Biochemical and Physiochemical Characterization of Chromatin Fractions with Different Degrees of Solubility Isolated from Chicken Erythrocyte Nuclei†

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ABSTRACT: Chicken erythrocyte chromatin was prepared according to two different methods [Fulmer, A. W., & Bloomfield, V. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5968–5972; Ausio, J., Borochov, N., Seger, D., & Eisenberg, H. (1984) *J. Mol. Biol. 177*, 373–398] to give three main common fractions, according to its solubility (S) or insolubility (I) in 0.15 M NaCl buffers or to its further solubility in 0.25 mM ethylenediaminetetraacetic acid (E). From the biochemical point of view, all of them have been found to be undistinguishable. Analytical ultracentrifugation shows that all these fractions can reversibly undergo the transition from the low to the higher order structure, through a nearly identical way of folding. Thermal denaturation profiles yielded three transitions having the same T_m's for the three fractions. The percentage of DNA melting in the first transition decreased in the order S > I > E, and the amount in the second transition increased in the same order. Together with the different solubility of these fractions in the presence of divalent ions, these results indicate that in the three fractions of chromatin studied, the amount of linker DNA bound to the nucleosome varied.

Most of our knowledge about chromatin comes from the studies on solubilized deoxyribonucleoprotein ("chromatin") samples. In order to achieve this solubilization, nuclei are digested with nucleases and subsequently exploded in order to disrupt the nuclear membrane. As a consequence of these drastic manipulations, the "in situ" structure of the chromatin is modified as reflected by the change of the solubility behavior (Skalka et al., 1976) of the resulting fractions. From this point of view, at least two classes of "chromatin" fractions can be clearly distinguished: one which has its lower solubility at around physiological ionic strength and one which remains fully soluble under these conditions. Rather than being ascribed to a particular biological system (Fulmer & Bloomfield, 1981), they seem to be present in all the "chromatin" preparations, independently of the biological source used. These differing solubility fractions have been found not only in chicken erythrocyte (Ruiz-Carrillo et al., 1980; Fulmer & Bloomfield, 1981) but also in calf thymus (Hollandt et al., 1979) and in rat liver (Brust & Harbers, 1981). Their presence may reflect either the intrinsic existence of different chromatin domains in the starting nuclei (Igo-Kemenes et al., 1982; Hancock, 1982) or the partial disruption or damage introduced with the biochemical manipulation (Hollandt et al., 1979) already mentioned. Both fractions were earlier reported to have a very close physicochemical behavior and also a nearly identical biochemical composition (Fulmer & Bloomfield, 1981, 1982; Ausio et al., 1984), so that no explanation has been given for their solubility difference. In the present investigation, we have combined highly sensitive biochemical and physicochemical approaches in one more effort to explain such a solubility phenomena and to examine whether or not there is a structural difference between these fractions of chicken erythrocyte chromatin.

Materials and Methods

Chromatin Fractionation. Chicken erythrocyte nuclei were prepared and digested with micrococcal nuclease either at 0 °C (Fulmer & Bloomfield, 1981) or at 37 °C (Ausio et al., 1984). Briefly in the first case (A), the erythrocyte nuclei were suspended in digestion buffer containing 0.045 M NaCl, 0.015 M KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 mM phenylmethanesulfonyl fluoride (PMSF)[1] and 5 mM Tris-HCl, pH 7.5 ($A_{260} \simeq 40$). The nuclear suspension was digested at 0 °C with 50 units/mL micrococcal nuclease (Worthington) for 2 h, digestion was terminated by adding $^1/_{20}$th volume of 0.1 M EDTA, pH 7.5, and soluble bulk chromatin was isolated by extraction with the digestion buffer containing 5 mM EDTA (Fulmer & Bloomfield, 1981). Fractionation of bulk chromatin into soluble chromatin (S) and insoluble chromatin (I) was achieved by dialyzing the bulk chromatin against 0.15 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, and 0.2 mM PMSF, pH 7.5. The fractions were collected as previously described (Fulmer & Bloomfield, 1981). The chromatin that remained insoluble by nuclease digestion was extracted with 0.25 mM EDTA, pH 7.5, to yield EDTA-extractable chromatin (E). In the alternative method (B) (Ausio et al., 1984), the nuclei were digested to about 1% acid solubility in 100 mM KCl, 50 mM Tris-HCl (pH 8.0), and 1 mM $CaCl_2$ ($A_{260} \simeq 120$). The digestion was carried out at 37 °C with 4.5 units/mL micrococcal nuclease (Worthington) for 4.5 min. The reaction was terminated by adding EDTA up to 5 mM, in the cold. Chromatin fragments were solubilized according to Scheme I.

The chromatin samples, so obtained, were concentrated in an Amicon ultrafiltration device, using XM-300 membranes, and they were further fractionated either by using 5–25%

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[1] Abbreviations: CD, circular dichroism; T_m, temperature of maximum dh/dT for each transition; % H_T, percent of total hyperchromicity found in a given transition; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Con A, concanavalin A; BSA, bovine serum albumin.

