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# Ultraviolet Light Induced Preferential Cross-Linking of Histone H3 to Deoxyribonucleic Acid in Chromatin and Nuclei of Chicken Erythrocytes†

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ABSTRACT: Histones have been cross-linked to DNA in chicken erythrocyte nuclei and chromatin by using ultraviolet light irradiation at 254 nm. Following irradiation, cross-linked histone–DNA adducts were isolated and purified by hydroxylapatite chromatography, and the DNA component was subjected to acid hydrolysis. Of several hydrolysis techniques investigated, trichloroacetic hydrolysis of the DNA component of the adducts was found to be most effective. Histones isolated from hydrolyzed histone–DNA adducts were characterized by gel electrophoresis and fingerprint analysis. No histone–histone protein adducts were observed. All histone fractions have been shown to cross-link DNA in nuclei or chromatin by utilizing the technique employed, but with different propensities. The order of observed cross-linking, deduced from kinetic experiments, is H1 + H5, H3 > H4 > H2A ≫ H2B. The preferential binding of the core histone H3, as compared to the other core histones, is discussed in light of recent data concerning histone–DNA interactions and nucleosome structure. The use of the ultraviolet light technique as a conformational probe to study chromatin is also discussed.

The fundamental unit of chromatin structure, termed the nucleosome, consists of about 170 base pairs of DNA associated with a core of histones [see McGhee & Felsenfeld (1980) for a review]. The nucleosome core is composed of two molecules each of four histones (H2A, H2B, H3, and H4) around which is coiled 146 base pairs of DNA. One histone molecule (H1) is bound to DNA where it enters and exits from the nucleosome core. The nucleosome structure of chicken erythrocyte chromatin has been shown to be the same as for other chromatins, except that varying amounts of a unique histone (H5) may replace H1 in the nucleosome (Shaw et al.,

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1976). As a class of proteins, histones are unusually resistant to evolutionary change (Elgin & Weintraub, 1975). This conservation of amino acid sequence presumably results from the need for each histone to interact specifically with other histones, as well as DNA, in the assembly of the nucleosome. A variety of experimental approaches have been utilized to elucidate the nature of histone–histone and histone–DNA interactions in chromatin [see McGhee & Felsenfeld (1980) for a review]. Recently, several investigators have irradiated chromatin with ultraviolet light as a probe to study histone–DNA interactions in chromatin, nuclei, or isolated nucleosomes (Cao & Sung, 1978; Mandel et al., 1979; Sperling & Sperling, 1978; Martinson et al., 1976, 1979a,b; Martinson & McCarthy, 1976; Kunkel & Martinson, 1978; Martinson & True, 1979a,b). The validity of this approach is based on evidence that ultraviolet light irradiation induces stable covalently linked protein–DNA adducts (Strniste & Rall, 1976), that these adducts are formed specifically between interacting groups in the native nucleosome structure (Havron & Sperling, 1977; Sperling & Sperling, 1978), and that protein–DNA adduct formation is essentially independent of the amino acid content of the interacting protein (Varghese, 1976).

In spite of several investigations using the ultraviolet light technique, data demonstrating which histone fraction(s) may cross-link to DNA have not be unequivocal [compare Sperling & Sperling (1978), Kunkel & Martinson (1978), and Mandel et al. (1979)]. The differences observed in these investigations may be related to the experimental strategies employed. For example, the level of histone–DNA adduct formation has been shown to be a function of the wavelength of light used (Mandel et al., 1979). In addition, histone–DNA adducts are difficult to isolate, and the histones present in such complexes are difficult to characterize by direct analysis (Sperling & Sperling, 1978; Kunkel & Martinson, 1978). To clarify this situation, we have devised a simple procedure employing irradiation of chicken erythrocyte chromatin with short-wavelength (254-nm) light to photochemically and selectively cross-link histones to DNA. Following isolation and hydrolysis of the histone–DNA adducts, we have rigorously characterized the histones present in the adducts by electrophoresis and fingerprint analysis. Our results indicate that all histone fractions may be induced to cross-link to DNA in chicken erythrocyte chromatin and that the order of cross-linking, deduced from kinetic experiments, is H5 + H1, H3 > H4 > H2A ≫ H2B.

## Materials and Methods

*Isolation of Erythrocyte Nuclei.* Following cardiac puncture, adult white leghorn chicken blood was withdrawn into a syringe containing an equal volume of 0.15 M NaCl–0.015 M sodium citrate, pH 7.2 (saline/citrate).[1] Erythrocytes were pelleted by centrifugation of the blood at 3000*g* (Sorvall HB4 rotor) for 10 min, isolated by removal of the plasma and buffy coat by aspiration, and purified by two wash–centrifugation cycles using saline/citrate as the washing solution. Purified erythrocytes were resuspended in a saline/citrate solution at $3 \times 10^8$ cells/mL and stored frozen at −60 °C until use. For preparation of nuclei, the frozen eryhrocyte solution was quickly thawed, diluted with an equal volume of ice-cold saline/citrate containing 0.5% Nonidet P-40, and placed on ice for 30 min to lyse cells. Nuclei were pelleted from the lysate by centrifugation at 3000*g* for 10 min. Depending on the level of contamination with hemoglobin, nuclei were washed and pelleted at least twice more by using saline/citrate as the washing solution.

*Preparation of Chromatin.* Chromatin was prepared from isolated nuclei essentially according to Shaw et al. (1976). Briefly, nuclei were lysed in hypotonic buffer (50 mM Tris, pH 7.5), and the chromatin was progressively hydrated by decreasing the Tris concentration in the buffer from 50 to 10 to 5 mM and finally to 1 mM, all at pH 7.5. At each step, the sample was homogenized with a Dounce homogenizer, centrifuged at 4000*g* for 10 min, and then hydrated in the next Tris solution. The DNA concentration in the chromatin was determined by absorbance measurements at 260 nm in 1% sodium dodecyl sulfate solution.

*UV Irradiation.* Monolayers of either nuclei or chromatin suspension ($A_{260}$ = 15) were placed in a 10-cm siliconized glass petri dish and irradiated for various times up to 4 h at 254 nm with a UV light (Ultra Violet Co. Model C-61 mineralight without filter). The nuclei or chromatin suspension was stirred continuously with a Teflon-coated stir bar to ensure uniform irradiation. The irradiated sample was maintained at 0 °C by using a metallic cooling plate through which refrigerated water was circulated. Under the standard irradiation condition, the sample was placed 10 cm from the light source, and the incident power of the UV light as measured by a UV dosimeter was $3.5 \times 10^{-3}$ W/cm$^2$. In control experiments (see Results), samples were irradiated with a 450-W Hanovia UV lamp filtered by Vycor glass. Following irradiation, phenylmethanesulfonyl fluoride (PMSF) in 2-propanol was added to a final concentration of 1 mM, and the sample was stored frozen at −20 °C.

