TABLE IV: Comparison of Data on Extent of Purification of $RNA^{Cys}$.

| tRNA | μmoles of Cys/ μmole of RNA |
|---|---|
| RNA (commercial) (Table I) | 0.0231 |
| RNA (purified) (Table III) | 0.9903 |
| RNA (commercial) (Table III) | 0.0229 |

tRNA recovered from the organomercurial–polysaccharide column with that for commericial tRNA. Experiment 1 contained commercial tRNA. Experiments 2 and 3 contained purified tRNA. A comparison of the net specific activities obtained in expt 2 and 3 shows that agreement was obtained for [$^{35}$S]cysteine transferred to RNA when either 0.0052 or 0.01 μmole of the purified $tRNA^{Cys}$ was used. From a comparison of the net specific activities of the purified RNA (expt 2 and 3) with the net specific activity of commercial RNA (expt 1), it was estimated that the $tRNA^{Cys}$ had been purified approximately 43-fold. In other studies not shown, a mixture of [$^{14}$C]amino acids (arginine, histidine, isoleucine, methionine, proline, threonine, and valine) could not be transferred to the purified RNA.

Calculations were made on the assumption that tRNA has a molecular weight of 25,000. Because 1.25 μmoles of tRNA per milliliter produces 1 unit of absorbancy at 260 mμ (Berg *et al.*, 1961), the amino acid acceptor activity and the absorbancy were used to estimate purity. Table IV shows the accumulated results of this purification procedure. The commercial RNA, from which the purified $tRNA^{Cys}$ was obtained, compared favorably with regard to the amount of cysteine transferred per μmole of RNA in expt 1 of Table III. Approximately 0.023 μmole of [$^{35}$S]cysteine/mole of RNA was transferred in each case. From these data, it was calculated that 2.3% of the commercial RNA was charged with cysteine. The recovered RNA could be charged with 0.9903 μmole of cysteine/μmole of RNA (Table III), indicating approximately a 43-fold purification of $tRNA^{Cys}$.

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# Mixed Conformations of Deoxyribonucleic Acid in Intact Chromatin Isolated by Various Preparative Methods†

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ABSTRACT: The optical and analytical characteristics of calf thymus nucleohistone (TNH) isolated by a variety of methods have been examined. In general, the values of $\epsilon_P$ (the extinction coefficient at the maximum) and the protein/DNA ratios of the products obtained from these various methods are comparable. The circular dichroism (CD) properties and the melting behavior, however, differ in a significant fashion. The circular dichroism data taken together with the thermal melting profiles are best interpreted in terms of the presence of two discrete conformations of DNA in TNH. Part of the DNA duplex is exposed or accessible to the solvent environment—either completely or partially—and is in the B conformation. The remainder of the base pairs and ribophosphate backbone is protected from the solvent environment by efficient histone coverage and is in the C conformation. Correlation with other data in the literature suggests that these latter sections, as a consequence of the C conformation and effective charge neutralization, may also be in a super-coiled form. Addition of divalent ions increases the per cent C character at the expense of the B, whereas the addition of NaEDTA has the reverse effect. These ions may be an integral part of the native structure of TNH *in vivo*. Variations in the CD and melting properties of the various preparations can be attributed to variations in the removal of ions from the TNH in the early stages of isolation from the tissue.

The structure of the chromatin of eucaryotes has recently become the subject of intense investigational activity. (For comprehensive reviews, see Bonner *et al.* (1968b); Stellwagen and Cole (1969), Hearst and Botchan (1970), and DeLange and Smith (1971).) These investigations have revealed that it is a multicomponent entity consisting of at least two classes

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of proteins, basic histones of limited heterogeneity and a more heterogeneous group of nonhistone proteins, in complex with the major nucleic acid, DNA, as well as a minor RNA component (Huang and Bonner, 1965). The weight ratios of these various constituents as well as the optical characteristics of the complex (Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov *et al.*, 1970; Wagner and Spelsberg, 1971) are variable and dependent on the tissue of origin as well as the method of isolation. On the average, purified chromatin, or "nucleohistone," as we shall refer to it in this communication, from calf thymus (TNH)[1] has a weight ratio of protein to DNA of 1.5 ± 0.2 g/g with histone protein constituting *ca.* 70% of the total protein complement. The RNA content of the complex is generally less than 1% of the weight of the DNA (Bonner *et al.* 1968b).

The structure of this complex is obviously a many faceted problem. In this present communication, we wish to report the results of some comparative optical studies at pH 7 conducted with preparations isolated by various methods with the dual aim of ascertaining the origin of the variability of TNH properties and establishing the conformation of the major nucleic acid constituent, DNA, in TNH and its relationship to the protein constituents of the complex. A preliminary report of this work has appeared previously (Hanlon and Johnson, 1971).

## Experimental Section

Calf thymuses were collected fresh at the slaughterhouse and immediately frozen upon a bed of Dry Ice within 10 min after extirpation. Upon returning to the laboratory, the tissue was stored in a freezer at −10°. Preparations isolated by a given procedure from tissue stored anywhere from 0 to 6 months showed no significant variation in their optical and analytical properties. All chemicals employed in the isolation procedures as well as in the subsequent preparation of TNH and DNA solutions were reagent grade.

The nucleohistone was isolated in the cold (*ca.* 6°) from these frozen calf thymuses by several procedures. One of these, the procedure of Maurer and Chalkley (1967), was followed precisely. In this isolation, the nuclei are first isolated by blending the tissue gently in 75 × $10^{-3}$ *M* NaCl–24 × $10^{-3}$ M EDTA, pH 8. The final purification step involves high-speed centrifugation of the crude chromatin through a sucrose gradient. The gelatinous pellet obtained from the latter step is exhaustively dialyzed against 0.01 M Tris buffer, pH 8. This procedure has been identified in the text as the "M–C" procedure. It is essentially identical with a standard procedure given by Bonner *et al.* (1968a) suitable for various organs and organisms.

Some of the TNH stock solution in 0.01 M Tris, pH 8, was dialyzed against 2.5 × $10^{-3}$ M Tris, pH 8, and subjected to further low-speed blending according to the method of Shih and Fasman (1970) to produce a lower molecular weight product. The TNH solution with an optical density (OD) of *ca.* 45 was blended three successive times for 1 min in a 115-V Waring blendor attached to Variac set at 40 V. It was then centrifuged in a SS-34 rotor for 10 min at 10,000 rpm at 0° in a RC-2 Servall centrifuge. The supernatant resulting from this process is referred to as "M–C, sheared."

A variation of the M–C procedure involved increases in the amount of shearing force to which the tissue was subjected in the initial nuclei isolation steps. A 220-V Waring blendor was employed in the place of the 115-V Waring blendor called for in the original M–C procedure. It was operated in the "Low" position when the M–C procedure called for 70 V or less, and in the "High" position when the procedure called for more than 70 V on the voltage regulator attached to the Waring blendor. Although we have no quantitative estimates of the differences in the shearing force which the use of the high-voltage blendor made, qualitatively it had to be greater. Its use resulted in the complete disruption of nuclei in the grinding medium, 75 × $10^{-3}$ M NaCl–24 × $10^{-3}$ M NaEDTA. This variation has been referred to as the "M–C, high-blend" procedure.

Another method employed was a combination of the M–C, high blend and a modification (Hanlon, 1961) of an older one by Crampton *et al.* (1954). The tissue was first subjected to the high-speed blending process in 75 × $10^{-3}$ M NaCl–24 × $10^{-3}$ M NaEDTA in the 220-V blendor called for in the M–C, high-blend procedure in the "nuclei isolation" step, followed by extraction and additional blending for *ca.* 30 sec in 4 × $10^{-4}$ M $NaHCO_3$ to solubilize the crude chromatin. A final purification step of this latter product involved the precipitation of the nucleohistone by making the medium 0.14 M NaCl by the addition of 5.77 volumes of 0.177 M NaCl. The precipitate was collected by low-speed centrifugation and redissolved in 4 × $10^{-4}$ M $NaHCO_3$. After a day on a low-speed magnetic stirrer, undissolved material was removed by low-speed centrifugation, to produce a clear stock solution. This procedure has been designated as the "H–C" procedure.

