The Photoaddition of Trimethylpsoralen to *Drosophila melanogaster* Nuclei: A Probe for Chromatin Substructure†

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ABSTRACT: Derivatives of the furocoumarin, psoralen, can penetrate intact cells or nuclei and cross-link opposite strands of the chromosomal DNA under the influence of long wavelength ultraviolet light. The potential of trioxsalen (4,5′,8-trimethylpsoralen) as a probe for chromatin structure has been investigated. The DNA in both embryo nuclei and tissue culture cells from *Drosophila melanogaster* was found to be about 90% protected from trioxsalen binding relative to purified DNA. Digestion of trioxsalen-treated nuclei by micrococcal nuclease and gel electrophoresis of the resulting DNA gave the same type of band pattern that is characteristic of native, untreated nuclei after digestion. Nuclease digestion was therefore used to examine the distribution of bound trioxsalen in the DNA. The resulting DNA fragments were analyzed both by radioactivity measurements and quantitative electron microscopy. The nuclease cleaved intact photoreacted nuclei in such a way that preferential excision of trioxsalen containing regions of the DNA occurred, but, when acting upon purified DNA that contained bound trioxsalen, it attacked the trioxsalen-free regions preferentially. It was thus concluded that trioxsalen binds at the sites corresponding to the regular nuclease sensitive regions of the chromatin in nuclei.

One approach toward elucidating the structure of eukaryotic chromatin has been the utilization of various "probe" molecules. Of these, cationic species such as polylysine (Itzhaki, 1971), Mg^{2+} (Schmidt et al., 1972), and dyes (e.g., toluidine blue [Miura and Ohba, 1967], ethidium bromide [Lurquin and Seligy, 1972], and quinacrine [Brodie et al., 1975]) have yielded data concerning the amount of DNA that is not bound directly to the histone proteins and the relationship of this to the degree of transcription that the chromatin template can support.

The use of endonucleolytic enzymes, though originally directed toward similar ends (Mirsky, 1971; Clark and Felsenfeld, 1971), has recently led to more detailed insights into chromatin structure by revealing the existence of a repeating structural unit (Burgoyne et al., 1974). Micrococcal nuclease digestion of nuclei from a wide variety of sources (Honda et al., 1974; McGhee and Engel, 1975; Piper et al., 1976) results in fragments of DNA about 200 base pairs long (and fragments that are multiples of this monomer length).

Nuclease digestion suffers from certain disadvantages. Firstly, the native chromatin structure is broken down and its relationship to the structure of the isolated "monosome" nucleoprotein particles is as yet unclear. Secondly, it is possible that enzymatic cleavage induces at least a certain amount of histone migration (Bakayev et al., 1975; Shaw et al., 1976). Thirdly, because of the absolute requirement of micrococcal nuclease for Ca^{2+}, the interesting question concerning the effect of divalent cation concentration on the native chromatin structure cannot be addressed.

Recently, experiments from this laboratory (Hanson et al., 1976) have indicated the utility of photochemical binding of derivatives of the furocoumarin, psoralen, as a new structural probe for chromatin. These molecules easily penetrate intact cells or isolated nuclei and then intercalate in the DNA helix. Upon irradiation with UV[1] light in the range 320–380 nm, covalent cross-linkage of the complementary DNA strands occurs if two pyrimidine bases are positioned on opposite strands adjacent to the intercalated furocoumarin (Cole, 1970). The relative positions of the cross-links in the DNA may then be observed in the electron microscope (Hanson et al., 1976). This ability to retain a stable linear record of cross-linked regions represents a potentially important advantage over the nuclease method.

The purpose of the following experiments was to investigate more closely the photoreaction of 4,5′,8-trimethylpsoralen (trioxsalen) with purified DNA, and with the DNA in whole cells and isolated nuclei. In particular, we wished to examine whether DNA in intact chromatin could be cross-linked to the same extent as purified DNA and, if it could not, to search for specificity in the cross-linking reaction using a combination of micrococcal nuclease digestion, gel electrophoresis, and electron microscopy.

Materials and Methods

Cells and Nuclei from Drosophila melanogaster. Drosophila melanogaster tissue culture cells (Schneider Line 2 cells) were grown in suspension culture in Schneider's medium (Schneider, 1972). The cells were radioactively labeled at an initial density of 4×10^6/mL with 0.5 μCi/mL of [^{14}C]thymidine (ICN Corp.) for 36 h. The final specific activity attained was 6940 cpm/μg. These cells were diluted with unlabeled cells of the same line to give lower specific activity DNA as required.

Nuclei were isolated from 24 h embryos of *Drosophila melanogaster* using a modification of the procedure of Kram et al. (1972). The embryos were homogenized in 25 mM KCl–1 mM $MgCl_2$–0.25 M sucrose–15 mM mercaptoethanol–50 mM Tris-HCl (pH 7.4) (TMKS) (Honda et al., 1974). The nuclei were pelleted twice at 1000*g* for 5 min at 4 °C and

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[1] Abbreviations used: TMKS, 25 mM KCl–1 mM $MgCl_2$–0.25 M sucrose–15 mM mercaptoethanol–50 mM Tris-HCl (pH 7.4); SSC, 0.15 M NaCl–0.015 M sodium citrate (pH 7.0); Tris, tris(hydroxymethyl)-aminomethane; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid.

resuspended in the same buffer, unless the irradiation was to take place in SSC (0.15 M NaCl–0.015 M sodium citrate (pH 7.0)) in which case two further washings and pelletings in SSC were carried out. The nuclei were used immediately in the cross-linking reaction.

