Relationship between Protein and DNA Structure in Calf Thymus Chromatin. I. Compositional Aspects†

Sue Hanlon,* Roger S. Johnson, and Aurelia Chan

ABSTRACT: Structural transformations in the DNA constituent of calf thymus chromatin have been followed by circular dichroism as proteins are removed from the native complex. Histones have been dissociated in sequentially different manner by subjecting the chromatin to increasing concentrations of NaCl and of sodium deoxycholate, according to the procedure of Ohlenbusch *et al.* (Ohlenbusch, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967), *J. Mol. Biol. 25,* 299) and of J. E. Smart and J. Bonner ((1971a), *J. Mol. Biol. 58,* 651). Complexes containing mainly the arginine rich histones were also prepared, using the method of R. J. Clark and G. Felsenfeld ((1972), *Nature (London) 240,* 226). The spectral properties above 250 nm of all these residual complexes as well as the intact controls can be adequately accounted for in terms of a mixed population of B and C conformations of the DNA base pairs. The per cent B character of the residual complexes in the NaCl and the sodium doxycholate experiments remains essentially that of the intact controls (36% B) until the protein content (W) has been reduced to *ca.* 0.9 g/g of DNA. Below this value, a linear increase in the per cent B occurs until a value of 100% is reached at 0.33 g/g. Reference to literature data on the histone content of chromatin complexes prepared under comparable experimental conditions reveals that the linear increase in the per cent B below $W = 0.9$ parallels the removal of the last half of the histone remaining in complex. Statistical analyses reveal that there is no significant differences in the dependence of the per cent B character of the complex on the protein or histone content of the complexes prepared by the two methods, despite the differences in the nature of the compositional classes removed in the two types of experiments. The arginine-rich histone complexes exhibit a per cent B which is less than 100% but higher than the value of intact purified calf thymus chromatin, thus revealing that this histone class cannot be solely responsible for the C character of the DNA in chromatin. These facts taken together lead to the conclusion that histone proteins are responsible for the maintenance of the C character of intact chromatin, but there is no one compositional class uniquely responsible for this function.

On the basis of the results of CD,[1] nuclease digestion, and thermal melting experiments, we have recently proposed a model for calf thymus chromatin in which the DNA constituent is in two discrete conformational states (Hanlon *et al.*, 1972; Johnson *et al.*, 1972). One population of base pairs, *ca.* 30–50% (depending on the method of preparation and solvent), is accessible to all solvent components and is in the B conformation. The remaining base pairs are protected from the aqueous environment and are in the C conformation. Protein is associated with both conformations. It is clearly crucial, in fact, to the maintenance of the C form since removal by nonspecific methods such as Pronase digestion results in the complete conversion of all of the base pairs to the B form.

With this background, the question to which we have next addressed ourselves is whether there is a unique class of proteins responsible for the maintenance of this C conformation in chromatin. To answer this, we have employed an approach identical with that of Smart and Bonner (1971a–c) who followed several properties of chromatin as proteins were dissociated by reagents which remove histones in different sequential orders. We have measured the circular dichroism properties of the residual macromolecular complexes which result from subjecting calf thymus chromatin to increasingly higher concentrations of NaCl and sodium deoxycholate, the reagents employed by Smart and Bonner in their experiments.

In addition, we have also examined the residual complex consisting of DNA and mainly the arginine-rich histones, prepared from intact chromatin by the method of Clark and Felsenfeld (1972). The changes observed in the CD spectra of these complexes have been analyzed in terms of the conformational properties of the DNA and the protein components. In the first of these two papers, we have compared the various methods in terms of the compositional effects of proteins in complex on DNA structure. In paper II of this series (Hanlon *et al.*, 1974), we have explored the conformational relationships between the DNA and the protein constituents of chromatin. A preliminary report of these investigations has previously appeared (Hanlon *et al.*, 1973).

Experimental Section

Purified chromatin (TNH)[1] was prepared from frozen calf thymuses by method i of Maurer and Chalkley (1967). The details of the tissue collection as well as the physical and chemical properties of the product have been previously described (Hanlon *et al.*, 1972; Johnson *et al.*, 1972). Most of the preparations used in this study were subjected to further low-speed blending, according to the method of Shih and Fasman (1970), in order to reduce the molecular size. No noticeable differences in optical and thermal melting characteristics were observed between the sheared and the unsheared preparations. No distinction is therefore made in the text between sheared and unsheared material.

For the protein dissociations effected by increasing concentrations of NaCl, we followed a slightly modified version of the procedure of Ohlenbusch *et al.* (1967). In our experiments,

† From the Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, Illinois 60612. *Received November 21, 1973.* This work was supported by a grant from the National Science Foundation (GB 24550-A). This work was taken in part from the Ph.D. Thesis of R. S. J.

[1] Abbreviations used are: CD, circular dichroism; NaEDTA, the sodium salt of ethylenediaminetetraaceticacid; TNH, purified calf thymus chromatin.

concentrated stock solutions of intact TNH were diluted with water and 4.0 M NaCl to a concentration of *ca.* 1×10^{-3} M TNH (on a nucleotide basis) and a desired final NaCl concentration. After stirring these solutions from 2 to 24 hr, the dissociated proteins were separated from the residual macromolecular complex by carefully layering the solution over 1 ml of 1.7 M sucrose in either a SW 39L or a type 50 fixed angle rotor at 36,000 rpm for 24 hr. All but the final 1-ml fraction in the bottom of the tube was carefully removed and discarded. The bottom 1-ml fraction was then mixed with any visible pellet and diluted with an appropriate low to moderate ionic strength solvent containing NaCl and Tris buffer. The particular choice of solvent was dictated by the desire to avoid low ionic strength denaturing conditions for those complexes which had been stripped of appreciable amounts of histone proteins.

In a few instances, the gelatinous pellets obtained in the final purification step of the Maurer and Chalkley (1967) procedure were dialyzed directly against the desired NaCl concentration for 20 hr. The separation of the dissociated proteins from the macromolecular complex proceeded as described above. Invariably, the residual complex obtained at a given NaCl concentration by this latter procedure had a higher protein to DNA weight ratio (W) compared to that obtained from the first procedure.

The removal of protein by dissociation with sodium deoxycholate followed, without modifications, the procedure of Smart and Bonner (1971a). A stock solution of 0.25 M sodium deoxycholate was prepared by titrating deoxycholic acid (Calbiochem) with NaOH to a final pH of 8.5–9. Thin-layer chromatography (Rouser *et al.*, 1967) revealed that the deoxycholic acid used in these experiments consisted of less than 10% impurities having an R_f similar to cholic acid. The final dialysis step to remove the reagent from the macromolecular complex was conducted against 2.5×10^{-3} M Tris (pH 8), as called for in the procedure.