A fourth method of isolation which we employed was the Zubay and Doty procedure (1959) in which the tissue is first exhaustively blended with 75 × $10^{-3}$ M NaCl–24 × $10^{-3}$ M NaEDTA, pH 8, and then extracted with a large volume of 7 × $10^{-4}$ M $KH_2PO_4$–$K_2HPO_4$ buffer, pH 7.2. The 220-V blendor was employed throughout this isolation. In our hands, we found that the final stock solution of TNH was turbid and required a low-speed centrifugation step to yield a clarified stock whose light-scattering properties were not excessive. This isolation procedure is referred to as the "Z–D" procedure.

All of the stock solutions prepared by the procedures described above were initially examined immediately after isolation. The remainder was frozen and stored at −8° until needed. The effects of freezing and thawing preparations in this manner are noted in the text, the former being identified by the term "fresh" and the latter by the designation, "thawed." Stock solutions which had been frozen and subsequently thawed exhibited variable amounts of insoluble and aggregated material, and had to be clarified by low-speed centrifugation prior to examination.

Absorption spectra were routinely obtained on all samples between 400 and 210 nm with a Cary Model 14 CMR recording spectrophotometer equipped with thermostatted adaptors. Temperature of the adaptor jackets was regulated by a Haake circulating water bath and monitored by means of a telethermometer and probe manufactured by Yellow Springs Instrument Co. Routinely, measurements were made at 25°, but in the case of the melting experiments, the temperature range included 6–95°. In these latter experiments, about 20 min was allowed for equilibration at each temperature. Full spectra were obtained after the absorbance at 260 nm, and the temperature of the cell contents (measured directly by inserting the probe in the solution at the top of the cell just under the stopper) ceased to change for a given

[1] The following abbreviation is employed in the text: TNH, calf thymus nucleohistone.

temperature setting on the Haake. Light-scattering corrections of all spectra (including the ones obtained in the melting experiments) were performed empirically in the manner suggested by Oster (1948) by plotting the log of the absorbance, $A$, measured between 400 and 330 nm, against the log of the wavelength, $\lambda$, and extrapolating the value of log $A$ to the wavelength of interest. This scattering correction at 250 nm generally amounted to 2–5% of the maximum absorbance at 260 nm.

Concentrations of all TNH and DNA solutions were determined from their absorbance at 260 nm after correcting for light scattering and are reported in terms of moles of phosphorus per liter. The extinction coefficients at 260 nm, $\epsilon_P$, employed in these calculations were 6600 $M^{-1}$ $cm^{-1}$ for DNA and 6760 $M^{-1}$ $cm^{-1}$ for TNH at 25°. These were averages of values taken from the relevant literature (Hanlon, 1961; Zubay and Doty, 1959) and our own determinations reported in Table I.

Phosphate determinations for the extinction coefficient determinations followed the methods of Ames and Dubin (1960) and Fiske and Subbarow (Leloir and Cardini, 1957). Both inorganic phosphate ($KH_2PO_4$) and 2′,3′-AMP (Sigma) were used as standards in these determinations. Concentrations of AMP in the standard solutions were determined spectrally using an extinction coefficient of $15.4 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 259 nm.

Protein content was determined chemically by a biuret method (Gornall *et al.*, 1949) and the Lowry procedure (Lowry, *et al.*, 1951) using bovine serum albumin (Sigma) as a standard. The concentration of the latter in standard stock solutions was determined spectrally using an experimentally determined extinction coefficient of 6.4 dl/g cm at 280 nm. Protein content was also measured spectrophotometrically by the method of Tuan and Bonner (1969) using the equation

$$\frac{\text{wt of protein}}{\text{wt of DNA}} = \frac{\epsilon_{P\,260}^{TNH}}{(30.99)(\epsilon_{230}^{1\%\,H})}\left[\frac{A_{230}^{TNH}}{A_{260}^{TNH}} - \frac{A_{230}^{DNA}}{A_{260}^{DNA}}\right] \quad (1)$$

where $\epsilon_{P\,260}^{NH}$ is the molar extinction coefficient of TNH, $\epsilon_{230}^{1\%\,H}$ is the extinction coefficient of histone protein on a g/dl basis, $A^{TNH}$ and $A^{DNA}$ are the absorbances of a TNH solution and a DNA solution, respectively, at the indicated wavelengths, 230 and 260 nm. $\epsilon_{230}^{1\%\,H}$ was taken as 41.5 $(g/dl)^{-1}$ $cm^{-1}$, the value of Jensen quoted by Smart and Bonner (1971a). The absorbance ratio for DNA was experimentally determined as 2.33. The protein/DNA ratio is reported in terms of the weight of the free acid form of DNA of which phosphorus constitutes 10.0%. In principle this method of assessment should yield values somewhat too low since it attributes all of the $A_{260}^{TNH}$ to DNA absorbance whereas the chromatin proteins do make a small contribution. The resulting error, however, is only *ca.* 2% which is negligible.

Circular dichroism (CD) spectra were recorded at 27° over the wavelength range of 350–210 nm with a Cary Model 60 spectropolarimeter equipped with a 6001 CD unit. The results are presented in terms of molar ellipticities, $[\theta]$, based on the molar phosphorus concentration of the solution of DNA or TNH. The latter was generally $2.5 \times 10^{-4}$ M and the path length, 1 cm. In the case of the TNH proteins, we have presented the data in terms of the molecular ellipticities per mole of amide residue multiplied by the (moles of amide/mole of nucleotide) ratio characteristic of the particular preparation from which the proteins were isolated. Average values of 120 and 310 for the peptide residue and the nucleotide residue molecular weight, respectively, were employed in these calculations. In all experiments, the total absorbance of the solution path did not exceed 2.

Ultracentrifuge studies were conducted with a Spinco Model E analytical ultacentrifuge equipped with absorption optics at polymer concentrations of *ca.* $1.2 \times 10^{-4}$ M. Films obtained from the latter system were scanned with an Analytrol densitometer. Top-loading cells were employed in order to avoid shear degradation of the samples by narrow-bore, syringe needles.

The nucleases employed in the digestion experiments with TNH were hog spleen deoxyribonuclease II and bovine spleen phosphodiesterase II, obtained from Worthington. These were especially selected for their lack of divalent ion requirement. The digestions were conducted with 6-ml aliquots of $3 \times 10^{-4}$ M TNH at a concentration level of 0.04 mg/ml for the deoxyribonuclease II and 0.28 unit/ml for the phosphodiesterase. This mixture was incubated in a dialysis sack for a 2-day period at 7°. For the first 24 hr, the contents of the sack were dialyzed against three changes of a sodium phosphate buffer at pH 5.5. During the second 24-hr period, the pH 5.5 buffer was replaced by a pH 7 one. The loss in nucleotides was monitored by the absorbance change in the bag contents, measured before and after dialysis. An appropriate control solution containing no enzymes was treated in a fashion identical with the experimental.

The protein digestion experiments employed the enzyme, pronase, obtained from Pentex. A pronase solution (0.05 ml) at 1 mg/ml was added to 2.8 ml of a TNH solution at $2.2 \times 10^{-4}$ M in 0.001 M Tris or NaCl at pH 7 in the CD cell, and the changes in the CD and the absorption spectra were taken as a function of time. After about 30 min, when the decrease in the molecular ellipticity at 225 nm revealed that a considerable fraction of the protein had been digested, a sufficient volume of saturated NaCl was added to make the final salt concentration 0.02 M. Experiments in which this step was omitted yielded spectra with somewhat lower ellipticities indicating that some denaturation of the exposed DNA in the low ionic strength medium suitable for the TNH studies had occurred.

## Results

The analytical, optical, and hydrodynamic data obtained on the various preparations are summarized in Table I. The extinction coefficients, $\epsilon_P$, given in the fourth column averaged as 6800 $M^{-1}$ $cm^{-1}$ for the high-blend preparations and 6680 $M^{-1}$ $cm^{-1}$ for the low-blend preparations. This difference is well within the variability of the determinations on individual preparations and hence an average value of 6760 $M^{-1}$ $cm^{-1}$ obtained by weighing all preparations equally was correspondingly used in the calculation of concentrations. The addition of NaEDTA to the extent of 0.003 M to all types of preparations after isolation had no effect on $\epsilon_P$.

