Volume 4 Number 6 June 1977 Nucleic Acids Research

Conformational states of chromatin ν bodies induced by urea

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Received 15 March 1977

ABSTRACT

Monomer chromatin nu bodies (ν_1) from chicken erythrocyte nuclei were exposed to 0–10 M urea plus 0.2 mM EDTA (pH 7). Alterations in ν_1 conformation were examined using hydrodynamic methods (i.e., S, η, and $\frac{d\rho}{dc}$), thermal denaturation, circular dichroism, reactivity of histone thiol groups to N-ethyl maleimide, and electron microscopy. The two domains of a ν body (i.e., the DNA-rich shell and the protein-rich core) appeared to respond differently to the destabilizing effects of increasing urea: DNA conformation and stability exhibited noncooperative changes; the core protein structure revealed cooperative destabilization between 4 and 7 M urea. Companion studies on the conformation of the inner histone "heterotypic tetramer" also revealed cooperative destabilization with increasing urea concentration.

INTRODUCTION

Recent studies have indicated the possibility that the fundamental nucleohistone subunit, known as the ν body or nucleosome (1-5), persists in altered conformation in chromatin presumed to be transcriptionally active (6-8). The present investigation employs urea as a model perturbant, with the goal of identifying some of the conformational states and transitions of the chromatin subunit. The availability of large amounts of well-characterized monomer ν bodies (9, 10) has permitted an extensive study of these urea effects by numerous biophysical techniques.

METHODS

Preparation of ν Bodies and Histones

Chicken erythrocyte monomer ν bodies (total ν_1) were obtained by zonal ultracentrifugation and stored frozen in 0.2 mM EDTA, pH 7.0 (9, 10). Most experiments were conducted on the KCl-soluble fraction of ν_1 (9, 10), which was also stored frozen in 0.2 mM EDTA (absorbance, A_{260} ~100–200). The term "ν_1" will be

employed throughout to denote these "core" particles composed of two each of H4, H3, H2A, and H2B histones associated with a DNA fragment of ~140 nucleotide pairs.

The inner histone "heterotypic tetramer" (one each of H4, H3, H2A, and H2B, devoid of DNA) was prepared by a modification of the original technique (11). Chicken erythrocyte chromatin was dispersed in 2 M NaCl, 10 mM Tris-HCl (pH 7.0), and 0.1 mM dithiothreitol (DTT) at a concentration of 1–2 mg DNA/ml. The swollen chromatin gel was centrifuged for 16 hr at 42,000 rpm and 4° to pellet DNA. The histone-containing supernate was concentrated to approximately 10 mg protein/ml, assuming that for a 1 mg/ml solution $A_{230} = 3.5$ (12). The heterotypic tetramer was fractionated from H1 and H5 by layering 0.5 ml of the concentrated histone extract onto 4.5 ml of a 5–20% sucrose gradient containing 2 M NaCl, 10 mM Tris (pH 7.0), and 0.1 mM DTT, then centrifuging in an SW 50.1 rotor at 48,000 rpm and 4° C for 48 hr. Gel electrophoretic analysis of the recovered fractions was in good agreement with the original method (11). The inner histone tetramer fraction was pooled and dialyzed against 2 M NaCl, 10 mM Tris (pH 7.0), and 0.1 mM DTT, then stored frozen at -20°C. The concentration of the inner histone stock solution (i.e., moles amino acids/ml) was determined by amino acid analysis.

Stock solutions of urea (~10 M, ultrapure grade, Schwarz/Mann) were made in 0.2 mM EDTA (pH 7), filtered through millipore filters, and examined for conductivity and pH. Freshly made solutions exhibited pH 7.0. Over a period of several weeks the pH remained constant, and the conductivity usually increased only slightly. Volumetric dilutions of the urea stock with filtered 0.2 mM EDTA (pH 7) yielded stock solutions from 0–10 M urea containing 0.2 mM EDTA. Each urea solvent was measured refractometrically against 0.2 mM EDTA at the same temperature. Urea molarity was calculated from the following analytical expression:

$$M = 4.800 \times 10^{-3} + 1.134[(N-N_o) \times 10^2] + 1.118 \times 10^{-2}[(N-N_o) \times 10^2]^2$$

where $(N-N_o)$ is the refractive index increment of the urea solution compared with 0.2 M EDTA, derived from published physical data (13, 14) and determinations in this laboratory.

Sucrose Gradient Ultracentrifugation in Urea

To demonstrate the binding of histones to DNA in 8 M urea, we layered separately ν_1 and inner histones onto linear 5–20% sucrose gradients containing 8 M

urea plus 0.2 mM EDTA, then centrifuged them in an SW 50.1 rotor for 14 hr at 50,000 rpm and 25°C. The samples were 50 µl of ν_1 in 0.2 mM EDTA (A_{260} = 250), and 50 µl of inner histones in the 2 M NaCl buffer (concentration = 100 mg histone/ml). Fractions were collected dropwise from the bottom of the centrifuge tubes, and each fraction was measured at 260 nm for DNA. Aliquots from each fraction were dried on filter paper, washed in trichloroacetic acid, and stained with naphthalene blue-black; the stain was eluted and measured at 610 nm (15).

Hydrodynamic Properties

Samples were dialyzed to equilibrium against pure solvent, and dilutions were made using the final dialysate. Concentrations of ν_1 were determined from measurements of absorption at 260 nm employing the extinction coefficient for a 1% solution, A_{260} = 93.12 ± 0.5 (10).

Intrinsic viscosity data were obtained at the zero-shear limit using a cartesian diver Couette-type viscometer described previously (16, 17). Copper drive rings used in the three rotors required to span the solution density range were machined from the same piece of electrolytically pure copper, and all rotors were carefully calibrated against the respective pure solvents. Density overlap of at least three urea molarity units was available between successive rotors, thus ensuring against rotor differences contributing appreciable error to the measurements. All runs were made at concentrations below 1.5 mg/ml. In this range negligible concentration dependence of the relative viscosity was observed. All determinations were made at 25.0°C.

Sedimentation data were obtained with a Beckman Model E analytical ultracentrifuge equipped for UV absorption scanning. All data were obtained at 25°C and 36,000 rpm in an An-G rotor. Solutions were stabilized against mixing by use of a 0.5% sucrose gradient as described earlier (10). Sedimentation velocities were calculated with standard methods. For all runs used, the statistical correlation of ln r/r° versus t was at least 0.999, and the standard error in the sedimentation coefficient, S, was well under 1%. Corrections to $S_{20,w}$ were based upon published solvent density and viscosity data (13) and our own determinations of partial specific volume. Corrections for the sucrose gradient were made with data supplied by Breillatt (18).