The complexes consisting of DNA and mainly the arginine-rich histones, III and IV, were prepared from purified chromatin by following, with minor modifications, the first step of the procedure of Clark and Felsenfeld (1972). Stock chromatin solutions at 3×10^{-3} M in 2.5×10^{-3} M Tris (pH 7.4) were diluted with an equal volume of a solvent consisting of 10 M urea, 2×10^{-3} M $CaCl_2$, and 2.5×10^{-3} M Tris (pH 7.5). This solution was stirred for 2 hr and then dialyzed overnight against 5 M urea–1×10^{-3} M $CaCl_2$–2.5×10^{-3} M Tris (pH 7.5). After this step, the chromatin solution was put through a sulfoethylcellulose column (SE cellulose, Bio-Rad) equilibrated with the dialysis fluid, in the manner specified by Clark and Felsenfeld (1972). The deproteinized chromatin emerged at or near the void volume of the column. The peak fractions were pooled and dialyzed against several changes of 0.01–0.02 M NaCl–0.01 M Tris (pH 7.5). In some instances, the initial dialysis fluid also contained 0.005 M NaEDTA.[1] This step appeared to have minimal effect on the properties of the final dialyzed product.

The complexes obtained by this procedure generally had a protein to DNA weight ratio, W, of 0.50 ± 0.05. This is reasonably close to the value 0.45 expected on the basis of a fractional content of arginine-rich histones of 0.39 (average) (Hnilica *et al.*, 1966) and a total histone to DNA weight ratio of 1.14 (Bonner *et al.*, 1968a,b). Hence, the second purification step involving chromatography on DNA-cellulose, called for in the original procedure (Clark and Felsenfeld, 1972), was correspondingly omitted. In this way, we hopefully ensured the fact that the complexes contained the full complement of arginine-rich proteins originally present in the intact chromatin preparations from which they were derived.

The complexes resulting from these various procedures are described in the text as residual complexes or residual TNH. The procedures themselves are referred to as the NaCl, the sodium deoxycholate, and the Ca–urea procedure, respectively. Control chromatin solutions, put through the same procedures in the absence of dissociating reagents, are referred to as intact chromatin or intact TNH.

In order to qualitatively confirm the patterns of histone removal by NaCl and sodium deoxycholate, the histones of a few complexes were extracted with 0.2 M H_2SO_4. Three volumes of cold ethanol were then added to the extract and the mixture was immersed in an acetone–Dry Ice bath for *ca.* 5 min or allowed to stand at −10° overnight. The histone precipitate was recovered by a quick centrifugation (*ca.* 3 min at 10,000 rpm) and redissolved in a few drops of 10 M urea. The resulting solutions were then applied to polyacrylamide gels and electrophoresed according to the method of Panyim and Chalkley (1969). Protein bands were stained with 0.02% Amido-Schwarz in 7% CH_3COOH–40% CH_3CH_2OH. Gels were destained in the same solvent, and the various histone fractions were identified by reference to the literature as well as by the pattern of bands obtained with commercial samples of calf thymus histones. This visual inspection provided only a crude estimate of whether a given histone fraction still remained in appreciable quantities or had been dissociated appreciably by the given concentration of NaCl or sodium deoxycholate.

Concentrations of the intact and residual complexes were determined from their absorption spectra, after correcting for light scattering by the method of Oster (1948). These concentrations are reported on a molar nucleotide basis. An extinction coefficient, E_P^{TNH}, of 6750 M^{-1} cm^{-1}, previously determined for intact chromatin (Johnson *et al.*, 1972) was used for all complexes. Differences due to the removal of protein from the residual complexes were ignored since it would contribute, at most, only a 2% error.

Protein content of all complexes was determined spectrally by the ratio of the absorbance at 230 and 260 nm using the equation of Tuan and Bonner (1969) as previously described (Johnson *et al.*, 1972). Protein content is expressed in the text and in legends as a weight ratio, W, of protein to DNA in complex. A few of the protein content values were also checked by Lowry determinations (Lowry *et al.*, 1951) using bovine serum albumin (Sigma) as a standard. These chemical determinations confirmed the fact, as previously observed (Johnson *et al.*, 1972), that the W values determined spectrally were valid within the combined experimental error of both measurements (±0.05 g/g).

Absorption spectra were measured in a Cary Model 14CMR recording spectrophotometer equipped with thermostated adaptors. Temperature was maintained by a Haake circulating water bath and monitored by a Telethermometer Bridge and Probe assembly manufactured by Yellow Springs Instrument Co. Unless otherwise specified, spectra were obtained at 25° in quartz cuvets of 1-cm path.

Circular dichroism spectra were determined at 27° in a 1-cm quartz cell with a Cary Model 60 recording spectropolarimeter equipped with a 6001 CD attachment. All spectral data are given in terms of molecular ellipticities, based on the nucleotide molar concentration in solution.

Calculations of the percentage of B, C, and A conformations of DNA present in the complexes were performed in the manner described in our earlier publications (Hanlon *et al.*, 1972; Johnson *et al.*, 1972). Basically, the CD spectrum of a given complex above 260 nm, which reflects only the DNA contribution, is assumed to consist of a linear combination of the contri-

butions of the B and C conformations. The observed molecular ellipticities, $[\theta]_\lambda^{obsd}$, at eight wavelengths across the positive band are thus assumed to be related to the fractions of base pairs in the B (f_B) and the C (f_C) conformation by

$$[\theta]_\lambda^{obsd} = f_B[\theta]_\lambda^B + f_C[\theta]_\lambda^C = f_B[\theta]_\lambda^B + (1 - f_B)[\theta]_\lambda^C \quad (1)$$

where $[\theta]_\lambda^B$ and $[\theta]_\lambda^C$ are the reference ellipticities at wavelength λ of the B and the C conformations of protein-free DNA. The latter were obtained in the manner previously described (Hanlon *et al.*, 1972) using the spectra of Tunis-Schneider and Maestre (1970) for identification purposes. Equation 1 can be solved for f_B at each of the several wavelengths and these eight values then averaged to yield an average f_B, which is reported as a percentage, % B.

The presence of a third component, the A form, can be tested by a linear least-mean-squares averaging of the expression

$$[\theta]_{\lambda_i}^{obsd} = f_A[\theta]_{\lambda_i}^A + f_B[\theta]_{\lambda_i}^B + f_C[\theta]_{\lambda_i}^C = (1 - f_B - f_C)[\theta]_{\lambda_i}^A + f_B[\theta]_{\lambda_i}^B + f_C[\theta]_{\lambda_i}^C \quad (2)$$

where f_A is the fraction of bases in the A conformation and $[\theta]_{\lambda_i}^A$ is the ellipticity of the reference A spectrum at wavelength λ_i.

