A Study of the Structure of Isolated Chromatin*

R. Chalkley† and R. H. Jensen‡

ABSTRACT: Calf thymus chromatin, isolated by chemically gentle means, can be partially disrupted by shear. The resulting nucleohistone is highly heterogeneous with respect to molecular size and can be fractionated by sedimentation into nucleohistones of increasingly complex structure. The large molecules are made up of smaller nucleohistone molecules linked together noncovalently by protein cross-links. Lysine-rich histone appears to play a role in maintaining the structure of such complexes.

The chemical and physical properties of large and small nucleohistones are of two classes. First, the properties in common are the general chemical composition, ultraviolet absorption spectrum, melting profile, and a compact conformation in low ionic strength buffers; second, those properties which are solely dependent upon the presence of a complex structure and are found only in large nucleohistones. This includes sensitivity to 4 M urea and to NaCl of concentration high enough to remove only a small amount of protein. Treatment with these materials invariably destroys the cross-linked structure of more complex nucleohistones. Fractionation of nucleohistone on the basis of sedimentation velocity also fractionates it with respect to *in vitro* template activity for ribonucleic acid synthesis. More complex nucleohistones direct ribonucleic acid synthesis at a reduced rate; destruction of the structure gives rise to an increased template activity.

In the nuclei of higher organisms, chromosomal DNA exists in close association with specific proteins, both histone and non-histone (Bonner and Ts'o, 1964). When isolated nuclei are disrupted, most of the chromosomal complement can be separated from the nuclear sap by differential centrifugation. The rapid sedimentation of isolated chromatin is not simply due to the presence of DNA of high molecular weight, but is a result of the complex structural nature of the chromatin itself which is subsequently isolated as a gel (Zubay and Doty, 1959).

Chemically, the preparation of chromatin gels is a gentle procedure, and inherent in work in this field is the concept that these gels may serve as worthwhile models for nuclear chromatin. We have been concerned with the physicochemical and biological properties of calf thymus chromatin (itself a gel). Because of the difficulty of studying a gel directly, we have partially disrupted chromatin by high-speed mixing and obtained nucleohistone (Bonner *et al.*, 1968) which has proved more amenable to physicochemical analysis. Nucleohistone prepared in this manner is heterogeneous in molecular size, and it can be fractionated by sedimentation. We have studied the properties of fractions of increasing molecular complexity in order to gain information concerning the nature of chromatin which is even more complex.

The results with nucleohistone suggest that isolated chromatin is composed of complexes of DNA and histone forming units which are linked together by protein. The nature of this linkage has been examined and is described. A relationship has been observed between the extent of cross-linking in nucleohistone and its ability to act as a template for *in vitro* RNA synthesis; the more extensively cross-linked the nucleohistone, the lower its template activity.

Materials and Methods

Preparation of Calf Thymus Chromatin. Calf thymus tissue was obtained within minutes of slaughter, and transported to the laboratory in ice. The tissue was cleared from surrounding fat, divided into *ca.* 15-g pieces, and stored at −60°. The isolation of thymus chromatin followed the method described by Maurer and Chalkley (1967) for calf endometrium chromatin. The calf thymus chromatin was homogenized in a final volume of 40 ml of 0.01 M Tris (pH 8.0) and dialyzed against the same overnight. The dialyzed chromatin which appears as an opalescent gel was diluted with 0.01 M Tris (pH 8.0) to a final A_{260} of 20 and sheared in a Virtis homogenizer at 40 V for 2 min. The homogenate was clarified by centrifuging at 18,000 rpm for 30 min. More than 95% of the DNA in the homogenate was recovered in the supernatant fractions.

In order to isolate nucleohistones of differing sedimentation coefficient, unfractionated nucleohistone (7 ml) was layered onto 45 ml of a 5–30% (w/v) linear sucrose density gradient in 0.01 M Tris (pH 8.0) and centrifuged for 4.7 hr at 25,000 rpm in a Spinco SW-25.2 rotor. A glass tube (2-mm i.d.) was inserted, penetrating about five-sixths of the gradient, and the solution was pumped out (suction) using a polystaltic pump (Buchler

* From the Division of Biology, California Institute of Technology, Pasadena, California. *Received July 19, 1968.* This work was supported by U. S. Public Health Service Grant GM-13762 and by the Herman Frasch Foundation. R. H. J. was supported by International Minerals and Chemical Corp.

† Present address: Department of Biochemistry, School of Medicine, University of Iowa, Iowa City, Iowa 52240.

‡ Present address: International Minerals and Chemical Corp., Libertyville, Ill.

TABLE I: Sedimentation Characteristics of Nucleohistone and DNA Isolated from Nucleohistone.

Material	Bulk Solvent (M)	$s^0_{20,w}$ (S)	$s^0_{20,w}$ (S) at Half-Peak Height	
			Rear	Front
30S nucleohistone (a)[a]	NaCl (0.01)–50% D_2O, Tris (0.001), pH 8	27.0	18.2	43.5
30S nucleohistone (b)	Same	32.2	25.1	49.4
130S nucleohistone (a)	Same	128	92.2	168
130S nucleohistone (b)	Same	132	99.7	159
Chromatin[b]	NaCl (3)–Tris (0.1), pH 8	25.2	9.6	39.6
DNA from chromatin[c]	Same	26.0	6.4	44.0
30S nucleohistone	Same	14.0	9.7	17.7
130S nucleohistone	Same	14.5	11.5	17.8

[a] The letters a and b in parentheses are two separate preparations of nucleohistone from different samples of calf thymus tissue. [b] Nucleoproteins dissociate fully into DNA and protein in 3 M NaCl; the $s^0_{20,w}$ recorded is therefore that of the DNA of the nucleoprotein. [c] The DNA from chromatin was prepared by the Marmur (1961) procedure followed by two phenol extractions and ether extraction. DNA was prepared from the other fractions in an identical manner, and in all cases $s^0_{20,w}$ was the same as the material directly layered onto 3 M NaCl.