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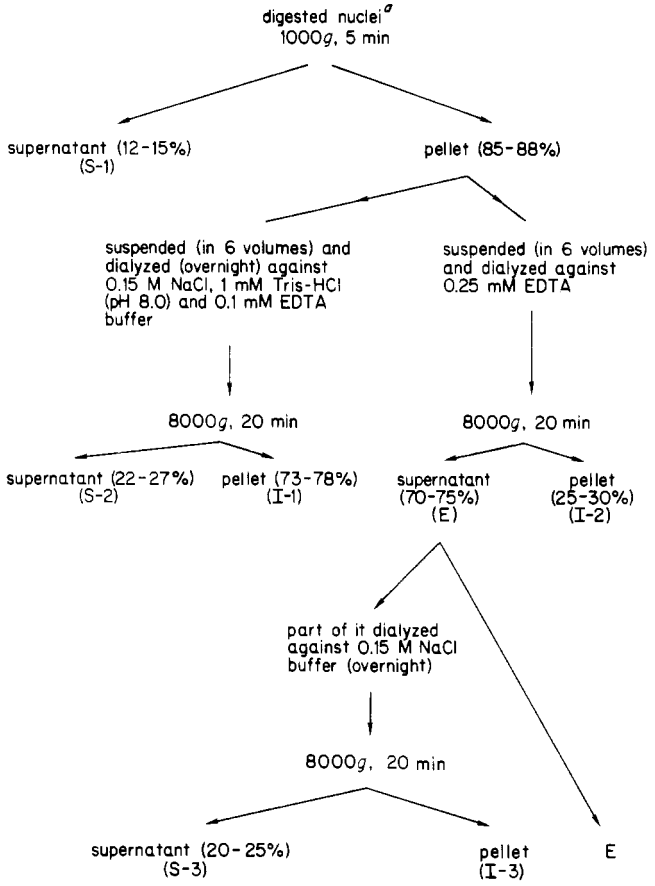
Scheme I



[a] All the steps were carried out at 4 °C, and all the yields refer to percentage of A_{260}.

sucrose gradients centrifuged at 25 000 rpm for 2.5 h at 4 °C in a SW 57 Beckman rotor or by gel filtration chromatography on a Bio-Gel A-15 m column (2.5 × 70 cm) (Bio-Rad, Richmond, CA). The sucrose gradients and the columns were prepared and equilibrated in 5–10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0, buffer containing different amounts of NaCl as required in each case.

Selective Depletion of Histones H1 and H5. Histones H1 and H5 were extracted together from chromatin by shaking chromatin at room temperature in 0.35 M NaCl, 1 mM Tris-HCl (pH 7.3), 0.2 mM EDTA, and 0.4 mM PMSF in the presence of AG 50 W-X2 (50–100 mesh) Bio-Rad resin as described by Ruiz-Carillo et al. (1980).

Histone H1 selectively depleted chromatin was prepared through the use of the same resin but following the procedure described by Muyldermans et al. (1980).

Gel Electrophoresis. Gel electrophoresis of histones was routinely performed in 15% polyacrylamide–SDS slab gels (Weintraub et al., 1975). High-resolution urea–acetic acid–polyacrylamide gels were also used. In this case, they were carried out according to Hurley (1975) but with several modifications to make it suitable for long vertical (32 × 16 × 0.15 cm) slab gels, and therefore, they were prepared in the following way: *Stacking* gels were made from solution A [20% acrylamide and 1% *N,N'*-methylenebis(acrylamide)], solution B (43.2% acetic acid), and solution C (10 M urea). Solutions for making gels consisted of 3.2 mL of solution A + 2 mL of solution B + 10 mL of solution C and 0.8 mL of H_2O. To this mixture is added 18 mg of thiourea and shaken until it becomes completely soluble. Finally 74 μL of 30% H_2O_2 is added, shaken, and *immediately* poured over the gel (polymerization usually takes 3–5 min at room temperature). The gel had been prepared by mixing 20 mL of solution A* [60% acrylamide and 2% *N,N'*-methylenebis(acrylamide)], 10 mL of solution B, and 50 mL of solution C, to which 70.5 mg of thiourea was added until they became soluble. Then 450 μL of 30% H_2O_2 was added and shaken, and the resulting mixture was immediately poured into the gel chamber. The running buffer is 0.9 N acetic acid, 5 M urea, 5% β-mercaptoethanol, and 0.1% pyronine. When proteins (histones) had to be dissociated from DNA in situ, the above sample solution was then replaced by 7 M urea, 0.9 N acetic acid, and 5% β-mercaptoethanol containing 10 mg/mL clupeine sulfate, as already described (Reczek et al., 1982). Electrophoresis was performed at 6 °C for 58–64 h at 350–370 V.

The gels were stained first with 0.2% amido black 10B in 5% acetic acid and 25% methanol and second with 0.4% Coomassie blue in the same solvent. The gels were finally destained by diffusion in the solvent without dye. The gels were analyzed as previously described (Fulmer & Fasman, 1979; Panyim & Chalkley, 1969).

For the radioactive staining with ^{125}I-labeled concanavalin A, the method described by Burridge (1976) was used, although in our case buffer A additionally contained 0.5 mM $CaCl_2$ and 0.5 mM $MnCl_2$ and BSA was also used as a carrier protein instead of hemoglobin.

Gel electrophoresis of DNA was carried out on 4% polyacrylamide gels according to Peacocke and Dingman (1967).

Determination of the Nucleosomal Repeat Distance. The various chromatin samples ($A_{260} \simeq 1.0$, volume 1.5 mL) were digested with 4 units of micrococcal nuclease/mL as a function of time. DNA from each fraction, at various times of digestion, was extracted and applied to a slab gel containing 1.5% agarose as described earlier (Fulmer & Fasman, 1979). Calibration of DNA mobility in these gels was done by coelectrophoresis of *Hin*cII–*Hin*dIII (New England Bio-Labs) restriction fragments of λ DNA (Miles).

Spectroscopic Measurements. Absorption spectra were recorded on a Cary 14 spectrophotometer at 23 °C. Circular dichroism spectra were recorded at 23 °C with a Cary 60 instrument as described previously (Fulmer & Fasman, 1979). DNA nucleotide residue concentrations were determined by absorption at 258 nm using $\epsilon_{258} = 6800$ cm^{-1} (mol of nucleotide)$^{-1}$.

