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Effect of Formaldehyde on the Circular Dichroism of Chicken Erythrocyte Chromatin†

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ABSTRACT: Formaldehyde (HCHO) fixation of chicken erythrocyte chromatin produces a marked decrease in its positive circular dichroism (CD), above 260 nm, and the appearance of a small negative ellipticity around 295 nm. The ultraviolet spectrum of chromatin is unaffected, nor does HCHO produce any changes in the uv or CD spectra of chicken erythrocyte DNA. The extent of the circular dichroism transition from the native chromatin to the suppressed spectrum is dependent on the concentration of HCHO and salt concentration. The kinetics of the reactions are complex, implicating at least two reactive species. Studies of the reaction of HCHO with chromatin in ethylene glycol and CD measurements of aqueous chromatin solutions with added glutaraldehyde preclude simple dehydration and general cross-linking effects as causes of the CD changes observed. The results are interpreted as indicating a conformational change of the DNA in chromatin caused by histone–DNA or histone–histone cross-linking.

The use of formaldehyde in the physical characterization of chromatin has increased in recent years (Miller and Beatty, 1969a,b; Miller et al., 1970; Ilyin and Georgiev, 1969; Hancock, 1970; Olins and Olins, 1974; Senior et al., 1975; Olins et al., 1975); however, there have been few investigations into the mechanism of the reaction between HCHO[1] and nucleoprotein (Romakov and Bozhko, 1967; Brutlag et al., 1969; Li, 1972; Siomin et al., 1973). Recently, chromatin particles (ν bodies) were isolated from H_2O-swollen, HCHO-fixed, sonicated chicken erythrocyte nuclei (Olins and Olins, 1974; Senior et al., 1975). To evaluate the effect of HCHO fixation on the structure of ν bodies, studies were initiated in this laboratory on the reaction of HCHO with chromatin, using the techniques of low-angle X-ray diffraction (Carlson and Olins, 1974; Olins et al., 1975) and circular dichroism (Senior and Olins, 1974). The results of the CD study are reported in this paper.

Materials and Methods

Preparation of Chromatin. Chicken erythrocyte nuclei were isolated as described previously (Olins and Olins, 1972). Soluble chromatin was prepared according to the method of Zubay and Doty (1959) as modified by Olins et al. (1975). Soluble chromatin in 0.7 m*M* phosphate buffer (pH 6.8) was exhaustively dialyzed vs. appropriate buffers. Solutions of A_{260} = 2 were clarified by centrifugation at 15,000 rpm for 30 min in a Sorvall RC2B. The resulting supernatants exhibited no light scattering detectable by ultraviolet absorbance spectroscopy.

Preparation of DNA. Chicken erythrocyte DNA was isolated according to procedures modified from Marmur (1961), Paul and Gilmore (1968), and Church and McCarthy (1968). The DNA was dialyzed into 0.1 × SSC for uv and CD studies. Solutions of DNA in 95% ethylene glycol were prepared by a quantitative dilution of stock DNA in 0.1 × SSC, with reagent grade ethylene glycol containing the indicated concentrations of NaCl. Calf thymus DNA from Worthington was dissolved in 10 m*M* NaCl and used without further purification.

Determination of Concentrations. Concentrations of chromatin and of DNA stock solutions in the various aqueous solvents were determined by phosphate analysis (Chen et al., 1956; as modified by Ames and Dubin, 1960). The concentration of chromatin in ethylene glycol was assayed using the diphenylamine reaction (Giles and Myers, 1965). Chromatin of known phosphate concentration, dissolved in 10 m*M* NaCl, was used as the standard for the diphenylamine reaction.

Fixation of Chromatin and DNA. Formaldehyde solutions were prepared by dilution from 37% formaldehyde (analytical reagent grade) on the day of the experiment. These HCHO solutions were brought to pH 7 with 0.2 *M* KOH. Generally, HCHO concentrations were such that 0.1 ml of HCHO solution was added to 1.0 ml of stock chromatin solution to give the desired final HCHO concentration. In determining the final CD spectra of chromatin solutions fixed with HCHO, samples were prepared as described above and allowed to fix in the cold for at least 48 hr. Kinetic experiments were performed at room temperature.