4,5′,8-Trimethylpsoralen. 4,5′,8-Trimethylpsoralen (trioxsalen) obtained from the Paul B. Elder Co. (Ohio) and tritiated in this laboratory (Isaacs et al., 1977) was dissolved in absolute ethanol to give stock solutions of about 0.8 mg/mL. The final concentration of trioxsalen in these solutions was measured from the absorbance at 249 nm, using an extinction coefficient of 31 008 M^{-1} cm^{-1} at this wavelength (determined in this laboratory). Aliquots of the stock solutions in 10 mL of Triton X-100 (1 L)–toluene (2 L)–Omnifluor (12 g) (New England Nuclear) scintillation fluid made 10% in H_2O were counted on a Beckman LS-230 scintillation counter, and the specific activity of the trioxsalen was determined. Stock solutions were generally adjusted to fall in the range of approximately 75 000 cpm/μg of trioxsalen, except when cross-linking ^{14}C-labeled DNA, where solutions with specific activities of over 1.5×10^6 cpm/μg were used to minimize ^{14}C to ^{3}H spillover corrections.

Irradiation. Trioxsalen was added to suspensions of cells, nuclei, or solutions of DNA to a final concentration of 5 μg/mL. The use of a final concentration above the saturation level in aqueous solutions was intended to ensure that the system remained saturated in trioxsalen as the photochemical binding reaction proceeded.

Irradiation of 0.5-mL samples took place at 6 °C in a water-cooled semimicrocuvette of 1 cm path length placed in the beam of a high-pressure mercury emission lamp (AH-6, General Electric). The incident light was filtered through a 5-mm glass filter, which allowed the passage of light between 320 and 410 nm (maximum at about 370 nm). The intensity incident on the cuvette was 25 mW/cm^2.

Isolation of DNA from Irradiated Tissue Culture Cells and Embryo Nuclei. After irradiation, nuclei were obtained from tissue culture cells by homogenizing in TMKS plus 0.5% Nonidet P-40 detergent (Shell Oil Ltd., London). The nuclei (either tissue culture cell or embryo nuclei) were then either lysed by addition of EDTA to 25 mM and Sarkosyl NL-97 (Geigy) to 1.5%, or made 1 mM in $CaCl_2$ and digested with micrococcal nuclease (Worthington) for periods of 1 to 30 min at 37 °C. The nuclease digestion was terminated by addition of EDTA and Sarkosyl as above. All samples were then subjected to extensive Pronase digestion (1 mg/mL Pronase, Calbiochem B grade, nuclease free, 5–7 h at 50 °C). The lysates were made 1.0 M in NaCl and extracted three times with chloroform–isoamyl alcohol (24:1) to remove excess unbound trioxsalen and polypeptide digestion products. The emulsion formed at each extraction was broken by centrifugation at 1500*g* for 15 min. The aqueous phase was removed and dialyzed into SSC overnight, then subjected to RNase B digestion (Calbiochem A grade) for 3 h at 37 °C, 150 μg/mL, α-amylase digestion (Calbiochem B grade) for 1 h at 37 °C, 150 μg/mL, and then by Pronase treatment for 4 h at 37 °C, 1 mg/mL. The extraction procedure was repeated three more times, and the samples were dialyzed into 10 mM sodium phosphate–1 mM EDTA (pH 6.8).

The concentrations of the DNA samples thus obtained were assayed spectrophotometrically using an extinction coefficient of 6600 M^{-1} phosphorus (Peacocke and Walker, 1962) or, in the case of ^{14}C-labeled DNA from tissue culture cells, by counting aliquots as described above. The amount of bound [^{3}H]trioxsalen was also determined by counting and the ratio of trioxsalen molecules to DNA base pairs was calculated.

Polyacrylamide Gel Electrophoresis. Analysis of the DNA fragments resulting from nuclease digestion of the trioxsalen-treated nuclei took place on 10-cm long by 0.4-cm thick 4% polyacrylamide analytical slab gels (Loening, 1967). Electrophoresis was at 40 V for 9–10 h. DNA bands were visualized by ethidium bromide staining and photographed. For ^{14}C-labeled DNA samples each longitudinal gel region corresponding to one sample loading was sliced transversely into 1.4-mm long fractions which were separately incubated in glass scintillation vials with 1 mL of a 9:1 mixture of NCS tissue solubilizer (Amersham/Searle) and water at 50 °C for at least 4 h. Ten milliliters of toluene (1 L)–2,5-diphenyloxazole (6 g)–1,4-bis[2-(5-phenyloxazolyl)]benzene (75 mg) (Amersham/Searle) scintillation fluid was then added and the fractions were counted.

Production of DNA fragments for subsequent electron microscopy took place on preparative slab gels (one sample per $14 \times 10 \times 0.4$ cm gel). The positions of the DNA bands were visualized by slicing and staining the left and right edges of the gel. The large unstained middle portion of the gel was sliced into pieces containing the monomer, dimer, and trimer fragments. Each piece was mashed with a mortar and pestle and then homogenized further in a Dounce homogenizer. The DNA was extracted three times with 10 volumes of 0.01 M sodium phosphate–1 mM EDTA (pH 7.0) buffer. Gel particles were pelleted at 1000*g* for 10 min, and the DNA was concentrated out of the supernatant by bulk hydroxylapatite chromatography.

Electron Microscopy. The purified DNA samples were denatured in 10% formaldehyde, 0.02 M sodium carbonate, 5 mM EDTA (pH 7.0) at 70 °C for 2 h (Hanson et al., 1976) and then spread for microscopy using the procedure of Davis et al. (1971). Microscopy was performed on a Philips 201 electron microscope operating at 60 keV. Nicked PM-2 DNA (prepared by the method of Wang (1971) and a gift from L. Liu) was used as an internal length standard for native DNA preparations.

Length measurements of DNA fragments were performed on enlarged prints or projections of 35 mm negatives using an electronic planimeter (Numonics Corp.) interfaced to a paper tape punch. A PDP 8/E computer was used to compile weight average histograms of DNA sizes.

Assay of the Digestion of DNA by Micrococcal Nuclease. ^{14}C-Labeled DNA and cold DNA were isolated from *Drosophila melanogaster* tissue culture cells as described above, except that the micrococcal nuclease digestion step was of course omitted.