Thermal Denaturation. Thermal denaturation measurements were made by using a Gilford UV–VIS instrument. A microprocessor-controlled spectrophotometer system 2600 with a thermal programmer 2527 accessory as previously discussed (Sasi et al., 1982) was used.

Analytical Ultracentrifugation. Sedimentation velocity experiments were carried out in a Beckman Model E analytical ultracentrifuge equipped with speed and temperature control and a photoelectric scanner. An-T titanium (four-hole) or an An-D aluminum (two-hole) rotors were used, together with 12-mm Kel-F double-sector centerpieces. Boundaries were recorded routinely at 260 nm. All experiments were performed at 5–10 °C, and the data were further converted to standard $s_{20,w}$ values.

Chromatin Solubility in the Presence of Divalent Ions. Chromatin solubility in $MgCl_2$ was determined by allowing solutions at about 0.8 A_{260} to stay overnight at 4 °C in 1 mM Tris-HCl (pH 8.0) at the appropriate $MgCl_2$ concentration and then centrifuging at 12000*g* for 10 min and subsequently measuring the optical density of the supernatant.

RNA Measurement. RNA determination was carried out by hydrolyzing the samples in triplicate in 0.4 N NaOH at

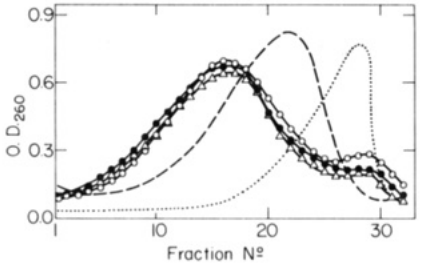


FIGURE 1: Sucrose gradient fractionation of the soluble chromatin samples in 150 mM NaCl: (○) S-1; (△) S-2; (●) S-3. For comparison, the same fractionation is shown for fraction E in 25 mM NaCl (---) and 5 mM NaCl (···). In all cases, the buffer was 1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA; the linear gradient in sucrose was 5–25% in a Beckman SW 27 rotor at 25000 rpm for 3 h at 4 °C. Procedure B isolation was used. S-1, S-2, and S-3 are as defined in Scheme I.

37 °C overnight. Afterward, the solution was diluted with 4 volumes of 1 M sodium phosphate (pH 7.0), and its absorbance at 260 and 290 nm was measured. Finally, the nucleotide concentration (in millimolar) was taken equal to $0.1015(A_{260} - A_{290})$ (Elson et al., 1979).

RESULTS

In the present investigation, soluble bulk chromatin from inactive chicken erythrocytes was fractionated into two classes, soluble (S) and insoluble (I), on the basis of their solubility in 0.15 M NaCl. The chromatin that remained insoluble by nuclease digestion was extracted with 0.25 mM EDTA to yield E chromatin. These three chromatin samples were further subjected either to gel filtration chromatography or to sucrose gradient fractionation to obtain the high molecular weight fractions.

Figure 1 shows the fractionation, on sucrose gradients, of the three soluble fractions obtained during the procedure of chromatin preparation according to Ausio et al. (1984) (method A) as described in Scheme I shown under Materials and Methods. As can be seen in this figure, all these samples are indistinguishable from the point of view of their size distribution. On the other hand, the yields of these fractions are highly concentration dependent. For instance, the larger the volume of 0.15 M NaCl buffer in which the nuclei are suspended after digestion, the higher the yield in S-2, the relationship being of an exponential type, reaching one limiting asymptotic value. The same is also true in the opposite situation, i.e., when the sample has to be concentrated. Again, precipitation increases upon increasing concentration of the chromatin sample. From this point of view, the "soluble" chromatin fractions behave similarly to the "insoluble" one. It is worthwhile, however, to mention that the yields obtained in their preparation, although lower than those obtained with the insoluble fractions, are clearly incompatible with the explanations ascribing the soluble difference as due to and/or reflecting differences in biological activity. Furthermore, chromatin can still be resolubilized with 0.15 M NaCl from I-1 and I-3 pellets as defined in Scheme I shown under Materials and Methods.

Table I: Histone Compositions of Chromatin Fractions[a]

sample	relative staining intensities[b]					
	H4	H2A	H2B	H3	H5	H1
S chromatin	1	0.82	1.05	1.18	0.78	0.21
I chromatin	1	1.0	1.17	0.99	0.69	0.23
E chromatin	1	0.92	1.29	1.10	0.88	0.30

[a] SDS–polyacrylamide gel electrophoresis was performed as discussed under Materials and Methods. [b] Stained with 0.1% amido black. Values reported represent the staining intensity of a given histone band vs. that of the H4 band of the same gel. Values represent the average of different samples and are reproducible within ±0.10.

After fractionation, we proceeded to characterize the different fractions. It is important to fractionate first, since as it can be seen in Figure 1 the starting samples contain some contamination with nucleosomes and oligonucleosomes (tubes 27–32) which are partially depleted of linker histones (histones H1–H5) (results not shown).