Glutaraldehyde (Polysciences, Inc., Warrington, Pa.) was purchased as a neutralized aqueous 8% solution and has been described previously (Olins and Wright, 1973). The fixation of chromatin with glutaraldehyde was the same as described above for HCHO.

Optical Studies. Ultraviolet spectra were obtained on a Cary 15 spectrophotometer. CD measurements were made on a Durrum-Jasco recording spectropolarimeter (Model ORD/UV-5) with CD attachment. The instrument was calibrated with *d*-10-camphorsulfonic acid (Urry et al., 1968). Results are reported as molecular ellipticity, $[\theta]$ (deg cm^2 dmol^{-1}), based on DNA phosphate concentration. Absorbances of the samples used in the CD studies were between 0.5 and 2 at 260 nm, and measurements were taken in cuvets of 0.5 or 1-cm path length.

Kinetics. The change in ellipticity with time was followed at 275 nm, using the time base of the CD recorder. Total

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[1] Abbreviations used are: HCHO, formaldehyde; SSC, standard saline citrate (0.15 *M* NaCl–0.015 *M* sodium citrate (pH 7.5)).

changes in ellipticity were estimated for each sample, either from the kinetic traces or from final CD spectra.

Reagents. All chemicals were reagent grade or better. All solutions were prepared from glass-distilled water.

Results

The CD spectra of samples of chromatin in water, fixed with various concentrations of HCHO, are presented in Figure 1. In the region of the spectrum above 240 nm there is a progressive transition with increasing HCHO concentration from a native chromatin spectrum (Simpson and Sober, 1970; Shih and Fasman, 1970; Boffa et al., 1971; Fric and Sponar, 1971; Hanlon et al., 1972; Johnson et al., 1972; Williams et al., 1972), to one which closely resembles the spectra of films of the Li salt of naked DNA[2] (Tunis-Schneider and Maestre, 1970). For purposes of discussion in this paper we shall refer to this as the "suppressed" spectrum.[3]

Below 240 nm, where the major contributor to CD is protein (Johnson et al., 1972), there is little change in the chromatin spectrum upon fixation with HCHO. There were no measurable differences in the uv spectra of native chromatin and chromatin which had been fixed with 1% HCHO for 48 hr. In addition, calf thymus DNA (in 10 m*M* NaCl) and chicken erythrocyte DNA (in 0.1 × SSC) exhibited no observable changes in CD or uv spectra when fixed for 48 hr with 1% HCHO.

Since many of the physical properties of chromatin depend upon salt concentration, it was of interest to examine the reaction of HCHO with chromatin in the presence of salt. Spectra similar to those shown in Figure 1 were obtained for chromatin in 5 and 10 m*M* NaCl and 0.035 m*M* phosphate buffer (pH 6.8). All samples showed the same trend from native to suppressed CD spectra with increasing HCHO concentration; however, the extent of the CD change, especially at low concentrations of HCHO, was greater in the presence of salt than in water. This is most easily seen if ellipticities at one wavelength are presented as a function of HCHO concentration, as shown in Figure 2A. At 275 nm the change in ellipticity observed from native to HCHO-fixed chromatin is largest.