*Extraction of Histones Not Cross-Linked to DNA.* Small samples of nuclei or chromatin (2 mL, $A_{260}$ = 1) were irradiated in a 50-mm glass Petri dish as described above. After irradiation, histones were extracted with 0.4 N $H_2SO_4$ at 0 °C for 2 h. Insoluble material was removed by centrifugation. The histones were precipitated overnight with 5 volumes of ethanol at −22 °C, collected by centrifugation, vacuum dried, and electrophoresed.

*Purification of Cross-Linked DNA–Protein Complexes.* DNA–protein complexes were prepared essentially according to Rickwood & McGillvery (1975). Briefly, irradiated nuclei or chromatin was dissolved in a dissociating medium consisting of 2 M NaCl, 5 M urea, and 0.01 M sodium phosphate, pH 6.8. Insoluble chromatin was removed from solution by centrifugation at 12000*g* for 10 min and was resuspended in the dissociating medium and sonicated. The resulting two soluble chromatin fractions were pooled and applied to a hydroxylapatite [(HAP) Bio-Gel DNA grade] column (2 × 10 cm), previously equilibrated with dissociating medium. The dissociated and sonicated chromatin ($A_{260}$ = 15) was applied to the column, and the top of the column was stirred from time to time with a Pasteur pipet to prevent clogging and compacting. The column was thoroughly washed with dissociating medium to remove proteins noncovalently bound to DNA. Covalently bound protein–DNA complexes were eluted from the column by increasing the concentration of sodium phosphate in the dissociating medium to 0.5 M. Fractions containing DNA were combined, dialyzed extensively against water to remove salts, and freeze-dried. All glassware used in the above operations was siliconized by pretreatment with dichlorodimethylsilane (5%) in chloroform.

[1] Abbreviations: saline/citrate, 0.15 M NaCl–0.015 M sodium citrate, pH 7.2; TLC, thin-layer chromatography; DABA, 3,5-diaminobenzoic acid dihydrochloride; $Cl_3CCOOH$, trichloroacetic acid; $CF_3COOH$, trifluoroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; UV light, ultraviolet light; PMSF, phenylmethanesulfonyl fluoride; HAP, hydroxylapatite; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

*Protein and DNA Determination.* The quantitation of the covalently bound DNA–protein complexes is difficult, since the complexes possess the characteristics of both nucleic acids and proteins, and high concentrations of salt and urea are present. To circumvent these problems, we adapted the fluorometric method of DNA determination by Kissane & Robbins (1958). This method is based on the reaction of the fluorogen 3,5-diaminobenzoic acid dihydrochloride (DABA) with the aldehyde group generated at the C-1 position of the deoxyribose during acid hydrolysis. In our adaptation, extra hydrochloric acid was added to effect hydrolysis in the presence of 0.5 M $Na_2HPO_4$ in the dissociating buffer used to elute the protein–DNA complex from the hydroxylapatite column. The fluorometric assay is unaffected by UV irradiation damage to the DNA bases. A microfilter determination of protein by the procedure of McKnight (1977) was used to assay the protein content. In this procedure, samples were spotted on glass fiber filters (Whatman GF/C), fixed with $Cl_3CCOOH$, and stained with Coomassie Brilliant Blue. Following destaining, the dye-binding protein was quantitated by elution from the filters and measured at 590 nm. The McKnight assay was not influenced by the presence of DNA cross-linked to protein, since dye retention on the filters was determined to be dependent only on the protein component present.

*Hydrolysis of the DNA and Analysis of Protein in the Cross-Linked Complexes.* In some studies, the DNA–protein complex were subjected to mild acid hydrolysis in 66% formic acid containing diphenylamine according to the method of Levina & Mirzabekov (1975). After incubation at 30 °C for 17 h, the samples were diluted with 0.5 volume of water and extracted with 6 volumes of ether. The ether layer was removed, and the extraction was repeated 2 more times. The solution was then dialyzed against water and freeze-dried, and the sample was dissolved in buffer (1% $NaDodSO_4$, 0.06 M Tris, pH 6.8, 5 mM EDTA, 10% glycerol, 5% $\beta$-mercaptoethanol, and 0.01% bromophenol blue) prior to $NaDodSO_4$–polyacrylamide gel electrophoresis.

In other studies, the hydrolysis of the DNA–protein complexes with volatile acids such as HCl or $CF_3COOH$ was performed. Briefly, aliquots of the complexes containing 250 $\mu$g of DNA were hydrolyzed by the addition of HCl or $CF_3COOH$ to a concentration of 10% (w/v) and incubated at either constant time or temperature. Following incubation, the samples were lyophilized and dissolved in electrophoresis sample buffer.

Hydrolysis of the complexes with 10% perchloric or trichloroacetic acid was also performed. Aliquots of samples containing 250 $\mu$g of DNA were hydrolyzed with the acids at various times and temperatures. After hydrolysis, each of the samples was adjusted to a final 20% (w/v) acid concentration and left on ice for at least 24 h to precipitate the protein. Protein was isolated by centrifugation (16000*g*) for 10 min, and the protein pellet was gently rinsed with cold 80% ethanol (−20 °C) to extract the acid. Samples were dried in vacuo and dissolved in electrophoresis sample buffer.

*Gel Electrophoresis.* To visualize proteins released from the DNA–protein complexes following acid hydrolysis, we subjected samples to polyacrylamide gel analysis. Electrophoresis was carried out in $NaDodSO_4$ discontinuous 15% polyacrylamide slab gels by using the buffer system of Laemmli (1970). The gels were stained with 0.1% Coomassie Brilliant Blue in 50% methanol–10% acetic acid and destained in 10% acetic acid. Densitometer scanning of gels at 560 nm was achieved by using a Varian Techtron spectrophotometer equipped with a linear transport gel scanner.