Instruments), and collected in 1-ml fractions. Desired fractions were pooled and dialyzed overnight against 0.01 M Tris (pH 8.0). That this technique gives samples of moderately homogeneous S-value range is shown by the data of Tables I and III.

Preparation of DNA. DNA was isolated from calf thymus nucleohistone by a modified Marmur procedure (Marmur, 1961), followed by two phenol extractions and subsequent removal of phenol with ether.

Preparation of RNA Polymerase. RNA polymerase was isolated from early log-phase cells of *Escherichia coli* strain D-10 (a ribonuclease-minus strain) following the method of Chamberlin and Berg (1962) up to their fraction 4 (F4).

Chemical Analyses. DNA and RNA were analyzed after separation using a modified Schmidt–Tannhauser procedure (Ts'o and Sato, 1959). DNA was determined by the diphenylamine assay of Burton (1956), and RNA was determined by the orcinol reaction (Dische and Schwarz, 1937). Purified calf thymus DNA (Worthington Biochemical Corp.) and yeast RNA (Sigma) were used as standards.

Protein was analyzed by the Lowry procedure (Lowry *et al.*, 1951) following separation into histone and nonhistone components. Histone was first extracted into 0.4 N H_2SO_4 (30 min at 2°), then precipitated with 25% trichloroacetic acid and finally redissolved in 1 N NaOH for analysis. Acid-insoluble material was treated with 10% trichloroacetric acid (100° for 10 min) and the residue of nonhistone protein was dissolved in 1 N NaOH for analysis. Calf thymus histones and bovine serum albumin (Sigma) were used as standards.

Ultraviolet Absorption and Thermal Denaturation. Ultraviolet absorption spectra were determined with a Cary 12M recording spectrophotometer. Thermal melting profiles were recorded using a Gilford Model 2000 spectrophotometer with a jacketed cuvet compartment and a linear temperature programmer. The rate of temperature increase was 0.5°/min.

Assay for RNA Synthesis. The incubation and assay systems were those described by Marushige and Bonner (1966) with the exception that spermidine phosphate was omitted from the incubation medium. After incubation, the sample was chilled in ice, and 0.5 ml of sheared calf thymus DNA (200 μg/ml) was added as coprecipitant followed immediately by 2 ml of cold 20% trichloroacetic acid. When using RNA polymerase fraction 4 and low levels of template, the addition of a coprecipitant is strongly recommended.

Sedimentation Analysis. Sedimentation velocity was studied using band-sedimentation techniques either on preformed sucrose gradients in a Spinco Model L ultracentrifuge or on self-generating density gradients in a Spinco Model E ultracentrifuge (Vinograd *et al.*, 1963). All sedimentation coefficients were calculated from the results of analytical ultracentrifugation. Distribution of sedimentation coefficients was calculated by the method of Vinograd and Bruner (1966).

Results

Calf Thymus Chromatin. Calf thymus chromatin, isolated by chemically gentle methods (see Materials and Methods), exists as a gel. It has a high sedimentation velocity and is completely sedimented at 2000*g* in 20 min. However, DNA isolated from calf thymus chromatin has $s^0_{20,w} = 25$, which corresponds to mol wt 12×10^6 (Studier, 1965), and the chemical composition of calf thymus chromatin by mass is DNA 1.0, histone 0.97, and nonhistone protein 0.52. It is unlikely that a simple complex of 25S DNA and protein could account for the rapid sedimentation of chromatin and we sus-

TABLE II: Chemical Composition of Calf Thymus Nucleohistones.[a]

Nucleohistone	DNA	Histone	Nonhistone Protein	RNA
30S	1.00	0.91 ± 0.02	0.32 ± 0.03	<0.005
130S	1.00	1.03 ± 0.03	0.31 ± 0.03	<0.005
Unfractionated nucleohistone	1.00	0.95	0.33	<0.005
Chromatin	1.00	0.97	0.52	0.01

[a] Date presented as mass ratio relative to DNA. Although histone is extracted and assayed as histone sulfate, the data are reported in terms of histone protein. Previous workers who have computed similar mass ratios have often reported histone together with HSO_4^-, in which case the histone contribution is increased by about 28%.

pect that the high-sedimentation velocity of chromatin is a result of linking between several or many DNA molecules.

Disruption and Fractionation of Calf Thymus Chromatin. We sought to partially disrupt the structure of chromatin in a manner which would cause minimal chemical changes. Mechanical shear reduces the sedimentation velocity of chromatin such that less than 5% of the DNA and histone is sedimented from 0.01 M Tris (pH 8.0) during centrifugation at 30,000*g* for 20 min. The material which is not pelleted under these conditions after shearing is called nucleohistone. That shearing does not permanently dissociate histone is shown by the fact that the DNA/histone ratio is unchanged after sedimenting sheared chromatin into a pellet (see also Table II).

The sedimentation profile of nucleohistone on a linear sucrose gradient (Figure 1) demonstrates its heterogeneity. The distribution of material is skewed with a long leading edge. The sedimentation coefficient at band maximum is 28–30 S. Increasing the time (2–10 min) or the rate (40–80 *V*) of shear leads to an increase in the amount of slower sedimenting (30S) material, though material of smaller size is not found.