Figure 2B shows the time course of the ellipticity change at 275 nm upon addition of HCHO (final concentration, 0.61 *M*) to solutions of chromatin in water and in 5 m*M* NaCl. The initial portion of the reaction appears to be faster in salt than in water. Kinetic data were analyzed according to a method for parallel first-order reactions (Frost and Pearson, 1961); the results of two such analyses are shown in Figure 3. The values of ellipticity change for the fast reaction were corrected for decreases due to the slow reaction in each case. Apparent first-order rate constants, evaluated from the slopes of least-squares lines similar to those shown in Figure 3, are given in Table I. Both the rate constants for the fast and slow reactions are dependent on the concentration of HCHO, indicating pseudo-first-order behavior under the reaction conditions employed. The fast process appears to occur more rapidly in salt than in water. Table I also lists the percentage of the total ellipticity change involved in the fast and slow processes at different concentrations of HCHO. The proportion of the chromatin involved in the slow reaction (S_0) decreases slightly with increasing [HCHO], while the amount involved in the fast reaction (F_0) increases. The net effect is a very rapid increase in the percentage of the chromatin involved in the fast reaction with increasing [HCHO].

Ethylene glycol is known to produce a suppressed CD spectrum in solutions of DNA (Green and Mahler, 1968, 1970; Nelson and Johnson, 1970). Dehydration of the bases, such as occurs in the presence of high salt (Studdert et al., 1972; Zimmer and Luck, 1974) and in Li-DNA films at low relative humidity (Tunis-Schneider and Maestre, 1970), has been suggested as the cause of the CD change. Therefore, it was of interest to compare the effects of ethylene glycol on chicken erythrocyte DNA and on chromatin.

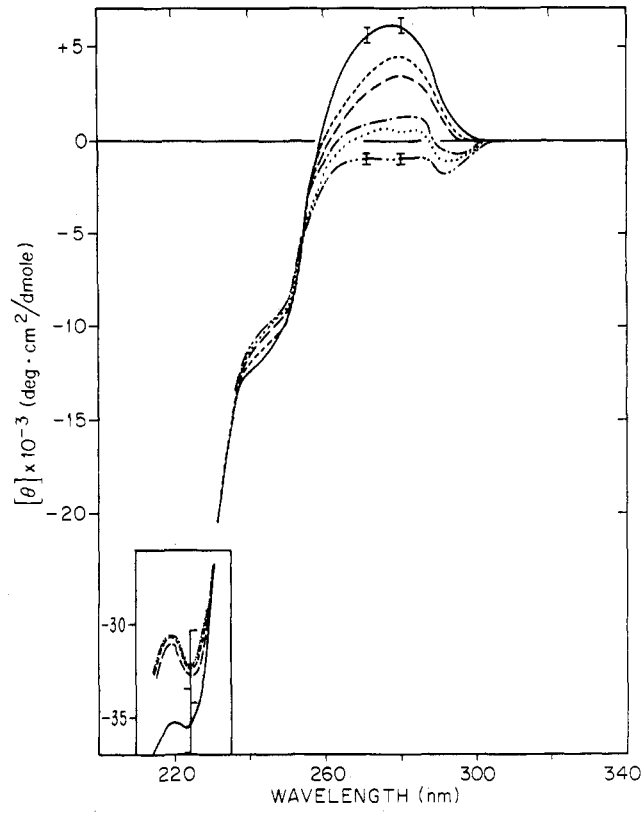

FIGURE 1: CD spectra of chicken erythrocyte chromatin in water, after 1 week of fixation at 4° with the indicated HCHO concentrations: (—) 0 *M*; (- - -) 0.031 *M*; (– –) 0.061 *M*; (– · –) 0.151 *M*; (· · ·) 0.303 *M*; (– · · –) 1.12 *M*. The concentration of chromatin was 1.94×10^{-4} *M* phosphate for unfixed material and 1.76×10^{-4} *M* phosphate for solutions with added HCHO. CD measurements were obtained at 25° in a cuvet with a 0.5-cm light path. 1% HCHO would be equivalent to 0.33 *M*.

[2] The similarity in CD spectra between Li-DNA films, presumably in the C conformation, and solutions of DNA in high salt have led to suggestions that this type of CD spectrum is peculiar to C form DNA. Recently, however, X-ray data of Maniates et al. (1974) have suggested that a reduction of positive ellipticity above 260 nm is not necessarily indicative of the presence of DNA in the C conformation. In the absence of wide angle X-ray data for the chromatin samples studied here, we cannot assign a conformation to the DNA within fixed or unfixed chromatin.