In contrast to the reported results of Tuan and Bonner (1969), we did not find that the $\epsilon_P$ for TNH isolated by any of the procedures employed was appreciably higher than that of DNA. This is demonstrated by the data in Table II which record the result of protein removal from the complex by two methods, digestion by pronase (as described in the Experimental Section) and dissociation in 3.6 M KCl. In these latter experiments, triplicate dilutions of a stock TNH solution were made into the control solvent and into the high-salt solvent which should dissociate the TNH complex completely to free protein and nucleic acid. The $\epsilon_P$ values of these

TABLE I: Analytical and Optical Characteristics of Calf Thymus Nucleohistone Isolated by Various Procedures.

| Preparation | Comments | Solvent | $\epsilon_P$ (260) ($M^{-1}$ $cm^{-1}$ ±200) | Protein:DNA Weight Ratio Lowry (±0.20) | Biuret (±0.10) | Spectral (±0.07) | $([\theta]^a \times 10^{-3})$ ± 0.07 (deg $cm^{-2}$ dmole) | % B (± 2%) | $(s_{20,w})_{50}$ at *ca.* 0.006% (S) |
|---|---|---|---|---|---|---|---|---|---|
| M-C | Fresh, average | $2.5 \times 10^{-3}$ M | 6680 | | | 1.69 | 2.88 | 34 | 32.1 |
| | Thawed, average | Tris buffer, | | | | 1.53 | 3.02 | 36 | |
| | Single soln, thawed | pH 7.5 | | 1.40 | | (1.60) | | | |
| M-C, sheared | Fresh, average | $2.5 \times 10^{-3}$ M | | 1.32 | 1.68 | 1.65 | 3.31 | 40 | |
| | Thawed, average | Tris buffer, | | | | 1.38 | 3.45 | 42 | |
| | Single soln, thawed | pH 7.5 | | (1.74) | | (1.46) | | | |
| M-C high blend | Fresh, average | $2.5 \times 10^{-3}$ M | 6830 | | | 1.74 | 3.52 | 43 | 32.7 |
| | Thawed, average | Tris, pH 7.5, | | | | 1.61 | 3.67 | 45 | |
| | Single soln, thawed | or $10^{-3}$ M NaCl, pH 7 | | (2.27) | (2.38) | (2.28) | | | |
| H-C | Fresh, average | $2.5 \times 10^{-3}$ M | 6760 | | | 1.40 | 4.03 | 48 | |
| | Thawed, average | Tris, pH 7.5, | | | 1.61 | 1.59 | 4.09 | 49 | |
| | Single soln, thawed | or $4 \times 10^{-4}$ M $NaHCO_3$, pH 7.8 | | | (1.53) | (1.55) | | | |
| Z-D | Fresh, average | $7 \times 10^{-4}$ M, | | | | 1.35 | 3.81 | 47 | 32.7 |
| | Thawed, average | $KH_2PO_4$– | | | | 1.45 | 3.88 | 48 | |
| | Single soln, thawed | $K_2HPO_4$ | | (1.55) | | (1.54) | | | |
| | | Buffer, pH 7 | | | | | | | |
| | | Avg $\epsilon_P$ = 6760 | | | | | | | |

[a] Molecular ellipticity at 282.5 nm.

dissociated or digested complexes differ, at the most, by only 2% from the control, thus demonstrating that in these preparations, at least, there is no marked hyperchromicity of the DNA in the TNH complex. The small difference between the $\epsilon_P$ averages for TNH and DNA is readily accounted for by the small contribution of the protein constituents at 260 nm (Hanlon, 1961).

The protein/DNA weight ratio varied somewhat with the method of isolation. The Maurer and Chalkley procedure, or any of its variations, in general, yielded somewhat higher ratios than the H–C or Z–D methods. The ratio for a given M–C preparation, however, generally decreased slightly if the stock solution had been frozen and thawed. The aggregated material removed from such thawed stock solutions presumably represented material enriched in chromatin protein. This effect was not observed for the H–C or Z–D solutions. This is not surprising in view of the fact that the fresh stock solutions from the latter two procedures initially had a significant fraction of aggregated insoluble material which had to be removed by low-speed centrifugation. It is likely that this insoluble fraction also had a higher protein/DNA ratio.

Generally speaking, the analytical determinations of protein content agreed reasonably well with the spectrophotometric determinations, although the precision of the former was poorer. The Lowry protein values, in particular, were very bad. It might be pointed out that the average deviation quoted for the biuret and the spectral determinations is dictated in part by the variability of the thawed samples. When individual stock solutions are compared, the average deviation within a given determination is only ±0.02 for the spectral data and ±0.04 for the biuret data. The Lowry determinations, in contrast, still show average deviations of ±0.2. This point is demonstrated by the single solutions entries in Table I.

A circular dichroism pattern of a typical M–C preparation of TNH over the wavelength range of 215–320 nm is shown in Figure 1 together with the patterns of its isolated constituents, DNA and chromatin proteins. The protein spectrum has been multiplied by the normalization factor, 3.88 = ((1.5 × 310)/120) (described in the Experimental Section), appropriate for this particular preparation. Since the protein spectrum is markedly dependent on ionic strength, we have displayed the spectrum obtained in 1.3 M NaCl in the hopes that this solvent will simulate the ionic environment provided by DNA in the native complex. It is apparent from the data shown in Figure 1 that the proteins of TNH do not make significant contributions to the CD spectrum of the TNH complex above 250 nm. Hence, the positive band in the CD spectrum is attributable entirely to nucleic acid, and, specifically, to DNA since the RNA content of purified chromatin from calf thymus should be less than 1% (Bonner *et al.*, 1968b). As has been observed by others (Permogorov *et al.*, 1970; Shih and Fasman, 1970; Simpson and Sober, 1970; Wagner and Spelsberg, 1971), the positive CD band in TNH differs markedly from that of DNA.

The CD patterns for all preparations of TNH which we have isolated are independent of concentration over a 20-fold range from $1.2 \times 10^{-4}$ M to $2.5 \times 10^{-3}$ M. Even gels of

TABLE II: Effects of Protein Removal or Dissociation on $\epsilon_P$ of Calf Thymus Nucleohistone.

| Preparation | Comments | Solvent | $A_{260}$[a] | $\epsilon_P$ (260) ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|---|
| M–C | Control | $10^{-3}$ M NaCl (pH 7) | 1.713 | 6680 |
| | Pronase digested | $10^{-3}$ M NaCl (pH 7) + pronase | | |
| | 25 min | | 1.705 | |
| | 12 hr | | 1.707 | 6650 |
| | Control | $4 \times 10^{-4}$ M $NaHCO_3$ (pH 7) | 0.872 | 6680 |
| | Salt dissociated | 3.6 M KCl, 0.005 M $PO_4$ buffer (pH 7) | 0.880 | 6740 |
| H–C | Control | $4 \times 10^{-4}$ M $NaHCO_3$ (pH 7) | 1.354 | 6760 |
| | Salt dissociated | 3.6 M KCl, 0.005 M $PO_4$ buffer (pH 7) | 1.377 | 6870 |
| DNA (commercial preparation) | | 0.10 M NaCl, 0.05 M $NaH_2PO_4$, $Na_2HPO_4$ | | 6600 |

[a] Absorbance at 260 nm corrected for light scattering.

TABLE III: % B Character for Individual Solutions of Various Chromatin Preparations.