The density increment $(\partial\rho/\partial c)$ (19) was obtained directly from plots of density (ρ) versus concentration (c). The value for the apparent specific volume,

$$\phi^* = \frac{1}{\rho_0}\left(1-\frac{\partial\rho}{\partial c}\right)$$

was obtained by use of a Paar DMA-02C digital density meter equilibrated to 25.00°C. Samples were diluted to appropriate concentrations in 4 and 8 M urea plus 0.2 mM EDTA (pH 7.0), respectively, and then dialyzed to equilibrium at room temperature. To avoid evaporation, samples were removed from the dialysis bag with a syringe without allowing air to enter and then immediately inserted directly into the clean, dry cell of the density meter.

Sample concentrations were obtained by measuring the A_{260} of weight dilutions of the samples used for density measurements. Since the extinction coefficient of the chromatin in urea is not known, weight dilutions of a sample of known concentration were made into 0.2 mM EDTA plus 4 M urea and 8 M urea solutions. The absorbance of these samples in urea was monitored, and the extinction coefficient was adjusted accordingly. The extinction coefficient for the 0.2 mM EDTA sample has been shown in a previous study (10) to be 93.12 A_{260} units for a 10 mg/ml solution, and the adjusted value was found to be 95.7.

Thermal Denaturation

Melting studies were performed in a Gilford automatic recording spectrophotometer equipped with a linear temperature programmer (19, 20). Solutions of ν_1 in 0.2 mM EDTA, or of chicken DNA in various salt solutions, were diluted to an A_{260} of approximately 1.0 with the proper urea-containing buffer. Derivative melting profiles (change in hyperchromicity/change in temperature, $\Delta H/\Delta T$) were calculated on an Olivetti Programma 101 by use of a program for determining the derivative at the midpoint of five successive points (21).

Circular Dichroism

Measurements were performed on a Jasco circular dichrograph SS-10, employed and calibrated as previously described (22). Chicken DNA and ν_1 were examined in 1-cm cuvettes at room temperature. The molar extinction coefficient at 260 nm DNA ($\varepsilon_p = 6500$) was assumed in calculations of the molecular ellipticity per mole of nucleotides, $[\theta]_p$. Inner histones were examined in 1-mm cuvettes at room temperature. Calculation of the molecular ellipticity per mole of amino acid residues, $[\theta]_{AA}$, was based upon the quantitative dilution of a calibrated (by amino acid analysis) histone stock solution.

Reaction with ^{3}H-labeled N-ethylmaleimide (NEM)

NEM [ethyl-2-^{3}H] was obtained as a pentane solution from New England Nuclear Corp. (150 mCi/mmole). The pentane was removed under vacuum and the crystals were redissolved in 100% ethanol at the initial concentration and stored in screw-cap vials at -20°C.

For the reaction of NEM with ν_1, 15 µl of ν_1 (A_{260} = 114–164) was added to 0.5 ml of urea buffer containing 0.2 mM EDTA (pH 7.0). Then 5 µl NEM (in ethanol) was added, and the mixture was incubated at 37°C for 90 min (prior experiments had indicated reaction completion by 60 min). The reaction was terminated by addition of 10 µl of 20 mM DTT. All samples were dialyzed against three overnight changes of large volumes of 0.2 mM EDTA (pH 7.0), recovered, measured at A_{260}, and counted in Aquasol (i.e., 0.1 ml of sample to 10 ml scintillation fluid). Maximum reaction of NEM yielded 20,000–40,000 cpm, compared with background values of about 30 cpm. Counting efficiency, determined employing ^{3}H-labeled water (New England Nuclear Corp.) as an internal standard, averaged 42%. The molarity of ν_1 was calculated from measured A_{260} by employing a molar extinction coefficient at 260 nm (ε_ν = 1.98 x 10^6) based upon the extinction coefficient of a 1% solution of ν_1 (i.e., 93.12 at 260 nm), and a molecular weight per ν_1 of 2.13 x 10^5 g/mole (10).

Electron Microscopy

A drop of ν_1 (A_{260} = 1.0) in 0.2 mM EDTA or urea buffer was placed on a freshly glowed carbon film for 30 sec. The grid was rinsed in Kodak Photoflo (pH 7.0) and dried. Contrast was achieved by negative staining with 5 mM uranyl acetate for 30 sec (10). The electron microscope was lined up for tilted-beam dark-field microscopy.

RESULTS

Hydrodynamic Properties of ν_1 in Urea

Several studies (23–26) have demonstrated that the histones remain associated with DNA in chromatin treated with up to 5 M urea, at low ionic strength. In the present investigation we have observed that treatment of ν_1 with at least 8 M urea plus 0.2 mM EDTA does not dissociate histone from DNA (Fig. 1). The possibility of

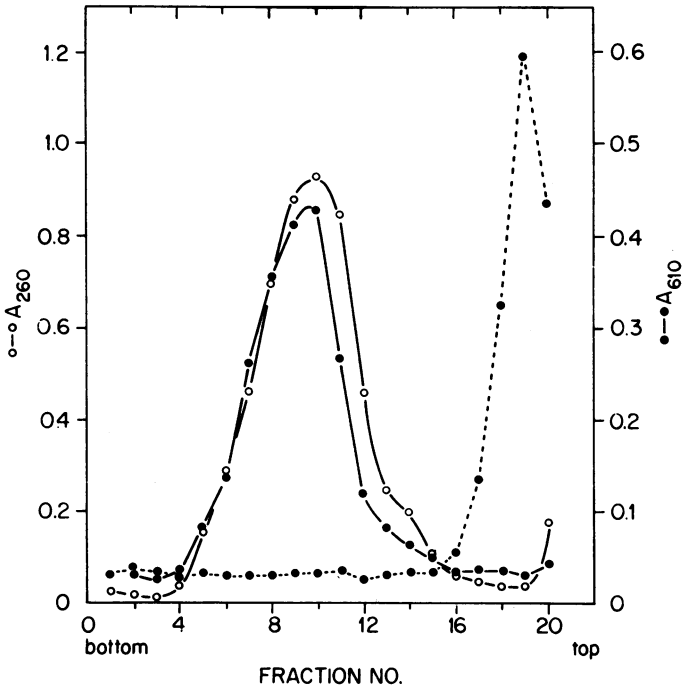

Figure 1. Sucrose gradient ultracentrifugation of ν_1 and inner histones in 8 M urea. Samples: ν_1 examined at 260 nm (O) and, for protein staining with naphthalene blue-black, at 610 nm (●); inner histones centrifuged in a separate tube, examined for protein at 610 nm (●----●).

migration of bound histone on the DNA fragments cannot be eliminated by this experiment.