In Figure 2, the histone composition of different "chromatin" fractions is shown. As can be seen, all of them contain the full histone complement and in the same stoichiometry (Figure 2 and Table I). No detectable selective loss, as that found when magnesium is included in the fractionation buffers (Pantazis & Sakamoto, 1980), has been found. In fact, even

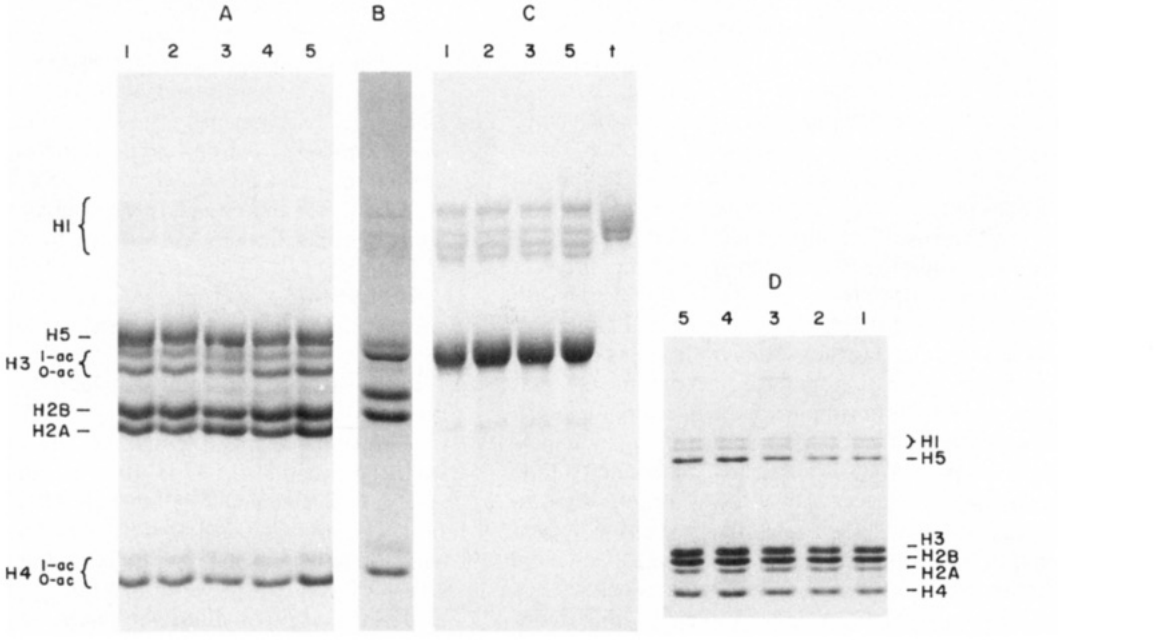


FIGURE 2: Gel electrophoresis of histones. (A) 0.4 N HCl extracted histones electrophoresed in a urea–acetic acid gel; (B) protamine-displaced histones in the same gel; (C) 5% perchloric acid extracted histones in the same gel; (D) SDS electrophoresis of histones. In all cases, lane 1 is S-1, lane 2 is S-2, lane 3 is S-3, lane 4 is I-3, lane 5 is E, and lane *t* is calf thymus 5% perchloric acid extracted histones.

the acetylation levels of histones H3 and H4 (which can be clearly distinguished in the acid–urea gels used) appear to be also identical. Moreover, and as reported earlier (Fulmer & Bloomfield, 1981), non-histone chromosomal proteins were not detected in any of these chromatin fractions. It has to be mentioned here that all these results were independent of the method used to prepare the chromatin, at 0 °C according to Fulmer and Bloomfield (1981) or at 37 °C according to Ausio et al. (1984). The same is also true for the method employed to obtain the histones, i.e., if they were either acid extracted (Figure 2A,C) or in situ displaced from the DNA by addition of competing protamine under saturation conditions (Figure 2B); in both cases, the results were the same. The reason for using acid extraction (preferentially) was due to the fact that the resolution in the urea gels used here was higher. Protamine displaced histones run in the same gel with lower resolution (Figure 2B).

In the search for some more subtle differences at the histone level, gels similar to those shown in Figure 2A–C were subjected to radioactive labeling by overlaying them with ^{125}I-concanavalin A in order to look for oligosaccharide compositional differences, which might account for the difference in solubility. This technique had been recently used (Levy-Wilson, 1983) to show the existence of some saccharides associated with the histones of the macronuclei from *Tetrahymena thermophila*. Working under similar conditions (i.e., amount of protein loaded in the gel and level of radioactivity of the iodinated Con A), we have been able to detect saccharides only in the protein used as a marker (ovalbumin), clearly indicating that, if there is any glycosylation in chicken erythrocyte histones, this is at a much lower level than that reported by Levy-Wilson (1983) in *T. thermophila* and therefore far beyond the scope of the solubility problem.

At the compositional level, the protein/DNA ratio was found to be very similar, in both the soluble (S) and insoluble fractions (I and E), as judged from their A_{260}/A_{230} ratios. This protein/DNA ratio was estimated from the A_{260}/A_{230} ratios on the basis of the following assumptions: (1) The amount of non-histone proteins is very low in chicken erythrocytes; (2) $A^{1cm}_{0.1\%}$ = 0.23 at 260 nm and 8.34 at 230 nm for DNA; for histones, $A^{1cm}_{0.1\%}$ = 0.23 at 260 nm and 4.2 at 230 nm for octameric (H2A, H2B, H3, and H4) histones (Stein, 1979); $A^{0.1\%}_{230nm}$ for histone H5 = $A^{0.1\%}_{230nm}$ for histone H1 = 2.03 (Aviles, 1978), and H1 + H5/histone core ratio = 1.3 (Bates & Thomas, 1981); (3) additivity of the extinction coefficients. Using these assumptions, we have estimated a protein/DNA ratio of 1.14 ± 0.07 and 1.09 ± 0.06 (w/w) for insoluble and soluble fractions, respectively. Actually, these values represent the average of at least five different preparations. It is worthwhile to mention the fact that in spite of the different method used, our values are in reasonable agreement with those of 1.05 ± 0.05 and 1.01 ± 0.05 reported by Fulmer and Bloomfield (1981), indicating the validity of the approach used. What is important, however, from these figures is the fact that they nearly coincide within the error of measurement, the protein/DNA ratio for the soluble fractions being systematically slightly lower (~4%), indicating once more how subtle the compositional differences can be. Concerning the protein/DNA ratio, we have observed that the fractions (S) which are soluble in 0.15 M NaCl are always associated, either before or after fractionation, with a small quite homogeneous fraction of oligonucleosome (Figure 3A, lanes 2 and 3) with a major band at ~14 ± 2 nucleosomes as measured on the acrylamide gel in Figure 3, containing a full complement of histones (Figure 3B, lane 1) but with an A_{260}/A_{230} ratio = 1.95 which

