoresis experiment. [c] From a gel in which no singly cross-linked DNA was analyzed.

present a larger target. This effect was even more pronounced with the larger λ DNA fragments: after only 10 s of photoreaction, most of the DNA of length ≥4.4 kbp had the reduced mobility of cross-linked DNA.

The M_r^{app} (apparent molecular weight) of each cross-linked DNA fragment was determined from a graph of log M_r vs. electrophoretic mobility. Un-cross-linked restriction nuclease

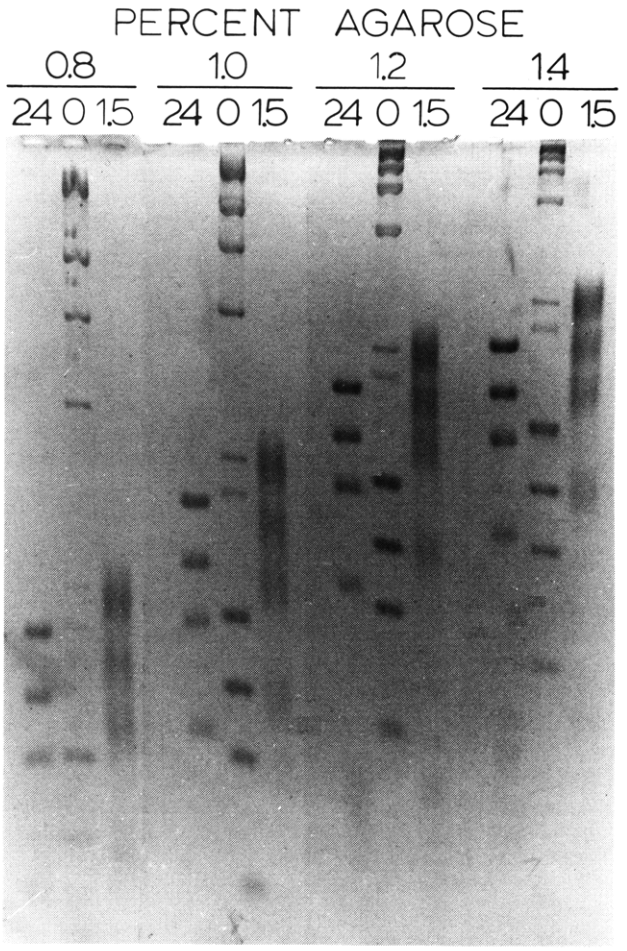

FIGURE 5: Alkaline electrophoresis of cross-linked DNA in different concentrations of agarose. The gel was poured between two vertical glass plates in four sections, beginning with the 0.8% agarose. One plate was then removed, the gel was placed in a horizontal position, the sample well template was placed against one edge of the gel, and the region behind the template was filled in with agarose. The same samples were run at each percent agarose. The samples are designated as (0) unirradiated mixture of the ϕX174 and λ DNA fragments, (1.5) ϕX174 DNA fragments photoreacted for 1.5 min to give ~3.6 cross-links/kbp, and (24) ϕX174 DNA fragments photoreacted for 24 min to give ~22 cross-links/kbp.

fragments of both ϕX174 and λ DNA were included in all gels as M_r standards. Some representative results are shown in Figure 4, and all the experiments are summarized in Table II. For each ϕX174 DNA fragment containing a single cross-link, M_r^{app} was equal to 2.0 ± 0.1 times $M(ss)$, the actual molecular weight of the corresponding single-stranded fragment. Thus, each fragment had the mobility expected for its cross-linked molecular weight. The 2-fold change in M_r^{app} was largely independent of DNA fragment size in the range 0.3–4 kbp but increased substantially with the λ DNA fragments greater than ~6 kbp in size (Figure 4b).

Each heavily cross-linked DNA fragment (12–36-min irradiation) had an M_r^{app} that was 1.4 ● 0.1 times the molecular weight of its single-stranded counterpart. Within the range of 0.6–10 kbp, the ratio of $M_r^{app}/M(ss)$ decreased slightly with increasing fragment length (Table II).