Measurements of intrinsic viscosity $[\eta]$ and $S_{20,w}$ as a function of urea molarity are given in Table 1 and presented graphically in Fig. 2. Experimental apparent specific volume data obtained were as follows (all solutions contained 0.2 mM EDTA, pH 7.0): $\phi^* = 0.661 \pm 0.003$ in EDTA only (10); $\phi^* = 0.672 \pm 0.006$ in 4 M urea; $\phi^* = 0.677 \pm 0.010$ in 8 M urea. The values of ϕ^* shown in Table 1 and used to correct to $S_{20,w}$ from experimental sedimentation velocity data were obtained from the above experimental results by linear interpolation or extrapolation. It is clear that $[\eta]$ increases continuously over the range 0–8 M urea, but it appears to reach a limiting value between 8 and 10 M. On the other hand, $S_{20,w}$ decreases monotonically over the entire urea range studied.

Some insight into the meaning of these changes in hydrodynamic properties can be obtained from the theory of Scheraga and Mandelkern (27), which can be

TABLE 1. Hydrodynamic Effects of Urea on ν_1

Urea	$S_{20,w}$[a] (Svedbergs)	$[\eta]$[a] (cm^3/g)	ϕ^*[b] (cm^3/g)	$\beta \times 10^{-6}$[c,d]	a/b[e]	$v_p \times 10^{18}$ (cm^3)	a[f] (Å)	b (Å)	δ_1 (g H_2O/g dry particle)
0	8.92 ± 0.06	11.2 ± 0.2	0.661	2.12 ± 0.03	1	1.60	72.6	72.6	3.79
1.90	7.84 ± 0.03	20.7 ± 0.2	0.666	2.29 ± 0.03	6.29	1.00	211	33.6	2.12
3.81	7.15 ± 0.20	28.7 ± 0.3	0.671	2.33 ± 0.08	7.43	1.14	247	33.2	2.51
5.69	6.40 ± 0.23	36.2 ± 0.4	0.674	2.25 ± 0.09	5.40	2.07	243	45.1	5.09
7.68	5.95 ± 0.31	41.1 ± 0.6	0.677	2.18 ± 0.12	3.50	3.58	219	62.5	9.29
9.84	5.55 ± 0.38	40.8 ± 0.5	0.679	2.03 ± 0.15	--	--	--	--	--

[a] Error limits determined as standard error in independent determinations on several samples

[b] Interpolated values from experimental data at 0, 4, and 8 M urea (see text).

[c] Based upon an assumed molecular weight of 216,000 daltons (10).

[d] Error limit determined from combined standard errors in $S_{20,w}$ and $[\eta]$.

[e] Corresponding value for anhydrous particle in 0 M urea: a = b = 38.4 Å (10).

[f] Prolate ellipsoid (see text).

represented by the equation

$$\beta = \frac{N_A S^0 [\eta]^{1/3}}{M^{2/3}} \frac{\eta_0}{(1-\rho\bar{v})} = 1.556 \times 10^6 \left(\frac{f_0}{f}\right) \nu^{1/3} \qquad (1)$$

where M is molecular weight, S^0 is sedimentation coefficient at infinite dilution, η_0 is solvent viscosity, N_A is Avogadro's number, (f_0/f) is the Perrin asymmetry correction to the translational frictional coefficient (28) and ν is the corresponding asymmetry correction in the Simha treatment of intrinsic viscosity (29); in this study the apparent specific volume ϕ^* was employed in the calculation of β in place of $\bar{v}$, the partial specific volume. The parameter β is a unique function of axial ratio for ellipsoidal particles, and hence this treatment circumvents the ambiguity of particle size versus asymmetry associated with analysis of sedimentation or viscosity data alone. Unfortunately, β is rather insensitive to axial ratio for prolate ellipsoids of revolution and almost independent of axial ratio for oblate ellipsoids. This fact reduces its quantitative utility in the present analysis, but it does permit us to distinguish qualitatively between these two models. Our data cannot be rationalized with a urea-induced oblate asymmetry in ν_1, and this conclusion is well outside the range of observed experimental error.

Table 1 lists values of β calculated from Eq. 1, assuming a fixed molecular

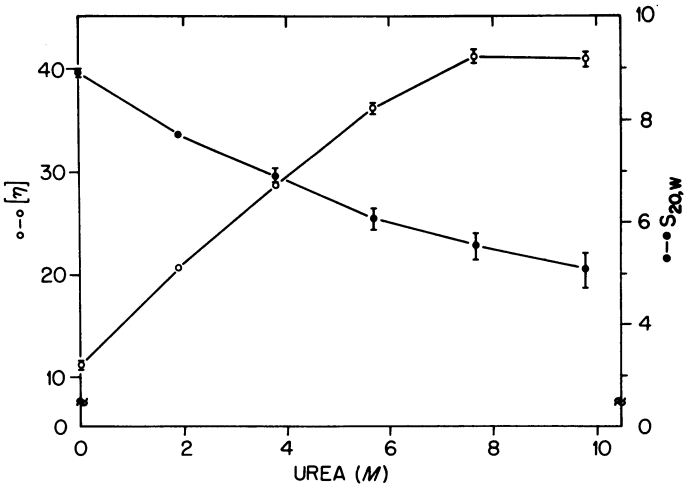

Figure 2. Hydrodynamic properties of ν_1 as a function of urea molarity. [η] units are cm^3/g; sedimentation coefficients corrected to $S_{20,w}$

weight for ν_1 of 216,000 daltons (10), for the various urea samples; corresponding prolate ellipsoid axial ratios are also shown. In 0.2 mM EDTA alone, $\beta = 2.12 \times 10^6$, which is (perhaps fortuitously) precisely the theoretical value for a hard sphere. β definitely appears to increase with increasing urea concentrations up to 4–6 M urea, then levels off and falls to a value below the theoretical lower limit of 2.12×10^6 near 10 M urea; however, we note that the upper error limit for the 9.84 M urea point is higher than the theoretical lower limit of β.

