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Specificity in the Association of Histones with Deoxyribonucleic Acid. Evidence from Derivative Thermal Denaturation Profiles*

Allen T. Ansevin and Barry W. Brown

ABSTRACT: Progressive stages in the heat denaturation of dehistonized rat thymus DNA reconstituted with each of the five major histone fractions from calf thymus were revealed as distinct peaks in plots of the temperature derivative of hyperchromicity *vs.* temperature. Nucleohistones containing F1, F2a1, F2a2, or F3 in each case increased their ultraviolet absorbance in three or more steps which are presumed to correspond to critical denaturation temperatures for free or weakly complexed DNA and two or more nucleohistone complexes having different binding energies. This is evidence for a more complicated interaction between histones and DNA than previously has been reported. Analysis of the derivative denaturation patterns for these nucleohistones, which were formed in 3.6 M urea solution at low ionic strength, suggests that complexes of the five histone fractions are distinctive with respect to (1) number of complexes formed by the fraction and the weight distribution among these complexes, (2) apparent binding strengths, and (3) complexing efficiency on a weight basis (F1 > F2b > F3 $\simeq$ F2a1 $\simeq$ F2a2). With the exception of the F1 complex, the nucleohistones approximated the stoichiometric relationship: (arginine + lysine) = DNA phosphate.

Reliability of the completely automated spectrophotometer system employed for these studies was demonstrated by determining the melting point and quantity of each component in a mixture of three bacterial DNAs.

Specific associations of histones with DNA have been examined from several aspects. These include chemical investigations of possible differences in the species or tissue distribution of major histone fractions (Stedman and Stedman, 1950; Hnilica, 1966; Hnilica *et al.*, 1966; Fambrough *et al.*, 1968), *in vitro* studies of the biological and biochemical effects of particular histones (Huang and Bonner, 1962; Allfrey *et al.*, 1963, 1965; Hnilica and Billen, 1964; Littau *et al.*, 1965; Liau *et al.*, 1965; Mirsky *et al.*, 1968), physical analysis of the elution of histones from chromatin (Giannoni and Peacocke, 1963; Ohba, 1966; Marushige and Bonner, 1966; Ohlenbusch *et al.*, 1967), and the interaction of histones with DNA (Akinrimisi *et al.*, 1965; Olins, 1969; Johns and Butler, 1964; Johns and Forrester, 1969; Huang *et al.*, 1964; Sponar *et al.*, 1967; Tuan and Bonner, 1969; Shih and Bonner, 1970). Surprisingly little species or tissues pecificity has been observed in the types and distribution of histones (Hnilica, 1967; Fambrough and Bonner, 1969). This has led to theories that other molecules such as RNA (Frenster, 1965a,b; Britten and Davidson, 1969; Bekhor *et al.*, 1969) or acidic proteins (Gilmour and Paul, 1969; Paul and Gilmour, 1968; Wang, 1968; Huang and Huang, 1969) may be primarily responsible for controlling the "readout" of genetic information. Presently, there is little indication that histones bind selectively to specific base sequences. However, the evidence presented here on multiple-stage binding patterns for most major histone fractions, and the results of other studies, indicate that histones show at least some degree of uniqueness in the manner of their attachment to DNA. These differences need to be included among the many factors influencing the unquestionably complex mechanisms for genetic control in advanced organisms.

The present experiments approach the question of whether different histones make specific contributions to nucleoprotein structure by examining the thermal stabilization of DNA complexed with purified preparations of each of the five major histones from calf thymus. A particularly revealing analysis is achieved in these studies by derivative thermal denaturation profiles which show transitions that normally would go undetected. Results from an independent but very similar investigation recently were reported by Shih and Bonner (1970). The present work differs from theirs in the variety of fractions employed, the method for preparing the DNA, and the way in which complexes were formed. All major histones of calf thymus are represented in the present investigation and the DNA has been isolated by a very gentle method. The nucleoprotein complexes were formed by the direct addition of histone to DNA in a medium of low ionic strength. The experiments to follow indicate a greater complexity in the structural organization of nucleohistones than has been demonstrated previously.

Materials and Methods

Equipment. Thermal transitions in DNA and nucleoproteins were observed at 260 nm with an automatic, temperature-programmed, digitally recording spectrophotometer. This

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automatic spectrophotometer was assembled from a number of components, as follows. Samples were illuminated with light from a Beckman DU monochrometer; the power supply, automatic sample changer, and photometer were manufactured by Gilford Instrument Laboratories; Beckman "thermospacers" were employed to heat the sample compartment and cool the photometer. Temperature was measured with a carefully calibrated Yellow Springs Instrument Co. "thermilinear" thermistor couple and bridge network having a voltage response that appeared linear with temperature over the range 20–100°, within the limits specified by the manufacturer (±0.2°); the bridge was powered by a monitored mercury cell. The termistor elements were located at the base of a thin-walled region of an elongated Teflon stopper machined to fit into the "blank" cuvet in a position that placed the thermistor about 4 mm above the optical path. The sides of the cuvet holder were extended upward 12 mm by a rectangular brass collar to favor temperature uniformity within the stoppered cuvets; the top of the sample compartment also was elevated to make more room for the flexing of thermistor leads threaded through a hole in the compartment lid. A programmer controlling the time delay between each series of readings also actuated an eight-channel signal multiplexer unit, kindly constructed and supplied by Dr. Peter M. Corry. Voltages were observed and converted into binary-coded digital form with a Non-Linear Systems, Inc., Model X-2 digital voltmeter interfaced with the signal multiplexer. Alternate temperature and optical density readings were recorded on paper in eight five-digit columns and simultaneously encoded onto punch tape by a Friden Flexowriter, controlled from the multiplexer unit.

The sample compartment was heated by the circulation of ethylene glycol at a rate of about 1 l./min from a temperature-programmed bath. A rapid flow of cooling water for the photometer module and a slow bleed of room temperature air through the cell compartment prevented an anomalous exponential rise in blank readings that otherwise often commenced at a temperature near 80°; even when an anomaly was observed, no errors in net optical density resulted after subtraction of the blank. Bath temperature was increased at the constant rate of 0.5°/min; temperature lag between the bath and the cuvets increased from an initial value of 6° to a final value of 10° in a linear fashion in the temperature range 35–100°.