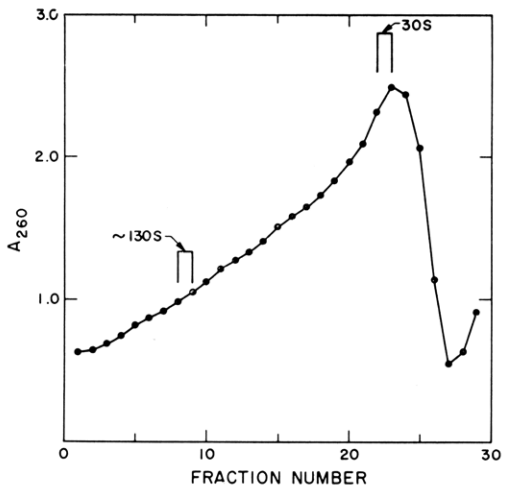

FIGURE 1: Sucrose gradient sedimentation of whole calf thymus nucleohistone. Whole nucleohistone (7 ml, A_{260} 20) was layered onto 45 ml of a 5–30% linear sucrose density gradient and centrifuged (25,000 rpm, 4.6 hr) in a Spinco SW 25.2 rotor. Three sucrose solutions were pumped out simultaneously as described in Materials and Methods and fractionated into 75-drop fractions. A_{260} of each fraction was recorded. 20S and 130S nucleohistone were collected as shown and dialyzed overnight against 0.01 M Tris (pH 8).

DNA isolated from calf thymus nucleohistone has a mean $s^0_{20,w}$ = 14 S. Neither increased rate nor extended periods of homogenization reduce the mean $s^0_{20,w}$ of such DNA appreciably. It appears that shear disrupts chromatin to a limiting subunit of 30S nucleohistone which cannot be further sheared in a Virtis homogenizer.

Sucrose gradient sedimentation of partially disrupted chromatin (Figure 1) affords a convenient means of obtaining nucleohistone of differing molecular complexity. It is a reasonable assumption that nucleohistone of higher S value may retain some of the structural features of chromatin. We customarily isolate fractions with $s^0_{20,w} \approx$ 130 and 30 S. The choice of 130 S was initially purely arbitrary, based only on the wish to study material of substantially higher S value than that of the peak (30 S). Material of intermediate S values has been studied and has properties in between those described for 130S and 30S nucleoproteins. Most of the following studies are devoted to comparing and contrasting the properties of 30S and 130S calf thymus nucleohistone.

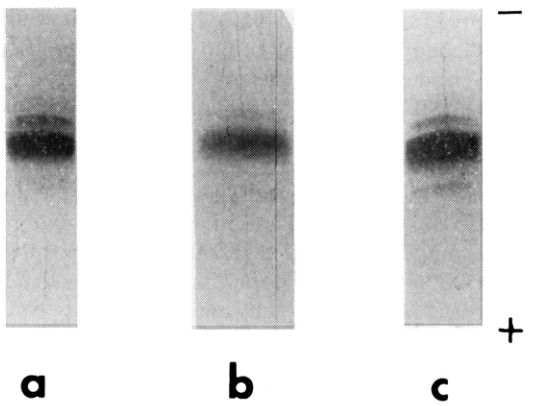

FIGURE 2: Photograph of acrylamide gels after electrophoresis of (a) whole histone, (b) histone isolated from 30S nucleohistone, and (c) histone isolated from 130S nucleohistone. Histones were extracted as described in Materials and Methods and electrophoresis gels were run parallel as described by Bonner *et al.* (1968).

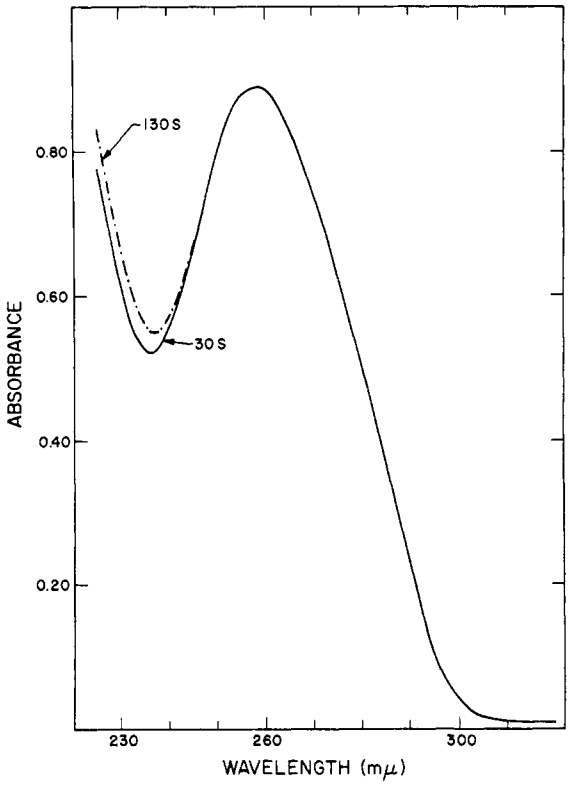

FIGURE 3: Ultraviolet absorption spectra of 30S and 130S nucleohistones. The curves are identical from 320 to 245 mμ and are drawn as a single line over this range. Light scatter has been subtracted.