Digestion mixtures contained about 24 μg of [^{14}C]thymidine-labeled DNA (sp act. = 2100 cpm/μg) cross-linked with tritiated trioxsalen to varying degrees (see Results), about 90 μg of cold DNA to act as carrier, and 72 units of micrococcal nuclease made up to a total volume of 6 mL in TMKS buffer plus 1 mM $CaCl_2$ (pH 7.4). Digestion was carried out at 37 °C (the samples being prewarmed to this temperature before addition of the enzyme) and 1-mL aliquots were withdrawn at different intervals. The digestion was stopped with ice-cold EDTA (25 mM). NaCl was added to a final concentration of 100 mM, and the DNA was precipitated by addition of 2 volumes of ice-cold ethanol. The mixtures were then passed through Whatman GF/C filters (presoaked in saturated sodium pyrophosphate and rinsed with 100 mL of 0.1 M HCl and then 100% ethanol), followed by two rinses with 1.5 mL of cold ethanol. The dried filters were assayed for radioactive content in 10 mL of toluene (1 L)–Omnifluor (4 g) scintillation fluid.

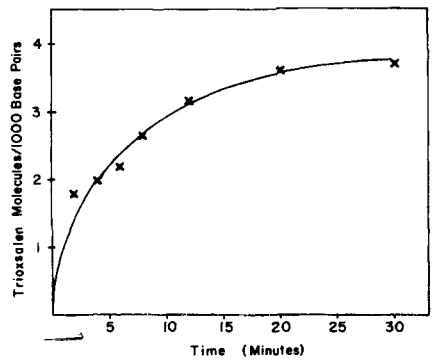

FIGURE 1: *D. melanogaster* embryo nuclei at an A_{650}^{1cm} of about 6 (equivalent to a DNA concentration of about 100 μg/mL under our conditions) plus one addition of trioxsalen at 5 μg/mL. Irradiation intensity in this and subsequent experiments was 25 mW/cm^2. After 30 min about 3% of the added trioxsalen was covalently attached to the DNA.

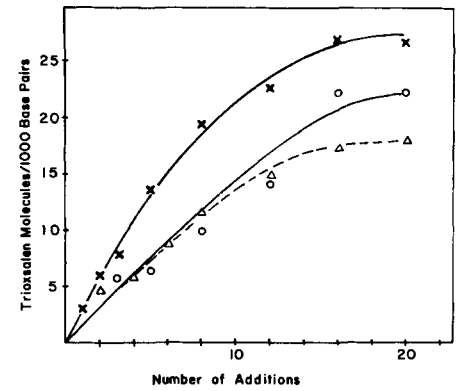

FIGURE 2: *D. melanogaster* nuclei and cells reacted with multiple additions of trioxsalen. (X) Embryo nuclei in SSC buffer; (O) embryo nuclei in TMKS buffer; (Δ) tissue culture cells in TMKS buffer. Nuclei were at an A_{650}^{1cm} of about 6. Tissue culture cells were at a concentration of about 2 × 10^8 cells/mL. Trioxsalen was added at 10-min intervals to a concentration of 5 μg/mL each time. The nuclei/cells were pelleted at 1500*g* after each addition and resuspended in fresh buffer.

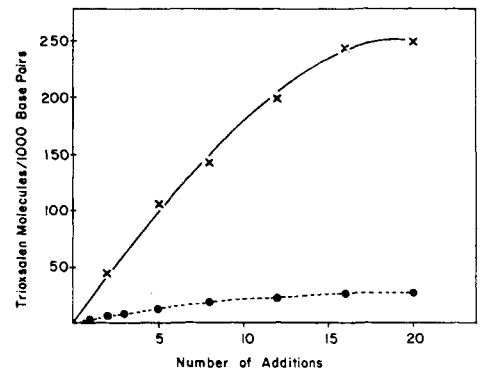

FIGURE 3: *D. melanogaster* purified DNA and nuclei reacted with multiple additions of trioxsalen. (X) Purified DNA in SSC buffer; (●--●) embryo nuclei in SSC buffer. DNA was at a concentration of 100 μg/mL. Nuclei were at an A_{650}^{1cm} of about 6.

Results

Micrococcal nuclease digestion studies on purified nuclei suggest that the DNA in nuclei is largely protected from digestion by protein–DNA interactions. The DNA is primarily susceptible to nuclease at regular intervals of about 200 base pairs (Burgoyne et al., 1974; Noll, 1974). It is important to determine whether these same regions are preferentially accessible to trioxsalen cross-linking. If only part of the DNA in chromatin is accessible to trioxsalen, then this DNA should react to a lower extent than purified DNA under the same conditions. If the regions susceptible to cross-linking correspond to the regions susceptible to nuclease, then digestion of cross-linked nuclei should preferentially remove trioxsalen. The following experiments test these predictions.

Treatment of DNA, Nuclei, and Cells from Drosophila melanogaster with Trioxsalen and UV Light. Upon irradiation at 320–380 nm of a mixture of trioxsalen and DNA (purified, in cells or in nuclei), two competing reactions occur: (a) the photoinduced covalent binding of trioxsalen to the DNA to yield adducts that are attached to either one or both of the DNA strands (Cole, 1970); and (b) the photodestruction of the trioxsalen itself. Irradiation with 25 mW/cm^2 UV light of 5 μg/mL trioxsalen in ethanol alone for increasing periods of time suggested that the trioxsalen was substantially broken down after about 10 min, as monitored by the change in the UV absorbance spectrum (data not shown). Consistent with this, a single addition of 5 μg/mL of trioxsalen to nuclei achieved near-maximal binding after about 15 min of irradiation at this level (Figure 1). The protocol for determining the maximum total amount of trioxsalen that could be bound to nuclei or purified DNA samples thus consisted of repeatedly adding fresh aliquots (5 μg/mL) of trioxsalen at 10-min intervals until a plateau was attained. Separate experiments indicated that allowing the concentration of ethanol to rise above 2% adversely affected the nuclei and cells, which were therefore pelleted at 1500*g* for 10 min after each 10-min irradiation, resuspended in fresh buffer plus trioxsalen, and then reirradiated. In this way, the ethanol concentration was kept below 0.5% at all times. (An ethanolic stock solution of trioxsalen must be used since the solubility in water is only ca. 0.6 μg/mL.) A similar protocol was followed in the control experiments using purified DNA, except that the ethanol could not be removed in this way between each irradiation. In this case a final ethanol concentration of 9.1% was reached with the most heavily treated sample.