| Preparation | | M–C | M–C | M–C | Low-blend M–C | M–C | M–C | M–C sheared | High-blend M–C | High-blend Z–D | H–C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Solvent | | $7 \times 10^{-4}$ M $PO_4$ buffer (pH 7.05) | $10^{-3}$ M KCl (pH 7.4) | $4 \times 10^{-4}$ M $NaHCO_3$ (pH 7.5) | $2.5 \times 10^{-3}$ M Tris buffer (pH 7.8) | $2.5 \times 10^{-3}$ M Tris– $2.5 \times 10^{-4}$ M Na-EDTA (pH 7.7) | $2.5 \times 10^{-3}$ M Tris– $2.5 \times 10^{-4}$ M $CaCl_2$ (pH 7.5) | $2.5 \times 10^{-3}$ M Tris (pH 7.8) | $10^{-3}$ M NaCl (pH 7.1) | $7 \times 10^{-4}$ M $KPO_4$ buffer (pH 7.1) | $4 \times 10^{-4}$ M $NaHCO_3$ (pH 7) |
| Condition | | Fresh | Thawed | Thawed | Thawed | Thawed | Thawed | Thawed | Fresh | Fresh | Fresh |
| Protein/DNA (g/g) | | 1.53 | 1.32 | 1.30 | 1.36 | 1.36 | 1.36 | 1.44 | 1.72 | 1.35 | 1.40 |
| | [θ]′ Limits:DNA in a | | | | | | | | | | |
| λ (nm) | B Solvent [θ] × $10^{-3}$ | C Solvent [θ] × $10^{-3}$ | % B at Given Wavelength Positions | | | | | | | | |
| 295 | 1.25 | −0.70 | 25.6 | 35.9 | 43.6 | 35.9 | 46.2 | 31.8 | 42.1 | 52.0 | 48.8 | 53.8 |
| 290 | 3.80 | −0.30 | 29.3 | 34.9 | 43.2 | 36.8 | 45.4 | 33.9 | 40.7 | 47.6 | 47.6 | 51.2 |
| 285 | 6.70 | +0.80 | 23.8 | 34.8 | 36.4 | 31.4 | 42.7 | 28.6 | 37.0 | 45.8 | 48.3 | 49.2 |
| 280 | 8.20 | −0.05 | 29.1 | 38.1 | 36.4 | 39.2 | 43.3 | 28.4 | 41.5 | 43.7 | 47.3 | 53.3 |
| 275 | 8.45 | −0.65 | 31.3 | 36.9 | 40.1 | 44.3 | 50.2 | 34.3 | 43.7 | 42.3 | 45.7 | 51.7 |
| 270 | 7.90 | −0.80 | 29.3 | 35.1 | 38.4 | 39.7 | 46.0 | 35.5 | 41.2 | 40.9 | 45.4 | 51.2 |
| 265 | 6.20 | −1.00 | 25.0 | 34.0 | 34.3 | 36.3 | 42.8 | 27.9 | 41.1 | 40.3 | 42.4 | 46.6 |
| 260 | 3.20 | −2.80 | 29.2 | 38.3 | 34.8 | 38.0 | 36.7 | 33.3 | 44.7 | 47.5 | 47.5 | 46.7 |
| Average % B for positive band | | | 27.8 | 36.0 | 38.4 | 37.7 | 44.1 | 31.7 | 41.6 | 45.0 | 46.6 | 50.5 |
| Avg deviation | | | ±2.3 | ±1.3 | ±2.9 | ±2.6 | ±2.8 | ±2.6 | ±1.5 | ±3.2 | ±1.6 | ±2.2 |
| Maximal % A | | | ≤1.9 | ≤0.7 | ≤1.8 | ≤1.8 | ≤1.8 | ≤2.2 | ≤1.3 | ≤1.9 | ≤1.2 | ≤1.7 |

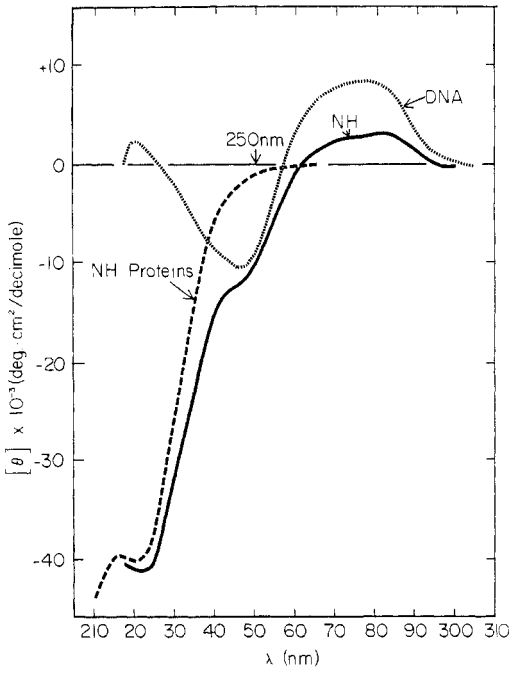


FIGURE 1: Circular dichroic spectra of calf thymus nucleohistone and its major constituents: (—) calf thymus nucleohistone in 2.5 × $10^{-3}$ M Tris-HCl buffer (pH 7.5), isolated by the method of Maurer and Chalkley; (■■■■■■) protein-free calf thymus DNA in 0.10 M NaCl–0.05 M $NaH_2PO_4$–$Na_2HPO_4$ (pH 7); (- - - -) proteins of calf thymus nucleohistone extracted by 1.3 M NaCl in that solvent at pH 7.3. This spectrum has been multiplied by the normalization factor, 3.88, described in the text.

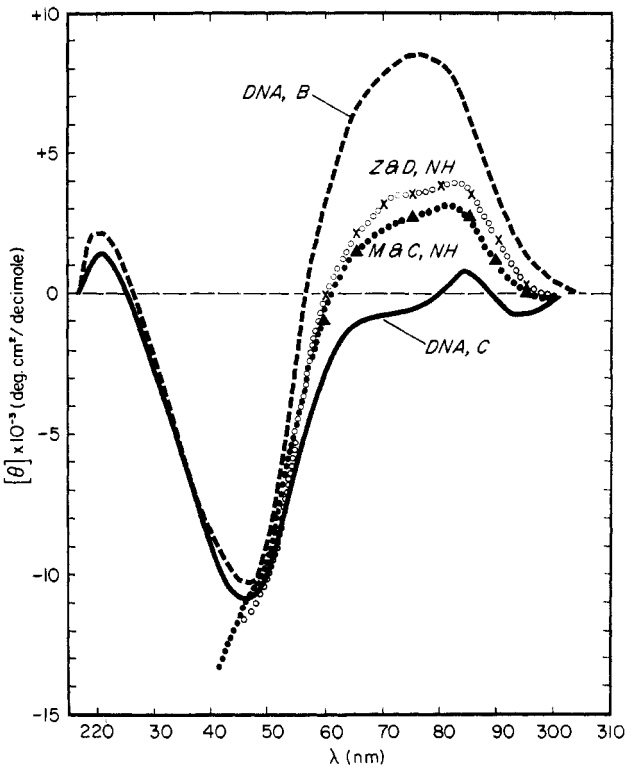


FIGURE 2: Comparison of the circular dichroic spectra of DNA and nucleohistone: (OOO×OOO) nucleohistone in 7 × $10^{-4}$ M $KH_2PO_4$–$K_2HPO_4$ (pH 7), isolated by the Zubay and Doty method, and (●●●▲●●●) nucleohistone in 2.5 × $10^{-3}$ M Tris (pH 7.5), isolated by the method of Maurer and Chalkley. The beaded curves represent the continuous experimental data whereas the points are the calculated values of $[\theta]_\lambda$, assuming a mixed conformation of B and C forms whose limit spectra are curves described below. The ×'s reflect a mixture of 47% B and 53% C and the ▲'s a mixture of 36% B and 64% C; (- - - -) protein-free DNA in 0.10 M NaCl–0.05 M $NaH_2PO_4$–$NaHPO_4$ (pH 7). This spectrum was taken as the limit of 100% B; (——) C reference spectrum taken from solution data in LiCl and $NH_4Cl$ (pH 7).

very concentrated solutions of TNH exhibit patterns with essentially identical shapes and values of certain characteristic normalization ratios such as ($[\theta]_{max}/[\theta]_{245}$). The rotational strength of the positive band, however, is influenced by other variables. Figure 2 shows a comparison between two different preparations, M–C (solid beaded curve) and Z–D (open beaded curve), which differ from each other as well as from DNA spectra in the solvents indicated. The values of $[\theta]_{282.5}$, which serves as an index of the rotational strength of the positive band for the several preparations under various conditions, are given in the eighth column of Table I. A small increase was consistently noted in the $[\theta]_{282.5}$ of solutions of the various M–C type preparations prepared from frozen stock solutions which had been thawed and then clarified by centrifugation. Although this effect is on the order of the average deviation in the experimental data, it is nonetheless felt to be real and related to the decreased (protein/DNA) weight ratio observed for these solutions compared to the product of the fresh solutions of the same preparation.

The larger variations in $[\theta]_{282.5}$ observed between the low-blend M–C procedure and any of the high-blend methods could not be explained by this factor but could be correlated with shearing force which the tissue received in the initial grinding medium. Those procedures in which the tissue received more high-speed blending (M–C, high blend, H–C, and Z–D) yielded preparations with significantly higher values of $[\theta]_{282.5}$. Since the initial grinding medium contained NaEDTA, this suggested that this larger variability could be attributed to variation in the ions bound to TNH, possibly picked up as contaminants or else carried along as an integral part of the structure of chromatin. In confirmation of this suspicion, the addition of NaEDTA to the isolated TNH preparations from the M–C method resulted in a marked increase in $[\theta]_{282.5}$, converting it to a value comparable to that observed for preparations resulting from the high-blend procedures. In contrast, NaEDTA had a much smaller effect on the spectra of the material from the latter procedures. These points are demonstrated by the values of $[\theta]_{282.5}$ given in Table IV. The effects on the entire spectrum are shown in Figure 3.