Physical and Chemical Characterization of Calf Thymus Nucleohistones. That nucleohistone fractions do not change their sedimentation patterns following isolation and dialysis is shown by further sedimentation either on a sucrose gradient or in the analytical ultracentrifuge. Table I contains the results of sedimentation analysis of 30S and 130S fractions. Of particular significance is the result that DNA whether isolated (under conditions of minimal shear) from 30S or 130S nucleohistone has an identical mean sedimentation coefficient and distribution about this mean.

Sedimentation equilibrium of these nucleohistone samples in a CsCl density gradient gives identical results in all cases; buoyant density at band center is 1.696 g/cc and a shoulder appears at $\rho^\circ = 1.704$ g/cc. These values are in good agreement with the values reported for whole calf thymus DNA (Schildkraut *et al.*, 1962).

Calf thymus nucleohistones are rich in DNA and histone; they contain some nonhistone protein and little RNA as shown in Table II. There is a small difference between the slower and faster sedimenting nucleohistones in the amount of histone present; faster sedimenting material contains slightly more histone. Fractions taken from intermediate points in the sucrose gradient fractionation of nucleohistone are consistent with a relationship between increasing sedimentation coefficient and increasing histone content. Although the quantity of histone present varies with the nucleohistone, all fractions of histone are present in all materials as shown by

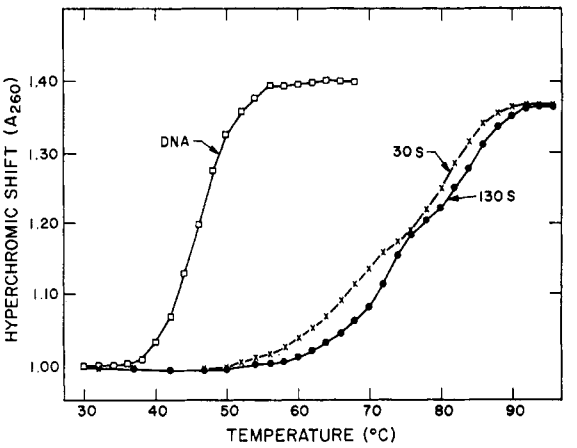

FIGURE 4: Thermal denaturation profiles of calf thymus DNA, 30S, and 130S nucleohistones. All samples were exhaustively dialyzed against 2.5×10^{-4} M EDTA (pH 8.0) before melting.

the acrylamide gel electrophoresis patterns of histones isolated from each fraction (Figure 2).

Differences in histone content are reflected in the ultraviolet spectra of these nucleohistones; samples containing more protein have an elevated molar absorptivity in the spectral region where protein absorbs (<245 mμ). Typical nucleohistone spectra are shown in Figure 3. There is a small but measurable degree of scatter, which has been subtracted using an empirically determined relationship between light scattering and wavelength (Leach and Scheraga, 1960).

The differences in histone content can be detected upon thermal denaturation as shown in Figure 4. There is a small difference in T_m (midpoint of thermal denaturation) of 1.5°. Thermal denaturat on profiles in solutions of low ionic strength are sensitive measures of the extent of stabilization of DNA by histone (Olivera, 1966; Ohba, 1966), and it is apparent from the shape of the melting curves that material with the melting behavior of free DNA is entirely absent, though there is a gradient change in the melting profile analogous to that reported for rat liver nucleohistone (Marushige and Bonner, 1966).

Molecular Size of 30S and 130S Nucleohistones. Using the technique of sedimentation equilibrium (Edelstein and Schachman, 1967) in a solvent consisting of 90% D_2O–0.005 M NaCl–0.01 M Tris (pH 8.0), we find that 30S nucleohistone has a molecular weight of approximately 2.5×10^6 daltons. Under the same conditions (2233 rpm) the bulk of 130S nucleohistone is slowly sedimented from solution. It is therefore concluded that the molecular weight of 130S nucleohistone is greater than that of 30S nucleohistone. It was not possible to obtain even an approximate molecular weight for the 130S nucleohistone using this technique because the required lower rotor speeds were unavailable to us. The 30S nucleohistone was too heterogeneous to obtain a precise molecular weight (*ca.* 30% was sedimented fully at equilibrium at 3617 rpm). In addition, a secondary ion effect (Van Holde and Baldwin, 1958) must be in operation because of the necessity to study nucleohis-

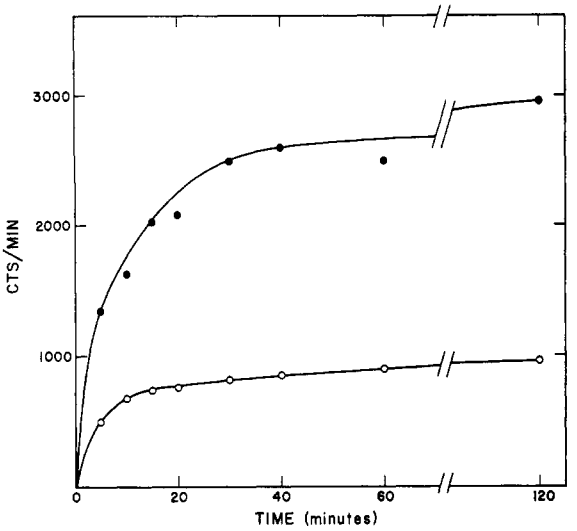

FIGURE 5: Incorporation of [^{14}C]ATP into RNA directed by calf thymus DNA and nucleohistone. A set of parallel incubations containing either DNA (—●—●—) or whole nucleohistone (—○—○—) was performed as described in Materials and Methods. At the times indicated an incubation was terminated and analyzed for RNA synthesis. The specific activity of the [^{14}C]ATP was 1 μCi/μmole.

tone in solutions of low ionic strength (<0.03 M) in which they are not aggregated (Jensen and Chalkley, 1968). In view of these obvious difficulties we cannot attach great confidence in the experimentally determined molecular weight for 30S nucleohistone, although it is clearly of the right order of magnitude for a 1:1 complex of histone and DNA of mol wt 1.8×10^6. However, the merit of the experiment is that it does establish a large qualitative molecular weight difference between 30S and 130S nucleohistone.