This averaging procedure, applied in this laboratory for a subsidiary problem (S. Hanlon, S. Brudno, T. Wu, and B. Wolf; manuscript in preparation), expresses the sum of the squares of the residuals as

$$R = \sum_{\lambda_i} \{[\theta]_{\lambda_i}^{obsd} - (1 - f_B - f_C)[\theta]_{\lambda_i}^A - f_B[\theta]_{\lambda_i}^B - f_C[\theta]_{\lambda_i}^C\}^2 \quad (3)$$

thus forcing the fractions of all components to sum to 1. This expression is then differentiated with respect to f_B and f_C. The corresponding partial derivatives are set equal to 0 in order to minimize the sum of the squares. The resulting two equations can then be solved unambiguously for f_B and f_C. The fraction of bases in the A conformation is obtained from the relationship, $1 = f_A + f_B + f_C$.

Statistical analyses were performed by standard methods (Snedecor and Cochran, 1967). In the linear regression analyses, the dependent variable was generally taken as W, for W values below 1.00, since this variable contained the greatest error in that range. Above $W = 1.00$, this procedure was reversed. Although small differences in the characteristics of the linear regressions were observed if the independent variable was consistently W over the entire range of W values, the conclusions drawn about the comparative behavior of the complexes resulting from the NaCl and the sodium deoxycholate procedures were unaffected as long as a consistent procedure was followed in the treatment of data from both sets of experiments.

Results and Discussion

As noted in previous experiments (Johnson *et al.*, 1972), the dissociation of proteins from intact TNH has virtually no effect on the absorption spectrum of the complex above 245 nm. In contrast, the positive band of the CD spectrum above this wavelength changes profoundly after the weight ratio, W, of protein to DNA has been reduced to about 0.9 g/g. This point is demonstrated by the data shown in Figures 1, 2, and3.

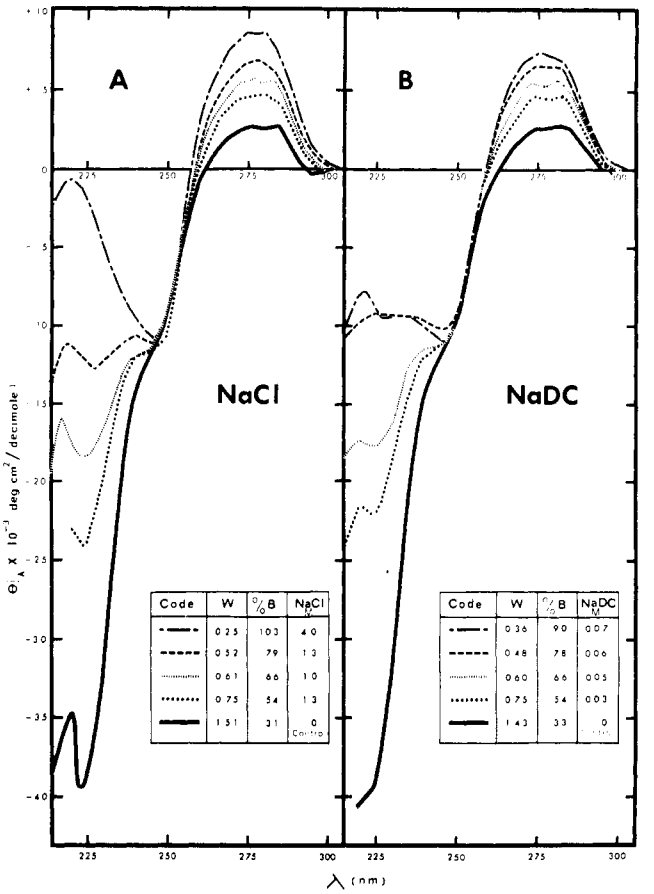

FIGURE 1: Circular dichroism spectra of intact controls and residual complexes prepared by the NaCl (A) and the sodium deoxycholate (B) dissociation procedures. The appropriate codes and characteristics of these preparations are given above in the figure. These data include the weight ratios, W, of protein to DNA, and per cent B character (calculated as described in the Experimental Section) and the molar concentration of the dissociating reagent to which the intact chromatin was subjected.

In Figure 1, we have displayed the CD spectra of some of the residual complexes from the NaCl and the sodium deoxycholate dissociation experiments. Figure 2 presents the spectra of complexes at comparable W values obtained from the three types of dissociation processes, NaCl, sodium deoxycholate, and Ca-urea. In this second figure, we have also included the reference spectra of calf thymus DNA (protein free) in the B and the C conformations. (These latter spectra were taken from the previously reported results of Hanlon *et al.* (1972).)

Although there are small differences in the shapes of the positive bands, the spectral characteristics of the various complexes exhibit a certain uniformity in behavior as their protein content is reduced. Complexes at comparable W values show equivalent decreases (on the average) in the magnitude of the negative band below 240 nm and increases in the positive band above 250 nm. The former effect might have been anticipated, in part, since the predominant contribution to the spectrum in this wavelength region is made by the protein constituent. As the protein content is reduced, one would expect the molecular ellipticity, which is on a molar nucleotide basis, to decrease. The magnitude of the decrease, which is governed by the conformation of the proteins being removed, will be treated in paper II (Hanlon *et al.*, 1974).

The increase in the positive band above 250 nm is consistent with a loss of C character of the DNA constituent, which makes the sole contribution to the spectrum in this wavelength

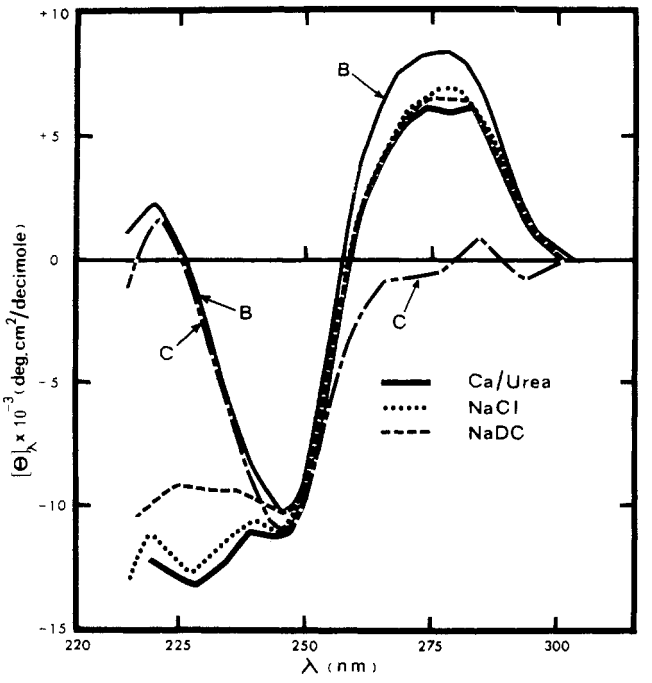

FIGURE 2: Comparison of the circular dichroism spectra of the residual complexes of similar protein content prepared by different dissociation procedures. TNH from the Ca-urea procedure, with a W of 0.50 and a per cent B of 74% (—); TNH from the NaCl procedure, with a W of 0.48 and a per cent B of 78% (• • •); TNH from the sodium deoxycholate procedure with a W of 0.52 and a per cent B of 79% (- - -). Also shown are the reference spectra of the B form (—) and the C form (---) of protein-free DNA, taken from the paper of Hanlon *et al.* (1972).