From the above results, it appears that ν_1 particles undergo some increase in asymmetry with increasing urea concentration, at least to those on the order of 6–8 M. At concentrations above 8 M, the apparent decrease in β could be attributed either to an increasing particle symmetry with further swelling or to the dissociation of a fraction of the particles leading to a somewhat reduced weight-average molecular weight. In support of the former, recent theoretical calculations (30) indicate that β values as low as 2.08×10^6 are predicted for a solvent-permeable, spherically shaped aggregation of hard spheres. Such a model might be generally consistent with a highly swollen but undissociated ν_1 structure. On the other hand, the latter hypothesis is clearly more consistent with our own electron microscopic observations. Because of the insensitivity of β to details of the model, the present hydrodynamic data are not sufficiently exact to permit a clear distinction to be made.

The final four columns of Table 1 show the effective particle volume, v_p; the

major and minor semiaxes, a and b; and the estimated hydration parameter, δ_1. These quantities are calculated from the observed $[\eta]$, β, and ϕ^* values using standard methods (31). Because of the uncertainties in the highest urea sample noted above, no calculations of these quantities were attempted at 9.84 M urea. The physical dismensions for hydrated ν_1 in 0.2 mM EDTA are consistently larger than reported previously in 0.1 M KCl (10), but this discrepancy is probably in large part an artifact due to differences in methods of calculation. Similarly, the hydration parameter reported here is larger, presumably for the same reasons, although the particles may in fact be more highly hydrated with no supporting electrolyte. As one goes from 0 to 10 M urea, v_p and the related values of δ_1 appear to first decrease at 2 M urea and then increase monotonically with increasing urea concentration. We do not feel that these results necessarily reflect true hydration changes, however.

Thermal Denaturation of ν_1 in Urea

Aliquots of ν_1 in 0.2 mM EDTA (pH 7) were diluted into urea solutions from 0 to 10 M urea plus 0.2 mM EDTA; the UV absorbance spectra were then measured. Identical volumetric dilutions revealed only small changes in extinction at 260 nm (i.e., nonsystematic variations of ± 1.0% over the entire urea range) and only slight changes in turbidity (i.e., A_{350}).

Thermal denaturation at 260 nm of ν_1 in 0.2 mM EDTA buffer revealed a major melting transition (T_m) at about 79°C, with less prominent transitions at approximately 62, 67, and 90°C (Fig. 3A). In 10 M urea, about 80% of the hyperchromicity transition centered around a T_m of 56°C; 10–20% centered around a T_m of 79°C. At intermediate urea concentrations (especially 3 to 7 M urea) turbidity transitions [i.e., increases in A_{350} with temperature (20)] occurred at about 80°C, complicating assessment of the relative amounts of hyperchromicity during thermal denaturation in different solutions. Nonetheless, we estimate that at least 2/3 of the total hyperchromicity of ν_1 in 0.2 mM EDTA exhibited significant destabilization by increased urea molarity. To develop an interpretation of these denaturation data we have conducted a study of the denaturation of DNA of varying base composition in solutions of different NaCl and urea composition (32). Examples of the effects of urea and salt on chicken DNA are shown in Fig. 3B and C. The destabilization of DNA melting by urea is linear with urea molarity in all cases. Our observations on DNA confirm and extend previous studies in the range 0–5 M urea (26, 33, 34). The

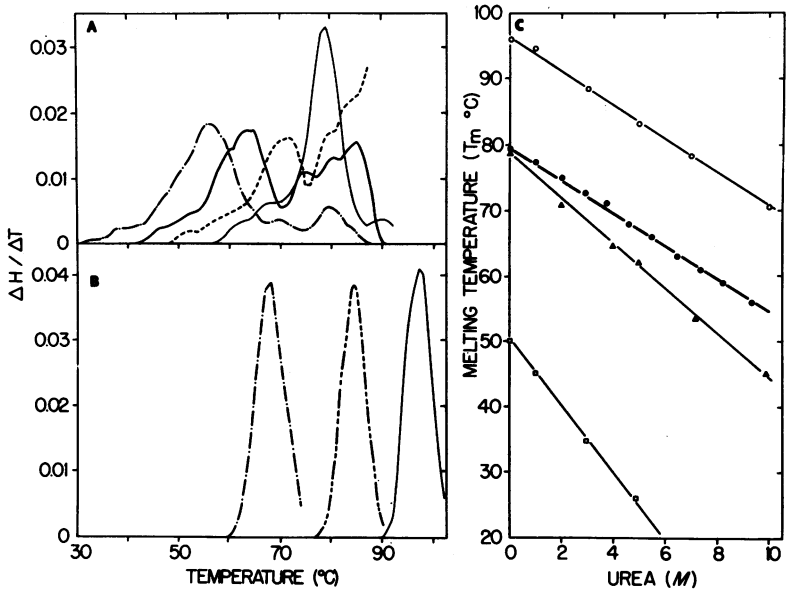

Figure 3. Thermal denaturation at 260 nm of ν_1 and chicken DNA as a function of urea molarity. All solutions contained 0.2 mM EDTA (pH 7.0). (A) Derivative melting profiles ($\Delta H/\Delta T$) of ν_1 in 0 (——), 4 (-----), 7 (-----), and 10 M (—— o ——) urea. (B) Derivative melting profiles of DNA in 2 M NaCl containing 0 (——), 5 (— - - —); and 10 M (—— o ——) urea. (C) Linear dependence of T_m on urea molarity: ν_1 in EDTA only (●); chicken DNA in 2 (O), 0.065 (△), and 0.0015 M (□) NaCl.

magnitude of the urea effect, however, is a function of NaCl concentration (Table 2). When these data are compared with ν_1, it is striking that a significant fraction (>2/3) of the DNA associated with histones in ν_1 in 0.2 mM EDTA shows the same magnitude of urea destabilization as DNA in 2 M NaCl. The melting temperature of ν_1 in 0.2 mM EDTA is the same as the melting temperature of DNA in 0.065 M NaCl, but ν_1 exhibits a destabilization by urea ($\Delta T_m/\Delta M$) comparable to that at the higher (2.0 M) NaCl concentration. Chicken DNA dissolved in 0.2 mM EDTA has a T_m similar to that in

TABLE 2. Urea Effect on T_m

Sample	Solvent	$\Delta T_m/\Delta M$ Urea (°C/M)
Chicken DNA	0.0015 M NaCl	- 5.0
Chicken DNA	0.065 M NaCl	- 3.4
Chicken DNA	2.0 M NaCl	- 2.5
ν_1	0.2 mM EDTA	- 2.5

0.0015 M NaCl (Fig. 3C) and exhibits comparable destabilization by urea. Thus, even though the thermal denaturation of DNA is considerably stabilized by association with the histones in ν_1, both naked DNA and histone-associated DNA can exhibit comparable susceptibility to the destabilizing effects of urea on thermal denaturation.