A normal observation cycle for the automated spectrophotometer started with a 15-sec recording period during which the absorbances of three samples and one blank were recorded alternately with four readings of the temperature in the blank cuvet; this was followed by an adjustable delay period, usually 1 min long.

Since the processing of a typical experiment required the consideration of over 1000 data entries, results were calculated with a digital computer. Net absorbance of samples was corrected to room temperature assuming the solvent expanded as pure water and the ratio of this absorbance to that at room temperature (hyperchromicity) was computed for each temperature.

One of the primary indicators used in this study was the derivative of hyperchromicity with respect to temperature. Particular care was taken in its computation. Because errors in experimental data tend to be magnified when a derivative is approximated by dividing an increment in a function by the corresponding change in the independent variable, a more refined approach was chosen. Hyperchromicity is a relatively smooth function of temperature and therefore can be well approximated in a short temperature range by the quadratic: $H = a + bT + cT^2$, where a, b, and c are constants to be determined from three or more observations of hyperchromicity (H) and temperature (T). The derivative was then taken to be: $2cT + b$, at the center of the temperature range considered. Values of the derivative of hyperchromicity with respect to temperature were computed for each temperature, except the three highest and lowest, by taking the data pair at that temperature along with the pairs at the three adjacent higher and lower temperatures to obtain a least squares determination of a, b, and c by multiple regression. An experiment typically had readings at around 125 different temperatures so that the seven points in each fit covered about 6% of the temperature range of the curve. A program option permitted a reduction in the temperature range to five or three points if desired.

These computations were carried out on an XDS Sigma 5 computer and graphs of hyperchromicity *vs.* temperature, and the temperature derivative of hyperchromicity *vs.* temperature were drawn by a Houston Instruments plotter driven by the computer.

Treatment of Samples for Thermal Denaturation. Samples were added to an appropriate buffer (described below) to give the desired final composition of the medium and an optical absorbance at 260 nm between 0.60 and 0.80 (about 35 μg of DNA/ml). Particulate aggregates were removed by centrifugation at 500*g*. Such low-speed centrifugation avoided the possibility of selective removal of a component of a preparation. Solutions were degassed in partial vacuum for 15 min and approximately 1-ml volumes were placed in Teflon-stoppered cuvets (Precision Cells, Inc., type 29Q) where they were overlaid with washed Dow-Corning silicone oil (type 200, specific gravity = 0.960 at 77°F). At the beginning and end of most experiments a record of solution turbidity was recorded every 10 or 20 nm from 320 to 400 nm, a wavelength region in which DNA and histones do not absorb. In many cases, periodic readings at 320 nm were recorded to detect possible transient changes in turbidity. Corresponding buffer was used as a blank in every experiment.

Buffers. Bacterial DNA samples were melted in 0.10 SSC.[1] Rat DNA and histone-complexed DNA were denatured in a buffer which, following addition of the sample, contained 3.6 M urea (Mann, Ultra Pure), 0.005 M sodium cacodylate buffer, 1.4×10^{-4} M sodium Versenate, and 0.011 SSC at pH 7.0. The stock buffer was stored at 4° for not longer than 1 month; properties of the buffer were found to change significantly if it was kept at room temperature.

Histones. Histone fractions from calf thymus tissue were kindly supplied by Dr. L. S. Hnilica and were prepared as described in a series of publications (Hnilica and Bess, 1964, 1965; Hnilica, 1965, 1966). The F1 (lysine rich) and F2b (moderately lysine rich) fractions were homogeneous by sedimentation analysis and only traces of minor components were revealed in urea acrylamide gel electrophoresis. Fractions F2a1 (glycine-arginine rich) and F2a2 (alanine-leucine rich) had only trace amounts of contaminating electrophoretic bands. F3 contained a large variety of electrophoretic bands (typical of most lyophilized arginine-rich fractions), all of which seem to be closely related chemically (Hnilica and Bess, 1965) and are believed to correspond to monomers, and disulfied-linked dimers and higher multimers (Fambrough and Bonner, 1968); the preparation was found to consist of both

[1] Abbreviations used are: SSC, 0.15 M NaCl–0.015 M trisodium citrate; Gdn·HCl, guanidine hydrochloride.

monomer and dimer molecules when analyzed by sedimentation equilibrium in concentrated Gdn·HCl. Significant features in the composition of fractions employed are indicated in Table I.

Nucleic Acids. Bacterial DNA standards were the gift of Dr. Manley Mandel. They were portions of the same highly purified stock solutions used in his laboratory for experiments correlating melting points and buoyant density in CsCl (Mandel *et al.*, 1970).

The mammalian DNA used for the reconstitutions reported here was supplied through the kind cooperation of Dr. Thomas C. Spelsberg. It was extracted from rat thymus chromatin at pH 8 using 2 M NaCl and 5 M urea to remove excess protein. This DNA was free of histones but contained about 20% of acidic proteins (weight of protein/DNA), and is believed to have the highest purity obtainable without employing strong protein denaturants or departing grossly from physiological pH. Thus, the DNA used here should have retained to a high degree any elements within the DNA itself that could influence the specific attachment of histones. The content of acidic proteins, although not negligible, was much lower than that found in whole chromatin from this tissue. These proteins did not influence the melting profile of the DNA.