As might be anticipated, the additions of divalent ions such as $Mg^{2+}$ or $Ca^{2+}$ lowered the value of $[\theta]_{282.5}$ for all types of preparations. At a given concentration, $Ca^{2+}$ was more effective than $Mg^{2+}$. The results of a few of the $Ca^{2+}$ addition experiments are also given in Table III and Figure 3.

In these latter experiments, concentrations of divalent ions of 4 × $10^{-4}$ M could not be exceeded due to the excessive turbidity and precipitation which occurred above that concentration. Sedimentation and viscosity data indicated considerable aggregation in the presence of these ions compared to controls containing 0.001 M NaEDTA. There was no correlation, however, between the degree of aggregation and the lowering of the value of $[\theta]_{282.5}$ in these as well as other solutions. As is demonstrated in the last column of Table I, all of the preparations (except the M–C, sheared) showed comparable values of $s_{50\%}$, which fall within the range of values normally observed for such preparations at this concentra-

TABLE IV: Effects of NaEDTA and $Ca^{2+}$ on the CD Spectra of TNH.

| Preparation | Condition | Protein/DNA (g/g) | Solvent, pH 7–7.5 | $[\theta]_{282.5} \times 10^{-3}$ $\pm$ ($0.1 \times 10^{-3}$) | % B ($\pm$2%) |
|---|---|---|---|---|---|
| M–C | Thawed | 1.40 | $2.5 \times 10^{-3}$ M Tris | 3.14 | 38 |
| | | 1.40 | $2.5 \times 10^{-3}$ M Tris<br>$2.5 \times 10^{-4}$ M NaEDTA | 4.04 | 46 |
| | | 1.40 | $2.5 \times 10^{-3}$ M Tris<br>$2.5 \times 10^{-4}$ M $CaCl_2$ | 2.93 | 32 |
| M–C, sheared | Thawed | 1.35 | $2.5 \times 10^{-3}$ M Tris | 3.40 | 41 |
| | | 1.35 | $2.5 \times 10^{-3}$ M Tris<br>$2.5 \times 10^{-4}$ M NaEDTA | 3.92 | 46 |
| | | 1.35 | $2.5 \times 10^{-3}$ M Tris<br>$2.5 \times 10^{-4}$ M $CaCl_2$ | 2.67 | 27 |
| M–C, high blend | Fresh | 1.72 | $1 \times 10^{-3}$ M NaCl | 3.67 | 45 |
| | | 1.72 | $1 \times 10^{-3}$ M NaEDTA | 3.67 | 45 |
| | | 1.72 | $1 \times 10^{-3}$ M NaCl<br>$3.3 \times 10^{-2}$ M $CaCl_2$ | 2.56 | 33 |
| H–C | Thawed | 1.55 | $2.5 \times 10^{-3}$ M Tris | 3.75 | 46 |
| | | 1.55 | $2.5 \times 10^{-3}$ M Tris<br>$1.0 \times 10^{-3}$ M NaEDTA | 4.01 | 47 |
| | | 1.55 | $2.5 \times 10^{-3}$ M Tris<br>$2.5 \times 10^{-4}$ M $CaCl_2$ | 3.11 | 36 |

tion (Zubay and Doty, 1959; Hanlon, 1961). Furthermore, treatment of the M–C preparation with 4 M urea, followed by dialysis to remove the urea, resulted in no change in $[\theta]_{282.5}$, although the sedimentation distribution curve was markedly sharpened because of the conversion of fast-sedimenting material to material of a lower sedimentation coefficient, as has been reported in the literature (Chalkley and Jensen, 1968).

Despite the dependency of the rotational strength of the positive band on the method of isolation or the solvent and solution conditions, the ellipticities throughout this band in all cases are markedly lower in TNH compared to DNA when the polymer is in solutions of moderate ionic strength. This latter DNA spectrum is shown as a dashed curve in Figure 2 and presumably reflects the characteristic spectrum of the B conformation of DNA (Tunis-Schneider and Maestre, 1970). On the other hand, the TNH spectra exhibit higher ellipticities in the positive band when compared to the CD spectrum of films of the Li salt of DNA obtained by Tunis-Schneider and Maestre (1970) under conditions where the nucleic acid should be in the C conformation. In concentrated solutions of $NH_4Cl$ and LiCl (*ca.* 6 M), we have obtained spectra of DNA which are almost identical with that reported by these authors for the C conformation. An average of the spectra obtained in these two solvents is displayed in Figure 2 as a solid line. This corresponds to a C limit spectrum obtained by a more detailed analysis of DNA's spectra in these solvents (Wolf and Hanlon, in preparation).

The intermediate character of the nucleohistone spectra suggested that they could be accounted for as a linear combination of contributions from the two DNA conformations, the B and C forms. Using the two experimental DNA curves displayed in Figure 2 as the limits of $[\theta]_\lambda$ for 100% B and 100% C, respectively, we calculated the values of $[\theta]_\lambda$ at various wavelengths throughout the positive band which one would expect to observe if the DNA conformation in TNH were a mixed one consisting only of B and C forms and there were no gross differences in base composition in the DNA in these two conformations. The results of these calculations are shown as *points* on the smooth curves which represent the actual

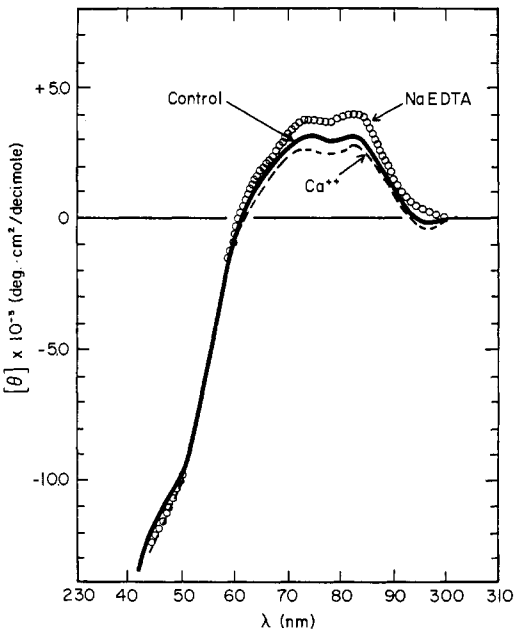


FIGURE 3: Effects of NaEDTA and $Ca^{2+}$ on the circular dichroic spectra of calf thymus nucleohistone. Spectra are presented for equivalent dilutions of a stock solution of nucleohistone prepared by the method of Maurer and Chalkley in (—) $2.5 \times 10^{-3}$ M Tris (pH 7.5), in (OOO) $2.5 \times 10^{-3}$ M Tris–$2.5 \times 10^{-4}$ M NaEDTA (pH 7.5), and (- - -) $2.5 \times 10^{-3}$ M Tris–$2.5 \times 10^{-4}$ M $CaCl_2$ (pH 7.5).