[3] It is apparent from the results of many workers that there is a wide range of suppressed spectra, the appearance of which depends on the nature of the salt and its concentration (Studdert et al., 1972; Hanlon et al., 1972, Ivanov et al., 1973; Zimmer and Luck, 1974), temperature (Studdert et al., 1972), solvent composition (Green and Mahler, 1968, 1970; Nelson and Johnson, 1970), relative humidity in DNA films (Tunis-Schneider and Maestre, 1970), and base composition (Zimmer and Luck, 1974). The general characteristics of the suppressed spectrum of DNAs of ca. 40% G-C content are (1) a decrease in the positive band from 275 to 280 nm, and a shift to longer wavelengths; (2) the appearance of a small negative band around 292 nm; (3) a small shoulder at approximately 267 nm; and (4) only small changes in ellipticity around 245 nm.

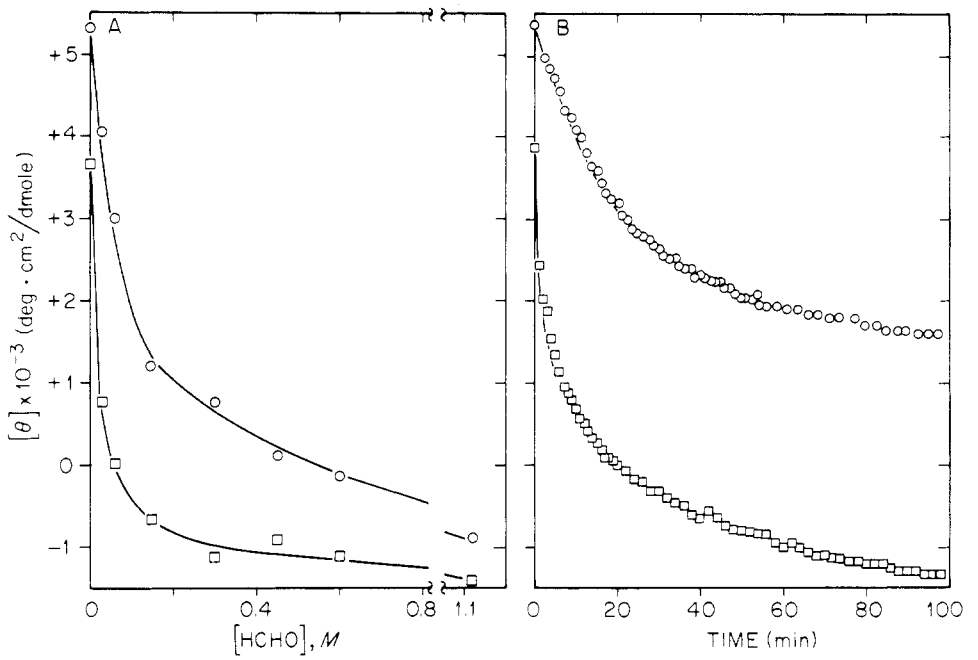

FIGURE 2: (A) Ellipticity at 275 nm as a function of HCHO concentration for solutions of chicken erythrocyte chromatin in (○) H_2O, and (□) 5 mM NaCl; fixed for 1 week at 4°. Chromatin concentrations were between 2.35×10^{-4} and 1.41×10^{-4} M phosphate, and measurements were made at 25° in a 0.5-cm cuvet. (B) Kinetics of the ellipticity change at 275 nm for chicken erythrocyte chromatin solutions in (○) H_2O, and (□) 5 mM NaCl; fixed with 0.61 M HCHO. Chromatin concentrations were 2.06×10^{-4} M phosphate in H_2O and 1.20×10^{-4} M phosphate in 6 mM NaCl. Measurements were made at 25° in a 0.5- or 1-cm cuvet.