Formation of Nucleoproteins. The binding of histones to DNA was accomplished by the very slow addition of a solution of histone at a concentration of 0.6 mg/ml (from dry weight), to a chilled DNA solution at the same initial concentration (absorbance assay: 20 A = 1 mg/ml); both were dissolved in urea buffer having the approximate composition of the thermal denaturation buffer for nucleoproteins. Protein was added with constant rapid stirring until the weight ratio of protein to DNA was 1:4. At this point, the first sample (0.25 X reconstituted) was withdrawn; protein addition was then continued to obtain 0.5 X and 0.75 X reconstituted samples. If fibrous products were observed at any of these stages, the aggregates were dispersed by gentle shearing. After reconstitution, solutions stood at least 2 hr at room temperature and usually 4–16 hr at 4° before being diluted for thermal denaturation assays. The dispersal of aggregates and subsequent standing of the solution resulted in reconstituted samples that were almost completely soluble when diluted for denaturation analysis. The loss of absorbance at 260 nm as a result of 15-min centrifugation at 500g typically was about 5% or less. Apparent absorbance at 320 nm or above was attributed to the scattering of light by large particles and amounted to less than 3% of the absorbance value at 260 nm for samples reconstituted with F1, F2a2, and F2b histones, and less than 5% for F2a1 and F3 nucleoprotein preparations. Since plots of log turbidity *vs.* wavelength (320–400 nm) (Englander and Epstein, 1957) were essentially the same at the end as at the beginning of the thermal analysis for all of the nucleoproteins reconstituted by this method, and no prominent anomalies indicative of scattering changes were observed, it was judged that scattering levels were not excessive for reconstituted preparations clarified only by low-speed centrifugation.

TABLE I: Composition of Histone Fractions from Calf Thymus.[a]

Amino Acid	Mole Fraction of Residues in Purified Preparations				
	F1	F2a1[b,c]	F2a2	F2b[d]	F3
Lysine	27.7	11/102	12.6	20/125	10.1
Histidine		2/102	2.8	3/125	1.8
Arginine	1.8	14/102	9.5	8/125	12.7
Asp + Glu	5.8	7/102	14.5	16/125	15.5
Lys + Arg	29.5	25/102	22.1	28/125	22.8
$\left(\frac{\text{Subunit } MW}{1000}\right)$	21.1[e]	11.3	14.4[f]	13.8	15.0[f]
Calculated net charge/10,000 daltons[g]	+21.9	+15.9	+8.7	+9.8	+6.9
Calculated net charge/subunit	+57.5	+18	+12.6	+13.5	+10.3
(Lys + Arg)/10,000 daltons	27.3	22.1	20.3	20.5	19.2

[a] Content of acidic and basic amino acids in calf thymus histone fractions. Data is abstracted from Table V of Hnilica (1967) except as otherwise indicated. Calculations of net charge and lysine plus argine residues per 10,000 daltons were made from the full data on amino acid content of each fraction. [b] DeLange *et al.* (1969). [c] Ogawa *et al.* (1969). [d] Iwai *et al.* (1970). [e] Teller *et al.* (1965). [f] A. T. Ansevin, unpublished data. [g] Net charge at neutral pH was assumed to be given by (lysine + arginine + histidine/2 − aspartic acid − glutamic acid).

Results

Reliability of Thermal Denaturation Profiles. Optical absorbance of nucleohistones was observed with an automatic spectrophotometer capable of recording a large number of optical absorbance readings (usually 120–130 points/sample) during the course of the gradual heat denaturation of samples. Since data were recorded in a form that could be read automatically, it was convenient to calculate results by digital computer. Furthermore, with the close spacing of data achieved by the present equipment, it was reasonable to carry calculations beyond the usual point of corrected hyperchromicities (absorbance at any temperature/absorbance at room temperature) and so the temperature derivative of the hyperchromicity was obtained. When this was done, unexpected details were revealed in the thermal denaturation profiles of nucleohistones, suggesting that denaturation of a complex nucleoprotein such as native chromatin takes place in a number of relatively discrete steps.

Because the process of differentiating a function containing experimental error can sometimes magnify unmeaningful variations, it was important to test the behavior of the entire spectrophotometer system with known samples. This was done by denaturing three bacterial DNA standards, both singly and in mixture, and comparing the melting points with the values obtained by other investigators (Mandel *et al.*, 1970). Results obtained with a mixture of approximately equal quantities of three different bacterial DNAs are presented in both integral and derivative form in Figure 1. It should be noted that in Figure 1, alone, every point observed is plotted. Subsequent figures are marked with a symbol only at every *fifth* point, to preserve simplicity when more than one sample is plotted on the same graph; however *all* points are used in drawing the line. Irregularities that may be seen in the integral curve of Figure 1 clearly indicate that the sample is complex, and the data cannot be used to calculate melting points in the usual

TABLE II: Identification of Thermal Transitions in Mixtures of DNAs.[a]

DNA Sample (in 0.10 SSC Buffer)	Mp in Mixture by Maximum Gradient Position Expt No.			Mp of Individual Nucleic Acid Samples	
	1	2	3	This Equipment (Continuous Heating) Maximum Gradient Position	Mandel *et al.* (1970) (Equilibrium Method) Midpoint Position
L. jugurti	68.95	68.95	68.85	68.20 68.50	69.1 ± 0.7
E. coli	76.60	76.64	76.55	75.67 75.52	75.4 ± 0.5
P. maltophilia	82.95	82.77	82.72	83.43	83.1 ± 0.5

[a] Melting points in 0.10 SSC were determined from the maximum of each peak in a plot of the temperature derivative of hyperchromicity (at 260 nm) *vs.* the temperature, for continuous-heating denaturation experiments conducted with equipment and techniques described in the Materials and Methods section. Values determined in this way with mixtures of three bacterial DNAs and also with each of the DNAs melted separately are compared to those obtained on the same individual samples by Mandel *et al.* (1970) according to the conventional method of determining boundary midpoints in plots of optical absorbance *vs.* temperature for data from discontinuous-heating experiments where thermal equilibrium is attained before absorbances are recorded.

manner (Marmur and Doty, 1962) since plateau regions are missing from one or both extremes for each of the three components. Melting points are well resolved, however, in the derivative plot where regions of maximum gradient in the hyperchromicity profile are displayed as separate peaks.

Table II compares the melting points determined in this way for mixtures to those determined individually with the present equipment and also to the values found individually by Mandel and associates (1970), who attained equilibrium before the sample was adjusted to a higher temperature.