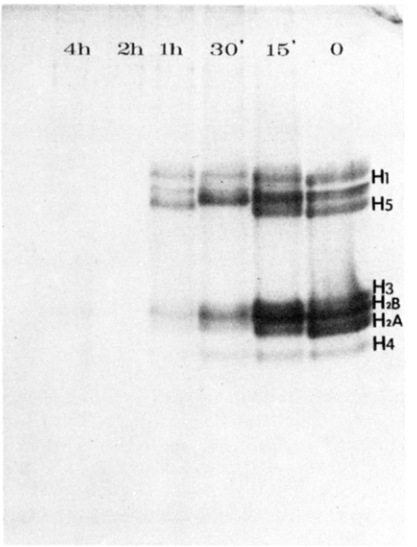


FIGURE 1: Electrophoretic profile of histones isolated from chicken erythrocyte chromatin irradiated at 254 nm for various time intervals. Identical samples of chromatin (1 OD $A_{260}$) in 2 mL were irradiated in a cooled glass dish placed 10 cm from a source of UV light (254 nm) of incident power $3.5 \times 10^{-3}$ W/cm$^2$. Histones were acid extracted from the chromatin and electrophoresed on $NaDodSO_4$–polyacrylamide gels.

*Labeling and Fingerprinting of Proteins.* The in situ radioactive labeling and enzymic digestion of protein in the $NaDodSO_4$ gel slices were performed by using a modification of the procedure of Elder et al. (1977). Individual stained zones were excised from the gel with a sharp razor blade. The gel slices were placed in siliconized tubes and washed extensively with 15% methanol and freeze-dried. To each of the dry gel slices 10 $\mu$Ci of [$^{14}$C]succinic anhydride dissolved in 20 $\mu$L of anhydrous dioxane (redistilled over Na) was added. The gels were allowed to absorb succinic anhydride solution for about 45 min, and then 100 $\mu$L of 0.2 M *N*-ethylmorpholine (pH 7.8) was added. Radioactive succinylation was allowed to proceed for 3 h at room temperature. The gel slices were then washed with at least four changes of 15% methanol and lyophilized. Labeled protein was digested in the gel slices by addition of 25 $\mu$g of thermolysin in 0.5 mL of 0.2 M *N*-ethylmorpholine buffer (pH 7.8). The hydrated gel slice was crushed with a spatula and incubated at 45 °C overnight. Following incubation, the buffer containing peptides released from the gel was separated from the gel by centrifugation. The gel was then subjected to two *N*-ethylmorpholine buffer wash–centrifugation cycles. The combined supernatants were lyophilized and the peptides were analyzed on cellulose-coated TLC plates (20 cm × 20 cm, Kodak no. 6064). Electrophoresis of the TLC plates was performed in a water-cooled thin-layer apparatus (O. Hiller Co., Madison, WI) in the presence of 6.7% formic acid (1 h at 500 V). After the plates were dried, they were developed by ascending chromatography using butanol–pyridine–acetic acid–water. The plates were again dried and autoradiographed for 10 days.

## Results

*Irradiation of Chromatin.* The $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of acid-extracted histones from chicken erythrocyte chromatin irradiated at 254 nm is displayed in Figure 1. All histones are present at short time periods of irradiation, but as the irradiation time is increased, stained zones in the electrophoretogram become negligible. It is known that UV irradiation at, or near, the DNA absorption maximum induces the formation of DNA–protein

Table I: Fractionation of Irradiated Chromatin on Hydroxylapatite[a]

| dissociation medium | phosphate concn (M) | yield of protein (%) | yield of DNA (%) | irradiation time (min) |
|---|---|---|---|---|
| 2 M NaCl | 0.01 | 65 | 0 | |
| 5 M urea | 0.05 | 6 | 0 | 0 |
| | 0.5 | 2 | 90 | |
| 2 M NaCl | 0.01 | 50 | 0 | |
| 5 M urea | 0.05 | 4 | 0 | 60 |
| | 0.5 | 7 | 70 | |
| 2 M NaCl | 0.01 | 27 | 0 | |
| 5 M urea | 0.05 | 1 | 0 | 240 |
| | 0.5 | 14 | 30 | |

[a] Recovery of input protein and DNA from hydroxylapatite chromatography of irradiated chromatin vs. time of irradiation. For conditions of irradiation, see section on UV irradiation under Materials and Methods. Irradiated chromatin was dissolved in 2 M NaCl, 5 M urea, and 0.01 M sodium phosphate (pH 6.8) and applied to a hydroxylapatite column equilibrated with the same buffer (dissociating buffer). The yields of protein and DNA, eluted by increasing phosphate concentrations in the dissociating buffer, are expressed as a percentage of the total material applied to the column.

adducts. Therefore, the apparent reduction in acid-extractable histones at increasing UV irradiation times suggests that histones are covalently attached to the DNA component in chromatin with greater efficiency at high UV irradiation doses. Similar results have been obtained by Martinson & McCarthy (1976); however, in contrast to their results, under the present irradiation conditions there is no observable H2A–H2B adduct formation.

*Isolation and Kinetics of the UV-Induced DNA–Protein Complexes.* Chromatin may be dissociated into its components of DNA and protein by using high salt and urea concentrations. Hydroxylapatite chromatography in the presence of dissociating buffer has been used successfully to separate DNA and its binding proteins (Rickwood & McGillvery, 1975). We have observed that histones, photochemically cross-linked to DNA, are retained on a hydroxylapatite column along with the DNA fraction, while free proteins may be washed off with dissociating buffer. The histone–DNA complexes may then be eluted from the column with high concentrations of phosphate buffer (0.5 M). Table I summarizes the quantitative data on the recovery of protein and DNA in the eluted fractions. The yield of protein and DNA recovered from chromatography of native nonirradiated chromatin is in good agreement with the published results of Rickwood & McGillvery (1975). As doses of irradiation are increased, the recovery of total protein and DNA decreases, such that following 4 h of irradiation only 42% of the protein and 30% of the DNA are eluted from the column. The diminished recovery of DNA and protein as a function of irradiation results from the inability of highly cross-linked protein and DNA adducts to elute into the column (see below). In spite of the decrease in recovery of total DNA and protein, an increase is observed in the percentage of protein recovered in the 0.5 M phosphate eluant: from 2% at zero irradiation dose to 7% at 1 h and to 14% at 4 h after irradiation. Similar results were obtained for chromatin prepared from irradiated nuclei (data not shown, but see Figure 2). When the protein recovered with the DNA fraction is plotted on the basis of protein:DNA weight ratio vs. time of irradiation (Figure 2), the kinetics of UV cross-linking may be studied. The rate of protein cross-linking deviates slightly from linearity, with an apparent lag at early time intervals. The observed lag is more pronounced when chromatin is irradiated as compared to nuclei. After