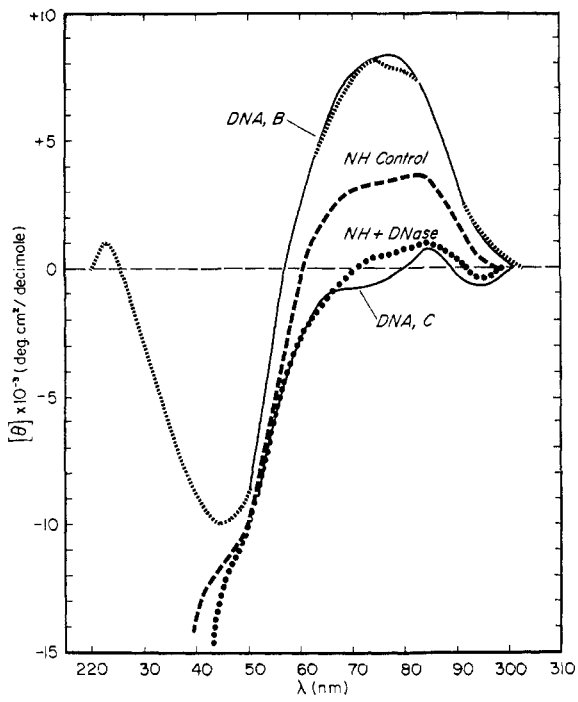


FIGURE 4: Changes in the circular dichroic spectrum of calf thymus nucleohistone upon digestion with proteases and nucleases: (- - -) control solution of TNH in $2.5 \times 10^{-3}$ M Tris (pH 7.5), isolated by the modified procedure of Maurer and Chalkley (M–C, high blend); (■■■■■) the control solution above subjected to pronase digestion for 90 min to 12 hr (as described in the text) in $2 \times 10^{-2}$ M NaCl–$2.5 \times 10^{-3}$ M Tris (pH 7.5); (●●●) the macromolecular product in $2.5 \times 10^{-3}$ M Tris (pH 7.5) resulting from nuclease digestion of the control solution of TNH above as described in the text. The limit spectra of DNA in the B and C conformations are drawn in as thin solid lines for comparison.

continuous experimental data obtained. The closed triangles represent a combination of 36% B and 63% C and fall on the solid beaded curve representing the spectrum of a M–C thawed preparation. The X's represent a combination of 47% B and 53% C and fall on the curve appropriate for the Z–D preparation. Conversely, the % B character was calculated from the experimentally observed ellipticities of the TNH solutions and the DNA limits at selected wavelengths across the positive band for these and other preparations under the various conditions cited. The detailed results for these calculations are given in Table III for eight wavelength positions. The per cent B values averaged across the band for a number of solutions of the same type are given in column 9 of Table I and column 6 of Table IV. The average deviation across a band or between solutions of the same preparation under the same conditions of solvent and solution is *ca.* ±2%. The increase in $[\theta]_{282.5}$ for the various reasons and conditions noted previously has a parallel effect on the per cent B.

The possibility that the other crystallographic form, the A conformation, was also present in TNH and contributing to these spectra was tested in the following manner. We assumed that the conformations of DNA in TNH included only the A, B, and C forms, and, hence, the fractions of each form present, $f_A$, $f_B$, and $f_C$, respectively, could be obtained by solving simultaneous equations of the type

$$[\theta]_\lambda = f_B[\theta]_\lambda^B + f_C[\theta]_\lambda^C + (1 - f_B - f_C)[\theta]_\lambda^A \qquad (2)$$

for each of the wavelength pairs, 260 and 280 nm, 265 and 275 nm, and 270 and 290 nm. $[\theta]_\lambda$ is the observed ellipticity at wavelength, λ, and $[\theta]_\lambda^B$, $[\theta]_\lambda^C$, and $[\theta]_\lambda^A$ are the limit ellipticities of the B, C, and A conformations, respectively, at wavelength, λ. The limit of the A conformation was obtained from the spectrum attributed to the A form in the paper of Tunis-Schneider and Maestre (1970). The results of these calculations for a few solutions, averaged for the three sets of wavelengths, are also given in Table III as maximal percentage contributions. This value could not have exceeded 2%, at the most, for any of the preparations.

There are various treatments which will result in dramatic changes in the per cent B contribution for a given solution. As would be expected, protein removal effects an increase in the per cent B content. (A detailed study of the relationship between this variable and the nature and amount of protein removed is the subject of paper II of this series.) A simple experiment of this type is demonstrated in Figure 4 by the spectrum, indicated by the beaded line, of a TNH solution 90 min to 12 hr after the proteolytic enzyme, pronase, had been added. The spectrum of the control solution of TNH to which no enzyme had been added is shown as a solid curve in this same figure. The CD spectrum of the pronase-treated solution is essentially identical with that obtained with DNA preparations at moderate ionic strength which have been deproteinized by more conventional methods. Since pronase would not be expected to digest RNA, this experiment demonstrates that the small amount of RNA which purified chromatin normally contains cannot be directly or solely responsible for the difference in CD patterns between the patterns for DNA and that observed for TNH.

At the other extreme, a spectrum close to the complete C form can be obtained by subjecting TNH to the endo and exo nuclease digestion conditions described in the Experimental Section. Figure 4 also shows the result of such a treatment. The nuclease-digested product had lost 34% of its bases as dialyzable nucleotides. Its per cent B content was 13% compared to the control solution whose per cent B was 46%. The B and C spectra of DNA are partially reproduced in this figure as thin solid lines for comparison.

In agreement with the observations of others (Li and Bonner, 1971; Smart and Bonner, 1971a), the melting profiles of these various preparations appeared to be composites of several distinct transitions. This is clearly seen in the plots of $R_T$, the ratio of the absorbance at temperature, $T$, to the absorbance at 25° (corrected for the slight volume dilution due to thermal expansion) as a function of temperature shown in Figures 5A and 6A. Figure 5A displays the data for an M–C solution (solid curve) and a Z–D solution (dashed curve) as well as a solution of DNA (beaded curve) in a solvent of comparable ionic strength. Figure 6A shows the melting curves of a solution of M–C TNH in the presence (dashed curve) and absence (solid curve) of NaEDTA. Other preparations exhibited curves with a similar degree of complexity.

Resolution of these transitions was facilitated by plotting these data in the derivative form, as has been suggested by Shih and Bonner (1970). Since our melting profiles were obtained in a noncontinuous manner, we arithmetically calculated the derivative by taking increments in $R_T$ at 0.5–1.0° intervals from the smoothed data of $R_T$ *vs.* $T$, and plotting the normalized function, $(100/\Delta R_T)(dR_T/dT)$, against the temperature midpoint of the interval. ($\Delta R_T$ is the total change in relative absorbance between 25° and 95–97°.) The results of this procedure are shown in Figures 5B and 6B for the profiles displayed in Figures 5A and 6A. Depending on the preparation and the solution conditions, anywhere from three

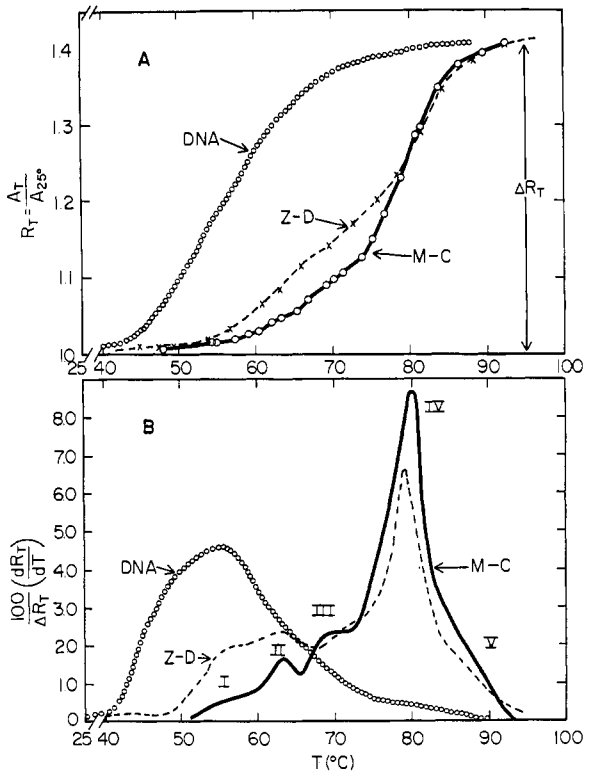


FIGURE 5: Melting profiles of calf thumus nucleohistone and DNA. A. Integral plots of the ratio, $R_T$, the absorbance at temperature, $T$, to that at 25° (corrected for thermal expansion of solvent), as a function of $T$, for TNH in (O—O) 2.5 × $10^{-3}$ M Tris (pH 7.5), isolated by the procedure of Maurer and Chalkley, and (×- -×) TNH in 7 × $10^{-4}$ M $KH_2PO_4$ (pH 7.0), isolated by the procedure of Zubay and Doty. The data for protein free DNA (OOOOO) in a solvent of comparable ionic strength ($10^{-3}$ M NaCl, pH 7) is included for comparison. B. Derivative plots of the data displayed in A plotted against $T$ and coded in the same manner as described above.