Circular Dichroism of ν_1 and Inner Histones in Urea

Numerous studies on the circular dichroic spectra of chromatin in low-ionic-strength buffers (22, 25, 26, 35–43) have revealed "suppression" of asymmetric absorbance in the region 260–300 nm relative to DNA under the same solvent conditions. More recent papers (44-46) have documented that the suppression is even more marked in isolated monomer ν bodies.

Recent studies employing laser Raman spectroscopy and circular dichroism on ν_1, DNA, and inner histones (47–48) have indicated that within the intact ν_1, in 0.2 mM EDTA, the DNA is predominantly in the "B-genus" and the histones consist of ~50% α-helix, ~50% "random" (i.e., not a regular 2° structure), and negligible amounts of β pleated-sheet. The large content of α-helix results in significant asymmetric absorbance at wavelengths below 240 nm.

For the present investigation circular dichroic spectra were obtained on ν_1 as a function of urea molarity (Fig. 4A). These spectra reveal that with increased urea molarity, the dominant α-helix contribution (<240 nm) is dramatically weakened, and the suppressed spectrum (260–300 nm) becomes like naked DNA. In comparison, in 0.1 M NaCl chicken DNA exhibited: $[\theta]_{P,240} = 9.1 \times 10^3$; $[\theta]_{P,280} = 7.4 \times 10^3$. Indeed, between 220 and 300 nm, ν_1 in 8, 9, and 10 M urea yielded circular dichroic spectra virtually superimposable on spectra of DNA in 0.1 M NaCl buffer (Fig. 4A).

The influence of urea on the circular dichroic spectra of inner histones in 2 M NaCl is represented in Fig. 4B. The strong α-helix contribution is markedly reduced in high urea–2 M NaCl. Similar experiments on the denaturing effects of urea were performed on the inner histones in 0.4 and 1.0 M NaCl.

Analysis of $[\theta]$ for ν_1 and inner histones as a function of urea molarity (Fig. 5B and D) demonstrates that the disruptive effect of urea on the α-helix is highly cooperative. For ν_1 in 0.2 mM EDTA, 50% denaturation of $[\theta]_{223}$ occurs at approximately 5 M urea, with no significant effect up to 3 M urea and complete denaturation by 7 M urea. The denaturation effect of urea on the inner histones is sensitive to NaCl molarity. Decreasing the NaCl concentration lowers the urea

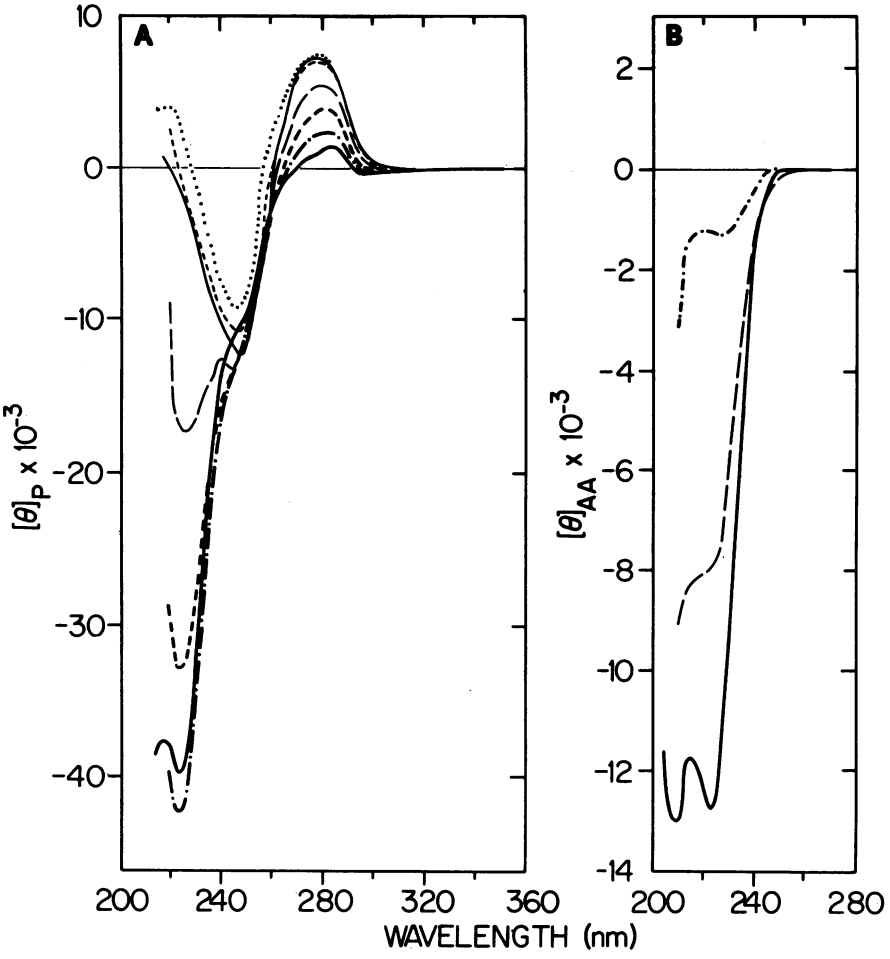

Figure 4. Circular dichroism of ν_1 and of inner histones at different urea concentrations. (A) ν_1 in 0 (——), 2 (——o——), 4 (-----), and 6 M urea (— — —), (thin lines), and in 8 (-----) and 10 M (——) urea (thick lines), containing 0.2 mM EDTA (pH 7.0). Chicken DNA (.....) in 0.1 M NaCl $[\theta]_P$, molecular ellipticity per mole nucleotides. (B) Inner histones in 0 (——), 6(--- ---), and 9 M (—o—) urea containing 2M NaCl, 0.1 mM DTT, and 10 mM Tris (pH 7.0) $[\theta]_{AA}$, molecular ellipticity per mole histone amino acid residues.

concentrations required for onset, 50%, and 100% completion of denaturation. The breadth of transition, in urea molarity, remains quite similar for ν_1 and for inner histones (i.e., a range of molarity of urea equal to about 4 between onset and completion of denaturation). Presumably, the increased electrostatic repulsion of the histone basic residues augments the disruptive effects of urea. Sufficient reduction in NaCl molarity itself leads to a small decrease in α-helix content (Fig. 5C), possibly