In Figure 4A the CD spectra of chicken erythrocyte DNA in aqueous and in ethylene glycol solution are presented. In agreement with the findings of Green and Mahler (1968), we observe a marked change in the CD spectrum of DNA to a nonconservative, suppressed spectrum in 95% ethylene glycol. In contrast, the CD spectra of chromatin (Figure 4B) show there are only slight differences between water and glycol. The major characteristics of the suppressed spectrum are undoubtedly absent from the spectrum of chromatin in 95% ethylene glycol. Dialysis of chromatin against 100% glycol does produce a CD spectrum with a $[\theta]_{max}$ which is reduced in magnitude and shifted to 285 nm, and with a cross-over at 262 nm. Nevertheless, the small negative peak at 295 nm, which is present in spectra of DNA in high salt or ethylene glycol and in spectra of chromatin fixed with HCHO in aqueous solution, is missing. It is clear that ethylene glycol does not produce as extensive a conformational change in chromatin as it does in DNA.

Since ethylene glycol has only a small effect upon the CD of chromatin, it was conceivable that the CD spectra of chromatin fixed in ethylene glycol with HCHO might resemble corresponding aqueous spectra. A comparison of the CD spectra of chromatin fixed with 0.61 M HCHO in H_2O and 91% ethylene glycol is shown in Figure 4B. Spectra, similar to those shown in Figure 1, of chromatin in 91% ethylene glycol fixed with different concentrations of HCHO reveal a progressive decrease and slight shift to longer wavelengths of $[\theta]_{max}$ with increasing HCHO concentration. However, the ellipticity from 310 to 290 nm increases slightly (Figure 4B), in contrast to the negative peak seen around 292 nm in aqueous solution. The extent of the transition from the native to the suppressed CD spectra is reduced in glycol solution.

In order to further examine the effect of aldehyde fixatives on the CD of chromatin, glutaraldehyde, which has been shown to cross-link the histone proteins of chicken erythrocyte nuclei (Olins and Wright, 1973), was incubated with chromatin in H_2O and 5 and 10 mM NaCl at 4° for 1.5 weeks. Even at glutaraldehyde concentrations of 80 mM, these CD spectra superimposed with spectra of native chromatin.

Table I: Kinetic Parameters of the Formaldehyde Reaction.[a]

Solvent	[HCHO] (M)	k_F[b] ($\times 10^5$ sec^{-1})	k_S[b] ($\times 10^5$ sec^{-1})	%F[c]	%S[c]
35 μM $NaPO_4$, pH 6.8	0.031	4 ± 1	0.8 ± 0.1	8	92
	0.15	15 ± 4	2 ± 1	43	57
	0.30	28 ± 10	4 ± 1	55	45
	0.61	43 ± 14	6 ± 1	61	39
5 mM NaCl	0.15	14 ± 2	2 ± 0.6	28	72
	0.61	41 ± 8	5 ± 1	44	56
H_2O	0.15	16 ± 6	1 ± 0.3	18	82
	0.30	20 ± 5	3 ± 1	40	60
	0.61	25 ± 6	5 ± 0.6	58	42

[a] Chromatin concentrations varied between 9×10^{-5} and 2×10^{-4} M phosphate. [b] Evaluated from the slopes of least-squares lines. F and S refer to the fast and slow reactions, respectively. The relatively large error limits in the data arise from the uncertainty in the final ellipticity values for the kinetic experiments, due to the long times involved and the noise level of the spectropolarimeter. [c] Evaluated from the intercepts of the least-squares lines; %F and %S represent the percentage of the fast and slow components in the original solution, respectively.

