Partial Fractionation and Chemical Characterization of the Major Nonhistone Chromosomal Proteins*

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ABSTRACT: The nonhistone chromosomal proteins (NHC proteins) have been little studied because of their tendency to aggregate and the consequent difficulty in isolating and fractionating them. We have prepared NHC proteins by dissociating chromatin in 25% formic acid–8 M urea–0.2 N NaCl, removing the DNA by centrifugation, and fractionating the chromosomal proteins by ion-exchange chromatography on Sephadex SE C-25 resin. Four fractions of NHC proteins are obtained; two of these are single purified proteins, and the others contain 4–6 and 4–7 different proteins. The NHC proteins show a ratio of acidic to basic amino acids from 2.7 to 1.2 and isoelectric points from apparently less than 3.7 to 8.0. The present results indicate that 10–15 proteins make up at least 70% of the NHC protein fraction.

Chromatin, the interphase form of the cell's hereditary material, is a complex of DNA, RNA, histones, and nonhistone chromosomal proteins (NHC proteins).[1] The latter component is probably the least studied; very little information concerning the complexity or role of the NHC protein fraction is available. This is primarily due to the difficulties in isolation of these proteins in a soluble form free of DNA and of histones. It has recently been shown by comparative sodium dodecyl sulfate gel electrophoresis that the NHC proteins appear to be a class of approximately 13–15 major protein species (Elgin and Bonner, 1970). A comparison of the major NHC proteins from several tissues of a given creature shows limited heterogeneity (Elgin and Bonner, 1970; Loeb and Creuzet, 1970; Shaw and Huang, 1970; MacGillivray *et al.*, 1971). This observation has recently been extended to the nonhistone chromatin phosphoproteins (Platz *et al.*, 1970). However, tissue-specific NHC proteins have been observed in all of the above-mentioned studies.

Our goal in this study has been to determine the chemical characteristics of the nonhistone chromosomal proteins and to assess the heterogeneity of the sodium dodecyl sulfate bands observed earlier. The method which we have developed permits the separation of the nonhistone chromosomal proteins from DNA and histone, and initially fractionates the NHC proteins into four groups, two of which appear to be single proteins. The method is such as to allow for further fractionation and is suitable for chemical studies of the NHC proteins. The low pH (2.5) used would not, however, allow one to assume that the isolated proteins could subsequently be used for biological studies. Our results continue to support the idea of a limited heterogeneity of the NHC protein fraction.

Methods

Preparation of Chromatin. All studies were carried out on rat liver chromatin prepared according to methods previously described (Elgin and Bonner, 1970). Chromatin was prepared, dialyzed against 0.01 M Tris (pH 8) at 0° for 4 hr, sheared, and dissociated without delay to avoid inherent problems of degradation (see Panyim *et al.*, 1968). The method used in isolating NHC proteins from chromatin is outlined in Figure 1.

Dissociation of Chromatin. Chromatin was dissociated and the bulk of the DNA removed by the following method: 1.5 volumes of 98–100% formic acid were added to the chromatin at 0° with rapid stirring. This preparation was stirred slowly for 0.5 hr. Sodium chloride (5 M) was added to a final concentration of 0.2 M and urea was added to a final concentration of 8 M. The solution was spun in a Ti-50 rotor (Spinco) for 18 hr at *ca.* 200,000*g*, 2–4°. This treatment resulted in the pelleting of *ca.* 95% of the DNA; the top 90% of the supernatant of each tube was removed by pipet and pooled.

Ion-Exchange Column Chromatography. Sephadex resin SE C-25 was prepared by soaking overnight in formic acid–urea solution (25% formic acid–10 M urea). The resin was then combined with the above supernatant (typically 150 mg of protein in 480-ml solution) and the mixture diluted with a tenfold excess of formic acid–urea solution with rapid stirring. This mixture was stirred at ambient temperature for 4 hr. The resin was collected by centrifugation at *ca.* 1000*g* and poured into a 2.5 cm × 30 cm column. All of the supernatant protein is bound to the resin by this technique. The chromosomal proteins are then eluted at ambient temperature by a sodium chloride concentration gradient according to the scheme shown in Figure 2 (steps of 0.1 and 0.2 N NaCl followed by a gradient from 0.2 to 0.8 N NaCl, all in 25% formic acid–10 M urea). The final protein fraction, V, is eluted with formic acid. The flow rate was maintained at 15 ml/hr. The column was assayed by absorbance of fractions at 280 mμ. Fractions were pooled as indicated in Figure 2 and either rerun on the same column or dialyzed and concentrated for further fractionation (see below and Figure 1). (Due to the slow destructive action of the formic acid on Sephadex, such a column can be used only once before discarding.[2]) This column was developed from the method of Ruoslahti as used by Smart *et al.* (1971). The yields of protein from the SE Sephadex column were determined by dialyzing aliquots

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[1] Abbreviation used is: NHC proteins, nonhistone chromosomal proteins.

[2] The formic acid–urea solvent is a highly dissociating agent. It can be safely used with glass, polyethylene, and similar plastics, or Teflon. While working with the solvent it is advisable to wear safety glasses and to wash one's hands frequently with dilute sodium bicarbonate.