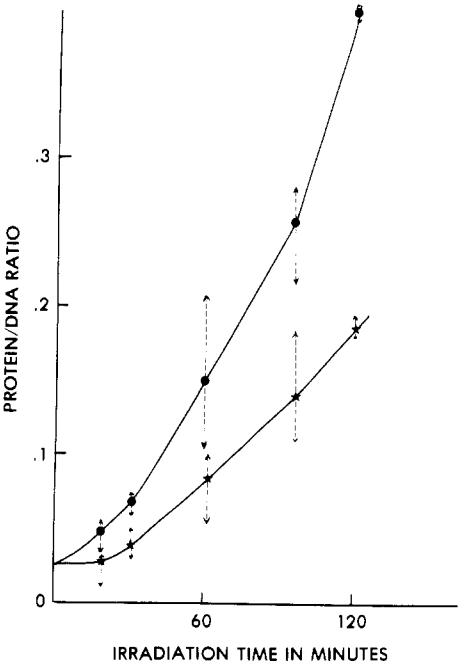


FIGURE 2: Protein:DNA ratio in complexes cross-linked by UV light vs. time of irradiation in a cooled glass dish placed 10 cm from a source of UV light (254 nm) of incident power $3.5 \times 10^{-3}$ W/cm$^2$. Protein:DNA cross-linked complexes were purified by hydroxylapatite chromatography. DNA and protein levels in the cross-linked complexes isolated from nuclei (●) or chromatin (★) were determined by modifications of the fluorometric method of Kissane & Robbins (1958) and the glass fiber filter method of McKnight (1977), respectively.

2 h of irradiation, the protein:DNA ratio increases to about 7 times the control value (0.18 vs. 0.025) for chromatin and to about 16 times the control value (0.40 vs. 0.025) for nuclei. The same trend is observed for both nuclei and chromatin for irradiation time periods up to 4 h (data not shown). At all doses of irradiation, the observed extent of protein–DNA cross-linking for nuclei is about twice that of chromatin. Except for the extent of observed protein to DNA cross-linking, complexes produced by direct irradiation of chromatin, or by irradiation of nuclei followed by preparation of chromatin, are indistinguishable from each other. The above results clearly demonstrate the reduction in dissociable protein isolated from chromatin at increasing UV light doses and suggest that the cross-linked protein–DNA complexes are present in the 0.5 M phosphate eluant. That the amount of DNA recovered by this technique decreases at high irradiation doses is disturbing. We have observed a dose-dependent formation of a yellow product left at the top of the HAP column after elution with buffer. It is known that UV light can induce the formation of photoproducts, which in turn can serve as reagents for further chain reactions leading to highly cross-linked polymers which are difficult to characterize. We have not attempted to characterize this material. In subsequent experiments, prolonged irradiation was avoided.

For visualization of the proteins present in the covalently bound complexes, the DNA moiety must be hydrolyzed without affecting the integrity of the protein component. Enzymatic hydrolysis of DNA with micrococcal DNase or pancreatic DNase I was tested, with no apparent success. Therefore, chemical hydrolysis of protein–DNA complexes isolated from irradiated chromatin or nuclei was performed. Figure 3 shows the $NaDodSO_4$–polyacrylamide gel electrophoresis profile of proteins released from the cross-linked complexes isolated from irradiated nuclei after hydrolysis of

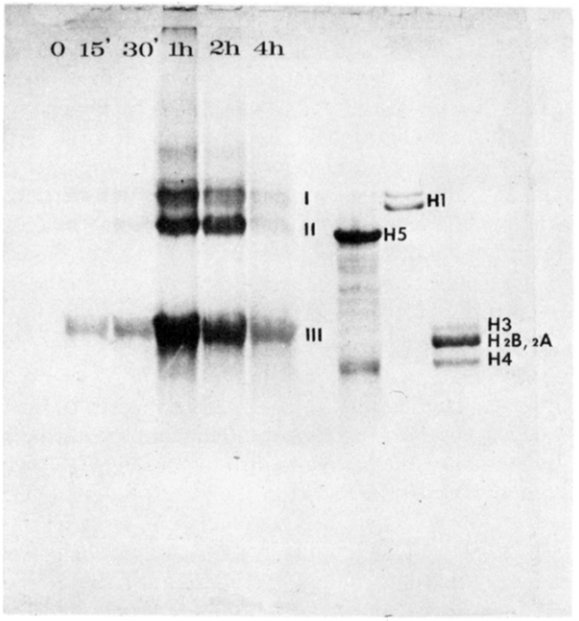


FIGURE 3: Electrophoretic profile of histones cross-linked to DNA in chicken erythrocyte nuclei by irradiation at 254 nm for various time intervals. Aliquots of nuclei were irradiated in a cooled glass dish placed 10 cm from a source of UV light (254 nm) of incident power $3.5 \times 10^{-3}$ W/cm$^2$. Cross-linked histone–DNA complexes were isolated by hydroxylapatite chromatography, DNA was digested with diphenylamine in formic acid, and the residual protein was electrophoresed on NaDodSO$_4$–polyacrylamide gels.

DNA with diphenylamine reagent in formic acid. In the control experiment (no irradiation), no stained zones are detected; however, three major (labeled I, II, and III) and a few minor components appear progressively in the electrophoretograms as irradiation time is increased. The same result was observed for chromatin irradiation (data not shown). By inspection, the total protein recovered for a given irradiation time period appears not to be linearly dependent on the UV dose. For samples irradiated from 1 to 4 h, instead of an increase in protein content present in the complex, as would be predicted from the data in Table I, there is a sharp decline in protein bands present in the gel (Figure 3). This result suggests that highly cross-linked DNA in the complexes resists hydrolysis by formic acid. The hydrolysis of DNA with various other dilute acids was therefore investigated in search for a more complete release of protein from the complex. Volatile and nonvolatile acids were selected to facilitate protein recovery following DNA digestion. Volatile acids such as hydrochloric and trifluoroacetic may be extracted from protein by lyophilization, whereas protein is precipitated following hydrolysis of DNA by trichloroacetic and perchloric acids. The recovered proteins in all hydrolysis experiments were evaluated by NaDodSO$_4$–polyacrylamide gel electrophoresis, and the results of representative experiments are shown in Figure 4. The results of digestion by 0.05 N HCl with temperature ranges from 25 to 45 °C (Figure 4a) and the digestion by 10% $Cl_3CCOOH$ at 35 °C (0–48 h) are displayed in Figure 4b. These results clearly indicate that $Cl_3CCOOH$ is far superior to HCl with respect to the amount and number of protein fractions released from the complex. As may be deduced from Figure 4, the loss of protein under the HCl hydrolysis condition becomes more pronounced at higher temperatures. The use of perchloric acid also leads to loss of proteins, and at 37 °C, in contrast to $Cl_3CCOOH$, no protein bands are detected in the gel (data not shown). We therefore turned our attention to $Cl_3CCOOH$ digestion. Some histone protein is released (see Figure 4b) from the complexes during the 24-h incubation