Discussion

Formaldehyde is known to react with primary amino groups in the bases of nucleic acids and with primary amino, imidazole, and other groups in proteins (Fraenkel-Conrat and Olcott, 1948a,b; Fraenkel-Conrat, 1954; Feldman, 1973). Generally, these reactions are accompanied by

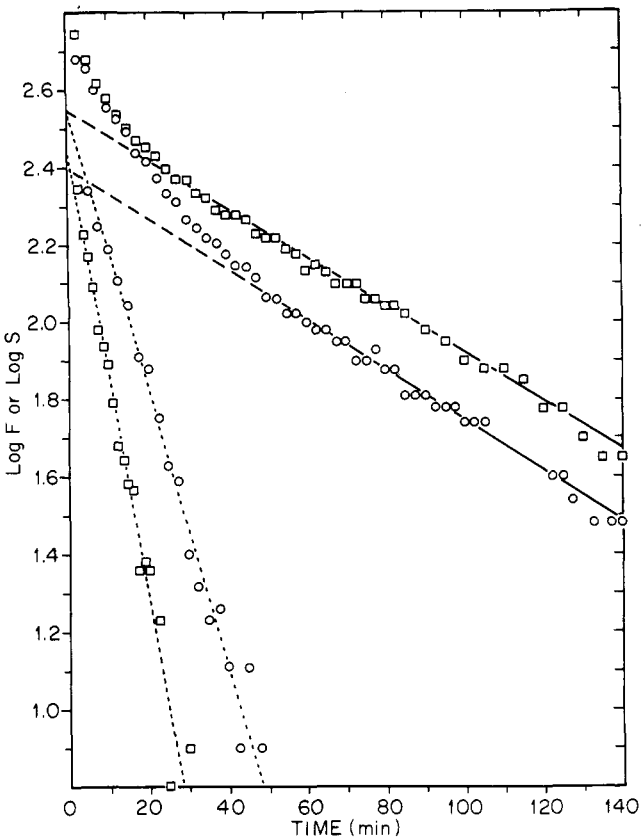

FIGURE 3: First-order kinetic analysis of the data from Figure 2B: (○) chromatin in H_2O; (□) chromatin in 5 mM NaCl. The plots of the fast reaction (···) were obtained from the initial, nonlinear portions of the slow reaction plots (—) by correction of the measured data for the ellipticity changes due to the slow process, using the following relationships (Frost and Pearson, 1961): $\log S = \log S_0 - k_S t/2.303$ and $F = E_\infty - E - S$, where S = ellipticity change due to slow component, S_0 = amount of slow component at zero time, = intercept of slow reaction plot, $k_S/2.303$ = slope of linear portion of slow plot, F = ellipticity change due to fast component, E_∞ = total ellipticity change, E = measured ellipticity change.