The Effect of Urea upon 30S and 130S Nucleohistones.

TABLE III: Effect of Urea on 30S and 130S Nucleohistone.

Bulk Solvent (M)[a]	$s^0_{20,w}$ (S) 30S Nucleohistone	$s^0_{20,w}$ (S) 130S Nucleohistone
NaCl (0.01)–Tris (0.001) (pH 8)–50% D_2O	27.0	128[b]
Urea (4)–Tris (0.001) (pH 8)[b]	17.9	22.7
NaCl (0.01)–Tris (0.001) (pH 8)–50% D_2O[c]	29.7	36.7

[a] An aliquot of 20 μl of sample (A_{260} 1.5) in 0.01 M Tris (pH 8) was placed in the cup of a type I centerpiece (20 mm). Bulk solvent was placed in cell sector. [b] Rotor speed = 20,410 rpm; all other samples were centrifuged at 35,600 rpm. [c] This sample was first diluted into 4 M urea, then dialyzed into 0.01 M Tris (pH 8). Since the concentration of this material was only A_{260} 0.75, 50 μl was placed into the cell cup.

TABLE IV: Effect of NaCl on $s^0_{20,w}$ of 30S and 130S Nucleohistones.

NaCl (M)[a]	$s^0_{20,w}$ (S) 30S Nucleohistone	$s^0_{20,w}$ (S) 130S Nucleohistone
0.01	32.2	132
0.40	30	(26)[b]
0.60	22	31
0.80	19	19
3.0	14	14.5

[a] Nucleohistone (20 μl, A_{260} 1.5) in 0.01 M Tris (pH 8) was put into the cup of a type I centerpiece (30 mm). The bulk solvent was 0.001 M Tris (pH 8)–50% D_2O–NaCl to give the molarity shown in the table. [b] At 0.40 M in NaCl, 130S nucleohistone spread rapidly throughout the cell, thus preventing measurement of sedimentation velocity at band maximum. The number reported is the $s^0_{20,w}$ of half-height on the trailing side of the very broad band. This side of the band was relatively sharp.

Treatment of 30S nucleohistone with 4 M urea reduces the $s^0_{20,w}$ as shown in Table III. When this material was returned to urea-free medium, the original sedimentation coefficient was regained. That no protein was dissociated by urea treatment has been shown by sedimenting the nucleohistone from 4 M urea into a 50% w/v sucrose underlayer (38,000 rpm, 16 hr) and chemically analyzing the sediment. Chemical analysis and thermal denaturation studies indicated no significant change from the untreated material. The reduction of $s^0_{20,w}$ might be the result of an increase either in the partial specific volume or in the frictional coefficient of the nucleohistone. Recent experiments support this latter interpretation (Bartley and Chalkley, 1968). The change in frictional coefficient is probably due to an extension of nucleohistone from its more compact conformation (Ohba, 1966; Giannoni and Peacocke, 1963) in dilute buffer.

The complex nature of 130S nucleohistone is destroyed in 4 M urea and the material sediments with $s^0_{20,w}$ = 23 S. This process is irreversible as is shown by the fact that, following removal of urea, sedimentation velocity increases to $s^0_{20,w}$ = 37 S in a manner analogous to the behavior of the 30S nucleohistone; the initial high $s^0_{20,w}$ is not regained.

The Effect of Salt upon 30S and 130S Nucleohistones. The effect of increasing NaCl concentration upon the $s^0_{20,w}$ of 30S and 130S nucleohistone is shown in Table IV. In 0.4 M NaCl the structure of 130S nucleohistone is modified, a process which is complete in 0.8 M NaCl, at which salt concentration both nucleohistones behave in an identical fashion, showing sedimentation behavior characteristic of partially deproteinized nucleohistone. In the concentration range 0.1–0.4 M NaCl, calf thymus nucleohistones are extensively aggregated (Jensen and