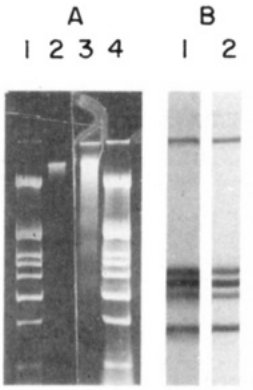


FIGURE 3: Electrophoretic analysis of DNA and histone composition of the small oligonucleosome fraction associated with soluble chromatin. (A) 4% acrylamide gel of DNA (lanes 2 and 3); pBr322 *Hin*fI digested (lanes 1 and 4 used as markers); (B) SDS histone gel of (1) histones from this fraction and (2) standard histones.

corresponds to a protein/DNA ratio of 0.5. No RNA, in significant amounts, was found to be associated with this oligonucleosomal fraction as revealed by chemical analysis. The existence of such a fraction is seen when soluble chromatin fractions are exhaustively dialyzed against 0.25 mM EDTA. After filtration in an Amicon XM-300, this fraction appears in the filtrate, a fact which apparently seems in contradiction with the above-mentioned value of 14 ± 2 nucleosomes but not with the $s_{20,w}$ value of 14.1 ± 0.2 S. The amount of this component was estimated from high molecular weight samples of already fractionated soluble chromatin (in order to avoid the contribution due to histone H1–H5-depleted oligonucleosomes which are also present in the unfractionated samples, as already mentioned) and was found to correspond to ~3.5% of the soluble chromatin. Such values, together with the protein/DNA ratio of ~0.5, may account for the slightly lower protein/DNA ratio found for the soluble fraction. The nature of this fraction is, however, not clear as it is also not obvious that it is the reason for the difference in solubility. Indeed, insoluble chromatin, which is mixed with this component under the same compositional proportions, still remains insoluble. Such a similar oligonucleosomal component was never found in the E fraction.

Following the biochemical characterization, the possible variations of the spacer length of DNA in the chromatin fractions were investigated by agarose gel electrophoresis. DNA fragments obtained from micrococcal nuclease digestion were extracted from chromatin samples (S, I, and E) and applied to 1.4% agarose gels as discussed earlier (Reczek et al., 1982). The nucleosomal repeats of all three fractions were found to be identical and corresponds to ≃200 ± 10 base pairs of DNA.

Further structural characterization was carried out by using circular dichroism, analytical ultracentrifugation, and thermal denaturation.

In the first case, different samples of S, I, and E chromatin fractions were dialyzed against 0.25 mM EDTA and 1 mM sodium phosphate, pH 7.0, and their CD spectra were recorded from 350 to 250 nm at 23 °C (data not shown). Identical CD spectra were obtained for all three fractions of chromatin with $[\theta]_{282.5}$ = 4000 deg cm^2 dmol^{-1}. To investigate the effect of ionic strength on the CD spectra of various chromatin samples, CD studies were also performed in a medium containing 60 mM NaCl, 1 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0. $[\theta]_{282.5}$ values of all three chromatin samples were decreased by 1000 deg cm^2 dmol^{-1}. This indicates either changes in the winding angle (Baase & Johnson, 1979) of the

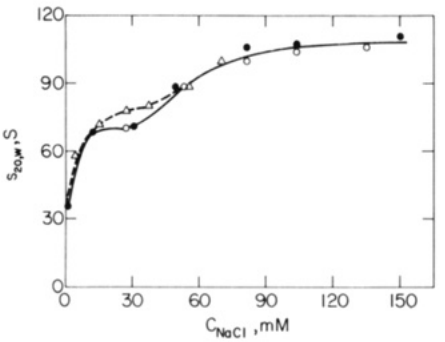


FIGURE 4: Sedimentation coefficients, $s_{20,w}$, as a function of NaCl concentration in a 1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA buffer of (△) insoluble chromatin (I) and (●, ○) soluble chromatin (S). In (●), the initial starting NaCl concentration (150 mM) was lowered and again increased back to its initial value (○). The number of nucleosomes in weight average (N_w) was ≃37 in both the soluble and insoluble fractions.

Table II: Transition Midpoints[a] and Relative Areas of Thermal Transitions[b] of Various Chromatin Fractions

	I		II		III	
sample	T_m	% H_T	T_m	% H_T	T_m	% H_T
S chromatin	64	29	72	15	82.5	56
I chromatin	63	13	72	27	82.8	60
E chromatin	63.4	9	72	31	83.1	60

[a] T_m, the temperature of maximum dh/dT for each transition; error ± 0.5 °C. [b] % H_T, the percent of total hyperchromicity found in a given transition; error ± 5%.

DNA or ψ DNA formation (Fulmer & Fasman, 1979) induced by high ionic strength buffer. The alteration is identical for all three fractions at 23 °C. In agreement with these results, when the data obtained for the hydrodynamical behavior in the ultracentrifuge were compared for both soluble and insoluble chromatin fractions as well as their ability for undergoing the low order → higher order structure transition as a function of the ionic strength, similar results were obtained, shown in Figure 4. As can be seen, once again both samples are hardly distinguishable, and both of them are able to undergo the compaction transition to the same extent and in concordance with previous reported results (Ausio et al., 1984).