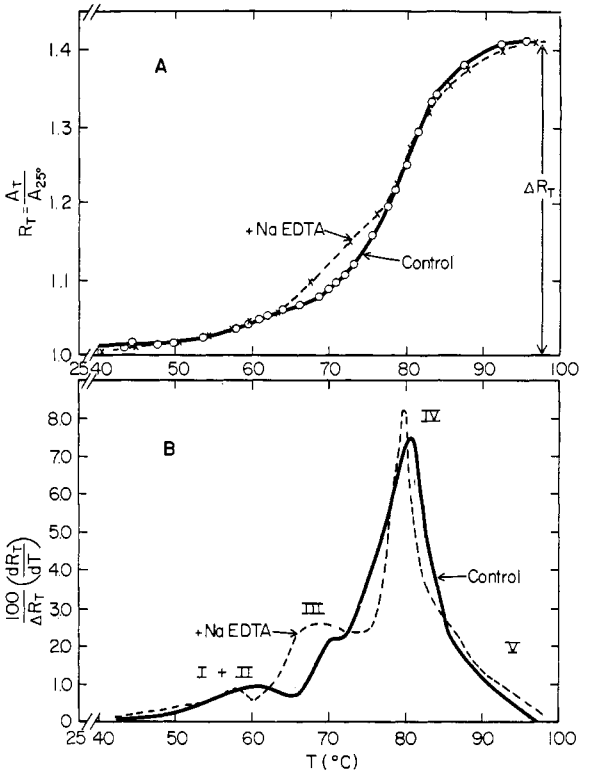


FIGURE 6: Effect of NaEDTA on the melting profile of calf thymus nucleohistone isolated by the procedure of Maurer and Chalkley. A. Integral plots of the ratio, $R_T$, of the absorbance at temperature, $T$, to that at 25° (corrected for thermal expansion of solvent), as a function of $T$, for the control solution (O—O) of TNH in 2.5 × $10^{-3}$ M Tris (pH 7.5) and the experimental solution (×- -×) in 2.5 × $10^{-3}$ M Tris–2.5 × $10^{-4}$ M in NaEDTA (pH 7.5). B. Derivative plots of the data displayed in A plotted against $T$ and coded in the same manner as described above.

to five transitions can usually be detected. In Figures 5 and 6, we have displayed some of our better profiles in which five are evident. We have numbered these I, II, III, IV, and V, in accordance with the system of Li and Bonner (1971). Resolution of these and other profiles into their individual transitions yields a set of $T_m$'s (the temperature at which 50% of the transition has occurred) which is only approximate. Calculation of the fractional contribution, $f$ (in per cent), which each transition makes to the total hyperchromic change, $\Delta R_T$, from these data is extremely hazardous for the individual transitions in all but the most ideal cases. The summed contribution of the first three transitions (I + II + III), however, can usually be obtained with little difficulty due to the relative simplicity and sharpness of the transition with a $T_m$ of *ca.* 80°. This sum together with the $T_m$'s is presented in Table V. The effect of NaEDTA on the M–C solutions is to convert some of the material melting in transitions IV and V into material melting in the lower transitions. Unfortunately, the parallel experiment in which $Ca^{2+}$ or $Mg^{2+}$ is added rather than NaEDTA could not be undertaken for this type of measurement as the divalent ion containing solutions became increasingly more turbid and precipitated as the temperature was raised above 25°.

The $T_{m,s}$ of transition I correspond, on the average, to the $T_{m,s}$ of protein-free DNA in media of comparable ionic strength. Thus, in agreement with Li and Bonner, we have assigned this transition to protein-free DNA stretches having one end free. Removal of histones by the procedure of Ohlenbusch *et al.* (1967) results in an increase in I and II and decreases in III, IV, and V. Hence, we assume that these authors' assignments for these various transitions are applicable to our preparations. On this basis, transition II reflects the melting of protein-free DNA in regions interspersed between two protein-covered regions, and transitions III, IV, and V are attributable to the melting behavior of histone-covered regions of DNA. Transition III, however, is reported to be almost as sensitive to ionic strength as is transition I or II (Li and Bonner, 1971) and, hence, the histone coverage of the DNA melting in this transition must either be minimal or very inefficient in terms of charge neutralization.

As the data in the last two columns of Table V show, there is a very good correlation between the sum of the contribution which the first three transitions, I, II, and III, make to the melting profile and the per cent B character calculated from the CD spectrum of the same solution. The figures in these two columns agree remarkably well considering the experimental error involved.

## Conclusion

In Figure 7, we have shown a model for the relationships between protein binding and DNA conformation in chromatin which ties together our own experimental observations as well as those of others (Li and Bonner, 1971; Smart

TABLE V: Correlation of Melting Profile and Circular Dichroic Characteristics of Calf Thymus Nucleohistone.

| Preparation | Solvent (pH 7–7.5) | *ca.* $T_m$'s ± 1° (°C) I | II | III | IV | V | $F$ (I + II) (%) | $F$ (I + II + III) (%) | % B |
|---|---|---|---|---|---|---|---|---|---|
| M–C | $10^{-3}$ M KCl | 57 | 63 | 70 | 80 | 88 | 14 | 34 | 34 |
| | $2.5 \times 10^{-3}$ M Tris | 56 | 61 | 71 | 81 | 92 | 16 | 37 | 42 |
| | $2.5 \times 10^{-3}$ M Tris–$2.5 \times 10^{-4}$ M NaEDTA | 57 | 63 | 69 | 80 | 87 | 12 | 49 | 47 |
| | $4 \times 10^{-4}$ M $NaHCO_3$ | | 61 | 65 + 72[a] | 80 | 89 | | 40 | 40 |
| | $4 \times 10^{-4}$ M $NaHCO_3$ | 53 | 60 | 65 | 79 | 88 | 20 | 41 | 41 |
| M–C, sheared | $2.5 \times 10^{-3}$ M Tris | | 60 | 72 | 80 | 88 | 12 | 35 | 34 |
| | $2.5 \times 10^{-4}$ M NaEDTA–$2.5 \times 10^{-3}$ M Tris | | 60 | 71 | 79 | 88 | 33 | 51 | 46 |
| M–C high blend | $10^{-3}$ M KCl | 57 | 65 | 72 | 79 | 89 | 20 | 36 | 45 |
| H–C | $4 \times 10^{-4}$ M $NaHCO_3$ | | 62 | 71 | 81 | 90 | 20 | 51 | 50 |
| Z–D | $7 \times 10^{-4}$ M $KH_2PO_4$–$K_2HPO_4$ | 57 | 65 | 72 | 81 | 88 | 28 | 50 | 48 |
| Z–D | $7 \times 10^{-4}$ M $KH_2PO_4$–$K_2HPO_4$ | 56 | 63 | 72 | 80 | | 15 | 51 | 52 |

[a] Doublet.

and Bonner, 1971a,b; Clark and Felsenfeld, 1971). Our major experimental observation is that the per cent B character of DNA in TNH corresponds to the fraction of bases melting out in transitions I, II, and III. Conversely, the per cent C character corresponds to the fraction of bases melting out in transitions IV and V. We propose, therefore, that the DNA of TNH, as isolated by the various procedures described, is in two discrete conformations whose presence reflects several different environments. In one of these, regions IV and V, shown schematically in Figure 7, the chromatin proteins—mainly histones—are complexed efficiently in such a way that the bases and ribophosphate backbone are protected from exposure and interaction with various solvent components. Part of this interaction is mediated by divalent ions bound to the nucleohistone. These ions may simply be bound to the DNA phosphates and filling in small gaps in between histones. Alternatively, they may be bound to histones and modulating the conformation in such a way as to enable these proteins to bind more effectively and compactly in the grooves and to the phosphates of the DNA duplex. Whatever the mechanism, this interaction between histone and DNA maximally stabilizes the DNA to thermal denaturation, prevents extensive nuclease digestion of DNA in these sections, and uniquely stabilizes the C conformation of DNA.

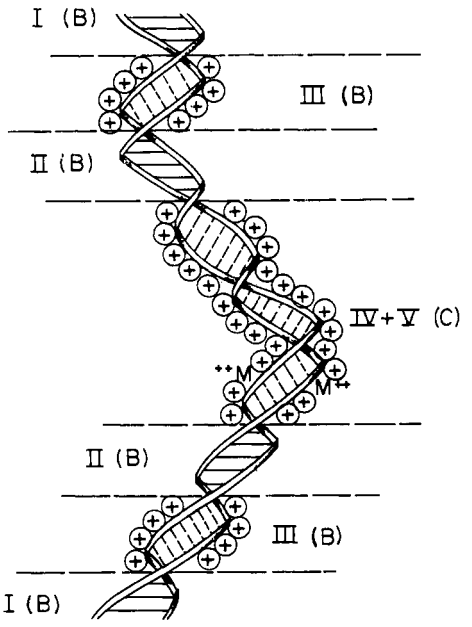


FIGURE 7: Schematic diagram of a model of TNH structure which accounts for the observed experimental results.