Figure 2 shows the amount of trioxsalen bound per unit amount of DNA for samples of cells irradiated in TMKS and of nuclei irradiated in both TMKS and SSC, with increasing numbers of 10-min dosages. The maximum amount bound in each case is about 18, 22, and 27 trioxsalen molecules per 1000 base pairs, respectively. These figures may be compared with that for the saturation of isolated DNA which, as seen in Figure 3, is about 250. Although the plateau levels are based on two points only, repeated experiments led consistently to figures within ±15% of those given above. Owing to the loss of a small fraction of the nuclei or cells at each centrifugation step, extension of the experiment beyond 20 additions of trioxsalen was not practical. The intranuclear DNA thus appears to be about 90% protected from covalent binding of the trioxsalen relative to isolated DNA, both in the presence and absence of divalent cation. Moreover, the close similarity of the binding curves for isolated nuclei and whole cells (Figure 2) suggests that the cytoplasm and cell membrane add no significant kinetic barrier to trioxsalen incorporation. Indeed, the fact that saturation of both nuclei and purified DNA requires about the same number of trioxsalen additions implies that the chromosomal proteins attached directly to the DNA are the sole factors involved in its protection.

Micrococcal Nuclease Digestion of Purified DNA after Photoreaction with Trioxsalen. Before using micrococcal nuclease to investigate in more detail the way in which trioxsalen binds to the DNA in cells and nuclei, it was necessary to

TABLE I: Comparison of Micrococcal Nuclease Digestion of [^{14}C]DNA with Varying Amounts of [^{3}H]Trioxsalen Bound.

No. of trioxsalen molecules per 1000 base pairs	0	6.6	14.7	42.7	172.4
Initial ^{3}H/^{14}C	0	0.8	1.7	5.7	30.8
Final ^{3}H/^{14}C (after 120-min digestion)	0	5.8	10.9	30.3	46.8
Enrichment [(final) (^{3}H/^{14}C)/ initial (^{3}H/^{14}C)]	—	7.2	6.4	5.3	1.5
Time to solubilize half of ^{14}C counts (min)	5.5	6.5	5.8	7.4	11.3
Fraction of ^{14}C counts precipitable after 120 min of digestion	0.03	0.03	0.04	0.04	0.16
Fraction of ^{3}H counts precipitable after 120 min of digestion	—	0.25	0.22	0.21	0.24

TABLE II: Comparison of the Specific Activities of *D. melanogaster* DNA from Trioxsalen Reacted Nuclei before and after Digestion by Micrococcal Nuclease.

No. of additions of trioxsalen	3	5	8	12	16	20
No. of trioxsalen molecules per 1000 base pairs of DNA for DNA isolated from undigested nuclei	5.6	6.2	9.8	13.9	22.0	22.0
No. of trioxsalen molecules per 1000 base pairs of DNA for DNA isolated from digested nuclei	2.6	3.6	6.3	8.7	16.6	16.0
(Sp act. of digested DNA)/ (sp act. of undigested DNA)	0.46	0.58	0.64	0.63	0.75	0.73

determine how the nuclease digestion of purified DNA was affected by the intercalated reagent.

Aliquots of [^{14}C]thymidine-labeled DNA (2100 cpm/μg) extracted and purified from *Drosophila melanogaster* tissue culture cells were irradiated with increasing amounts of [^{3}H]trioxsalen, and the excess unbound reagent was removed by extensive extraction and dialysis. Four samples were produced containing respectively 6.6, 14.7, 42.7, and 172.4 covalently bound trioxsalen molecules per 1000 base pairs. These samples were subjected to micrococcal nuclease digestion as described in Materials and Methods, and the DNA retained on the filter after ethanol precipitation was assayed for relative trioxsalen content as digestion proceeded.

The results are shown in Table I. In each case, the DNA precipitated on the filter becomes progressively enriched in trioxsalen with increasing time of digestion, maximum enrichment being observed for the sample originally with the least amount of bound trioxsalen. The initial digestion rate is relatively unaffected when there is a low density of trioxsalen bound to DNA, but the initial rate is decreased by about a factor of two for the most heavily reacted sample (1 trioxsalen per 5.8 base pairs). The enzyme thus preferentially digests areas of the DNA that are free of trioxsalen.

The fraction of [^{14}C]DNA that is retained on the filter after 120 min of digestion increases as the amount of trioxsalen bound to the DNA increases. This increase is rather small for the DNA samples with least trioxsalen bound, but almost 16 percent of the DNA sample with the most trioxsalen bound is retained.

The fraction of [^{3}H]trioxsalen that is retained on the filter after 120 min of digestion does not change as the amount of trioxsalen bound to the DNA increases. Thus a constant fraction (~0.23) of the trioxsalen appears to be bound to the DNA in such a way as to make the DNA resistant to micrococcal nuclease digestion. It has been shown (Cole, 1971) that about 30% of the trioxsalen bound to λ bacteriophage DNA is in the form of a cross-link. The remaining 70% of the trioxsalen are monoadducts (covalently attached to just one of the DNA strands). This result, then, suggests the possibility that a trioxsalen cross-link makes the DNA totally resistant to micrococcal nuclease digestion.