Finally, the S, I, and E chromatin samples were thermally denatured in 0.25 mM EDTA and 1.0 mM sodium phosphate, pH 7.0. Thermal denaturation profiles, dh/dT vs. T, of these samples are shown in Figure 5. The denaturation envelopes were resolved into three-component transitions. The transition midpoint (T_m) as well as the relative area of the thermal transitions (% H_T) is given in Table II. The three T_m's of the three samples were found to be the same. The percentage of DNA melting in the I transition originating from the linker regions decreased in the following order: S > I > E (29–9%). The amount of DNA in the second transition, originating from the core region immediately adjacent to the linker DNA, increased in the same order (15–31%). Thus, in I and E chromatin, the amount of DNA melting in the linker region is depleted, and this DNA is transferred to the core region. This indicates that more DNA, present in the linker regions of I and E chromatin, is bound to the histone core compared to S chromatin, probably making it insoluble at physiological ionic strength. In transition III, the amount of DNA remained constant in all three chromatin samples. These results are consistent with the data shown in Figure 6 which show the solubility behavior, in the presence of magnesium [i.e., using divalent instead of monovalent ions (Borochov et al., 1984)],

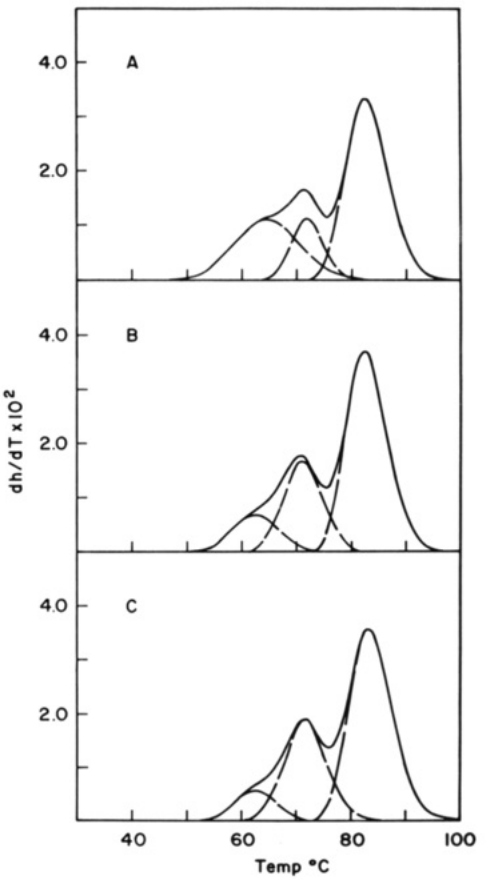


FIGURE 5: Thermal denaturation profiles (dh/dT vs. T) of S chromatin, I chromatin, and E chromatin (in 1 mM sodium phosphate and 0.25 mM EDTA, pH 7.0). (A) S chromatin; (B) I chromatin; (C) E chromatin. Cell path length, 1 cm; initial absorbance of chromatin samples, $A_{258} \simeq 1.0$; heating rate, 0.25 °C/min.

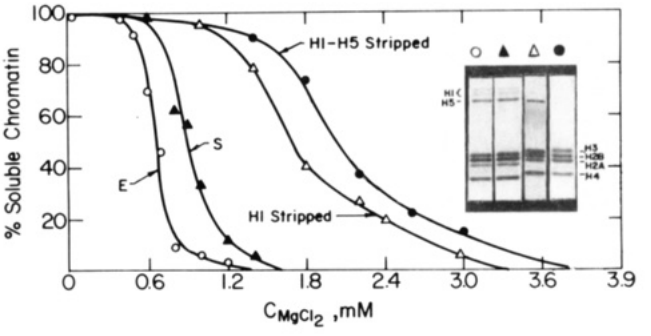


FIGURE 6: Solubility of different chromatin samples as a function of $MgCl_2$ concentration in 1 mM Tris-HCl (pH 8.0) buffer as determined by centrifugation and optical density determination of the supernatant. In all cases, the original chromatin absorbance was $A_{260} \simeq 0.8$. (○) E fraction; (△) H1-stripped chromatin; (●) H1/H5-stripped chromatin.

of the chromatin samples studied in this work when compared to artificially "linker-histone" stripped chromatin.

In Figure 6 is seen the solubility profile as a function of $MgCl_2$ concentration for various chromatin samples. The sample which aggregates at the lowest $MgCl_2$ concentration is sample E (that which had to be extracted from the original pellet, i.e., the originally least soluble). The S chromatin precipitates at the next higher $MgCl_2$ concentration. The chromatin which has been stripped of H1 requires still higher concentrations of $MgCl_2$ to cause precipitation while the sample stripped of both H1 and H5 requires the highest concentration of $MgCl_2$ to decrease its solubility. The insert of Figure 6 shows the gel electrophoretic patterns indicating

which histones were present in these samples. These curves are indicative of the fact that as one increases the amount of linker DNA available to the divalent ions in soluble (S) vs. insoluble (I and E) fractions, greater amounts of divalent ions are necessary to cause precipitation.

Discussion

It is quite evident that chromatin solubility, as a function of ionic strength in buffers containing monovalent salts, may be mostly related to and dependent on the linker histones (i.e., histone H1) and not on the degree of charge neutralization as initially thought (Davies & Walker, 1974). Indeed, far below the physiological ionic strength range, histone H1 can move, either by exchanging between different segments of chromatin (Caron & Thomas, 1981), or by moving to a site of higher affinity (Lasters et al., 1981), or by being in the presence of a competitive polyanion (Ansevin et al., 1975; Skalka et al., 1967b). In the presence of competitive polymers, chromatin increases its solubility (Skalka et al., 1976) and can eventually come to full solubility at ~150 mM NaCl even in the presence of very low quantities of the competing polyanion (Lasters et al., 1981).