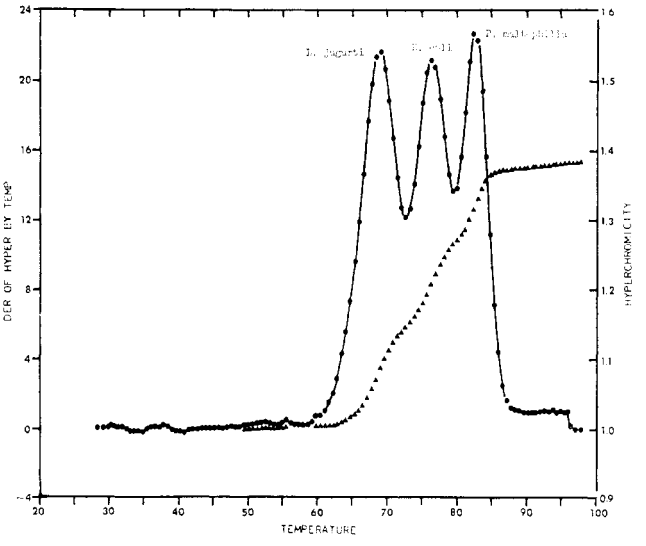


FIGURE 1: Superimposed integral and derivative thermal denaturation profiles for a mixture of roughly equal parts of three different bacterial DNAs dissolved in 0.10 SSC. The left ordinate and plotted circles refer to the temperature derivative of hyperchromicity at 260 nm multiplied by a factor of 1000 (derivative profile). Hyperchromicity was found from the ratio of absorbance at any elevated temperature to that at room temperature, after correcting for the thermal expansion of water. The right ordinate and triangles refer to the hyperchromicity curve (integral profile) of the DNA mixture. All observed points have been plotted. Data were recorded automatically during continuous heating of the sample by a bath having a programmed temperature rise of 0.5°/min.

Reproducibility of results is shown by the close correspondence from experiment to experiment for melting points within the mixture of three DNAs (maximum deviation from the mean was 0.3°). Reproducibility in estimating the amount of each component is demonstrated also by a similarity of areas under each peak, since variations within any peak were less than 3% of the total area, for the above three experiments. In addition, the results of Table I indicate that the absolute accuracy for melting points determined in mixtures was good since discrepancies between this and either of the individual methods were not greater than about 1°. The reproducibility of results for test samples demonstrates, therefore, that it is possible to obtain meaningful derivative thermal denaturation curves with the equipment and data treatment described here.

Dynamic Response of the Urea Buffer during Heating Cycle. Urea (3.6 M) was included in the denaturation medium to lower the melting temperature of DNA and also to favor protein solubility. This gave the solvent a unique dynamic character that was a consequence of the molecular rearrangement of urea in aqueous solution to form ionized ammonium cyanate (Warner, 1942; Dirnhuber and Schutz, 1948). The timing and extent of changes in both pH and ionic strength were measured by removing small samples of buffer directly from cuvets during a mock denaturation experiment at the normal heating rate of 0.5°/min. As shown in Figure 2, little change occurred during the initial part of the heating at temperatures where free DNA would be melted. However, the concentration of ions rose exponentially in a temperature region that corresponds to the second-half of the range in which nucleohistones denature. Conductivity was equivalent to 0.008 M NaCl at 70°, doubled at 80°, and doubled again by 90°. A rise in pH paralleled the conductivity change, reaching pH 8 at 90°. The dynamic property of this buffer allows DNA to be denatured at low temperature and low ionic strength, while at higher temperature it assists the destabilization of nucleohistone.

Stabilization Profiles of Selectively Formed Nucleohistones. When histones are added to DNA in a medium with low ionic strength, nucleohistone complexes are formed and the DNA is rendered more stable to heat denaturation. Plots of hyperchromicity *vs.* temperature (thermal denaturation profiles) furnish a very convenient means for detecting the formation of nucleoprotein, provided the solvent used produces a substantial separation in temperature between the melting of free DNA and of nucleoprotein. However, when the transitions are complex, as they are in native chromatin, the subtle irregularities in the usual integral thermal denaturation profile are difficult to recognize or evaluate. The derivative curve, however, is relatively easy to interpet in terms of temperature and magnitude for transitional events. Because of its special utility for the analysis of stabilized nucleoprotein structures, the temperature derivative of a thermal denaturation curve will be referred to as a stabilization profile when nucleoproteins are examined.

In the present work, purified calf thymus histone fractions were individually combined (reconstituted) with dehistonized rat thymus DNA at three levels of complexing as described under Materials and Methods, and the heat denaturation in low ionic strength urea buffer was observed. The curves of Figure 3, which reproduce the data from one experimental series exactly as gathered, are qualitatively representative of results obtained in other reconstitution experiments with the same histone-free DNA preparation. In many cases, they show striking similarities to profiles obtained with a more highly purified DNA preparation from which acidic proteins had been fully removed. (The latter experiments and others with native chromatin will be described in more detail elsewhere.) The temperature for the denaturation of weakly complexed DNA (first peak in the stabilization profile) varied noticeably, presumably because of small differences in the ion content of the denaturation medium from one preparation to another; stabilized areas were less influenced by this factor. Relatively small quantitative differences were found with most fractions on repetition of reconstitution experiments under the same conditions. Exposure to higher ionic strength (0.3 M NaCl), however, caused portions of certain of the preparations to precipitate and modified some of the patterns. F2b nucleoproteins were the least affected by such treatment.

It may be seen that each of the five histone fractions produced a characteristic stabilization profile and that four of them have more than one nucleoprotein peak in addition to a region in which denaturation approximates that for uncomplexed native DNA. Only one stabilized peak is immediately obvious in F2b nucleohistones at these protein concentrations. Even here, curve fitting procedures suggest that the simplicity is only apparent, and stabilization profiles at a weight ratio of 1:1 have been found to exhibit more than one peak, in addition to that for DNA (unpublished data).

All reconstituted samples, except 0.75 X F1, retained some uncomplexed or weakly complexed DNA. This is summarized in Table III, prepared from data on the size of the first peak, where the thermal transition was close to the temperature for free DNA in the same sample of solvent. The amount of DNA that was not tightly complexed was determined with a duPont curve resolver by finding the largest curve, having the shape of uncomplexed DNA (see dashed line of Figure 3) that could be fitted to the extreme left portion of the stabilization profile. (This was accomplished on the duPont curve resolver by decreasing the vertical amplification of the curve for DNA and displacing this curve very slightly to the right of its usual position, if necessary, until the left-hand edge of the DNA curve coincided to the maximum possible extent with the left edge of the melting profile for the nucleohistone without extending above the perimeter of the latter curve.) The area of the fitted curve was then divided by the total area under the profile to give the fraction of uncomplexed DNA. Figures in the table are the mean and maximum deviation from the mean for determinations of three separate reconstitution experiments.