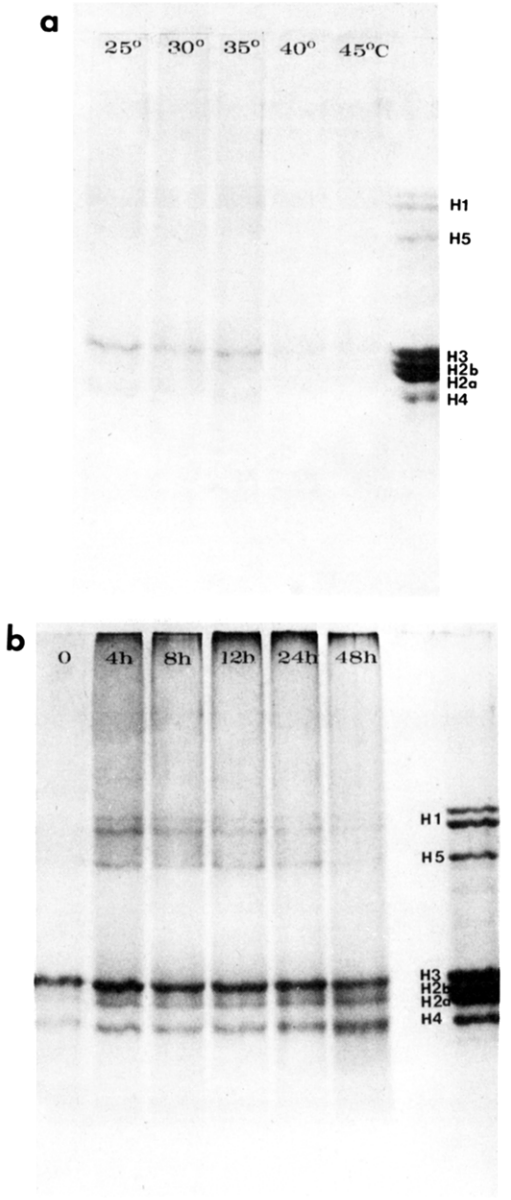


FIGURE 4: Comparison of the effectiveness of HCl and trichloroacetic acid for the hydrolysis of the DNA component of DNA–protein complexes. Identical samples of cross-linked DNA–protein complexes containing 250 μg of DNA were obtained by hydroxylapatite chromatography of nuclei irradiated in a cooled glass dish placed 10 cm from a source of UV light (254 nm) of incident power $3.5 \times 10^{-3}$ W/cm$^2$. (a) The DNA-containing samples were hydrolyzed in 0.05 N HCl at 25, 30, 35, 40, and 45 °C for 16 h, lyophilized, and electrophoresed, relative to erythrocyte histone standards, on NaDodSO$_4$–polyacrylamide gels. (b) The DNA-containing samples were incubated at 0, 4, 8, 12, 24, and 48 h in 10% w/v trichloroacetic acid at 35 °C, and the protein was precipitated on ice for 24 h in 20% w/v trichloroacetic acid solution. Protein samples were electrophoresed on NaDodSO$_4$–polyacrylamide gels.

period at 0 °C in $Cl_3CCOOH$ (see zero time) performed to ensure precipitation of the histone protein adducts following incubation in $Cl_3CCOOH$ at 35 °C. This release of protein from the adducts may be a reflection of DNA hydrolysis occurring during the 0 °C incubation period in $Cl_3CCOOH$. Alternatively, it may be due to a low level of reversal of histone–DNA cross-linking with time as observed by Kunkel & Martinson (1978). Temperature studies with 10% $Cl_3CCOOH$ suggest that 35 °C is optimum (data not shown), with protein release becoming complete after digestion for 4 h. The protein released does not show any sign of degradation after

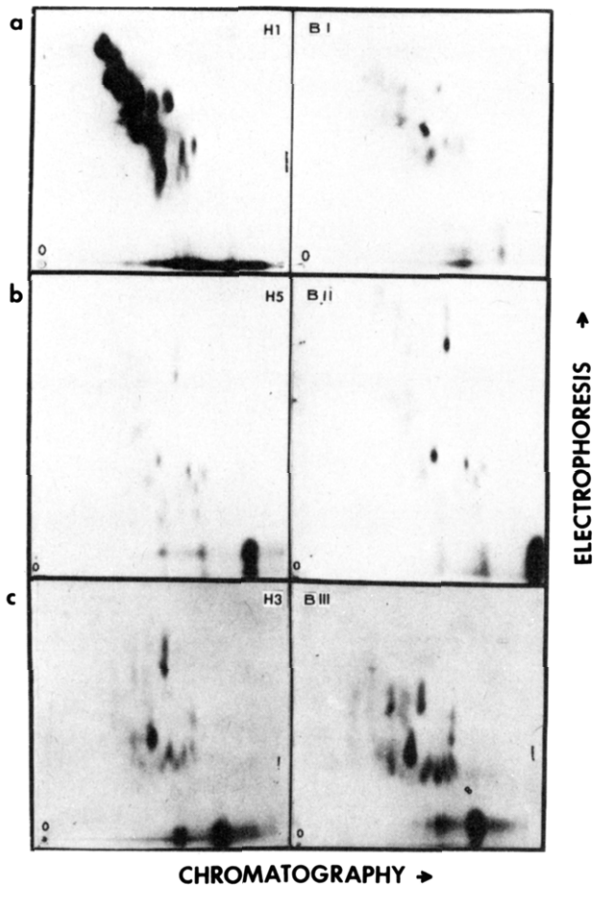


FIGURE 5: Autoradiographic fingerprint comparisons of histones H1, H5, and H3 with proteins corresponding to bands I, II, and III of the electrophoretic profile of cross-linked histone–DNA complexes presented in Figure 3. Protein bands to be analyzed were excised from $NaDodSO_4$–polyacrylamide gels, radioactively succinylated, digested in situ with thermolysin, and eluted from the gel. Following lyophilization, the peptides were analyzed by two-dimensional chromatography on cellulose-covered TLC plates, by electrophoresis in the presence of 6.7% formic acid, and by subsequent development by ascending chromatography using butanol–pyridine–acetic acid–water buffer. (a) Comparison of band I of Figure 3 and H1; (b) comparison of band II of Figure 3 and H4; (c) comparison of band III of Figure 3 and H3.