Despite the amount of information available on this subject, all the efforts to date to elucidate the reason for the existence of two well-defined chromatin fractions, according to its solubility or insolubility at physiological ionic strength, have been unsuccessful. The preparation of these two fractions has been carried out by using different methods and different biological systems (Ruiz-Carrillo et al., 1980; Fulmer & Bloomfield, 1981; Hollandt et al., 1979; Ausio et al., 1984). In order to avoid ambiguities arising from the preparative method, we have used two different preparative procedures involving different temperatures of chromatin digestion and different systems of fractionation (Fulmer & Bloomfield, 1981; Ausio et al., 1984).

It has been found that three main chromatin fractions, S, I, and E (as defined previously), can be obtained independently of the different pathways followed in their preparation. The relative yield of such fractions depends, however, on the detailed experimental conditions used, such as temperature and concentration (Ausio et al., 1984).

In agreement with previous reports (Fulmer & Bloomfield, 1981, 1982), we have not been able to find any significant biochemical difference between any of them, as shown in Figure 2 and in Table I. The use therein of a high-resolution electrophoretic system has allowed a more detailed analysis. In doing so, it has been possible to distinguish, at least, between six different subtypes or modifications of histone H1 from chicken erythrocyte (see Figure 2B) (five of these fractions have been recently characterized) (Dupressoir & Sautiere, 1984) and to show that all of them are present in S, I, or E fractions in exactly the same stoichiometry (Figure 2C).

At this point, it should be noted that all the results shown in Figure 2 or in Table I are the average of at least five different and independent chromatin preparations and that they correspond to chromatin that had already been fractionated either through sucrose gradients or through gel filtration. This later point is very important, since, as already mentioned previously, the starting samples are usually contaminated with small portions of oligonucleosomes which in turn are partially depleted of linker histones (histones H1 and H5).

The electrophoretic techniques used here have also led to the conclusion that excluded the possibility of differences in the acetylation of core histones (Perry & Chalkley, 1982), variation of non-histone chromosomal proteins, and ADP-ribosylation or glycosylation as the factors responsible for the solubility differences of chromatin in 0.15 M NaCl buffers.

Nevertheless, the thermal denaturation results show that S, I, and E chromatins behave differently. These data indicate that more of the DNA present in the linker regions of I and E chromatin is bound to the core regions compared to S chromatin. Thus, the higher stability and compaction of DNA in the linker regions of I and E chromatin explains the insolubility of these chromatins at physiological ionic strength.

This finding is very important, since it represents the first striking difference reported to exist between the 0.15 M NaCl soluble and the corresponding insoluble fractions.

Such a difference is corroborated by the solubility behavior of these samples in the presence of magnesium (Figure 6). Although the interpretation and the details of chromatin solubility in the presence of divalent ions are not yet well understood (Borochov et al., 1984), this is not the case with the data presented in Figure 6. Comparison of the solubilities of chromatin samples artificially stripped to a different extent of H1 and H5 clearly indicates the important role, in the precipitation phenomena, of the side-chain DNA phosphate groups, in particular those of the linker regions. The precipitation is most probably achieved through cross-bridging of the negative charges of the free phosphates from neighbor DNA molecules via their interaction with Mg^{2+}. Whatever the mechanism is, the enhanced solubility, in the presence of magnesium, of the soluble fraction, when compared to the insoluble one, once again indicates that in the first case the DNA in the linker region becomes more exposed to the solvent, thus requiring more Mg^{2+} to undergo full precipitation.

Let us now take into account the already mentioned fact that the composition of histones H1 and H5 remains the same qualitatively and quantitatively in all the fractions. When this results is taken together with the result which showed that all three fractions have the same repeating length, the following question may then be asked: Can we alter the position of some of the linker histones by exhaustive dialysis or repeated extractions with 0.15 M NaCl buffers, so that some of them move to a second binding site as that described by Nelson et al. (1979)? The answer to that question is difficult to assess, but such a movement or just displacement of part of the molecule ("tails"), either of histone H1 or of histone H5, toward a region closer to the core region of the nucleosome could explain and account for all the above-reported results. Indeed, it is known that histone H1 binds to DNA differently at 150 mM NaCl than at lower ionic strength (Glotov et al., 1978).

The solubility may be due to a competitive behavior, with the oligonucleosomal component with the lower protein/DNA ratio found associated with the soluble fractions (Figure 3), and accounts for an alternative or complementary explanation. Nevertheless, it has not been possible to find any evidence for this possibility, as has already been mentioned previously. Furthermore, the low content (3–4%) of such nucleosomes cannot account, by itself, for the melting differences found. The function of such a component remains unclear and requires further investigation. Finally, the fact should be emphasized that the data presented herein clearly show, at least in chicken erythrocytes, that the difference between the different degree of solubility of the chromatin fractions is not a specific one. In other words, the differences in solubility cannot be attributed to differences in the biological activity degree (i.e., transcription or translation). Indeed, the insoluble fraction yield is variable depending on physical parameters such as concentration, temperature, etc. In fact, previous

reported yields for this fraction [≃40% (Fulmer & Bloomfield, 1981)] are obviously far greater than the biological degree of activity of such inactive cells. All these considerations do not preclude the fact that the active chromatin fragments appear or cofractionate with the first soluble fractions such as S-1 (see Scheme I shown under Materials and Methods) but indicate that the solubility phenomena, described here, arises from some more general physical features, such as those mentioned above and which in turn seem to be intrinsic to this biological complex, termed chromatin.