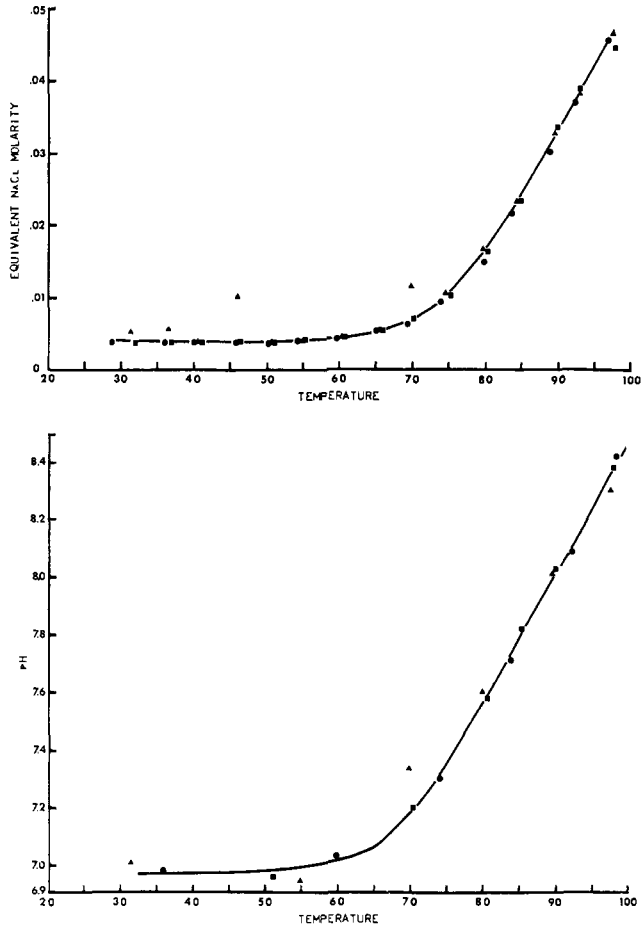
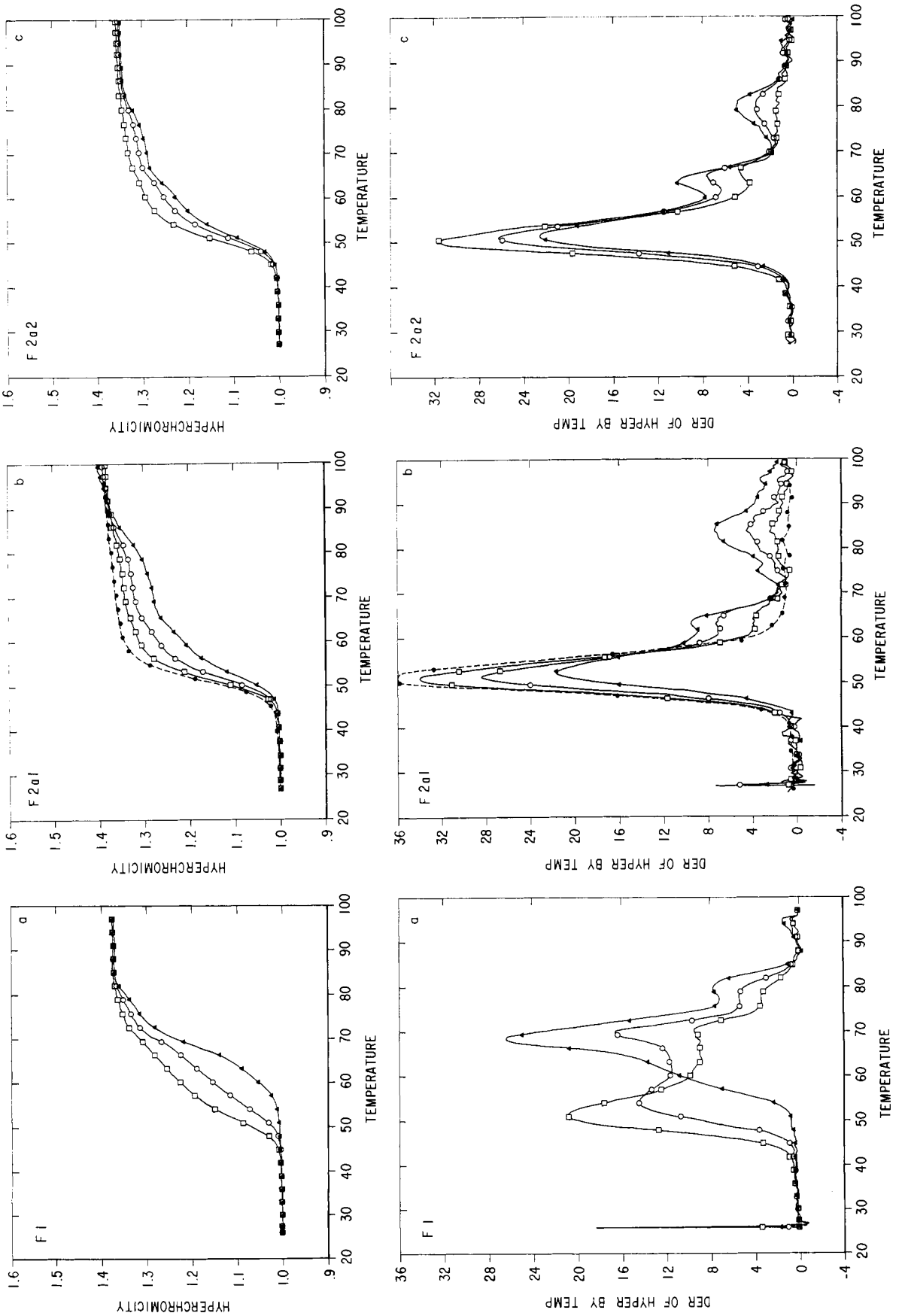


FIGURE 2: Changes in the 3.6 M urea buffer used for thermal denaturation of nucleoproteins. Samples for pH and conductivity determinations were taken directly from cuvets in the spectrophotometer during a typical heating cycle in which the bath temperature was raised at the rate of 0.5°/min. Conductivity has been interpreted in terms of the amount of NaCl producing the observed conductance in a 3.6 M urea solution. Triangles and squares: buffer solutions prepared with two different lots of Mann Ultra Pure urea; circles: buffer solution prepared with Fisher reagent grade urea.