Micrococcal Nuclease Digestion of Nuclei after Photoreaction with Trioxsalen. In order to determine the effect of the cross-linking reaction on the native chromatin structure in nuclei, trioxsalen-treated nuclei and an untreated control sample were digested under identical conditions. As shown in Figure 4, the characteristic DNA band pattern is still obtained upon gel electrophoresis of the treated sample, showing the same size distribution of the DNA fragments as the control

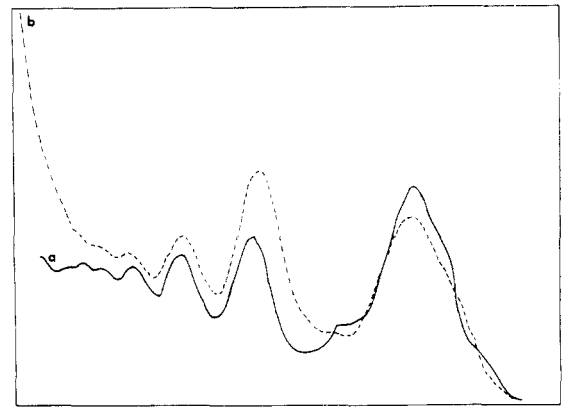

FIGURE 4: Four percent polyacrylamide gel traces of (a) micrococcal nuclease digested *D. melanogaster* embryo nuclei and (b) micrococcal nuclease digested *D. melanogaster* embryo nuclei previously reacted with trioxsalen in TMKS buffer to the extent of 5 trioxsalen molecules per 1000 base pairs. Each of the samples of nuclei was digested with 120 units of nuclease for 2 min. The direction of electrophoresis is from left to right.

sample. The difference in relative intensity of the monomer and dimer bands in the two samples is probably related to the difference in kinetics for digestion of normal DNA and trioxsalen bound DNA observed above.

To test for the specificity of the trioxsalen reaction, ^{3}H-labeled trioxsalen was used to cross-link DNA to increasing extents in samples of isolated *Drosophila melanogaster* nuclei. A portion of each treated sample was digested with micrococcal nuclease for the same time and a portion left intact. The DNA was isolated from each portion by the procedure described in Materials and Methods during which small oligonucleotide digestion products are lost by dialysis. The specific activities of the remaining digested DNA and the undigested DNA from each parent sample were determined. Table II shows that the specific activity of the digested sample is lower than that of the undigested sample in each case. The nuclease therefore preferentially digested away the trioxsalen-containing regions of the DNA. The largest drop in the specific activity of the digested sample relative to that of the undigested material occurs in the DNA containing least trioxsalen and diminishes somewhat as the amount of bound trioxsalen increases. This is again consistent with a progressive inhibition of the nuclease as the density of bound DNA sites increases.

The analysis was taken further by electrophoresing the DNA fragments from digested trioxsalen treated nuclei and determining the specific activity of the various size classes of DNA

TABLE III: Decrease in Trioxsalen Content of DNA Isolated from Tissue Culture Nuclei Digested for Increasing Time with Micrococcal Nuclease.

Time of digestion (min)	0	1.5	5	30
No. of trioxsalen molecules per 1000 base pairs of DNA for DNA isolated from tissue culture tissue	9.8	8.0	6.0	4.2
(Sp act. of digested DNA)/(sp act. of undigested DNA)		0.82	0.61	0.43
Av $^3H/^{14}C$ ratio over the major DNA peak after electrophoresis	7.5	3.9	1.5	1.1
($^3H/^{14}C$ ratio of digested major peak)/($^3H/^{14}C$ ratio of undigested DNA sample)		0.52	0.20	0.15

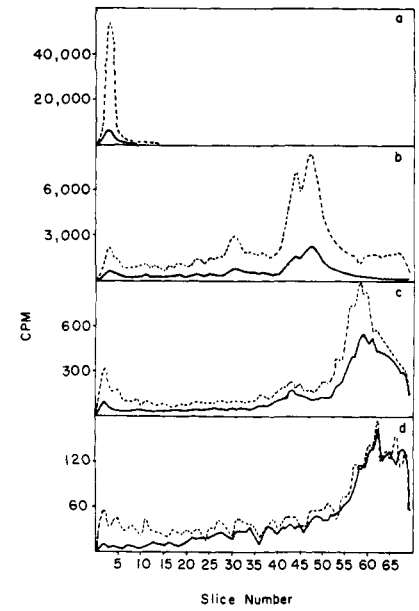

FIGURE 5: Four percent polyacrylamide gel traces of micrococcal nuclease digested nuclei from *D. melanogaster* tissue culture cells previously reacted with trioxsalen. (—) [^{14}C]Thymidine-labeled DNA; (- - -) ^3H-labeled trioxsalen. (a) DNA from undigested nuclei. (b) DNA from nuclei digested for 90 s. (c) DNA from nuclei digested for 5 min. (d) DNA from nuclei digested for 30 min. (After 30 min digestion the yield of DNA was 50% of that from undigested nuclei.) a, c, and d were run on the same slab gel for 10 h; b was run for 8 h on a separate gel. The direction of electrophoresis is from left to right.

that are separated on the gel. In this case, advantage was taken of the fact that [^{14}C]thymidine could be used to label beforehand the DNA of *Drosophila melanogaster* tissue culture cells and that then the cells could be reacted directly with tritiated trioxsalen since these cells show a trioxsalen saturation curve closely similar to that of isolated embryo nuclei (see Figure 2). This enabled the relative amounts of DNA and bound trioxsalen in a given gel slice to be easily monitored. After irradiation, a portion of the cells was lysed and the nuclei were digested with nuclease. Table III shows the amount of bound trioxsalen per base pair of the DNA isolated from the digested nuclei before separation of the fragments on gels. The amount bound per base pair decreases as a function of digestion time. Thus, in this system also, the trioxsalen-containing regions of the nuclear DNA are preferentially digested by micrococcal nuclease. This of course is in direct contrast to the case of trioxsalen-treated purified DNA, where the trioxsalen free regions are preferentially attacked.

Gel electrophoresis of the digestion mixtures represented in Table III was carried out and the gels were sliced and analyzed for radioactive content (Figure 5). In Figure 5a, essentially all of the DNA isolated from undigested nuclei is in the top six slices of the gel. The average $^3H/^{14}C$ ratio for these slices is 7.5. In Figure 5d, 70% of the ^{14}C counts are in the monomer peak (slices 49-69). The average $^3H/^{14}C$ ratio for these slices is 1.1. Thus extensive digestion of trioxsalen reacted nuclei leaves monomer size fragments which have a trioxsalen content 6.8-fold lower than undigested DNA (Table III).