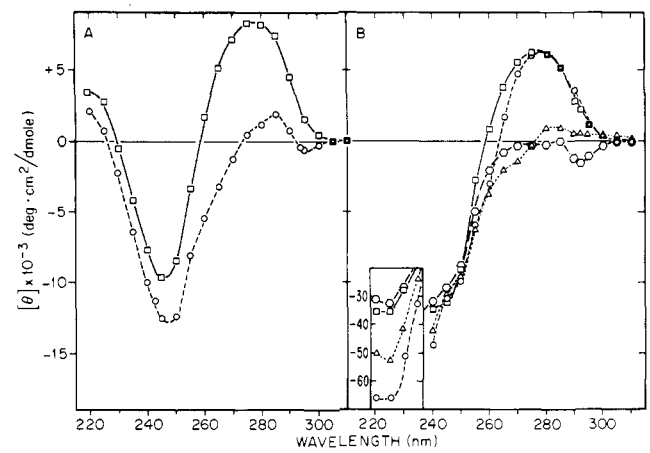

FIGURE 4: (A) CD spectra of chicken erythrocyte DNA in (□) 0.1 × SSC, and (○) 95% ethylene glycol containing 5 mM NaCl. DNA concentrations were 2.01×10^{-4} M in aqueous and 2.29×10^{-4} M in glycol solution. Measurements were made at room temperature in 0.5-cm cuvets. Spectra of DNA in 95% ethylene glycol solution containing 10 and 15 mM NaCl superimpose with the 5 mM spectrum. (B) CD spectra of chicken erythrocyte chromatin in (□) H_2O, (○) 95% ethylene glycol, (○) H_2O containing 0.61 M HCHO, and (△) 91% ethylene glycol containing 0.61 M HCHO. Samples containing HCHO had been fixed at 4° for 1 week or more. Chromatin concentrations were between 1×10^{-4} and 2×10^{-4} M phosphate, and measurements were made at room temperature in a 0.5-cm cuvet.

changes in the uv spectra of these macromolecules (Fraenkel-Conrat, 1954; Feldman, 1960; Haselkorn and Doty, 1961; Martin and Marini, 1967). We have not observed significant changes in the uv spectrum of fixed chromatin; however, due to the sharply increasing uv absorbance of chromatin between 230 and 220 nm, we cannot exclude the possibility that changes occur around these wavelengths caused by the reaction of HCHO with protein. The HCHO reaction with the exocyclic amino groups of adenine, guanine, and cytidine is accompanied by uv spectral changes above 260 nm. Von Hippel and Wong (1971) have calculated that the change in absorptivity at 278 nm due to the reaction of HCHO with calf thymus DNA is 1.7×10^3/mol of nucleotide. The limit of the resolution in the uv spectra reported here is 0.02 absorbance unit. Therefore, assuming that the optical properties of calf thymus and chicken erythrocyte DNA are comparable,[4] we calculate that, at most, 10% of the bases in chromatin could have formed reaction products with HCHO. Evidence supporting the possibility that the DNA in chromatin may, to a very limited extent, react with HCHO comes from the results of Brutlag et al. (1969), who found 7.5% of the bases in chromatin irreversibly bound by HCHO. Nevertheless, the HCHO reaction with chromatin does not promote denaturation of the double-stranded DNA. Li (1972) found that HCHO raises the melting temperature of chromatin and stabilizes the double-helical structure. Additionally, the CD spectra of heat-denatured DNA and denatured DNA which had been fixed with HCHO for 24 hr do not resemble the suppressed spectra. We believe, therefore, that the CD changes reported in this paper are caused neither by the presence of additional chromophores caused by reaction with HCHO nor to denaturation of the DNA.

CD spectra of macromolecules are known to be affected by light scattering, both isotropic particle scattering and anisotropic scattering (Gordon and Holzwarth, 1971; Gordon, 1972; Litman, 1972; Dorman and Maestre, 1973; Holzwarth et al., 1974). The effects generally lead to circular dichroism in nonabsorbing regions of the spectra, magnified CD amplitudes, and CD spectra which resemble absorption spectra, but with bands having a "hook" in the opposite direction (Holzwarth et al., 1974). The CD spectra presented here have none of these characteristics. Also, samples of water-swollen, HCHO-fixed, sonicated chicken erythrocyte nuclei, which had been fractionated on sucrose gradients and were shown by electron microscopic examination to be enriched in monomer chromatin particles (ν bodies) which are approximately 70 Å in diameter (Senior et al., 1975), have CD spectra identical with Li-DNA films (Tunis-Schneider and Maestre, 1970) in the spectral region from 340 to 240 nm. The dimensions of these particles, even considering possible hydration effects, are small in comparison to the wavelength of light, and scattering would not be expected to be significant.

Protein conformational changes may occur when chromatin is fixed with HCHO. However, the major CD changes observed are between 300 and 240 nm, the region of the CD spectrum where DNA is the predominant contributor. We consider it unlikely that protein conformational changes alone would cause such large changes in the

[4] Calf thymus DNA is composed of 42% G-C residues, and chicken erythrocyte DNA contains 41% G-C residues.

wavelength range 300–240 nm while producing only minor changes from 240 to 220 nm.