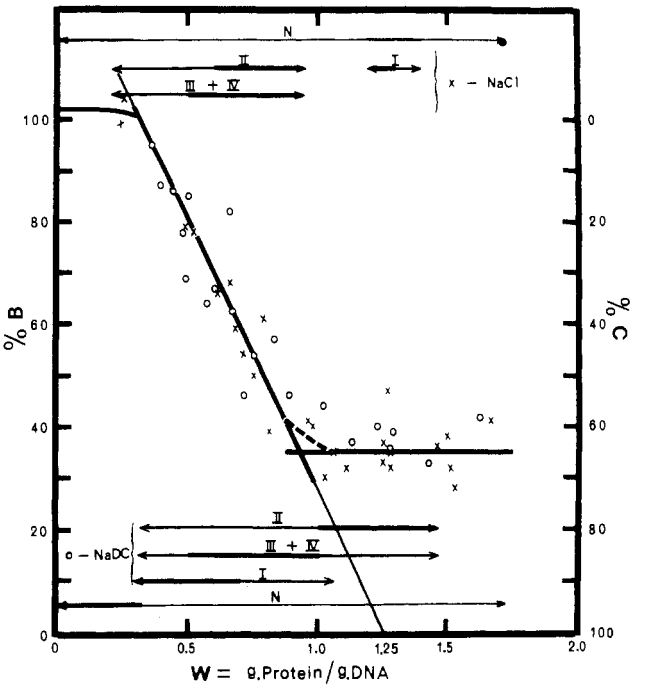

FIGURE 3: The dependence of the secondary structure of DNA on protein content of the residual complexes prepared by the NaCl and the sodium deoxycholate procedures. The percentage of bases in the B and in the C conformations are plotted on the left- and the right-hand ordinates, respectively, against the weight ratio, W, of protein to DNA on the abscissa. The crosses represent the data for the NaCl experiments and the open circles those for the sodium deoxycholate experiments. Above $W = 0.2$, the set of straight lines through the data points represents the results of the linear regression analyses of the combined data from the two sets of experiments. The set of horizontal lines above and below the data points represents the ranges over which the various protein fractions in intact chromatin dissociate in the two types of experiments. The histone proteins are designated by their conventional Roman numeral notation whereas N stands for nonhistone. The heavy portions of the lines indicate the ranges in which a sizable portion (~50%) of the given protein dissociates. These ranges were calculated from the data from the existing literature (Ohlenbusch *et al.*, 1967; Smart and Bonner, 1971a) as described in the text.

region. Detailed numerical results for some typical complexes are given in Table I. As is demonstrated by the entries in this table, the standard deviation, σ, in per cent B increases significantly as the W values decrease (with corresponding increases in per cent B). This effect is not attributable to the presence of or conversion to an A conformation as proteins are removed. Our calculations actually yield negative values for the per cent A contributions, regardless of whether the A reference spectrum of calf thymus DNA or *Escherichia coli* DNA (Tunis-Schneider and Maestre, 1970) is substituted for $[\theta]_\lambda^A$. The values given in Table I are those calculated for the *E. coli* limits, as the latter spectrum is somewhat closer to that which we have recently obtained in this laboratory by an analysis of calf thymus DNA spectra at high salt concentrations (S. Hanlon, S. Brudno, T. Wu, and B. Wolf, manuscript in preparation). In any case, these calculations indicate that the maximal per cent A cannot exceed 6% for the complexes of lower W, no matter what reference spectra are employed. This figure is within the standard deviation of the per cent B value for these complexes.

This increase in σ is more likely attributable to experimental error coupled with small errors in the reference limits, which would tend to magnify errors in per cent B as the latter value approached 100%. In addition, it is also probable that the base composition of the C regions in some of these complexes may be slightly different from the B regions. This must certainly be true, for instance, for the Ca-urea complexes, since the arginine-rich proteins are associated preferentially with G + C rich portions of the genome (Clark and Felsenfeld, 1972). As long as the average value of per cent B is utilized, however, any asymmetry in the base composition of the C and the B regions should not seriously affect the interpretation of the data.

The behavior of these average values of per cent B of the residual complexes prepared by the NaCl and the sodium deoxycholate procedures is shown in Figure 3 as a function of W. The crosses represent the data for the NaCl experiments and the open circles that from the sodium deoxycholate experiments. The set of horizontal lines drawn above and below the plot illustrates schematically the ranges in W over which various protein fractions dissociate in these experiments. These limits are based on the data taken from the literature and described in detail in the subsequent sections of this paper. The heavy portions of these lines denote those regions over which the major share of the particular fraction of protein is removed (≥50%).

The plot of per cent B *vs.* W has been approximated by a set of lines which are linear, within the limitation of our experimental error. The characteristics of the linear regression analyses appropriate for the regions of $W > 1$ and W between 0.25 and 1.0 are given in Table II. The slope and intercept of the regression functions given in this table are designated by the symbols m and b, respectively. The letter r represents the correlation coefficient while the letter t represents the t value for Student's "T" test. Although the slope and intercept for the data on the complexes from the two types of dissociation experiments differ somewhat in the range of W values between 0.25 and 1.0, a covariance analysis reveals that these differences are

TABLE I: Per Cent B Character for Various Residual Complexes of Calf Thymus Chromatin.