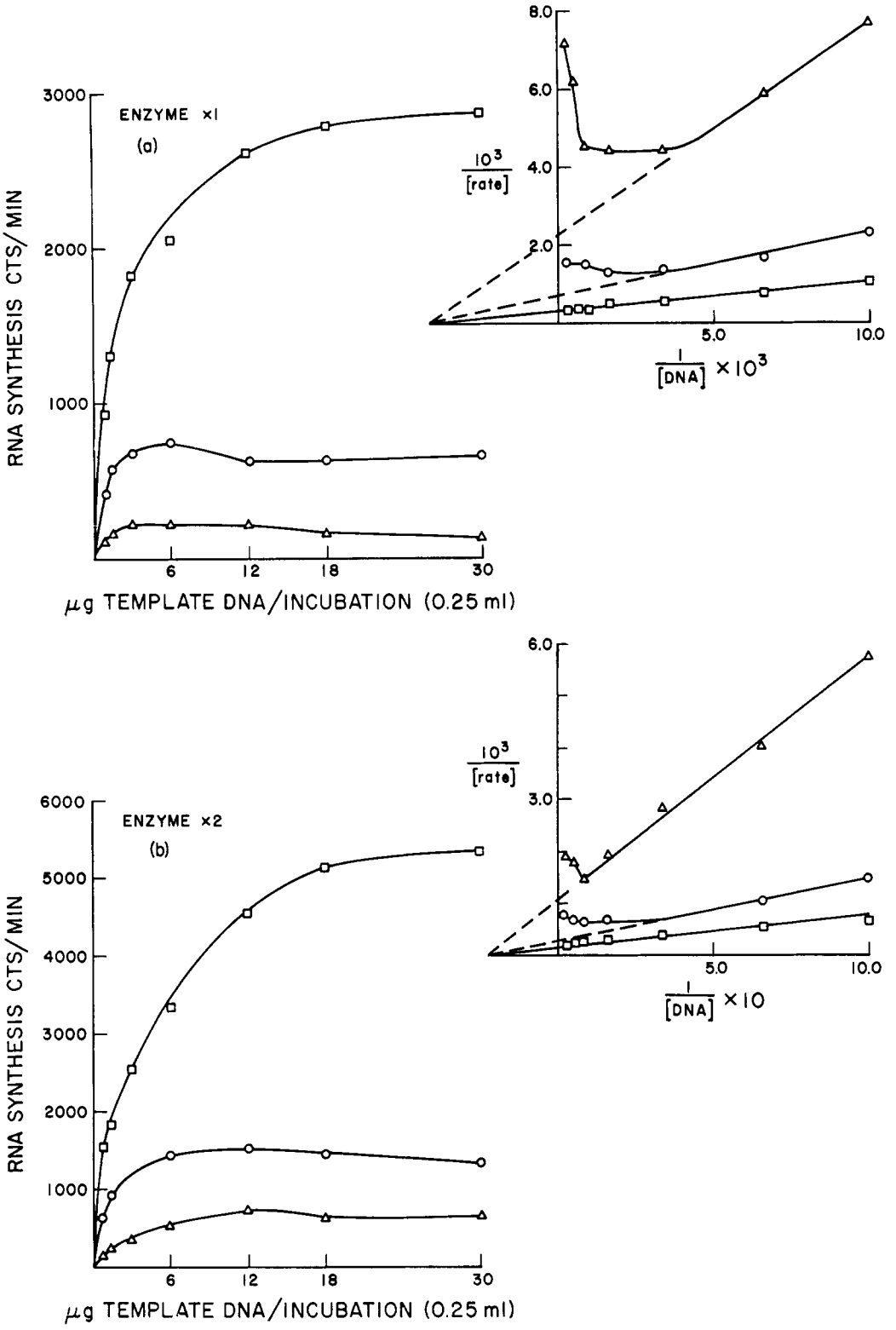

FIGURE 6: Dependence of RNA synthesis upon template concentration at two different enzyme concentrations. (a) Enzyme × 1; (b) Enzyme × 2. Incubations were performed in the presence of increasing concentrations of DNA (--□--□--), 30S (—○—○—), or 130S (—△—△—) nucleohistone as template. Lineweaver–Burk reciprocal plots of the data are shown in the inset, in which the same notation is employed.

TABLE V: Template Activity and Sedimentation Velocity of Calf Thymus Nucleohistone.[a]

Template	Amt of Template Present (μg of DNA/0.25 ml)	Pretreatment	$s^0_{20,w}$ (S)	Incorp of [^{14}C]-AMP into RNA (μμmoles/0.25 ml)
Calf thymus DNA	3	A	14	1980
Calf thymus DNA	6			2780
30S nucleohistone	3	A	30	700
30S nucleohistone	6			990
130S nucleohistone	3	A	128	480
130S nucleohistone	6			650
30S nucleohistone	3	B	30	720
30S nucleohistone	6			990
130S nucleohistone	3	B	32	750
130S nucleohistone	6			900
Calf thymus DNA	6	A	14	2800
Chromatin	6	A	>500	380
Chromatin	6	C	30–200[b]	1090
Chromatin	6	D	57	810

[a] Incubation conditions were as described in Materials and Methods. Pretreatment: (A) no pretreatment; (B) ionic strength elevated to 0.6 M NaCl followed by dialysis against 0.01 M Tris (pH 8); (C) chromatin was sheared (30 V/90 sec); there was no further fractionation; (D) ionic strength raised to 2.0 M NaCl followed by dialysis against 0.01 M Tris (pH 8). [b] Sheared chromatin has a heterogeneous distribution of $s^0_{20,w}$ (see Figure 1).

Chalkley, 1968); however, upon dialyzing to solutions of lower ionic strength the nucleohistones redissolve, showing an $s^0_{20,w}$ identical with that before treatment. However, removal of salt by dialysis from nucleohistone treated with concentrations of 0.4 M NaCl or higher causes a return to $s^0_{20,w}$ = 30 S in all instances. The disruption of the complex structure of 130S nucleohistone by salt above 0.4 M is irreversible.

RNA Synthesis Directed by Nucleohistone Templates. DNA and nucleohistone both direct RNA synthesis *in vitro* although at different rates. The data of Figure 5 show that the rate of RNA synthesis decreases with time of incubation, and furthermore that this decrease is the same for both templates. Thus the relative ability of nucleohistone and DNA to direct RNA synthesis is the same at all times during an incubation. The requirements for DNA-dependent RNA synthesis from calf thymus nucleohistone are strictly analogous to those reported for rat liver chromatin (Marushige and Bonner, 1966) and for free DNA (Chamberlin and Berg, 1962). RNA synthesis from thymus nucleohistone requires template, RNA–polymerase, divalent cations and the four ribonucleoside triphosphates.