24 h of incubation at 0 °C (Figure 4b). We defined the standard $Cl_3CCOOH$ digestion conditions as 10% $Cl_3CCOOH$ at 35 °C for 12 h.

*Characterization of the Proteins Cross-Linked to DNA.* As discussed above, formic acid digestion of cross-linked complexes gives rise to three electrophoretic zones, I, II, and III (Figure 3). These zones have mobilities identical with histones H1, H5, and H3, respectively. To confirm the identity of these histones, we performed fingerprinting analysis of the bands I, II, and III and compared them to the peptide maps of the histones H1, H5, and H3. The comparative peptide maps are presented as Figure 5.

The peptide maps for the irradiated samples compare favorably with the maps of control histones and therefore help to identify histones H1, H5, and H3 as being photochemically cross-linked to DNA by UV irradiation of the avian erythrocyte nuclei and chromatin. Nevertheless, some minor differences in the peptide maps of irradiated samples vs. controls are noted. This may be due to DNA fragments which resist acid hydrolysis and remain covalently attached to the peptide fragments. Alternatively, it may be due to minor contamination of other nuclear proteins. These and other alternatives need further investigations to clarify the nature and origin of the differences in the peptide maps.

When the $Cl_3CCOOH$ digestion method was used (Figure 4b), two additional components were released from the complex, and their identities were also verified by fingerprinting as being histones H4 and H2A (data not shown). Histone H2B was not observed to be cross-linked in amounts great enough to permit fingerprinting analysis; nevertheless, its presence in the complex can be inferred from the electrophoretogram (examine Figure 4b and left panel of Figure 6). Taken together, the above data demonstrate that all the major avian erythrocyte histones are cross-linked, to DNA by UV irradiation of nuclei or chromatin.

*Histone H3 Is the Major Adduct in the DNA–Protein Complexes.* The very low level of H2B, compared to the high level of H3 observed in DNA–protein complexes generated during the same time interval of irradiation, suggests that the histones may have different kinetics of cross-linking to DNA. To confirm this, we studied the course of cross-linking of histones to equivalent weights of DNA in nuclei exposed to short time periods of irradiation (Figure 6). After 30 min of irradiation, histone H3 is the major adduct, followed by H5 and H1, while H4 and H2A are barely visible. As the doses of irradiation are increased, the H3 adduct continues to be the predominant species, but H5 and H1 adducts also increase. In avian erythrocytes, H5 partially replaces H1 (Sotirov & Johns, 1972; Sung, 1977), and because both are located in the linker region on the nucleosome (Kornberg, 1977; Felsenfeld, 1978), we have combined their values. Histones H4 and H2A are clearly visible after 1 h of irradiation, and after 2 h, they have become prominent cross-linked species. On the other hand, H2B is the least reactive species, since even at 2 h of irradiation it is present in low yield. By inspection, the order of cross-linking of the avian erythrocyte histones is H3 > H5, H1 > H4 > H2A ≫ H2B. Because the nonrandom frequency of H3–DNA adduct formation is significant, we attempted to quantitate this interaction as a function of irradiation by scanning the stained gels (right panel of Figure 6). When the degree of adduct formation in arbitrary units is plotted vs. the time of irradiation (Figure 7), the rate of H3–DNA adduct formation, at all doses of irradiation, is observed to be about 3 times higher than the formation of adducts of other histones with DNA. Thus, when the comparison of adduct formation is made, the rate of histone cross-linking falls into three distinctive classes: (1) H1 + H5, H3; (2) H4, H2A; and (3) H2B. Histones H3 and H1 + H5 both show rapid and apparently linear kinetics of cross-linking, whereas class 2 cross-linking shows an initial lag followed by an exponential increase, and the rate of cross-linking of class 3 is too low to be estimated.

*Accessibility of Core–Histones to UV Irradiation.* Additional control experiments were performed to determine whether the irradiation conditions may have contributed to the observed differences in histone cross-linking to DNA in nuclei. Specifically, the effect of light intensity was studied by varying the distance between the UV lamp and the surface of the nuclei monolayer, while keeping the dose of irradiation constant by adjusting the time of exposure. At several light intensities from $2 \times 10^{-3}$ to $44 \times 10^{-3}$ W/cm$^2$ at constant irradiation dose, the same pattern of protein bands with histone H3 as the predominant species was observed. This result was also obtained when irradiation was performed with a 450-W Hanovia UV lamp filtered by Vycor glass, which delivers 10-fold more intense light than the standard mineralight (results not shown). This propensity of H3 to cross-link DNA remained unchanged when the kinetic measurements were