Ref Spectra[a]	λ (nm) 295	290	285	280	275	270	265	260
$[\theta]_\lambda^B \times 10^{-3}$	1.25	3.80	6.70	8.20	8.45	7.90	6.20	3.20
$[\theta]_\lambda^C \times 10^{-3}$	−0.70	−0.30	0.80	−0.05	−0.65	−0.80	−1.00	−2.80

Residual Complexes			% B at λ (nm)									Least-Mean-Square Anal.		
Diss. Reagent	*W*	Solvent	295	290	285	280	275	270	265	260	Av % B	σ	% A	% B	% C
0 M NaCl	1.46	2.5×10^{-3} M Tris	35.9	38.8	32.9	36.2	38.4	38.9	32.5	38.0	36.4	±2.6	0.7	37.1	62.2
0.5 M NaCl	1.25	2.5×10^{-3} M Tris	42.6	37.1	34.6	35.6	39.3	38.7	32.6	35.3	37.0	±3.2	−0.8	37.1	63.7
0.7 M NaCl	1.11	2.5×10^{-3} M Tris	35.9	35.6	29.7	31.5	36.7	32.6	24.9	27.3	31.8	±4.3	−2.7	32.7	70.0
1.0 M NaCl	0.96	2.5×10^{-3} M Tris	35.9	47.1	42.5	44.0	46.5	43.6	36.5	32.3	41.1	±5.4	−3.5	55.8	47.7
1.3 M NaCl	0.71	2.5×10^{-3} M Tris	53.8	55.1	56.6	56.0	56.5	55.3	50.3	46.7	53.8	±3.5	−3.5	55.8	47.7
2.5 M NaCl	0.49	2.5×10^{-3} M Tris	85.1	83.9	80.8	79.8	82.7	79.8	74.3	69.2	79.4	±5.3	−4.7	80.4	24.3
0 M NaDC[b]	1.29	2.5×10^{-3} M Tris	40.5	45.4	37.6	37.2	39.9	41.8	35.6	36.7	39.3	±3.2	−0.9	39.3	61.6
0.01 M NaDC[b]	1.13	2.5×10^{-3} M Tris	35.9	39.0	36.4	37.8	38.6	36.1	32.6	35.8	36.5	±2.0	−1.5	37.6	63.9
0.02 M NaDC[b]	0.89	2.5×10^{-3} M Tris	47.7	49.8	44.7	46.4	49.6	47.4	42.4	43.5	46.4	±2.7	−2.0	46.8	55.2
0.03 M NaDC[b]	0.75	2.5×10^{-3} M Tris	54.4	64.9	58.6	54.1	57.4	53.9	45.3	44.3	54.1	±6.8	−4.6	55.1	49.5
0.05 M NaDC[b]	0.60	2.5×10^{-3} M Tris	73.8	74.6	70.0	69.3	67.3	64.0	59.6	58.3	67.1	±6.1	−5.3	66.8	38.5
0.07 M NaDC[b]	0.39	2.5×10^{-3} M Tris + 2×10^{-2} M NaCl	96.9	89.8	84.1	85.4	84.5	80.1	88.2	78.7	86.0	±5.8	−1.7	84.7	17.0
1 mM $CaCl_2$ 5 M urea	0.50	1×10^{-2} M Tris + 2×10^{-2} M NaCl	81.4	77.5	79.1	74.6	74.3	73.7	70.1	62.6	74.2	±5.8	−4.1	74.7	29.4

[a] Taken from Hanlon *et al.* (1972). [b] Sodium deoxycholate.

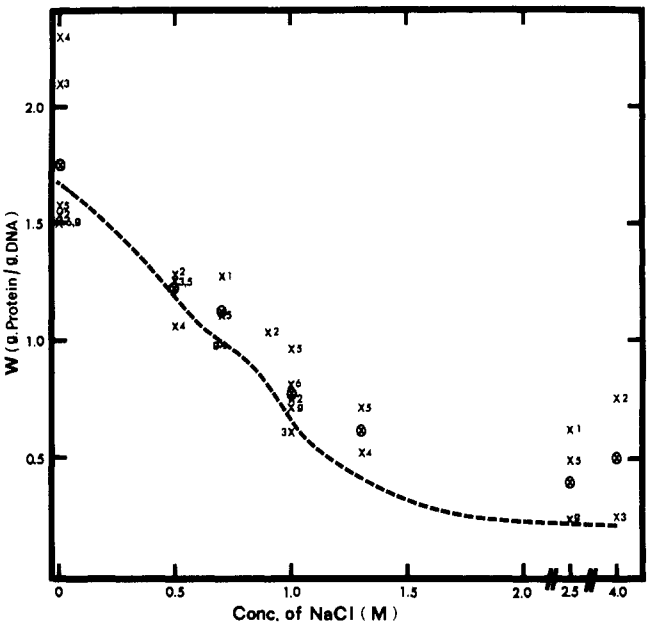

FIGURE 4: The dependence of total protein content of the residual complexes on the concentration of NaCl used in the dissociation procedure. The value of W for each complex is plotted on the ordinate against the molar concentration of NaCl on the abscissa. The crosses represent the data for the individual complexes and their controls, numbered according to the control intact chromatin sample (shown at 0 M NaCl) which was used in their preparation. The average of the values of W for the several preparations subjected to a given NaCl concentration is indicated by a circled cross. The dashed curve represents data taken from Ohlenbusch *et al.* (1967) for similar experiments with calf thymus chromatin.

TABLE II: Characteristics of the Linear Regression Analysis of the Dependence of DNA Conformation on Protein Content of Calf Thymus Chromatin.[a]

Characteristics of Function	Dissociation Procedure: NaCl	NaDC[b]	Combined Data
$0.25 \leq W \leq 1.00$	$W = m(\%\ \mathrm{B}) + b$		
m	−0.01006	−0.00894	−0.00953
b	1.304	1.221	1.267
r	−0.916	−0.870	−0.904
t	−6.85	−6.10	−10.14
Significance	Yes; $P < 0.01$	Yes; $P < 0.01$	Yes; $P < 0.01$
$F_{\mathrm{slope}} = 0.268$ (N.S.); $F_{\mathrm{intercept}} = 0.0295$ (N.S.)			
$1.00 \leq W \leq 2.25$	$\%\ \mathrm{B} = mW + b$		
m	5.02	−3.702	1.251
b	28.4	43.3	34.7
r	0.198	−0.190	0.050
t	0.670	−0.434	0.212
Significance	No	No	No
$\%\ \bar{B}$ (mean)	35.1	38.6	36.3
σ	±5.0	±3.9	±4.8

[a] The linear regressions of the combined data intersect at a W value of 0.93 g/g. The W value at 100% B for the linear regression of the combined data in the range $0.25 \leq W \leq 1.00$ is 0.33 g/g. [b] Sodium deoxycholate.

not significant. The line shown for this range in Figure 3 thus represents the linear regression of the combined data. Above $W = 1$, the t values and correlation coefficients for the linear regressions of per cent B on W are too low to be significant. Construction of a zone of confidence at the 95% level reveals that the upper limit of the increase in per cent B as a function of W cannot be greater than 50% at $W = 1.0$. We feel that, realistically, these facts taken together mean that there is no dependence of per cent B on W in this range of W values. Although the mean per cent B for the sodium deoxycholate experiments in this W range is somewhat higher than that for the NaCl experiments, again these differences are nonsignificant, and hence, the solid line in Figure 3 in this range represents the average of the combined data.