It may be seen from Table III that F1 complexes DNA much more efficiently than does any of the other fractions. For the reconstitution method used here, the completeness of complexing by histones had the order, F1 > F2b > F3 $\simeq$ F2a1 $\simeq$ F2a2, when products were analyzed in a 3.6 M urea buffer as described above.

Discussion

The temperature derivative of hyperchromicity has been utilized to emphasize details present in denaturation profiles that otherwise might not have been noticed. Previously undocumented binding heterogeneity for F1, F2a1, F2a2, and F3 histone fractions was shown to be a regular feature of their respective derivative denaturation curves. The analytic power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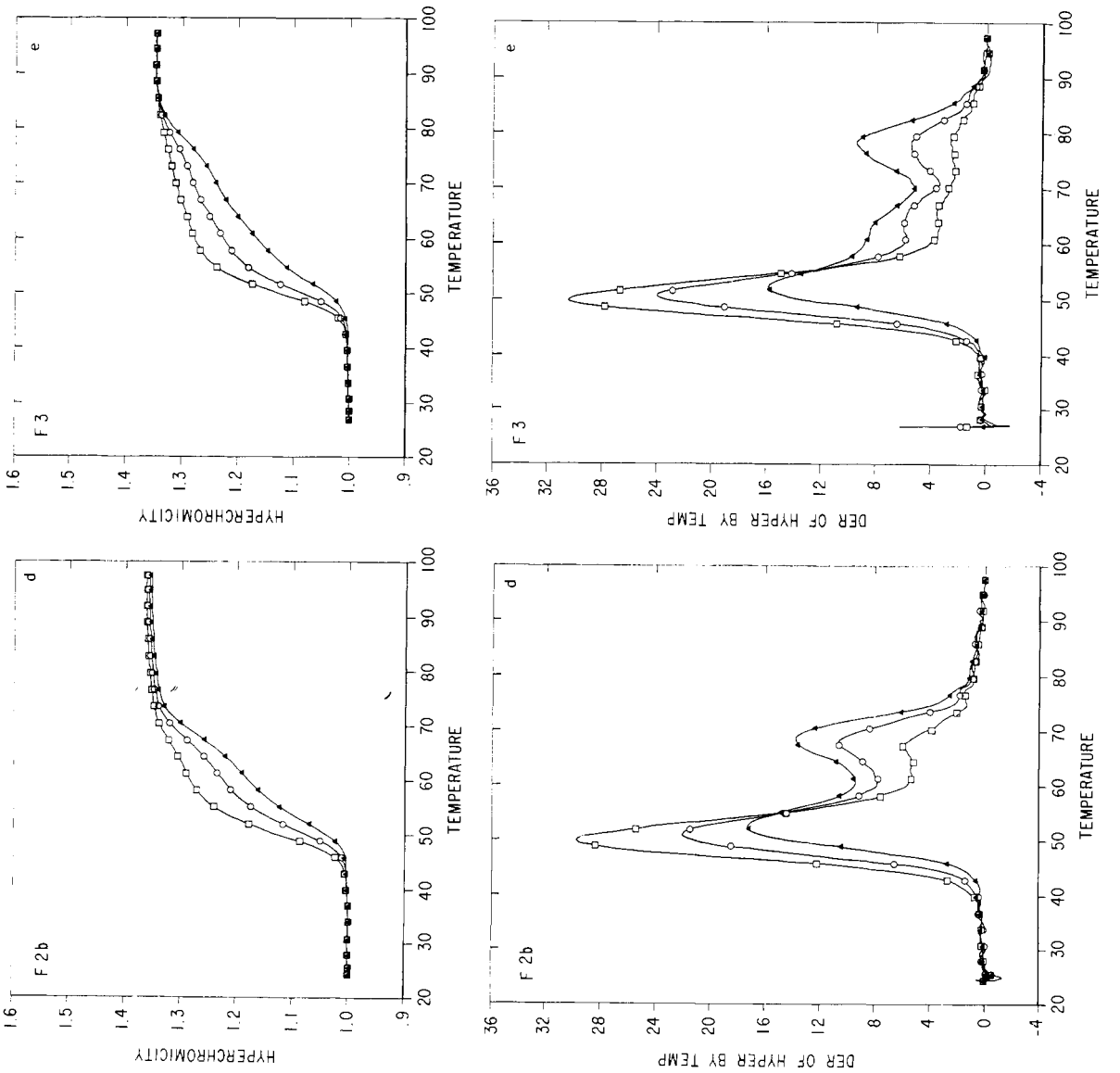

FIGURE 3: Corresponding integral (upper figure) and derivative (lower figure) thermal denaturation profiles of nucleoproteins formed from rat DNA and five different purified fractions of calf thymus histone at three levels of reconstitution; nominal extent of reconstitution is indicated by the value of the ratio, weight of protein/weight of DNA: □—□, 0.25; ○—○, 0.50; ▲—▲, 0.75. The type of histone used is recorded at the top of each graph. The thermal denaturation profile of histone-free DNA is shown as a dashed line with filled circles in part b. Denaturation was produced by continuous heating (programmed increase of 0.5°/min) in low ionic strength urea-containing buffer at pH 7; data were recorded and processed automatically. Only every *fifth* point in all graphs is indicated by a symbol, but all observations are used in drawing the continuous curves. The ordinate for derivative profiles is the temperature derivative of hyperchromicity at 260 nm times a factor of 1000. From the position of the DNA curve shown in part b, it can be noted that the first peak in the derivative denaturation profile of each nucleoprotein, except 0.75 F1, should correspond to uncomplexed or weakly complexed DNA and that peaks occurring at higher temperatures are due to histone-stabilized DNA. Records of turbidity at 320 nm (not shown here) indicated that no prominent peak is produced merely by an increase in scattered light. Reconstitution was by the slow addition of individual histone fractions to dehistonized rat thymus DNA in a low ionic strength buffer containing 3.6 M urea at pH 7.