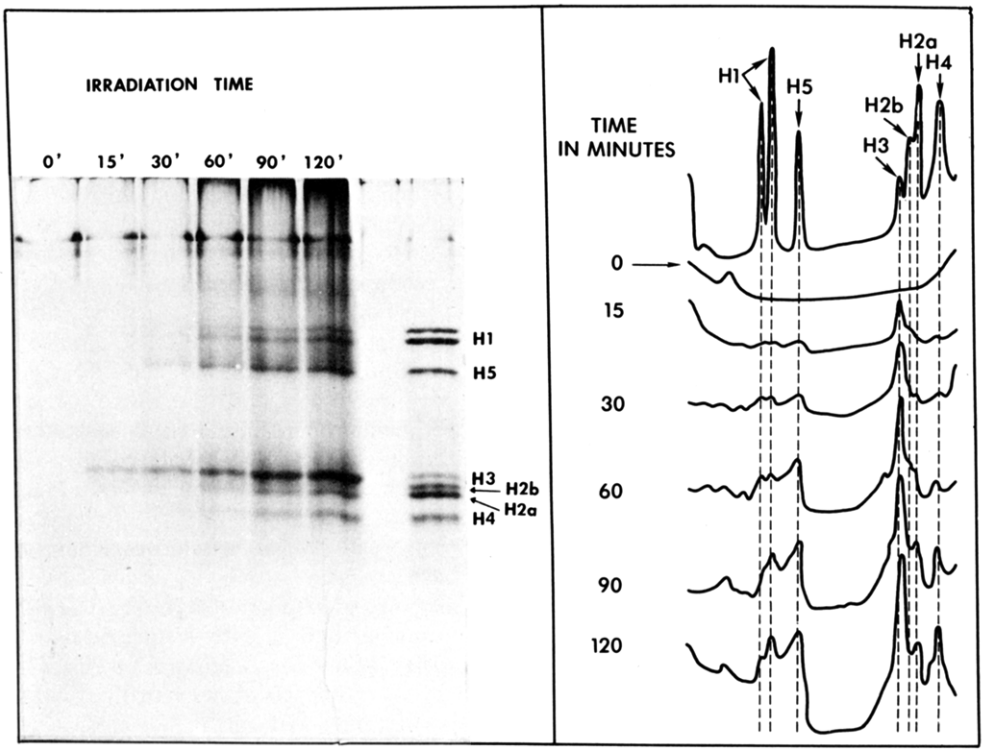


FIGURE 6: Electrophoretic profile and densitometer tracing of $NaDodSO_4$–polyacrylamide gels of histones released by acid hydrolysis from DNA–protein complexes prepared from irradiated nuclei. Chicken erythrocyte nuclei were irradiated at 254 nm in a cooled glass dish 10 cm from a source of UV light (254 nm) of incident power $3.5 \times 10^{-3}$ W/cm$^2$, for various time intervals. Following isolation by hydroxylapatite chromatography, equivalent amounts of complexes (500 $\mu$g of DNA) were acid hydrolyzed by using trichloroacetic acid at 35 °C for 12 h. For each irradiation time interval, 0, 15, 30, 60, 90, and 120 min, histones were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Gels were stained with Coomassie Brilliant Blue, destained in 10% acetic acid, and photographed (left) or scanned (right) at 560 nm by using a Varian Techtron spectrophotometer equipped with a linear transport gel scanner.

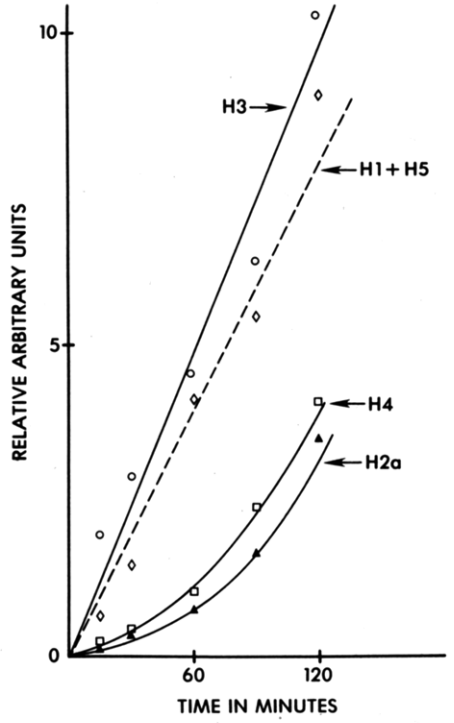


FIGURE 7: Kinetics of UV-induced histone–DNA cross-linking in nuclei irradiated at 254 nm. Relative arbitrary units of cross-linking to DNA for each histone were determined from the densitometer scans of Figure 6. Units were calculated by cutting out and weighing the individual peaks in the densitometer scan and were verified by measuring the width of the stained zone in an enlarged photograph of the densitometer scan and multiplying half the width times the height of the densitometer peak (triangulation method). The histones analyzed were H3 (○), H1 and H5 (◇), H4 (□), and H2A (▲). H2B cross-linking was insignificant.

made on UV irradiation of native chromatin. Further, chromatin which was "stripped" of H1 and H5 by 0.75 M NaCl treatment exhibited the same pattern of histone adduct formation: H3 > H4 > H2a ≫ H2b.

The dramatic differences in histone propensity of cross-linking to DNA may be related to the accessibility of histone–DNA interacting regions in the nucleosome, just as the susceptibility of nucleosomal DNA toward DNase I digestion is a measure of DNA accessibility. Alternatively, it may be an intrinsic property of the individual histones in that the nature and proximity of the amino acids at the histone–DNA combining sites are responsible for the various ranges of heteroadduct formation. These two alternatives were tested by performing irradiation of chromatin in the presence of 6 M urea. Under this denaturing condition, nucleosomes are unfolded, and chromatin is in an extended conformation; however, the histones are still complexed with DNA through ionic bonding. The result, as presented in Figure 8, shows that all the core histones are cross-linked to the same extent. No histones are cross-linked in a control experiment when chromatin is irradiated in the presence of urea and high salt. The results of the above experiments appear to support the hypothesis that the kinetics of histone–DNA adduct formation reflect histone accessibility to DNA.

## Discussion

The interpretation of data from irradiation experiments is based on previously published evidence that protein–DNA adducts are formed only between interacting amino acid and nucleic acid residues in nucleoprotein complexes, as well as by evidence that protein–DNA adduct formation is essentially independent of the amino acid residues and nucleotides at the interacting sites. It has been reported that a large variety of

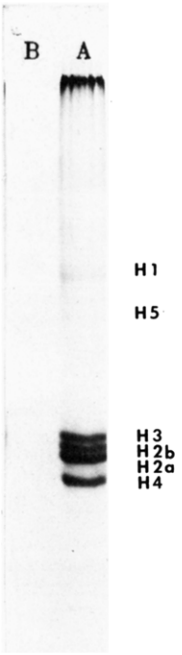


FIGURE 8: Effects of urea and urea plus salt on UV-irradiated chromatin. Chromatin dissociated in (A) 6 M urea or (B) 6 M urea and 2 M NaCl was irradiated at 254 nm in a cooled glass dish placed 10 cm from a source of UV light (254 nm) of incident power $3.5 \times 10^{-3}$ W/cm$^2$, for 90 min. Cross-linked histone–DNA complexes were isolated by hydroxylapatite chromatography and subjected to trichloroacetic acid hydrolysis at 35 °C for 12 h, and the histone protein was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The intensities of H3, H2A, H2B, and H4 observed in the gel profile are nearly identical.

amino acid residues form covalent linkages with the pyrimidine moieties of DNA upon irradiation (Varghese, 1976). In addition, photochemical cross-linking of protein to purine residues has been demonstrated in the photoaffinity labeling of membrane protein with cGMP (Antonoff et al., 1976) and in the binding of ATP with histone H4 (Sperling & Havron, 1976). Bovine serum albumin (Braun & Merrick, 1975), DNA polymerase (Markowitz, 1972), *lac* repressor, aminoacyl-tRNA synthetase (Schoemacher & Schimmel, 1974), and ribosomal proteins of *Escherichia coli* (Ehresman et al., 1975) have also been covalently attached to nucleic acids by using ultraviolet irradiation at 254 nm. Evidence that specifically interacting amino acid and nucleic acid sites are cross-linked during such irradiation has been provided by experiments which demonstrated that ultraviolet light induced cross-linking of an RNase pyrimidine nucleotide inhibitor involved only amino acid residues at the enzyme binding site (Sperling & Havron, 1976; Havron & Sperling, 1977). Additionally, photochemical cross-linking of ATP to isoleucyl-tRNA synthetase was shown to involve the cross-linking of only a single peptide (Yue & Schimmel, 1977). Taken together, these experimental results provide overwhelming support for the arguments that the photochemically induced cross-linking which we have observed in chromatin is the result of specific amino acid and nucleic residue interactions and that one histone fraction is not preferentially cross-linked to DNA on the basis of its amino acid composition. As such, the photochemically induced formation of histone–DNA adducts by irradiation of chromatin at 254 nm is a reflection of the structural organization of histones vis-ã-vis DNA in the nucleosome.