Effect of Agarose Concentration. It is apparent from Table II that the shifts in M_r^{app} observed for cross-linked DNA of size 0.3–2.3 kbp are not highly dependent on the percentage of agarose in the gel. Samples of cross-linked and un-cross-linked DNA were each run at four different agarose concentrations on the same slab gel (Figure 5) to further investigate the effect of agarose concentration. The M_r^{app} of the heavily

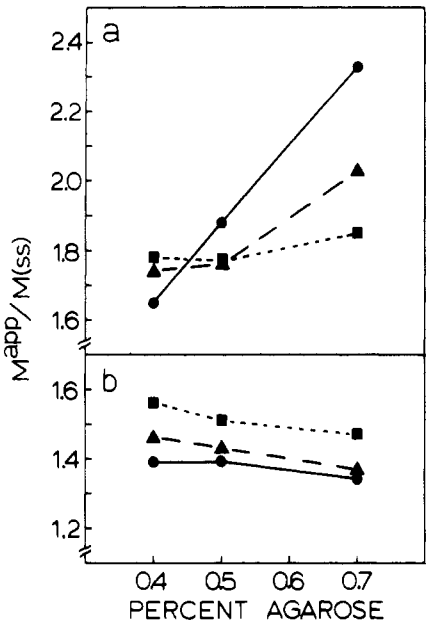

FIGURE 6: Apparent molecular weight of cross-linked λ DNA restriction fragments as a function of agarose concentration. The data were taken from a gel similar to that shown in Figure 5, with portions of the same samples being run at three different concentrations of agarose. (a) Lightly cross-linked DNA produced by 0.5 min of photoreaction. (b) Heavily cross-linked DNA produced by 24 min of photoreaction. The λ DNA fragments are represented as (●) B, (▲) C, and (■) D.

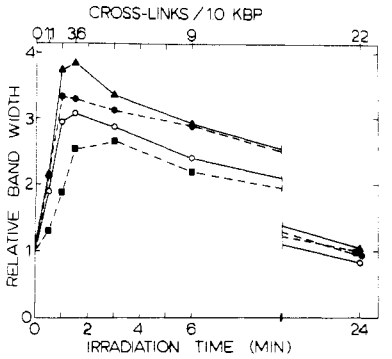

FIGURE 7: Dependence of DNA bandwidth on extent of cross-linking. The φX174 DNA fragments are represented as (○) A, (●) B, (▲) C, and (■) D. Each point is the average of two values, one obtained from a 1.0% agarose gel and one from a 1.4% gel. "Relative bandwidth" is the width at half peak height of a cross-linked DNA band divided by the width of the un-cross-linked DNA band formed by the same DNA restriction fragment. The widths of the un-cross-linked DNA bands were dependent on the DNA fragment size but were independent of DNA concentration over the range used. The abscissa is linear with irradiation time. The number of cross-links per kbp (Table I) is given for some of the samples.

cross-linked DNA (22 cross-links/kbp) did not change while the M_r^{app} of the lightly cross-linked DNA (3.6 cross-links/kbp) increased by 6% over the 0.8–1.4% agarose range.

As the molecular weight of the lightly cross-linked DNA increased, the effect of agarose concentration on its M_r^{app} became more pronounced (Figure 6a). The same DNA when heavily cross-linked showed very little dependence of M_r^{app} on agarose concentration (Figure 6b), as was seen with the smaller DNA fragments.

Bandwidth of Cross-Linked DNA in Alkaline Gels. With all sizes of DNA fragments (0.3–23.5 kbp), the width of the DNA band observed after alkaline gel electrophoresis was substantially greater for DNA containing a low density of cross-links than for the un-cross-linked DNA. The maximum bandwidth did not occur with the introduction of the first cross-link into the DNA, but rather after 1.5 min of photoreaction, when the DNA had ~1 cross-link every 300 bp (Figure 7). With further cross-linking, the bandwidths decreased until they were approximately equal to those of the un-cross-linked DNA molecules.