TABLE III: Distribution of Protein and DNA in Partially Reconstituted Nucleohistones.[a]

Nominal Extent of Reconstitution (Wt of Protein Added/Wt of DNA)	App Fraction of DNA in Uncomplexed Or Weakly Complexed State (%)					
	F1	F2a1	F2a2	IIb[b]	F2b	F3
0.25	54 ± 3	83 ± 11	84 ± 1		81 ± 2	88 ± 4
0.5	33 ± 1	68 ± 8	67 ± 6		62 ± 1	70 ± 4
0.75	0	53 ± 11	56 ± 9		46 ± 1	51 ± 7
	Wt of Protein/Wt of Complexed DNA					
0.25	0.54	1.5	1.6		1.3	2.1
0.5	0.75	1.6	1.5		1.3	1.7
0.75	0.75	1.6	1.7		1.4	1.5
Extrapolated limit[c]						
Shih and Bonner (1970)	0.8	1.9		1.8		
Excess[d]						
Johns and Forrester (1970)	0.7	2.1	2.4		1.5	1.8
Huang *et al.* (1964)	1.37	1.35		1.32		1.45
Nominal Extent of Reconstitution	Histone (Arginine + Lysine)/DNA Phosphate[e]					
0.25	0.5	1.1	1.1		0.9	1.3
0.5	0.7	1.2	1.0		0.9	1.1
0.75	0.7	1.2	1.2		1.0	1.0

[a] The fraction of uncomplexed or weakly complexed DNA in nucleoproteins reconstituted to various extents in low ionic strength buffer containing 3.6 M urea was determined from plots of the derivative of hyperchromicity (260 nm) *vs.* temperature. A duPont curve resolver was used to determine the total area under the derivative pattern as well as the fraction of this area in the first peak, which is attributed to DNA not tightly associated with histones; only that portion of the peak that could be fitted to the shape of the DNA derivative melting profile, which was almost Gaussian, was counted as uncomplexed. Percentages given for uncomplexed DNA in each preparation are the average of three separate reconstitution experiments, except for F2a1 histone which was studied in four experiments; the ± symbol is used to indicate the maximum deviation of any of the observations from the mean. [b] Fraction IIb is believed to contain a mixture of F2b and F2a2 and thus does not correspond exactly to any of the isolated fractions studied here. [c] The extrapolated limit for the weight of protein/weight of complexed DNA, is taken from the intercept in the plot of per cent hyperchromicity found in the first peak (DNA) *vs.* the ratio of histone to DNA in the reconstitution mixture. [d] Nucleohistones were collected under conditions in which equivalent or excess amounts of protein were likely. Figures for Johns and Forrester (1970) are approximate extrapolated values for complete precipitation of DNA in a 0.14 M NaCl solution. Values of Huang *et al.* (1964) are the result of chemical analysis on pellets obtained from sedimented soluble nucleohistones. [e] Calculated from present data using information from Table I and assuming a value of 335 daltons/DNA phosphate.

of this technique is perhaps best illustrated by a comparison between the derivative curves and the integral plots for reconstituted F3 nucleohistones; one component consistently observed in the derivative is essentially indistinguishable in the hyperchromicity profile. From this illustration, as well as other comparisons that may be made in Figure 3, it is concluded that the practice of specifying a midpoint transition temperature, or melting temperature, for nucleoproteins that denature over a range of temperatures in inappropriate and discards important information. Related to this, is the conclusion that a broad thermal denaturation profile is not synonymous with indefinite association of the complexed protein.

A particularly interesting observation to be made from the data of Table III is that different fractions apparently have different efficiencies for DNA stabilization. Capacity for thermal stabilization does not seem to be a simple function of the net charge per subunit, or the net charge per unit weight of histone. However, a fairly close stoichimetry appears to hold for most fractions between the number of arginine plus lysine positive charges and the phosphate negative charges on the combined DNA (F2b = 0.9, F2a1 = 1.2, F2a2 = 1.1, F3 = 1.0–1.3); the deviation from this relationship in the binding of F1 is probably significant (F1 = 0.5–0.7). It is quite possible that the amount of DNA stabilized per unit weight of histone is influenced by the shape of the bound histone molecules and their orientation with respect to the polynucleotide chain. Such a proposal is consistent with other indications that the lysine-rich fraction may be bound as an extended chain (Olins, 1969; Tuan and Bonner, 1969).

It is noteworthy that the ratios of histone to complexed DNA in the more fully reacted nucleohistones (0.75 X) show many similarities to the values obtained by Johns and Forrester (1970) for complexes formed by precipitation in 0.14 salt and to those found by Huang *et al.* (1964) and Shih and Bonner (1970) for salt gradient reconstitution products. Since the nucleohistones studied by Johns and Forrester and by Huang *et al.* were analyzed by methods other than thermal denaturation (and in the absence of urea) the general correspondence with present results partially supports the validity of interpreting thermal stabilization profiles in terms of complexes found at room temperature. With this in mind, an important deduction can be made from Table III: complexes of F2a1, F2a2, F2b, and F3 at 1:1 weight ratios to DNA should have detectable amounts of DNA in a free form. The predicted fractions of free DNA in nucleohistones of the type investigated here fall in the order, F2a1 $\geq$ F3 > F2b > F1 = 0, allowing for a possible trend in F3 binding. This order is similar to that observed by Huang and associates for the

RNA templating activity of their complexes with partially corresponding fractions (Huang *et al.*, 1964) at higher protein to DNA ratios. A prediction that some DNA would remain free in 1:1 (weight of protein/weight of DNA) complexes formed by gradient dialysis is indicated also by the thermal profiles of Shih and Bonner (1970). Within the somewhat limited extent that comparisons can be made between the complexes in Table III and those of Shih and Bonner, it would appear that gradient dialysis and "direct" reconstitution methods used here produce roughly similar results with respect to the binding capacity of histones for DNA.