It has been demonstrated that ultraviolet irradiation of chromatin produces both histone–DNA and histone–histone adducts and that the degree of formation of each type of adduct may be influenced by the wavelength of light used to irradiate the chromatin. For example, Mandel et al. (1979) observed that irradiation of chromatin at 254 nm favors the formation of histone–DNA adducts, whereas the proportion of histone–histone adduct formation may be increased by irradiation at 280 nm. The most commonly employed experimental approach to study histone–histone and histone–DNA interactions, using ultraviolet irradiation techniques, has been to extract and examine the free histone fractions from cross-linked chromatin. This subtractive approach provides direct evidence for histone–histone interactions, especially when chromatin is irradiated at ≥280 nm, but such data can only be utilized to suggest indirectly what histone fractions are cross-linked to DNA. For this reason, nonsubtractive experimental approaches have been performed to directly investigate histone–DNA-induced cross-linking. In nonsubtractive experiments, histone–DNA adducts are examined for histone content following selective hydrolysis of the DNA component. Using this technique, Kunkel & Martinson (1978) have irradiated nuclei with ultraviolet light between 230 and 290 nm and have reported that histone H2A–H2B dimers are formed as both histone–histone adducts and histone–DNA adducts. Similarly, Sperling & Sperling (1978) have irradiated isolated mononucleosomes with ultraviolet light at wavelengths greater than 290 nm, in the presence of a UV sensitizer (acetone), and have reported that preferential H2A and H2B histone–DNA adducts are formed. The results of these experiments have not always corresponded to those obtained by subtractive procedures [compare, for example, Sperling & Sperling (1978) and Mandel et al. (1979)]. Our results demonstrate that the confusion regarding ultraviolet cross-linking experiments can be cleared up by utilizing irradiation conditions which exclusively produce either histone–histone or histone–DNA adducts. When only histone–DNA adducts are formed, and the resulting adducts are characterized, it is clearly demonstrated that all of the histones present in chromatin interact intimately with DNA and thus may be cross-linked.

The rate of histone cross-linking to DNA falls into three distinctive classes: (1) H1 + H5, H3; (2) H4, H2A; and (3) H2B, which can be discussed in terms of the locations of the histones in the nucleosome. The observed preferential cross-linking of histone H1 and H5 to DNA is consistent with data from recent studies which place these histones in intimate contact with DNA at sites where the DNA enters and exits from the nucleosome core (Thoma et al., 1979; Finch et al., 1977). On the other hand, H3 is a core histone, yet H3–DNA cross-links are formed by ultraviolet light at levels 10 times that observed for other core histones (see Figure 6 and Figure 7; Cao & Sung, 1978). This result suggests that, in native chromatin and nuclei, the H3 to DNA interacting domain (or at least a component of it) is as exposed to DNA as are the histone H1 and H5 combining sites. Histone H3 has been placed near the 5′ and 3′ ends of the 146 base pair nucleosome core (Simpsom, 1976; Mirzabekov et al., 1978; Schick et al., 1980). Recently, using three different cross-linking reagents, Ring & Cole (1979) demonstrated that histone H3 was the most favored of the core histones to cross-link with H1, although cross-links to each of the other core histones were found. Based on the putative juxtaposition to H1, as well as on other data, it is suggested that histone H3 binds to DNA at the sites near the origin of the DNA path around the core histones. Klug et al. (1980) have proposed a model for the histone core of the nucleosome based on 22-Å resolution of the histone octamer. Their model shows the octamer to have

a 2-fold axis of symmetry and the overall shape of a left-handed helical spool on which is wound two turns of a flat superhelix of DNA in the nucleosome. Aided by various cross-linking experiments, Klug et al. (1980) have proposed that the $(H3)_2$–$(H4)_2$ tetramer forms a dislocated disk which defines the central turn of DNA. Interestingly, this model positions H3 near the entry and exit points of the DNA from the nucleosome. It is tempting to speculate further along the line that the H3–DNA combining region interacts with DNA at or near H1 binding sites in the nucleosome. During trypsin digestion of chromatin, Weintraub & VanLente (1974) have shown that the H3 N-terminal arm is the first core particle histone to be cleaved. If the N-terminal arm of histone H3 extends near the entry and exit points of the nucleosomal DNA in chromatin, it could explain both the rapid kinetics of cross-linking to DNA and the adduct formation with H1 observed by Ring & Cole (1979).

In the present work, we have demonstrated that UV-induced histone–DNA cross-linking is a sensitive conformational probe for the study of chromatin. The adducts produced in irradiated nuclei do not differ from chromatin, but the observed extent of histone to DNA cross-linking in nuclei is about twice that observed for chromatin. This result suggests that, although the relative degree of hydration of chromatin influences cross-linking quantitatively, the organization of chromatin is not significantly influenced by the hydration process. This conclusion is supported by, and is consistent with, current views of chromatin organization. Furthermore, qualitatively different organizations of chromatin are detected by the UV probe, as indicated by the lack of histone to DNA cross-linking in chromatin irradiated in the presence of urea and high salt, and in the nonpreferential nature of histone to DNA cross-linking in the presence of urea only. Thus, any alteration in nucleosome conformation as a result of in vitro or in vivo perturbations can be "photographed" by the UV probe and analyzed. For example, the UV probe could be used to examine the altered nucleosome conformation observed in transcriptionally active chromatin (Oudet et al., 1977; Bina-Stein & Simpson, 1977). The amino acid sequences of the histones have largely been determined. Thus, characterization of DNA cross-linked histone peptides can no doubt provide a more comprehensive picture for the nature and sites of histone binding to DNA. These studies are of central importance in determining the structure and function of eukaryotic chromatin.

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