The DNA in the monomer peaks in Figures 5b (39-57), 5c (49-69), and 5d (49-69) shows a continuous decline in $^3H/^{14}C$ ratio. The average $^3H/^{14}C$ ratios for the monomer peaks in Figures 5b, 5c, and 5d are 3.9, 1.5, and 1.1, respectively. Therefore, as the time of digestion increases, the trioxsalen content of the remaining monomer size pieces decreases. In addition, the $^3H/^{14}C$ ratio declines slightly across each of the monomer peaks. This implies that shorter monomer pieces contain less trioxsalen.

Gel b in Figure 5, which was run for a somewhat shorter time than the other gels, shows evidence of DNA fragments that electrophorese close to the dye front (fractions 58-69 in gel 5b). These are about 10 to 40 base pairs in size and presumably correspond to larger oligonucleotides cut out by the nuclease that cannot pass through dialysis tubing. Their $^3H/^{14}C$ ratios, which are higher than the control undigested DNA, are consistent with this idea.

The DNA fragments that are larger than monomer size in Figures 5b (1-38), 5c (1-48), and 5d (1-48) contain less trioxsalen per base pair than the undigested sample (Figure 5a); moreover the $^3H/^{14}C$ ratios drop continuously as a function of time of digestion. There are at least three possible explanations for this result. There may be some nonspecific absorption of monomer pieces to the gel as the monomer DNA migrates through the gel leaving a background of DNA with the same $^3H/^{14}C$ ratio as monomer. Secondly, there may be some "beads" that are less accessible to both trioxsalen and nuclease, perhaps due to nonhistone protein-DNA interactions at some sites that would otherwise be accessible to trioxsalen. The third possibility is that of some DNA not being in a "bead" structure at all. This would leave long regions of DNA that were easily accessible to both trioxsalen and nuclease.

Analysis of the Products of Micrococcal Nuclease Digestion of Trioxsalen-Treated Nuclei by Electron Microscopy. Electron microscopy was also used to analyze the specificity of the trioxsalen cross-linking reaction on the DNA of isolated nuclei. Nuclei into which a level of about 5 trioxsalen molecules per 1000 base pairs had been incorporated by photoreaction were partially digested with micrococcal nuclease. The nuclear DNA was purified and electrophoresed in a 4% polyacrylamide gel. Monomer, dimer, and trimer lengths of DNA were isolated from the gel as described in Materials and Methods. The purity of each fraction was determined by visualization in the electron microscope. Figure 6 shows the relevant length histograms. The means and standard deviations are 160 ± 25, 325 ± 35, and 495 ± 45 base pairs for the monomer, dimer, and trimer species, respectively. Each fraction is ≥90% pure.

The dimer and trimer fractions were denatured and then examined in the electron microscope. The types of molecules seen in the dimer denatured fraction are shown in Figure 7. Most of the molecules were linear. Dimer length linear molecules are interpreted as resulting from native dimer fragments with no cross-links. Tetramer length linear molecules and X and Y shaped molecules are interpreted as being dimer fragments with one cross-link. Similarly molecules with a single loop have two cross-links, while two loops indicate the presence

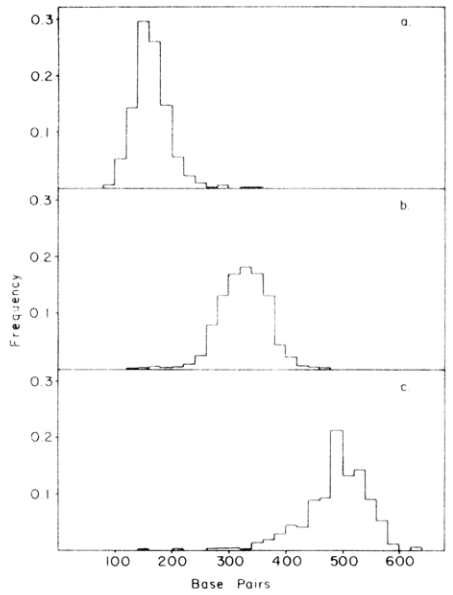

FIGURE 6: Histograms of digested DNA fragments isolated from 4% preparative polyacrylamide gels. (a) Monomer fragments; (b) dimer fragments; (c) trimer fragments. Molecular weights were determined relative to PM-2 DNA (6.5×10^6 [Kriegstein and Hogness, 1974]).

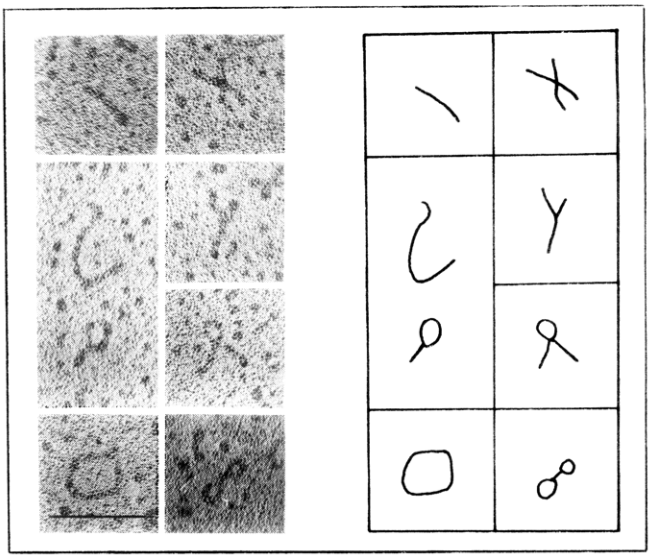

FIGURE 7: Examples of all of the types of figures seen in electron micrographs of isolated and denatured DNA dimer fragments. The bar represents the length of 500 bases of single-stranded DNA.