Discussion

Sensitivity of Method. The results described above provide the basis for a new method of detecting Me_3psoralen cross-links in DNA. With many of the methods used previously, such as electron microscopy, alkaline sedimentation, or the determination of the amount of radioisotopically labeled psoralen that is covalently bound to DNA, the smallest signals (and therefore the least reliable measurements) are obtained at low levels of photoreaction. The alkaline gel electrophoresis patterns, on the other hand, are very sensitive to the difference between 0 and 1 cross-link/DNA molecule. With DNA fragments of size 10 kbp the detection of 1 cross-link/100 kbp should easily be possible: 90% of the 10 kbp DNA would have the un-cross-linked mobility, and 10% would have the reduced mobility.

With the addition of subsequent cross-links to a DNA molecule, its electrophoretic mobility increases while its bandwidth goes through a maximum and then decreases. These changes provide a basis for estimating the number of cross-links per DNA molecule. In general, however, alkaline gel electrophoresis is less sensitive to the extent of cross-linking than the other methods mentioned above.

At short irradiation times, when only a portion of the DNA fragments have been cross-linked, the un-cross-linked molecules are accumulating Me_3psoralen monoadducts (Johnston et al., 1977). The electrophoretic mobilities and bandwidths of the monoadduct-containing DNA were indistinguishable from those of the unirradiated DNA (Figure 3). This observation is consistent with the electrophoretic behavior of the DNA being unaffected by low levels of monoadducts.

Explanation of Alkaline Gel Electrophoretic Patterns. The electrophoretic mobility shift produced by light cross-linking of 0.3–4 kbp DNA was exactly that expected for doubling the molecular weight of the denatured DNA. The mobilities of both un-cross-linked and cross-linked DNA were found to be similarly affected by changes in agarose concentration (Figure 5), so the ratio $M_r^{app}/M(ss)$ was not greatly affected. These observations support the idea that cross-linked DNA molecules of size 0.3–4 kbp migrate through the gel in a manner similar to that of un-cross-linked molecules of the same molecular weight, i.e., twice the $M(ss)$.

With DNA of higher molecular weight, the introduction of a cross-link produced a much greater than 2-fold increase in M_r^{app}. This can be explained if the dimensions of the large X forms are about equal to the dimensions of the pores in the gel, such that in some of its transient configurations the DNA is temporarily stuck [cf. Mickel et al. (1977)]. The increased dependence of $M_r^{app}/M(ss)$ on the agarose concentration that was observed for the larger DNA molecules (Figure 6a) is consistent with such a view. The large mobility decrease of the high molecular weight DNA (Figure 4b) and the pronounced dependence on agarose concentration (compare parts a and b of Figure 6) were both lost with further cross-linking of the DNA, which greatly restricted the average width of the molecule.

The breadth of the DNA bands in the alkaline gels did not reach its maximum value with the introduction of the first cross-link, but rather at a density of about 2–4 cross-links/kbp (Figure 7). The breadth should be a measure of the hete-

rogeneity of frictional factors exhibited by a population of cross-linked molecules. The appearance of the molecules in the electron microscope seems to reflect in some way their shape in solution, because the sample exhibiting the greatest variety of shapes (Figure 1c) had the greatest electrophoretic bandwidths.

Applications of Method. Alkaline gel electrophoresis should provide a good method for the routine assay of psoralen cross-links in DNA. It should also be useful for testing other compounds that are suspected to be DNA cross-linking agents, provided that the compounds do not produce single-strand breaks in the DNA. In studies where psoralen cross-linking is used to probe DNA–protein interactions in vivo, the alkaline gel electrophoresis patterns will be most informative if different regions of a DNA molecule are cross-linked to quite different extents. One system that has this property, the *Drosophila melanogaster* mtDNA (Potter et al., 1980), is currently being investigated.

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