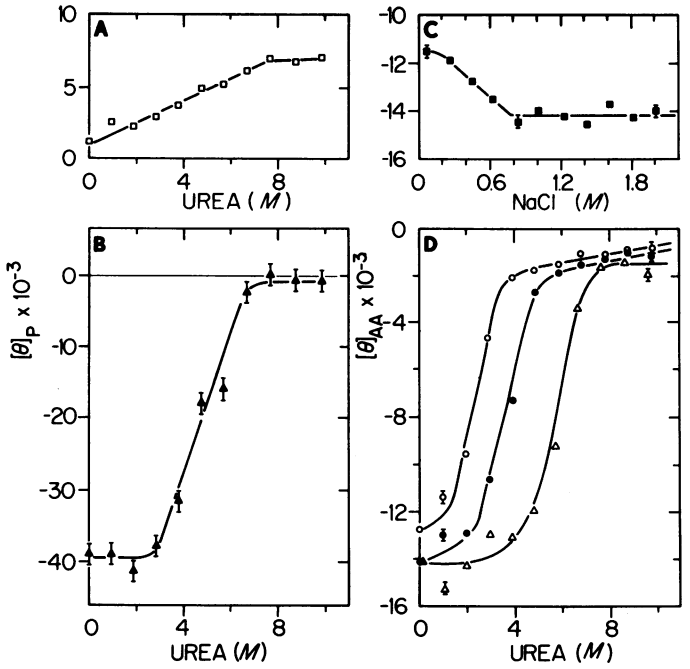

Figure 5. Circular dichroism of ν_1 and inner histones at different urea concentrations. (A) $[\theta]_{P,280}$ for ν_1. (B) $[\theta]_{P,222}$ for ν_1. (C) $[\theta]_{AA,223}$ for inner histones as a function of NaCl molarity. (D) $[\theta]_{AA,223}$ for inner histones as a function of urea molarity, in 0.4 (O), 1 (●), and 2 M (△) NaCl containing 0.1 mM DTT and 10mM Tris (pH 7.0).

corresponding to the dissociation of tetramer (H4, H3, H3B, H2A) into dimers (H4, H3) and (H2B, H2A) (11). However, the salt dependence of urea denaturation is clearly seen above 0.8 M NaCl, where $[\theta]_{223}$ is independent of NaCl concentration. Further, it is worth noting that Figs. 5B and D indicate that the inner histones associated with DNA in ν_1 behave toward the disruptive effect of urea much as they do in an environment of about 1.5 M NaCl.

Analysis of $[\theta]_{280}$ for ν_1 as a function of urea molarity demonstrates loss of the suppressed spectrum (Fig. 5A) but does not indicate any clear cooperation similar to that observed for the denaturation of the α-helix. Several previous studies (25, 26, 43–45) have observed the disruptive effects of urea on the circular dichroic spectra of chromatin or ν bodies up to 5 or 6 M urea. The present investigation extends these earlier observations to higher urea molarities and, in so doing, underscores the cooperative loss of α-helical structure due to urea.

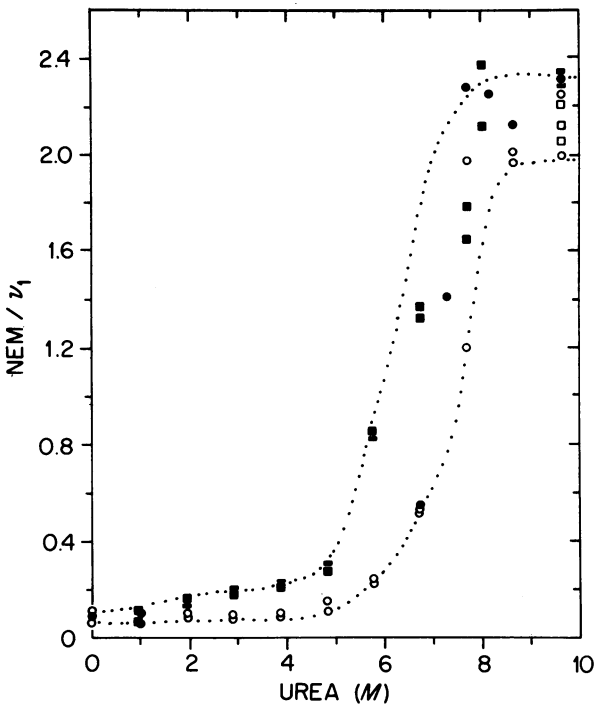

Figure 6. Reaction of ^{3}H-labeled NEM with ν_1 as a function of urea molarity. Samples: ν_1 (■), different preparations of total ν_1 (O, O, □). NEM/ν_1 measured in moles of NEM/mole of ν_1.

NEM Labeling of ν_1 in Urea

NEM is a highly specific reactant to protein sulfhydryl groups and has been employed extensively to probe the accessibility of cysteine residues in native and denatured states (49).

In the present investigation the reactivity of [^{3}H]NEM with ν_1 in 0.2 mM EDTA was markedly affected by urea molarity (Fig. 6). ν bodies exhibited little reaction until concentrations of > 5 M urea were reached, dramatically increasing to about 2.2 moles NEM/mole ν_1 by 8 M urea. Based upon current models of ν_1 which postulate that each ν body has two H3 molecules and upon sequence studies (3) indicating that there is one cysteine residue per chicken erythrocyte H3, the observed extent of reaction is in very good agreement. Sodium dodecyl sulfate gel electrophoresis of the histones following reaction in 10 M urea plus 0.2 mM EDTA demonstrated that most of the [^{3}H]NEM migrated in the region of H3 histone. A fraction of the cpm (i.e., ~20% of the total radioactivity) migrated just slower than H4. It is not clear whether

this represents reaction with H3 breakdown products or low levels of nonhistone proteins associated with the ν bodies.

The present study suggests that the H3 cysteine groups are "buried" in the "native" state. Since the onset and midpoint of NEM reaction are somewhat higher than the onset and midpoint of α-helix denaturation, it appears likely that significant loss of histone secondary (2°) structure must precede exposure of sulfhydryl groups.

Preliminary results in this laboratory indicate that the H3 cysteine residues also become reactive when ν_1 is in 1.5 –2.0 M KCl or NaCl. Since the native 2° structure of the histones is preserved in such solutions (11, 47, 48), denaturation of α-helix is not necessary for exposure of the thiol groups. Rather, it is possible that the H3 cysteine residues are "buried" between the surfaces of the associated histone tetramers and DNA. Further, the laser Raman studies (48) of ν_1 and inner histones indicate that the thiol groups are not disulfide bonded, but are apparently H-bonded in the native histone configuration.