Since the work herein was initiated, there have been several papers published directed at or pertinent to the solubility phenomena of chromatin. Huang and Cole (1984) have indicated that the difference in solubility of chromatin fractions was due to differences in the amount of H1 present in the two fractions, the higher H1-containing fraction being the more insoluble one. Thus, it is stated that H1 is nonuniformly distributed in chromatin in a stable state. They also found that different subtypes of H1 give definite solubility properties; e.g., H1c disfavors aggregation. Previously, Liao and Cole (1981) had demonstrated that different subtypes of H1 cause degrees of solubility when associated with naked DNA. Caution should be exercised when using such models of chromatin, as the sites available in chromatin and naked DNA are considerably different. Jin and Cole (1985) have studied the H1° distribution in soluble and insoluble brain chromatin and find there was no preferential binding to either fraction, in contrast to the nonuniform distribution of H1 previously reported. The subtype H1ab was heavily favored in the aggregated chromatin from both calf brain and thymus. Recently, Huang and Cole (1985) have retracted their earlier claim that the differences in solubility were due to H1 depletion and now attribute this phenomena as due to separation of classes of chromatin that differ in sensitivity to salt-induced aggregation. A careful study by Komaiko and Felsenfeld (1985) on chicken erythrocyte chromatin concluded that the difference in solubility was also due to extremely small differences in the amounts of H1/H5. Differences in solubility were also found due to the size of the DNA. This latter factor would not have relevance to an in situ situation. They concluded that the partitioning of chromatin into soluble and insoluble pools in 0.15–0.25 M NaCl arises from redistribution of a limiting amount of H1 and H5 to the chromatin fractions containing the longest DNA. As little is known concerning the DNA lengths in situ, the relevance of these findings to in vivo situations is not clear. Weintraub (1984) has published data which bear significantly on this problem. It was shown that both active and inactive genes contain H1/H5 but active genes in supernucleosomal particles (obtained by micrococcal nuclease digestion), in contrast to inactive supernucleosomal particles, are not held together after cleavage of linker DNA. This suggests that H1 cross-links adjacent nucleosomes in inactive regions and H1 is bound differently in expressed regions. Thus, the difference is due to their assembly. They attribute this either to H1 being altered or modified in these regions or to the associated chromatin being modified. Active chromatin has defined properties, such as the presence of HMG 14 and 17, DNase I and S1 hypersensitivity, histone modification, etc. However, in chicken erythrocyte chromatin, a minimal number of such differences pertain. Studies by Rocha et al. (1984) on salt fractionation of micrococcal nuclease digestion of adult chicken erythrocyte nuclei showed that selectivity enriched fractions of β^0-gene globin domains eluted at low salt. Other nonexpressed genes are concentrated in less salt soluble fractions. This study concluded that the low-salt soluble fraction was depleted in H1/H5, enriched in HMG 14/17, and contained more highly acetylated histones. In summary, these recent papers have explanations for solubility differences which are frequently contradictory. The solubility differences have been attributed to depletion of H1/H5, differences of subtypes of H1, and differences of supernucleosomal particle assembly. The work presented herein has not found differences in the H1/H5 content of the two fractions and in the DNA length in the two fractions but has found significant differences in solubility. The melting profile data strongly support the views of Weintraub (1984) that these two fractions are assembled in a different manner, yielding different stabilization factors.

Further work is necessary to fully explain the intrinsic differences between soluble and insoluble (in 0.15 M NaCl) chromatin, and the results herein would indicate the direction should be toward understanding the assemblies of the two fractions rather than looking for different components in the two fractions.

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Characterization of Human Platelet Basic Protein, a Precursor Form of Low-Affinity Platelet Factor 4 and β-Thromboglobulin†

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ABSTRACT: Platelet basic protein (PBP) was purified from the supernatant of thrombin-stimulated, washed human platelets by ion-exchange, affinity, molecular sieve, and high-performance liquid chromatography (HPLC). The NH_2-terminal amino acid sequence was determined by automated Edman degradation, revealing 9 unique residues followed by 10 residues of the established low-affinity platelet factor 4/β-thromboglobulin (LA-PF_4/βTG) sequence. Among the nine were three basic residues, accounting for the high isoelectric point of PBP. Additional evidence for precursor status includes the immunological cross-reactivity of all three species and the ability of plasmin and trypsin to produce from PBP a species resembling βTG in charge, hydrophobicity and size. Tryptic peptide maps of PBP and LA-PF_4 obtained by reverse-phase HPLC were very similar, and from each protein, a peptide was isolated which showed the amino acid composition predicted for the COOH-terminal tryptic peptide of βTG. Normal platelets contained predominantly LA-PF_4, with PBP ranging from 10% to 30% of total βTG antigen. This was true even when fresh platelets were lysed with trichloroacetic acid in order to provide the most complete and rapid inhibition of proteolytic activity. βTG itself was never detected in this situation or in the release supernatant of stimulated platelets, and only rarely in unprotected lysates. In agreement with earlier results, crude preparations of PBP were mitogenic for 3T3 cells, but highly purified preparations of PBP and LA-PF_4 were free of this activity.

Upon stimulation, human platelets secrete a number of proteins from their α-storage granules. Several of these are platelet specific and bind reversibly to insolubilized heparin [for a review, see Holt & Niewiarowski (1985)]. Major platelet-specific proteins are β-thromboglobulin (βTG),[1] eluted from heparin-agarose at 0.5 M NaCl (Moore et al., 1975; Rucinski et al., 1979), and platelet factor 4 (PF_4), which is displaced only by 1.2-1.5 M NaCl [e.g., see Levine & Wohl (1976)]. Both βTG and PF_4 have been sequenced, yielding subunit molecular weights of 7800 (PF_4) and 8800 (βTG)

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[1] Abbreviations: PF_4, platelet factor 4; βTG, β-thromboglobulin; PBP, platelet basic protein; LA-PF_4, low-affinity platelet factor 4; PDGF, platelet-derived growth factor; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)-aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SDS, sodium dodecyl sulfate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; KIU, kallikrein inactivator unit(s); Cbz, carbobenzoxy.

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