In both types of dissociation experiments, therefore, the per cent B of the residual complexes remains unaffected until a W value of 0.9 is attained. At this point, the per cent B begins to increase in a linear fashion, reflecting the loss of the C character, until a value of 100% is attained at a W of 0.3. Further protein removal presumably does not affect the conformation of DNA.

The per cent B values are plotted against W since the latter are our actual experimental measurements. What is of primary interest, however, is the transformation in the B character as the various protein fractions in intact chromatin are removed. At the simplest level we would like to ascertain which of the two types of proteins in complex, histone or nonhistone proteins, are more important in this regard.

The relationship between the fractions of various proteins present in complex and the total W values of the complexes can be obtained from data available in the literature if certain adjustments and assumptions are made. It is first instructive to compare the W values of our complexes obtained at a given concentration of dissociating reagent with the reported W values of others obtained at similar concentrations of salt. The data for the residual complexes obtained in the NaCl and sodium deoxycholate experiments are shown in Figures 4 and 5, respectively, as points. The numbers beside the points represent the complexes derived from a given control of intact chromatin. The dashed lines represent the data from similar experiments taken directly from the literature. In the NaCl experiments, these are the data for calf thymus chromatin reported in Figure 1 of the paper of Ohlenbusch *et al.* (1967). For the sodium deoxycholate experiments, we have displayed the data for pea bud chromatin taken from Smart and Bonner (1971a). The dashed line in Figure 5 represents the sum of the weight fractions of nonhistone and histone proteins obtained at a given sodium deoxycholate concentration reported in Figure 1 in the paper of Smart and Bonner (1971a).

In our NaCl experiments shown in Figure 4, the average W, represented by a cross in a circle, is somewhat higher than the reported literature value for that given NaCl concentration. There is no correlation, however, between the position of the W value of a given residual complex and its intact control, relative to either the line or the average value at a given NaCl concentration. For example, the (5) series had an intact control with a W value lower than the average of the controls examined, whereas the W values of the residual complexes derived from this control were generally higher than the average. The reverse is true for the (1) series. It is likely that these variations in the relative positions of W between controls, residual complexes, and the reported literature values are attributable to variable contamination of readily removed cytoplasmic proteins coupled with minor variations in the experimental proce-

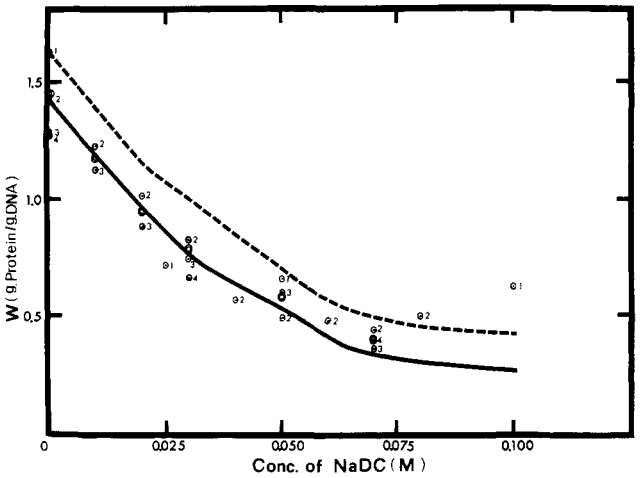

FIGURE 5: The dependence of the total protein content of the residual complexes on the concentration of sodium deoxycholate used in the dissociation procedure. The value of W for each complex is plotted against the molar concentration of sodium deoxycholate on the abscissa. The circles represent the data for the individual complexes and their controls, numbered according to the control intact chromatin sample (shown at 0 M sodium deoxycholate) which was used in their preparation. The average value of W for the several preparations subjected to a given sodium deoxycholate concentration is indicated by a double circle. The dashed curve represents data taken from Smart and Bonner (1971a-c) for similar experiments with pea bud chromatin. The solid curve represents the same data of these authors, adjusted for the presumed differences in the nonhistone protein content of our average intact calf thymus chromatin controls and the value given for pea bud chromatin by Smart and Bonner (1971a).

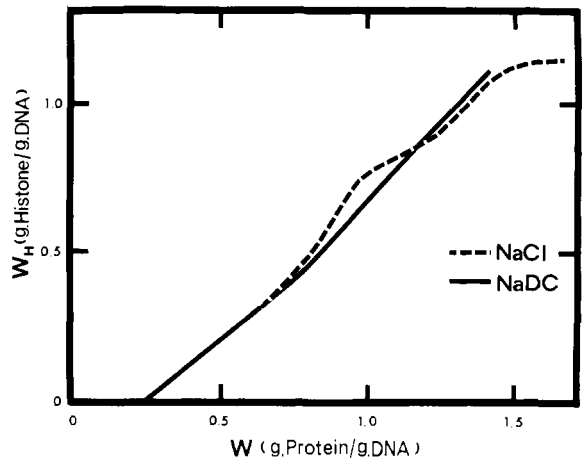

FIGURE 6: Relationship between histone content, W_H (grams of histone/gram of DNA), and total weight of all proteins in complex, W, in the residual complexes prepared by the NaCl (- - -) and the sodium deoxycholate (–) procedures. The NaCl data were taken directly from that reported by Ohlenbusch *et al.* (1967). The sodium deoxycholate data were obtained from the data of Smart and Bonner (1971a) after the latter were adjusted for the difference in the nonhistone protein content of our calf thymus preparations.

dure and errors in the protein determination. The average W and most of the W values of the complexes used in our analysis are close enough to the reported literature values to justify the assumption that, at a given W value, the residual complex will consist of approximately the same proportions of histone and nonhistone proteins reported for the complexes obtained by Ohlenbusch *et al.* (1967). This assumption thus permits us to specify the total histone content, W_H, in terms of a weight ratio of histone in complex to DNA, for given W values of the residual complexes. Using the data displayed in Figure 1 of Ohlenbusch *et al.* (1967), we have constructed a plot of W_H *vs.* W for the NaCl dissociation experiments. This relationship is shown in Figure 6 as a dashed line.