TABLE IV: Comparison of Observed and Calculated Frequencies of Cross-links in Dimer DNA Fragments.[a]

No. of Cross-links	Frequency	
	Obsd	Calcd
0	0.34	0.34
1	0.41	0.44
2	0.21	0.19
3	0.01	0.03

[a] Total number of molecules scored = 823.

of three cross-links. Each of the eight types of denatured dimer fragments found could thus be interpreted as having 0, 1, 2, or 3 cross-links and 98% of the molecules could be placed into one of these eight categories.

The relative percentages of dimer molecules with 0, 1, 2, or 3 cross-links could be determined simply by counting the number of denatured dimer fragments in each category (remembering that one native dimer fragments gives rise to *two* dimer length single strands if it contains no cross-links). These observed percentages are shown in Table IV.

If it is assumed that trioxsalen cross-links chromatin in the same regions at which micrococcal nuclease cleavage occurs, then there should be three accessible sites on each native dimer fragment, one in the middle and one at each end. Qualitatively, the kinds of molecules seen in the denatured dimer fraction are thus entirely consistent with those expected if the above assumption is correct.

Quantitatively, the problem can be approached as follows. If the probability of a cross-link occurring at any one site in the chromatin is P, then the probability of not having a cross-link at any one site is clearly $(1 - P)$. If cross-linking events at different sites are independent and if the micrococcal nuclease attack at any site is independent of the presence or absence of a cross-link at that site, then the probability of having a dimer fragment with zero cross-links is $(1 - P)^3$. The probability P can therefore be determined from the experimental denatured dimer data of Table IV. When this was done, P was found to equal 0.299.

This value of P can now be used to calculate the expected percentage of molecules with 1, 2, or 3 cross-links. The probability $f(n)$ of having exactly n ($n = 0, 1, 2$, or 3) cross-links in a dimer (where $K = 3$) is:

$$f(n) = \frac{K!}{n!(K-n)!}(P)^n(1-P)^{K-n} \qquad (1)$$

The calculated probabilities of having 1, 2, or 3 cross-links in a dimer fragment (using $P = 0.299$) are compared in Table IV with the experimental frequencies. The agreement is seen to be good.

A similar analysis of denatured trimer fragments can be carried out. These fragments can be grouped into 44 categories each of which is interpreted as having 0, 1, 2, 3, or 4 cross-links. Some examples of various categories are shown in Figure 8. The observed percentages of native trimer molecules with 0, 1, 2, 3, and 4 cross-links are shown in Table V.

Adopting the same approach as was used for the dimer fragments, the fraction of trimer molecules with no cross-link must equal $(1 - P)^4$; the value of P determined from the trimer data is 0.292, essentially identical with the value derived from the dimer data. Using this value of P, the probability of having 1, 2, 3, or 4 cross-links can be calculated from eq 1 (now $K = 4$). The results are shown in Table V. Once again, a good agreement between the calculated and experimental percentages was observed. This analysis is strong evidence for the proposal that the trioxsalen cross-linking reaction is confined to the nuclease sensitive sites between the DNA regions protected by chromosomal proteins.

The above, however, does not take into account the possibility of formation in these "covered" regions of monoadducts (i.e., trioxsalen molecules bound to one strand only) which might be prevented from undergoing the second photoreaction to give a cross-link by an alteration in the DNA conformation due to bound protein (Rill and Van Holde, 1973). To check this possibility, samples of DNA purified from treated nuclei (which show a characteristic loop pattern in the electron microscope after denaturation [Hanson et al., 1976]) were reirradiated in the native state and examined for evidence of an increase in cross-linking density compared with control samples. No such "zippering" effect was found.

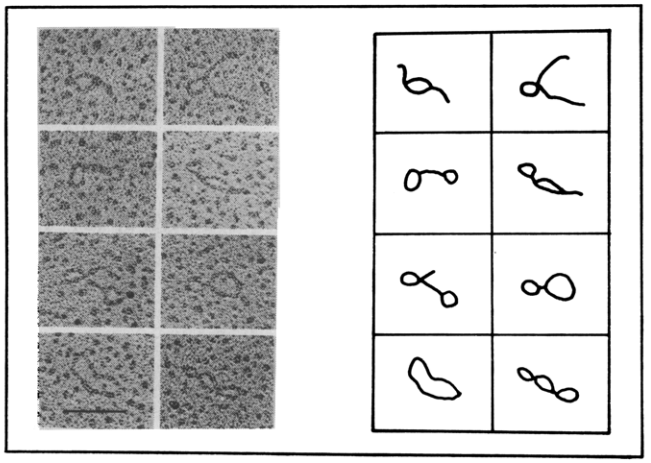

FIGURE 8: Examples of some of the types of figures seen in electron micrographs of isolated and denatured DNA trimer fragments. The bar represents the length of 500 bases of single-stranded DNA.

Discussion

The protection of chromosomal DNA from various chemical and enzymatic reactions by protein–DNA interactions is a well-studied phenomenon (see references in introductory section). The DNA in calf thymus chromatin (Clark and Felsenfeld, 1974) and in duck reticulocyte chromatin and nuclei (Sollner-Webb and Felsenfeld, 1975) has been found to be about 50% digestible by micrococcal nuclease. When the DNA of chicken erythrocyte chromatin is enzymatically methylated to saturation, again about 50% of the DNA undergoes modification (Bloch and Cedar, 1976). The sites of methylation in these experiments have been identified as being the same as the site of micrococcal nuclease action.

Noll (1974) has proposed on the basis of his studies of rat-liver nuclei partially digested by micrococcal nuclease that at least 87% of the nuclear DNA in that system is arranged in a nucleosome structure. That is, there exists an array of regularly spaced nucleoprotein complexes or monomer subunits, each containing about 200 base pairs of DNA, 140 base pairs of which form a nuclease resistant "core" particle (Van Holde et al., 1974), while the remaining 60 base pairs are accessible to nuclease attack. The length of this nuclease susceptible region appears to be different in chromatin from different sources (Noll, 1976; Morris, 1976). If all of the nuclear DNA were arranged according to this picture, then only 30% should be digestible. The observed limit digest value of 50% perhaps reflects the possibility that not all of the DNA is uniformly packed in monomer subunits of equal resistance to nuclease attack. The result obtained here, that only about 10% of the nuclear DNA is accessible to trioxsalen photoreaction, suggests that the sites of trioxsalen incorporation are at least as specific as those of nuclease attack.