Electron Microscopy of ν_1 in Urea

Monomer ν bodies revealed dramatic ultrastructural changes at urea concentrations ≥4 M (Fig. 7). The morphology of ν_1 dispersed in 2 M urea plus 0.2 mM EDTA was virtually unchanged from that of ν_1 in 0.2 mM EDTA. From 4 to 10 M urea, the particles exhibited evidence of swelling and unraveling, ultimately resembling short rodlets (in 8 and 10 M urea). Measurements of the ν_1 in 6 M urea (Fig. 7C) indicated that the particles had enlarged to rings or crescents of 200–250 Å diameter, with the circumferential uranyl staining equal to 322 ± 11 Å. The rodlets in 10 M urea were 379 ± 17 Å long, corresponding to approximately 80% of the length expected for 140 base pairs of DNA. Similar observations on the disruptive effects of urea on the ultrastructural morphology of ν_1 have been previously reported (50). This loss of characteristic morphology at ≥4 M urea is in agreement with our previous study (51) on the loss of chromatin periodic structure by 5 M urea.

DISCUSSION

A number of previous studies have documented the disruptive effects of urea on chromatin as assayed by hydrodynamics (23, 24), thermal denaturation (26, 34, 43), circular dichroism (25, 26, 43–45), electron microscopy (50–52), nuclease digestion (53, 54) and low-angle X-ray scattering (51). Although few of these investigations

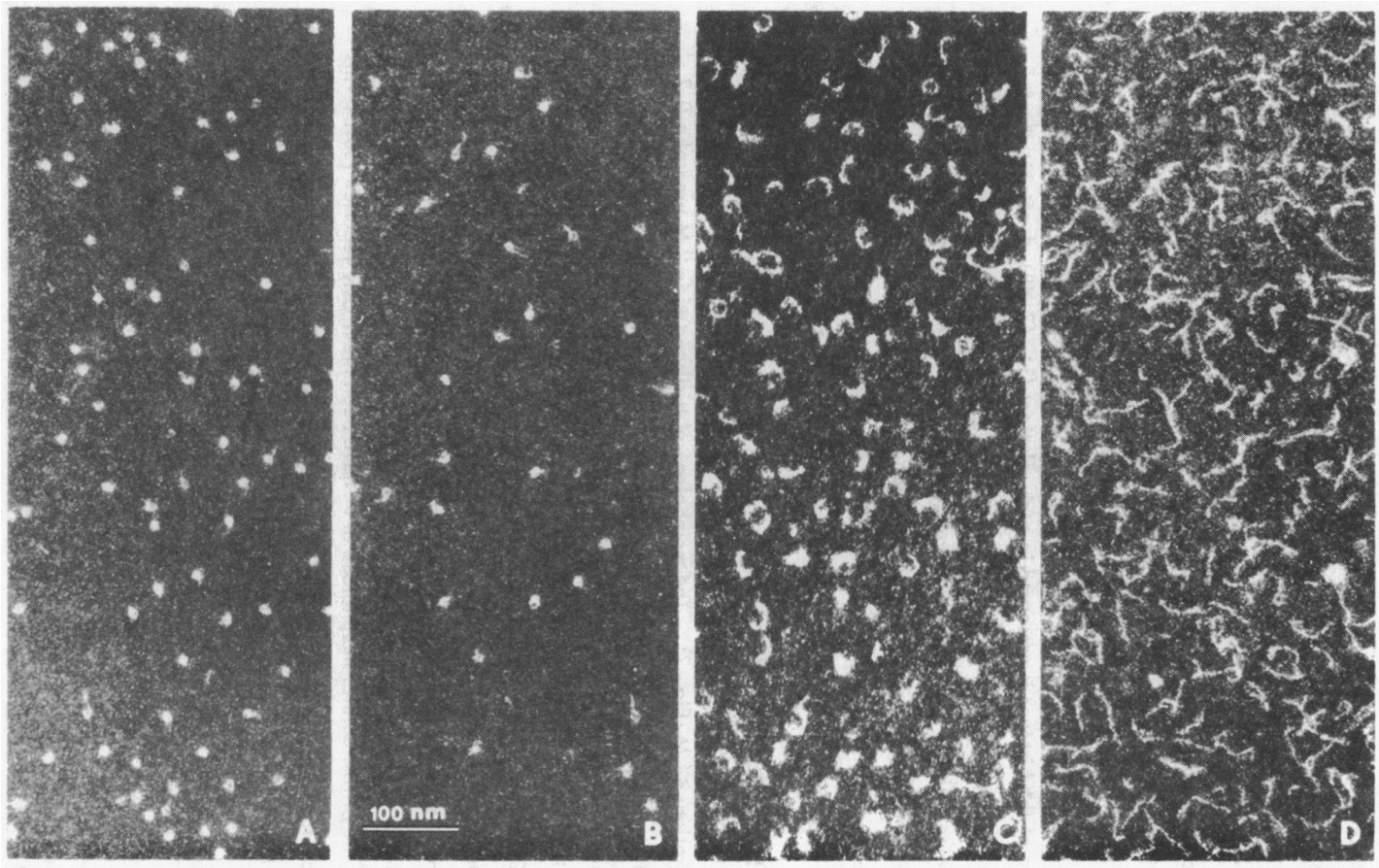

Figure 7. Dark-field electron microscopy of ν_1 exposed to varying urea concentrations. ν_1 in 0.2mM EDTA (pH 7.0) plus (A) 0, (B) 2, (C) 6, and (D) 8 M urea.

considered structural changes above 5 M urea, the present data indicate large changes between 5 and 10 M urea. Furthermore, the present investigation employs a variety of biophysical techniques to study the effects of urea upon well-characterized preparations of ν_1 (9, 10).

Increased urea concentration produces on ν_1 diverse physical effects which fall into two categories: noncooperative (i.e., gradual effects, occurring over most or all of the urea concentration range) and cooperative effects (i.e., abrupt structural transitions occurring over a narrow range of urea molarity). Noncooperative effects of urea were illustrated in hydrodynamics (Fig. 2), melting (Fig. 3), and circular dichroism ($[\theta]_{P,280}$; Fig. 5A); cooperative transitions were illustrated in circular dichroism ($[\theta]_{223}$; Figs. 5B and D), thiol reactivity (Fig. 6), and ultrastructure (Fig. 7).

Consideration of current models of ν_1 (3, 4, 9, 55), namely, a DNA-rich shell surrounding a protein-rich core, furnishes a basis for interpreting the diversity of urea effects. Noncooperative changes appear to relate to the stability and conformation of the DNA and to the overall size and shape of the particles — presumably all are a consequence of transitions in the outer DNA-rich shell. Cooperative changes could principally reflect structural transitions of the protein-rich core of ν_1.