In contrast to the behavior of the NaCl complexes, the complexes from the sodium deoxycholate experiments had W values, at a given sodium deoxycholate concentration, consistently below the data reported for pea bud chromatin by Smart and Bonner (1971a), indicated by the dashed line in Figure 5. Furthermore, there was generally a correlation between the position relative to either the average W value or the dashed line of the W value for the residual complexes and the intact control from which it was derived. As is apparent from the data shown in Figure 5, the intact controls of higher W values generally produced residual complexes of higher than average W. These discrepancies were almost constant over the entire range of reagent employed. Since the nonhistone protein in complex is only minimally dissociated by this reagent, this behavior suggests that the discrepancy between the reported values of W for pea bud chromatin and our preparations of calf thymus chromatin is attributable to differences in the nonhistone protein content and not the histone content. (In fact, the histone content of the pea bud preparations examined by Smart and Bonner matches the average histone content of calf thymus chromatin reported by Bonner *et al.* (1968a,b).) We have attempted to adjust the data of Smart and Bonner for this presumed difference in nonhistone protein content in the following manner. The weight ratio of total protein to DNA, W, of intact pea bud chromatin is taken as the sum of the weight fraction of histone proteins, 1.1, and nonhistone proteins, 0.5, reported in Figure 1 of Smart and Bonner (1971a) at 0 M sodium deoxycholate. We have assumed that the difference in average W value, 1.4, for our intact controls in the sodium deoxycholate experiments and the W value, 1.6, for intact pea bud chromatin is attributable solely to a difference in the nonhistone protein content of the two samples. Thus, the average nonhistone protein content of our calf thymus preparations is 0.3 g/g. We have further assumed that the attern of removal of this nonhistone protein is the same as that for pea bud chromatin. At a given sodium deoxycholate concentration, the W value of our residual complexes should therefore be given by $W = W_H + 0.3(w_N/0.5)$, where W_H is the weight ratio of histone protein to DNA and w_N is the weight ratio of nonhistone protein at the same sodium deoxycholate concentration, in Figure 1 of Smart and Bonner (1971a).

The values of W so calculated are shown as the solid line in Figure 5. This line now falls remarkably close to the average W values of the complexes obtained at a given concentration of reagent, thus justifying our assumption that the histone content dissociated by a given concentration of sodium deoxycholate was essentially the same for our preparations.

With this approach, we have constructed a plot of W_H *vs.* the W value appropriate for our complexes prepared by the sodium deoxycholate method. This relationship is represented in Figure 6 by the solid line. It is now obvious why the structural transformation of the DNA constituent of the residual complexes as a function of total protein content was the same for both NaCl and the sodium deoxycholate dissociation procedures. Both reagents essentially result in equivalent reductions of the histone protein content at a given W value below 0.9.

Using these relationships between W_H and W we can then replot our per cent B values from the several types of experiments against W_H. These results are shown in Figure 7, with the crosses again representing the data from the NaCl experiments and the open circles that from the sodium deoxycholate

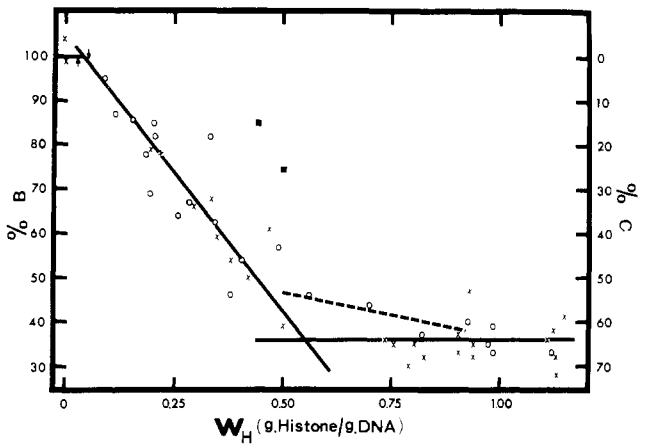

FIGURE 7: The dependence of the secondary structure of DNA on the histone content of the residual complexes. The percentages of bases in the B and in the C conformations are plotted on the left- and right-hand ordinates, respectively, and the histone content, W_H (grams of histone/gram of DNA) on the abscissa. The crosses and circles represent the data from the NaCl and the sodium deoxycholate experiments, respectively, while the two squares are complexes prepared by the Ca–urea procedure. The set of solid straight lines through the data points represents the results of the linear regression analyses of the combined data from the NaCl and the sodium deoxycholate experiments. The dashed line above W_H of 0.5 represents the linear regression analysis of the data points from the sodium deoxycholate experiments alone, as well as the upper limit of the 95% zone of confidence for the line through the NaCl points.

experiments. A covariance analysis, as expected, reveals no significant difference between the lines generated by the separate data. The dashed line represents the upper limit of the confidence interval for the data points at $W_H \geq 0.6$. As is seen in Figure 3, there is a range of W_H values in which the per cent B of the complexes changes dramatically. This is between W_H of 0.04 and 0.5–0.6. The former value could very easily be 0, within the limitations of our experimental errors and assumptions, thus justifying the conclusion that it is some portion of the histone complement which is crucial for the maintenance of the C conformation. Clearly all the histone content is not useful in this regard, as approximately half can be removed without marked changes in the average B character.

In Figure 7 we have also added the data from the Ca–urea experiments, as solid squares, although these points have not been utilized in the regression analysis. In placing these points, the entire complement of protein in complex has been attributed to histone protein. This is a reasonable assumption which should not be in error by more than 0.05 g/g, which is comparable to the error in our protein determination.

We realize that the assumptions employed in these adjustments and calculations of W_H are hazardous. An error of ±10%, or an absolute error of ±0.1 g/g in W_H, would not, however, seriously jeopardize our conclusions. We have used essentially the same methods of isolation as those employed in the experiments reported in the literature and it is difficult for us to believe that the error in W_H could be larger than this. An obvious criticism, for example, is that the data reported for the sodium deoxycholate dissociation experiments with pea bud chromatin are not applicable to calf thymus chromatin. Smart and Bonner (1971a), however, state quite clearly that they have observed the same pattern of protein dissociation in calf thymus chromatin. This is reasonable in view of the similarity of the histone content and composition of the two types of chromatin. We have verified this in a crude fashion for our own chromatin preparations by identifying the histones left in complexes prepared by 0.50 M NaCl, and in 0.02 and 0.055 M sodium deoxycholate. As expected, the lysine-rich fraction I is missing from the complex prepared in 0.50 M NaCl. In the gel patterns for the histones from the 0.02 M sodium deoxycholate complex, the slightly lysine-rich fraction II is severely diminished and in the patterns for the histones from the 0.055 M sodium deoxycholate complex, the lysine-rich I band is the most pronounced.