In conclusion, the combined results of saturation experiments, the retention of the regular nuclease digestion pattern after cross-linking, the preferential excision of trioxsalen by nuclease, and the electron microscope analysis of denatured DNA fragments from isolated dimer and trimer fractions strongly suggest that trioxsalen can only react with DNA in nuclei at specific sites, despite the small size of the molecule. Furthermore, those sites correspond closely to regions that are accessible to micrococcal nuclease digestion. These experiments establish the validity of the photo-cross-linking reaction as a probe of chromatin substructure and provide a basis for more detailed studies involving cross-linking of isolated "nucleosomes", chromatin from different sources, reconstituted histone–DNA complexes, and also the ionic strength and pH dependence of histone–DNA interactions.

TABLE V: Comparison of Observed and Calculated Frequencies of Cross-links in Trimer DNA Fragments.[a]

No. of Cross-links	Frequency	
	Obsd	Calcd
0	0.25	0.25
1	0.34	0.41
2	0.27	0.26
3	0.12	0.07
4	0.02	0.01

[a] Total number of molecules scored = 954.

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Nucleoside Adducts from the in Vitro Reaction of Benzo[*a*]pyrene-7,8-dihydrodiol 9,10-Oxide or Benzo[*a*]pyrene 4,5-Oxide with Nucleic Acids†

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ABSTRACT: The covalent binding of benzo[*a*]pyrene 4,5-oxide and benzo[*a*]pyrene-7,8-dihydrodiol 9,10-oxide isomer I and isomer II to nucleic acids in aqueous acetone solutions has been investigated. Benzo[*a*]pyrene 4,5-oxide reacted preferentially with guanosine residues. On the other hand, benzo[*a*]pyrene-7,8-dihydrodiol 9,10-oxide isomer I and isomer II reacted extensively with guanosine, adenosine, and cytidine residues. Time course studies showed that the reactivity of isomer I or isomer II with homopolyribonucleotides followed the order poly(G) > poly(A) > poly(C). Alkaline or enzymatic hydrolysis of the modified nucleic acids and subsequent chromatography on Sephadex LH-20 columns yielded benzo[*a*]pyrene-nucleotide adducts. These were enzymatically converted to the corresponding nucleosides which were resolved into several distinct components by high-pressure liquid chromatography. Evidence was obtained for the presence of multiple nucleoside adducts of guanosine, adenosine, cytidine, deoxyguanosine, deoxyadenosine, and deoxycytidine. The HPLC profiles of adducts formed with isomer I were different from the corresponding profiles of adducts formed with isomer II. Structural aspects of these nucleoside adducts are discussed.

Many polycyclic aromatic hydrocarbons[1] (PAHs) are potent carcinogens and mutagens which are widely distributed pollutants in the human environment. Following metabolic activation the compounds bind covalently to cellular proteins (Abell and Heidelberger, 1962; Heidelberger and Moldenhauer, 1956; Miller, 1951) and nucleic acids (Brookes and Lawley, 1964; Goshman and Heidelberger, 1967) in the intact animal and in cell culture (Baird et al., 1973; Brookes and Heidelberger, 1969; Duncan et al., 1969). This covalent interaction is probably critical to the carcinogenic process (Miller, 1970; Gelboin et al., 1972). In order to react with these cellular targets the PAHs must be metabolized to reactive intermediates. The microsomal enzyme system, aryl hydrocarbon hydroxylase, oxidizes PAHs to a variety of derivatives (Jerina and Daly, 1974). Covalent binding of PAHs to nucleic acids in vitro has been achieved by incubation in the presence of microsomes and NADPH (Borgen et al., 1973; Gelboin, 1969; Grilli et al., 1975; Grover and Sims, 1968; Pietropaolo and Weinstein, 1975; Thompson et al., 1976).

Boyland (1950) proposed that oxides are the reactive intermediates which form covalent bonds with nucleophilic centers in cellular macromolecules. Incubation of parent PAHs with microsomes, oxygen, and NADPH produces arene oxide intermediates including the K-region oxides of 7,12-dimethylbenz[*a*]anthracene (DMBA) (Keysell et al., 1973) and benzo[*a*]pyrene (BP) (Grover et al., 1972; Wang et al., 1972; Selkirk et al., 1975). These K-region oxides react covalently with nucleic acids in vitro (Baird et al., 1973, 1975; Blobstein et al., 1975, 1976; Swaisland et al., 1974a), are mutagenic in bacterial (McCann et al., 1975) and mammalian (Huberman et al., 1971) cells, and transform various cells in culture (Sims and Grover, 1974; Marquardt et al., 1976). However, there is evidence that the K-region oxides are not the major reactive intermediates in vivo (Baird et al., 1973, 1975). Evidence has been presented that in vivo the major reactive derivatives of benzo[*a*]anthracene (Swaisland et al., 1974b) and benzo[*a*]pyrene (Borgen et al., 1973; Daudel et al., 1975; Ivanovic et al., 1976; Sims et al., 1974) are dihydrodiol oxides.

In the case of BP, two isomers of the dihydrodiol oxide have

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[1] Abbreviations used: BP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbon; DMBA, 7,12-dimethylbenz[*a*]anthracene; poly(G)-BP-7,8-dihydrodiol 9,10-oxide, poly(G)-BP 4,5-oxide, etc. are the nucleic acid products obtained from reaction with the indicated BP oxide and this does not indicate that the epoxide form remains intact in the product; λ_{max}, wavelength of maximum absorbance in the ultraviolet (UV) spectrum; HPLC, high-pressure liquid chromatography; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.