Our current conception of the diverse effects of increasing urea (and of high NaCl) on ν_1 is schematically represented in Fig. 8. In the "native" state ~140 nucleotide pairs of DNA are shown wrapped around the outside of the α-helical-rich globular domains of (H4, H3, H2A, H2B)$_2$ close-packed with dihedral-point-group symmetry (9). A postulated true dyad axis (9, 57) relates the heterotypic tetramers and relates the portions of DNA associated with each heterotypic tetramer. The path of the DNA is arbitrary in this scheme, but preserves the equivalent single superhelical turn (58) and the postulated dyad axis. The H3 thiol residues are buried and H-bonded to some other histone residues (48). In "low urea" concentrations (3–4 M) the ν_1 have swollen and increased particle asymmetry, DNA has become more B-like, the α-helical content remains essentially unchanged, and the H3 thiols remain buried. Two possible low urea states are diagrammed — one with the histone globular regions not swollen (i.e., maintaining native 2°, 3°, and 4° structure), the other indicating a swelling of the globular regions (i.e., loss of 3° and 4° structure) with no loss of 2° structure. The present observation that ν_1 treated with 2 M urea, and prepared for electron microscopy by brief washing in solutions not containing urea, looks similar to untreated ν_1 suggests that the preservation of histone 2° structure permits rapid return to normal morphology.

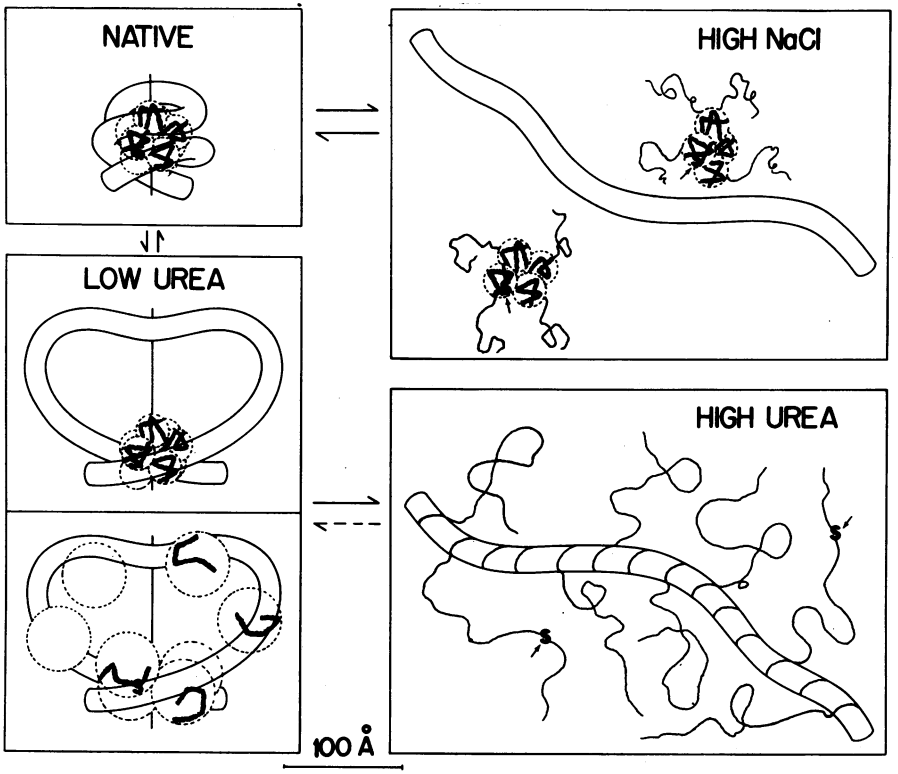

Figure 8. Scheme of the effects of urea and of high NaCl on ν_1. The postulated true dyad axis is represented as a vertical line penetrating the "native" and "low urea" structures. The equivalent single superhelical turn of DNA per ν_1 is conceived of as being stabilized at the ends of the DNA fragment, presumably by interaction with H3 and H4 (56). In the native ν_1 model, additional folding of the DNA can be accomplished while the single equivalent superhelical turn is still maintained, by twisting the DNA clockwise or counterclockwise around the particle dyad axis and binding to the histone core. α-helical segments are represented in the globular domains of the histone molecules. Two different possible low urea states are represented with the DNA coil untwisting and expanding into a more asymmetric and open structure. In "high urea," the histone globular regions have become random cells, while the basic regions are still attached to the extended DNA rod. The H3 thiol groups are exposed and reactive (⟶). In "high NaCl" (pH 7.0), ν_1 dissociates into the DNA fragment and two heterotypic tetramers.

In "high urea" solutions (8–10 M) particle swelling and unraveling has been completed, the DNA looks by circular dichroism like its "normal" B configuration (except that its T_m is still considerably greater than that of naked DNA in the same low-ionic-strength buffer), the α-helix is disrupted, and the H3 thiol groups are exposed and reactive. Preliminary studies have indicated that ν_1 treated with urea can exhibit some renaturation if the urea is dialyzed out prior to examination. Also shown in Fig. 8 is

the probable consequence of treatment of ν_1 with "high NaCl" (e.g., 2 M NaCl): the particles dissociate into heterotypic tetramers and DNA, 2° and 3° structure of the histone globular regions are retained, and the H3 thiol groups are exposed, although still H-bonded. Reconstruction of ν_1 from 2 M NaCl by gradient dialysis to 0.2 mM EDTA can be performed with extremely high yields.

The present suggestion that the different domains of a ν body (i.e., the DNA-rich shell and the protein-rich core) exhibit different responses to a simple chemical perturbant (urea) offers a basis for viewing the apparently altered conformation of transcriptional chromatin (6–8). Modulations of the DNA-rich shell (e.g., expansion and destabilization of the DNA) by nonhistone proteins or histone modification might occur with only minor alterations in the conformation of the protein-rich core, permitting rapid renaturation of the transcriptionally inactive chromatin configuration.

ACKNOWLEDGEMENTS

We are grateful to E. B. Wright and M. Hsu-Hsie for superb assistance. This research was sponsored by the Energy Research and Development Administration under contract with Union Carbide Corporation, by an NIH research grant to DEO (GM 19334), by an NSF research grant to ALO (PCM 76-01490), and by an NIH research grant to WEH (GM 17436).

This investigation has been published in preliminary form in the abstracts of the 21st Annual Meeting of the Biophysical Society (59).

This research was sponsored by the Energy Research and Development Administration under contract with Union Carbide Corporation, by a National Institute of Health research grant GM 19334 to DEO, by a National Science Foundation research grant PCM 76-01490 to ALO, and by a National Institute of Health research grant GM 17436 to WEH.

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