The dependence of rate of RNA synthesis upon template concentration is shown in Figure 6 for calf thymus DNA, 30S, and 130S nucleohistone. The ability of calf thymus nucleohistone to support RNA synthesis is less than that of calf DNA. The faster sedimenting nucleohistone is less template active than the slower sedimenting species. The rate of RNA synthesis from nucleohistone reaches a maximum at lower template concentration than DNA; until this level is reached the template activity of the 30S nucleohistone is 50% of that of an equal amount of DNA. However, the bulk of the DNA in 30S nucleohistone is complexed with histone as is evidenced by the melting behavior of 30S nucleohistone. We conclude that, on occasion, even that DNA which is associated with histone may be available for transcription.

When concentration curves are converted into standard Lineweaver–Burk plots in Figure 6, it is apparent that for nucleohistone the enzyme kinetics are complex. At low template concentrations DNA and both types of thymus nucleohistones have the same K_m as described by Marushige and Bonner (1966) for rat liver nucleohistone, but there is a marked shift in the reciprocal plots of nucleohistone at higher template concentrations leading to a lower over-all K_m. The complexity of the mechanism of RNA synthesis prevents a physical interpretation of the empirical constant K_m. However, the general character of the Lineweaver–Burk plots implies that at high concentrations of nucleohistone, available template is not proportional to added template. Among the possible interpretations of this fact, the most plausible one is that high concentration of nucleohistone causes inactivation of template by intermolecular aggregation.

The differential incorporation into acid-precipitable material transcribed from 30S or 130S nucleohistone is not a result of differential breakdown of the synthesized RNA. To show this, 30S and 130S nucleohistone were incubated for RNA synthesis, after 10 min actinomycin D (50 μg/ml) was added, and the slow decrease in acid-precipitable radioactivity was recorded (over

60 min). The rate of RNA breakdown was small compared with the rate of synthesis and each nucleohistone fraction lost a comparable fraction of incorporated radioactivity.

The Relation between Structure and Template Activity. We have examined the changes in template activity of chromatin and nucleohistone (sheared chromatin) following modification of the structure. This was achieved either using elevated salt concentrations followed by dialysis to low ionic strength or, in the case of chromatin, by shear forces. The data of Table V show that the template activity of chromatin or 130S nucleohistone (both having complex structures) was increased following treatment which reduced the $s^0_{20,w}$ of the material. It is clear that shear forces alone are capable of achieving this end, as shown by the increase in template activity of chromatin after shearing at 30 V for 90 sec. There was no centrifugation step in this experiment and it is emphasized that the over-all chemical composition of the solution was unchanged throughout. On the other hand, template activity of 30S nucleohistone was unchanged by salt treatment and subsequent dialysis, as was its sedimentation constant.

Discussion

The structure of nuclear chromatin is obviously complex, and most probably consists of several levels of organization. Recent studies by Pardon *et al.* (1967) using X-ray diffraction of stretched, moist nucleohistone fibers have indicated that a primary organization is at the level of supercoiling of individual nucleohistone molecules. A secondary level or organization, a cross-linking between nucleohistone molecules, is deduced from the observations of Littau *et al.* (1964) who examined the appearance of sectioned nuclei in the electron microscope after various chemical modifications.

The properties of the nucleohistones we have described indicate that both types of structure are present in the more complex (130S) material, but that the 30S material lacks the intermolecular cross-linked structure. Both structural forms are changed by urea treatment, but the forces responsible for supercoiling nucleohistones are modified reversibly, in contrast to those governing the cross-linked structure of 130S nucleohistone. This difference in the nature of the structural forces is further demonstrated by the high sensitivity of 130S nucleohistone to proteolysis compared with that of 30S material.

The cross-links in the more complex nucleohistones are ruptured irreversibly by the methods we have employed. This observation argues against the possibility that they are an artifact of the isolation procedure, and we suspect that the more complex nucleohistones are retaining some of the structural features of the isolated gels. In this context it is of interest that although the $s^0_{20,w}$ of isolated chromatin is too high to measure conveniently, chromatin DNA has $s^0_{20,w}$ = 25 S (see Table I), suggesting that chromatin itself is extensively cross-linked.

The ability of nucleoproteins of increasing complexity to direct RNA synthesis at reduced rates indicates that there may be a relationship between structure and template activity. However, it is not possible at this time to determine whether this is a biologically meaningful effect. We have shown that 130S nucleohistone aggregates to a greater extent than 30S nucleohistone in 0.15 M NaCl (Jensen and Chalkley, 1968) and a similar state of affairs may exist in our incubation system for RNA synthesis. Also, Sonnenberg and Zubay (1965) have reported the aggregation of nucleohistone in these systems.

Acknowledgment

The skillful assistance of Miss M. Thomas is gratefully acknowledged. The authors are indebted to Professor J. Bonner for continuing support and advice.

References

Bartley, J. A., and Chalkley, R. (1968), *Biochim. Biophys. Acta 160*, 252.

Bonner, J., Chalkley, G. R., Dahmus, M., Frambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B. M., and Widholm, H. (1968), *Methods Enzymol.* (in press).

Bonner, J., and Ts'o, P. O. P., Ed. (1964), *in* The Nucleohistones, San Francisco, Calif., Holden-Day.

Burton, K. (1956), *Biochem. J. 62*, 315.

Chamberlin, M., and Berg, P. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 81.