Despite the similarities noted above between the reconstitution products here and those described elsewhere, a striking difference may be seen in the complexity of the thermal denaturation profiles of Figure 3 and those reported by investigators using the gradient dialysis method of reconstitution. This difference is most apparent in the derivative profiles, where more than one stabilized product is seen for all histones, with the possible exception of F2b.

Since the complexity of these patterns suggests the possibility of a new order of specificity for histone interaction with DNA, it is important to examine the validity of the data at hand. A number of objections might be raised against a proposal that the complexity of thermal stabilization profiles is meaningful. These include the possibilities that: (1) some peaks are scattering artifacts; (2) extra products are thermodynamically unstable; (3) complexity is merely an expression of inhomogeneity in the histone fractions; (4) complexity is due to gross inhomogeneity of the DNA; (5) extra peaks in the stabilization profile are due to reaction products of DNA or proteins with ammonium cyanate produced by the hydrolysis of urea at higher temperatures; and (6) the products are uninteresting because they are not formed under physiological conditions. Most of these points can be answered at least partially. First, although scattering effects are present, considerable effort has gone into minimizing or accounting for scattering, including the choice of a solvent favoring protein solubility and the periodic recording of optical densities at 320 nm during denaturation experiments. From small changes that were found to occur in 320-nm readings, it can be concluded that terminal peaks in many fractions include a minor turbidity contribution, but the peaks cannot be attributed to light scattering alone. A second point, concerning the thermodynamic stability of each product, seems especially pertinent in the case of F1 nucleohistone and was raised earlier by Olins (1969). He considered that complexes that denatured gradually when heated were due to loose associations of F1 with DNA. The more sensitive derivative thermal profiles utilized here reveal patterns with a discreteness in the thermal transitions of F1 and other nucleohistones that would be most difficult to ascribe to random interaction. However, variations in the distribution of histone between alternative stabilized areas were observed in this laboratory from experiment to experiment with certain histones, and some patterns were changed considerably by exposure to hypertonic salt solution. This is evidence for possible thermodynamic instability of some interaction products but does not indicate that this type of stabilized structure could not occur *in vivo*.

That complexity in stabilization profiles might be due to microheterogeneity in histone fractions must be seriously considered since the fractions F1, F2a1, F2a2, and F3 have all been shown to contain more than one electrophoretic component (Panyim and Chalkley, 1969). The presence of dimers and higher polymers might well introduce greater complexity into the denaturation profiles of F3 nucleohistone, but this is not expected for other fractions. From a chemical standpoint, the differences within each fraction are very minor, and Shih and Bonner (1970) observed but one stabilized component even with mixtures of distinctly different fractions. Of course, gross heterogeneity within the DNA also might explain the existence of products with differing thermal stabilities. This objection was the reason for choosing rat DNA for the present study in preference to bovine DNA; thermal denaturation experiments in this laboratory show that rat DNA has a melting profile that is considerably more homogeneous than that of bovine DNA. Although this source of variety seems unlikely as an explanation for the occurrence of complexity in stabilization profiles where the character of the pattern differs from one histone fraction to another, a definitive answer cannot yet be given.

The gradual accumulation of ammonium cyanate during denaturation experiments conducted with a buffer containing 3.6 M urea was documented in Figure 2. The possibility that the reaction of cyanate with either DNA or histones could cause additional peaks in stabilization profiles recorded in urea solvents must be considered. Experiments to be reported in a subsequent publication (A. T. Ansevin, L. S. Hnilica, and T. C. Spelsberg) show that the addition of potassium cyanate to a denaturation buffer (without 3.6 M urea) to give an initial concentration similar to that attained in a urea buffer at about 90°, tends to destabilize nucleoprotein complexes. The denaturation of DNA alone is little influenced by cyanate at this concentration (0.035 M). In short, no evidence has been found in these or other experiments that cyanate introduces spurious peaks into derivative thermal denaturation profiles.

Finally, it is possible that the complexity observed in the stabilization profiles of this study is uninteresting because the nucleohistones were formed at low ionic strength, rather than in a medium corresponding to the cell nucleus. Of course, a similar criticism applies to all current methods for reconstitution and a truly satisfactory biological answer is not readily attainable. It is concluded, therefore, that the complexity of the interaction of histones with DNA is real but its biological significance is still undetermined.

Studies to be reported elsewhere show that the sensitivity achieved by derivative thermal denaturation profiles in urea buffer can be applied advantageously also to "native" and modified chromatin preparations. With the new technique, discrete transitions in stabilization profiles of reconstituted and natural nucleohistones can be characterized more fully with respect to temperature and magnitude than previously was possible with the familiar integral profile or alternative probability plot (Knittel *et al.*, 1968).

Results presented in this paper show a specificity in the binding of histones to DNA that distinguishes one histone fraction from another and presumably reflects different distributions of binding strengths in the present solvent. These experiments do not indicate whether the areas of DNA that are complexed first by each fraction are distinctive, as could be expected if interactions with DNA were base sequence selective. From the data of Table III it can be said that F1 histone must be capable of interacting with virtually all regions of rat DNA because no normally melting DNA remained in the 0.75 X reconstituted product. Furthermore, each of the other fractions increased its extent of reaction as the histone concentration was raised beyond the level in native chromatin, without showing a sharp cutoff of available binding regions. Thus, these experiments give no evidence of a specificity in attachment that could be related even to average base composition of the nucleic acid, although they cannot be taken to

exclude it. The studies do indicate that each fraction investigated is specific with respect to physical properties of the complexes formed under the present conditions. With the acceptance of certain assumptions, these properties include: (1) number of complexes and weight distribution among the complexes; (2) apparent binding strength of complexes; and (3) average packing density of histone along the DNA. Exact statements about these properties must be considered tentative, because the asumptions involved are not trivial. Nevertheless, differences among the histones are observed and additional investigations by this method would seem in order. Even if it should be found that histones do not determine by themselves the regions of DNA to which they attach *in vivo*, they may well fulfill specific biological roles, wherever they become deposited, as a result of distinct binding strengths and unique steric effects.

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