Another criticism which might be advanced is that uncontrolled differences in experimental circumstances have created differences in extent of proteolysis occurring in our preparations, compared to the ones reported in the literature. Our electrophoresis experiments do indeed indicate that some proteolysis has ensued. Obviously some degradation must also have occurred in the histones of the complexes in the experiments reported in the literature. We deliberately excluded $NaHSO_3$ in our preparative procedures in order to make our experimental conditions comparable. Again, it is difficult for us to believe that the differences in the extent of proteolysis between our complexes and those prepared by Ohlenbusch *et al.* (1967) and Smart and Bonner (1971a) could result in a loss of histone protein which exceeded a W_H value of 0.1 g/g.

An accurate portrayal of the various histone fractions which are dissociated in the NaCl and the sodium deoxycholate experiments is, of course, impossible without quantitative data of our own. It is useful, however, to indicate the various ranges in W over which the complexes in the literature are reported to lose the various classes of histone and nonhistone proteins. These ranges are shown as a set of horizontal lines in Figure 3, with the heavier portions representing the ranges over which a sizable fraction (50%) of the given protein is lost. The ranges shown for the NaCl complexes were constructed from the data in Table II and Figure 1 of Ohlenbusch *et al.* (1967). The patterns shown for the sodium deoxycholate complexes were constructed from the data found in Figures 1 and 2 of Smart and Bonner (1971a), adjusted for the differences in the nonhistone content, as previously described. Again, the limits of these ranges could be in error by 0.1 g/g along the W axis.

Conclusion

It is clear from these experimental results that histone proteins play a very crucial role in the stabilization of the C conformation of DNA in intact chromatin. If the nonhistone proteins participate at all in this process it must necessarily be in conjunction with the histones. Yet it is equally apparent that not all of the histone complement is involved in this function. As can be seen in Figure 7, approximately half of the histone proteins can be removed before a marked increase in the per cent B is observed. These facts taken together would imply that either there is a cooperative loss of C conformation once a critical amount of histone has been removed or, alternatively, there is a unique class of histones crucial to the maintenance of C structure whose pattern of removal is common to the two types of dissociation experiments.

The first of these possibilities is deemed unlikely on the basis of the linear dependence of per cent B on W_H with an intercept of *ca.* 0 at 100% B. (Subsequent analysis of the melting profiles and the protein conformations present in these complexes (see paper II, Hanlon *et al.*, 1974) also mitigates against this interpretation although we cannot, admittedly, discard it altogether.)

The second possibility of a unique single histone class being solely responsible for the C form is a more feasible explanation. The "class" distinction, however, cannot be solely on the basis of composition, since at least some combinations of the argi-

nine-rich and slightly lysine-rich histones are involved directly with a possible indirect contribution of the lysine-rich I as well. This conclusion can be deduced from the pattern shown in Figures 7 and 3 and arguments similar to those given by Smart and Bonner (1971a–c) who concluded from the results of their experiments that all histone fractions appeared to be involved in maintaining the difference in certain physical properties between chromatin and protein-free DNA. In terms of the conformational character of the DNA in chromatin, it is apparent from the patterns of histone dissociation shown in Figure 3 that the removal of the same histone fraction may have different effects on the C character. For instance, all of histone I can be dissociated in the NaCl experiments above $W = 1.0$ without an accompanying profound change in the C character. In the sodium deoxycholate experiments, on the other hand, it makes up a sizable fraction of the total histone complement left at $W = 0.4$ where the per cent B is still transforming at the same rate in a linear manner with respect to total content of histone. The reverse argument holds for histone fraction II which is mainly removed above $W = 1$ in the sodium deoxycholate experiments and below $W = 1.0$ in the NaCl experiments. It is also possible, of corse, that *neither* histone I nor a substantial subfraction of histone II—possibly IIb2—plays a role in maintaining the C character of the DNA. These two components could therefore be removed in any W range without affecting the structure or the structural conversion of the nucleic acid constituent.

The ranges of dissociation of the arginine-rich proteins (III and IV) shown in Figure 3 do coincide, more or less, in the two types of dissociation experiments. These proteins, however, cannot be totally effective in maintaining the C conformation of the nucleic acid since the per cent B character of the residual complexes from the Ca–urea experiments is significantly higher than the values of the intact controls from which they were derived. (This effect cannot be attributed to the irreversible denaturation of the associated proteins by the 5 M urea solvent since the controls and other residual complexes subjected to the same solvent showed only minimal increases (*ca.* 5%) in the per cent B upon removal of the urea.) Since the per cent B for these Ca–urea complexes is significantly different from 100%, these data can also be cited as supporting evidence for the fact that a subfraction of histone II cannot be solely responsible for the maintenance of the C character.

In any event, more than one histone fraction appears to be involved in the stabilization and maintenance of the C conformational regions in intact chromatin. There also appear to be two distinct classes of histones which participate in this process. In paper II, other aspects of these class differences are explored.

References

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B., and Witholm, J. (1968a), *Methods Enzymol. 12,* 3.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C. C., Marushige, K., and Tuan, D. Y. H. (1968b), *Science 159,* 47.

Clark, R. J., and Felsenfeld, G. (1972), *Nature (London) 240,* 226.

Hanlon, S., Johnson, R. S., and Chan, A. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 608 Abstr.

Hanlon, S., Johnson, R. S., and Chan, A. (1974), *Biochemistry 13,* 3972.

Hanlon, S., Johnson, R. S., Wolf, B., and Chan, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3263.

Hnilica, L. S., Edwards, L. J., and Hey, A. E. (1966), *Biochim. Biophys. Acta 124,* 109.

Johnson, R., Chan, A., and Hanlon, S. (1972), *Biochemistry 11,* 4347.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Maurer, H. R., and Chalkley, G. R. (1967), *J. Mol. Biol. 27,* 431.

Ohlenbusch, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967), *J. Mol. Biol 25,* 299.

Oster, G. (1948), *Chem. Rev. 43,* 319.

Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337.

Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967), *Lipid Chromatogr. Anal. 1,* 147.

Shih, T. Y., and Fasman, G. D. (1970), *J. Mol. Biol 52,* 125.

Smart, J. E., and Bonner, J. (1971a), *J. Mol. Biol. 58,* 651.

Smart, J. E., and Bonner, J. (1971b), *J. Mol. Biol. 58,* 661.

Smart, J. E., and Bonner, J. (1971c), *J. Mol. Biol. 58,* 675.

Snedecor, G., and Cochran, W. (1967), "Statistical Methods," 6th ed, Ames, Iowa, Iowa State University Press.

Tunis-Schneider, M. J., and Maestre, M. F. (1970), *J. Mol. Biol. 52,* 521.