Dische, Z., and Schwarz, K. (1937), *Mikrochim. Acta 2*, 13.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Frenster, J. H., Allfrey, V. G., and Mirsky, A. E. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 1026.

Giannoni, G., and Peacocke, A. R. (1963), *Biochim. Biophys. Acta 68*, 157.

Jensen, R. H., and Chalkley, G. R. (1968), *Biochemistry 7*, 4388 (this issue; following paper).

Leach, S. J., and Scheraga, H. A. (1960), *J. Am. Chem. Soc. 82*, 4790.

Littau, V. C., Allfrey, V. G., Frenster, J. H., and Mirsky, A. E. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 93.

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

Marmur, J. (1961), *J. Mol. Biol. 3*, 208.

Marushige, K., and Bonner, J. (1966), *J. Mol. Biol. 15*, 160.

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

Mauritzen, C. M., Starbuck, W. C., Saroja, I. S., and Taylor, C. W. (1967), *J. Biol. Chem. 242*, 2240.

Ohba, Y. (1966), *Biochim. Biophys. Acta 123*, 76.

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

Olivera, B. M. (1966), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Pardon, J. F., Wilkins, M. H. F., and Richards, B. M. (1967), *Nature 215*, 508.

Schildkraut, C. L., Marmur, J., and Doty, P. (1962),

J. Mol. Biol. 4, 430.
Sonnenberg, B., and Zubay, G. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 415.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 625.
Ts'o, P. O. P., and Sato, C. (1959), *Exptl. Cell Res. 17*, 227.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Vinograd, J., and Bruner, R. (1966), *Biopolymers 4*, 131.
Vinograd, J., Bruner, R., Kent, R., and Weigle, J. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 902.
Zubay, G., and Doty, P. (1959), *J. Mol. Biol. 1*, 1.

The Physical State of Nucleohistone under Physiological Ionic Strength. The Effect of Interaction with Free Nucleic Acids*

R. H. Jensen† and R. Chalkley‡

ABSTRACT: Nucleohistone aggregates in 0.05–0.30 M NaCl. The extent of aggregation depends upon ionic strength and increases with nucleohistone concentration and molecular size. At a given ionic strength aggregation is greater at pH 5 than at pH 8. Aggregated nucleohistone regains its original properties when redissolved in low ionic strength buffer. We propose that nucleohistone aggregates because its negative charge is effectively neutralized, thus leading to decreased hydration and increased interaction between nucleohistone molecules. Free deoxyribonucleic acid and ribonucleic acid inhibit nucleohistone precipitation by complexing lysine-rich histones, thus leaving the nucleohistone molecules more negatively charged.

Chromosomal material isolated from higher organisms is a disperse gel in solutions of low ionic strengths (<0.1 M) but is precipitated from solution at ionic strengths in the range 0.15–0.40 M (Oth and Desreux, 1957). This is true even if the nucleoprotein is of low (<30S) sedimentation coefficient.

The monovalent cation concentration in animal cell nuclei is in the region of 0.2 M (Langendorf *et al.*, 1961). A low level of divalent cations is also present. Chromatin should therefore be present *in vivo* largely in the precipitated state. We have studied the properties of calf thymus nucleohistone in solutions 0.15 M in NaCl. The properties of isolated nucleohistone under these conditions are expected to be similar to those of nucleoprotein *in vivo*.

Materials and Methods

Materials. Calf thymus nucleohistone was prepared by the method previously described (Chalkley and Jensen, 1968). At least 90% of the nuclear DNA of thymus cells is recovered complexed with histone and nonhistone protein in the mass ratios DNA, 1.0; histone, 1.0; and nonhistone, 0.30.

Our preparations contain a continuous distribution of nucleohistone molecules which range in sedimentation coefficient from 25 to about 200 S in 0.01 M Tris (pH 8.0). As previously reported, we normally isolate two fractions with $s_{20,w}$ of 30 and 130 S, respectively. The $s_{20,w}$ of DNA isolated from each of the two fractions is 14 S. We have shown that 130S nucleohistone consists of units of 30S nucleohistone linked together by protein. Therefore, its chemical properties are very similar to those of 30S nucleohistone.

DNA was isolated from calf thymus nucleohistone by a modified Marmur procedure (Marmur, 1961), followed by two phenol treatments and subsequent ether extraction of phenol. M. Dahmus kindly donated the tRNA which was isolated from ascites tumor cells by phenol extraction and purified by methylated albumin Kieselguhr column chromatography. rRNA was isolated from *Escherichia coli* strain MRE600 and was a gift of J. Sedat. All nucleic acid solutions were extensively dialyzed against 0.01 M Tris (pH 8.0) before use. Melting profiles were determined on samples dialyzed against 2.5×10^{-4} M EDTA (pH 8), immediately prior to study.

Histones were extracted into 0.4 N H_2SO_4 for 30 min. The precipitate of DNA plus nonhistone protein was removed by centrifuging at 31,000*g* for 20 min. Four volumes of 95% ethanol were added to the supernatant and the solution was held at −20° for 24 hr. The precipitated histone was collected by centrifugation at 23,500*g* for 20 min and washed four times with 95% ethanol. Eth-

* From the Division of Biology, California Institute of Technology, Pasadena, California. *Received July 19, 1968.* This work was supported by U. S. Public Health Service Grant GM-13762 and by the Herman Frasch Foundation. R. H. J. was supported by International Minerals and Chemical Corp.

† Present address: International Minerals and Chemical Corp., Libertyville, Ill. 60048.

‡ Present address: Department of Biochemistry, School of Medicine, University of Iowa, Iowa City, Iowa 52240.