Therefore, we believe that conformational changes in the DNA cause the CD changes observed. That the action of HCHO upon the DNA in chromatin is the result of a chemical reaction rather than a solvent effect is demonstrated by the fact that the suppressed CD spectrum is maintained in HCHO-fixed isolated chromatin particles which have been extensively dialyzed against aqueous buffers and purified on sucrose gradients. The reaction of formaldehyde with chromatin could lead to extensive cross-linking of chromatin components and a displacement of bound H_2O. The failure of 95% ethylene glycol to produce significant changes in the conformation of chromatin, however, suggests that a simple dehydration of the DNA in chromatin is not the cause of the CD changes reported here.

Glutaraldehyde (4 m*M*) is known to rapidly cross-link the histone proteins of chicken erythrocyte nuclei (Olins and Wright, 1973). The failure of 80 m*M* glutaraldehyde to produce any changes in the CD spectrum of chromatin suggests that the suppressed CD spectrum cannot be generated by all aldehyde cross-linking reagents.

We believe, therefore, that these changes in the CD spectra of chromatin are produced by specific formaldehyde reactions, involving histone–histone and histone–DNA cross-linking.[5] The theoretical basis for the CD spectrum of DNA in its various conformations is obscure at present. Nevertheless, Moore and Wagner (1973, 1974) and Studdert and Davis (1974a–c) have recently suggested that movement of the DNA bases away from the helix axis, such as results from a B → C conformational change, may cause changes in the CD spectrum of the same magnitude and in the same direction as those observed here. It is tempting to speculate that cross-linking of histone to DNA might cause such base movement. Additionally, the decreased reactivity of HCHO toward chromatin in ethylene glycol could be due to an increase in H-bonding strength between the DNA bases in the nonpolar solvent, preventing the HCHO reaction (Utiyama and Doty, 1971; Von Hippel and Wong, 1971). The cross-linking of histone to DNA can occur only infrequently along the DNA, however, since a maximum of 10% of the bases react with HCHO. In addition, the kinetics of the reaction implicate at least two types of reactive species, both producing a decrease in CD at 275 nm. The nonlinearity of the first-order rate constants with formaldehyde concentration suggests that HCHO is being used up in other reactions, probably protein–protein cross-linking. The dependence on HCHO concentration of the proportion of the total CD change included in the fast and slow reactions (%*F* and %*S* in Table I) must result from a complex mechanism. Therefore, we cannot rule out that a DNA conformational change is induced by a protein–protein cage formed by cross-linked histones.

Finally, we wish to suggest that the CD changes reported here, caused by HCHO fixation, may have some relevancy to the structure of native chromatin. Low-angle X-ray diffraction studies of chicken erythrocyte nuclei and chromatin show only minimal perturbation of periodic structure due to HCHO fixation (Olins et al., 1975). Furthermore, Rill and Van Holde (1973) and Johnson et al. (1972) have observed the suppressed DNA CD spectrum in unfixed chromatin which had been digested by nuclease. Additionally, Johnson et al. (1972) found a relationship between the amount of shearing to which chromatin had been subjected and the content of B-like DNA reflected in the CD spectrum. (They postulated that nuclease digested B-DNA regions, leaving C-DNA regions intact.) It is conceivable that shearing or stretching of unfixed chromatin might disrupt the native structure by unfolding some of the nucleohistone particles (ν bodies). Unfolded regions would be expected to be more susceptible to nucleolytic cleavage than intact ν bodies, and might also exhibit altered melting profiles (Johnson et al., 1972). If unraveled particles exhibit a higher ellipticity, above 260 nm, than do intact particles, then the reaction of HCHO with chromatin may enhance the suppressed spectrum by inducing a refolding of the particles. Thus formaldehyde may promote the formation of structures similar to those found in native chromatin.

[5] Since chicken erythrocyte nuclei prepared as described above contain negligible amounts of non-histone proteins (Olins et al., 1975), they are not considered in the ensuing discussion.

Acknowledgments

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