In the remaining regions, III, II, and I, DNA is in the B conformation. On the basis of Li and Bonner's results as well as our own (Johnson and Hanlon, manuscript in preparation), region III DNA must be partially associated with histones. This histone coverage is ineffectual, either because of partial denaturation and/or loss of divalent ions, or because the stretches of coverage are too short, to provide marked protection against nuclease digestion. Perhaps, as has been suggested by Smart and Bonner (1971b), the lysine-rich histone I makes a major contribution to this region III, and cannot, because of its high proline content, pack efficiently in the grooves of the helix.

In regions II and I, DNA is fully exposed to the solvent, either in stretches (II) sandwiched in between two regions of higher thermal stability, or as free ends (I).

This model satisfactorily accounts for the effects of protein removal by pronase and the increase in the C character due to nuclease digestion in a straightforward fashion. In fact, the per cent B (13%) of the nucleased product agrees very well

with a predicted value of 18% if all nucleotide loss and digestion had occurred in B regions. The effects of NaEDTA and $Ca^{2+}$ or $Mg^{2+}$ addition require further speculation. Experimentally, we observe that the removal of ions by the addition of NaEDTA results in a loss of material melting in transitions IV and V (which we have identified as regions IV + V in our schematic diagram), accompanied by an increase in DNA melting out in transitions II and III. On the other hand, addition of $Ca^{2+}$ increases the per cent C character which we attribute to an increase in the fraction of bases in regions IV and V. We interpret these results in terms of a structural role of $Ca^{2+}$ or $Mg^{2+}$ (the divalent ions which would naturally be present in the cell nucleus). We propose that these ions act in concert with some of the histones to increase the effectiveness of the packing arrangements and charge neutralization of DNA *in vivo*.

The variability in the per cent B character among the products of the various isolation methods is now understandable in terms of the removal of these ions in the initial tissue grinding stage. In addition, the extensive blending in all but the low-blend M–C procedure probably results in some partial denaturation and loosening of histone coverage thus increasing the B content of the complex. Even the M–C procedure, which is the gentlest one used, results in a per cent B character (34%) and a fractional estimate of completely exposed bases (*ca.* 10–20%, corresponding to DNA melting out in transitions I and II) which is much too high in terms of the amount of genetic message which would normally be expected to be turned on in a tissue as specialized as the thymus. One suspects that even the M–C procedure did not yield "native" TNH and that structural proteins originally present in this complex *in vivo* have been lost during the isolation procedure.

The identification of a C conformation in the DNA of TNH rests upon the spectral assignment of Tunis-Schneider and Maestre (1970). If that assignment is incorrect, then the conclusion herein concerning the presence of this conformation in TNH is also incorrect. We have no reason to believe, however, that an error has been made. It is true that Bram and Beeman (1971) stated that the result of their wide-angle X-ray scattering data indicated that the DNA of TNH prepared by the Zubay–Doty method was in the B form. This statement has been subsequently modified by Bram (1971) who suggested that the conformation of DNA of TNH is in the "B family." An examination of some of the characteristic dimensions of his DNA structure, however, reveals that they are very similar to the values for the C form reported by Marvin *et al.* (1961). We also feel that the precision of this technique is insufficient to distinguish between the pure forms of two such closely related conformations of DNA and roughly equimolar mixtures.

The validity of the calculation of the per cent B content is dependent on the assumption that there are no marked differences in base composition of DNA in the various regions of the model shown in Figure 7. The results of Clark and Felsenfeld (1971) support this assumption. On the basis of the results of nuclease digestion experiments with calf thymus chromatin, these authors have found that there is no significant difference between the base composition of nuclease-susceptible regions of TNH (which they have identified as protein-free or solvent-accessible DNA regions) and nuclease-insensitive regions (which they identify as protein-covered DNA regions). Their model of protein distribution in TNH is similar to both our own and that of Li and Bonner (1971).

A more serious challenge to our conclusions are the results of investigations on reconstituted complexes of DNA and various histone fractions (Fasman *et al.*, 1970; Li *et al.*, 1971; Olins and Olins, 1971; Shih and Fasman, 1971, 1972). Of special interest are the results of Fasman *et al.* (1970) who observed continuous lowering of the positive band in the CD patterns of DNA as increasing amounts of f-1 histone were complexed. Although some of the patterns are similar to those observed for TNH, it is our opinion that the final curve obtained reflects a conformational process different from that produced by simple histone–DNA interaction in TNH. We have obtained similar patterns to that observed by Fasman *et al.* (1970) with DNA at very high LiCl concentrations (11 *m* at 27°) under conditions where hydrodynamic data indicate that the molecule is undergoing a profound conformational change and/or is aggregating extensively prior to precipitation (Wolf and Hanlon, in preparation).

The question of the relationship of our results to the possibility of supercoiling of TNH in solution is an interesting one (Pardon *et al.*, 1967; Richards and Pardon, 1970; Bram and Ris, 1971). Certainly, the dimensions of the supercoiled structure of TNH proposed by Pardon *et al.* (1967) (*ca.* 100 Å in diameter and 120 Å in pitch) are such that one would hardly expect it to have a marked effect on the optical properties of DNA in dispersed form in solution (*i.e.*, unaggregated) as long as the B conformation were maintained. It is possible, however, that the C conformation of DNA coupled with the more effective charge neutralization of DNA in regions IV and V is more conducive to supercoiling and that the fraction of bases in these regions reflects that fraction of TNH in the supercoiled state. Since divalent ions increase the amount of C character, it is tempting to propose that these ions play a significant biological role as a switch mechanism. Upon being bound, the TNH structure tightens up, perhaps by supercoiling, and thus becomes less susceptible to interaction with the environment.

In conclusion, we feel that our data support a model of a mixed conformation of DNA in TNH consisting of B and C forms. The latter is stabilized by intimate binding of chromatin proteins and modulated by ionic factors. The relative proportion of each is, within limits, a function of the isolation procedure employed.

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# Conformational Changes of Transfer Ribonucleic Acid. Equilibrium Phase Diagrams†

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ABSTRACT: We report systematic studies of the thermal denaturation of $tRNA_1^{Tyr}$, $tRNA^{fMet}$, $tRNA_2^{Phe}$, and $tRNA_1^{Val}$, all from *Escherichia coli*. Measurements at the absorbance maximum of 4-thiouridine show multiphasic melting curves at high salt (0.17 M $Na^+$, for example, no $Mg^{2+}$), and predominantly monophasic curves at low salt (0.005 M $Na^+$, for example, no $Mg^{2+}$). The variation of these transition midpoints with the logarithm of $Na^+$ concentration defines a phase diagram of four different conformational zones, with temperature and ion concentration as independent variables. There is no evidence for a separate $Mg^{2+}$ zone of the phase diagram. When the low-salt conformation is converted to the high-salt form, the process has an activation energy between 25 and 61 kcal/mole, depending on the tRNA. We also report some characteristic spectral differences between the various conformational phases. In order to rationalize the conformational behavior, we propose a phase diagram in which the high-salt, low-temperature form is the "native" conformation, with tertiary structure. As the temperature is raised, the tertiary structure is lost, and the conformation at high salt and medium temperature is hypothesized to be "cloverleaf or close variant." At low temperature and low salt concentration, the tertiary structure is also lost; however, the product is not a "cloverleaf" but rather an "extended form" that has noncloverleaf secondary structure. When the temperature is raised sufficiently at both low and high salt concentration, the product is the randomly coiling single strand. From systematic examination of the influence of $Mg^{2+}$ on thermal transitions, we conclude that it causes a marked stabilization of the tertiary structure present in high $Na^+$, but that it does not produce a new separately melting cooperative interaction unit.

Ribonucleic acids have complex conformational properties. For example, tRNA can exist in a metastable "denatured" conformation (Lindahl *et al.*, 1966; Gartland and Sueoka, 1966), from which biological activity can be recovered by heat treatment. Or, when native tRNA is gradually heated, a number of intermediate states are observed before the randomly coiling, single-strand conformation is reached at high temperatures (Riesner *et al.*, 1969). Another transition of particular interest is the loss of tertiary structure postulated by Fresco *et al.* (1966).

Study of these conformational changes is hampered by the lack of rigorous proof of any RNA conformation. Nucleic

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