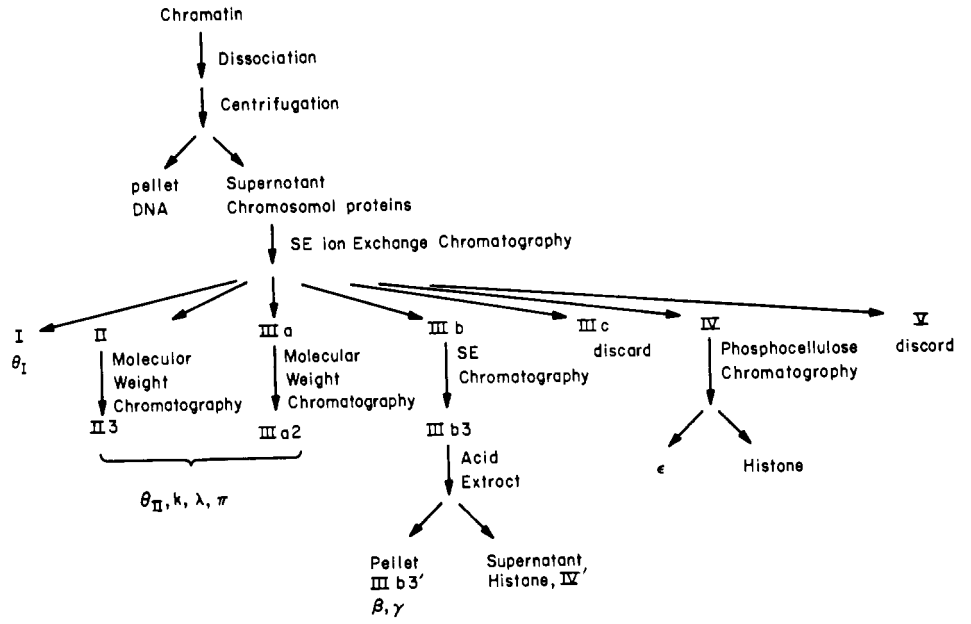

FIGURE 1: Summary of fractionation procedure.

of each fraction twice against 0.1 N NaOH and once against 0.1 N NaOH, 2% sodium carbonate (Lowry C reagent). The protein concentrations were then established by the method of Lowry *et al.* (1951), using bovine serum albumin (Sigma) as a standard.

General Methods of Chromatography. Fractions II, IIIa, and IV from the SE column were dialyzed against two changes of LiCl–urea solvent (Ozaki *et al.*, 1970), concentrated by ultrafiltration over a PM-10 membrane (Amicon, Inc.), and further dialyzed against LiCl–urea solvent before they were applied to columns. The solvent is 6 M urea–0.01 M H_3PO_4 neutralized to pH 8 with methylamine–3 $\times$ 10^{-3} M β-mercaptoethanol–0.1 M lithium chloride. Recovery of concentrated protein was 60–80%. Fractions II and IIIa were further purified on a 1.5 $\times$ 90 cm column of Sephadex G-75 or G-100. Columns were run at ambient temperature; flow rates on the order of 6–10 ml/hr were maintained. Fraction IV, similarly dialyzed and concentrated, was further separated into a histone component and a nonhistone component, ϵ, by chromatography on phosphocellulose (Mannex-P, Mann Research Lab.) utilizing a lithium chloride gradient from 0.1 M to 0.6 M according to the methods of Ozaki *et al.* (1970). Unfortunately, the relative elution positions of the proteins varied depending upon the lot of phosphocellulose. Generally, a 1.2 $\times$ 62 cm column running at 5–10 ml/hr was most satisfactory. In all the above purifications, column fractions were analyzed for protein by the method of Lowry *et al.* (1951). Final protein preparations from fractions II, IIIa, IIIb, and IV were lyophilized following extensive dialysis (100-fold volume, 6 changes) at 0° against 0.01 M acetic acid. Fraction I was similarly lyophilized from 0.05 M ammonia. All urea used was either obtained in highly purified form (Schwarz–Mann, Ultra Pure) or was purified by passage of a 10 M solution through a mixed-bed ion-exchange column (Barnstead D0803).

Disc Gel Electrophoresis. Samples were qualitatively analyzed by sodium dodecyl sulfate disc gel electrophoresis as described by Elgin and Bonner (1970). All sodium dodecyl sulfate gels were stained with coomassie brilliant blue R-250 (Mann–Schwarz). Unfortunately, due to the S-shaped response of coomassie brilliant blue absorption to protein concentration, the relative concentrations of the various proteins cannot be accurately assessed from such gels. [The binding of this dye is proportional to the protein content of a band only over the range of 1–20 μg. Different proteins bind dye to different extents; the dye-binding coefficient appears to vary by a factor of 2 or less (Elgin, 1971; see also Fairbanks, 1969).] Split sodium dodecyl sulfate gel electrophoresis was used to confirm the identification of isolated bands (Spiegel *et al.*, 1970). Samples were further analyzed by dissolving in 10 M urea and running on urea–polyacrylamide gels at pH 4.3 (15% gels) (Bonner *et al.*, 1968) or at pH 8.9 (7.5% gels) (Toevs and Brackenbury, 1969). The former were stained with Amido-Schwarz 10B (Matheson Coleman & Bell) and the latter with coomassie brilliant blue. Purified gel reagents (Bio-Rad Laboratories) were used throughout. Sodium dodecyl sulfate (Sipon WD) was obtained from Alcolac Chemical Corp. (Baltimore, Md.) and was recrystallized once from 80% ethanol before use. Gels were photographed using an orange filter and/or scanned at 600 mμ on a Gilford 2000 spectrophotometer equipped with a 0.05-mm slit and linear gel transport. All gels photographed together or directly compared were run at the same time with the exception of sample IIIb3' (Figures 6 and 9).

Labeling and Counting of Nonhistone Chromosomal Proteins. Labeled preparations of NHC proteins were obtained by injecting rats (male, albino, Sprague–Dawley, *ca.* 200 g body weight) intraperitoneally (under ether anesthesia) with tritiated leucine (Schwarz–Mann) *ca.* 50 Ci/mmole, 1.0–1.5 mCi/rat, 24 hr prior to sacrifice; and with inorganic phosphate, carrier-free, 2–6 mCi/rat, 3 hr prior to sacrifice. The animals were sacrificed by exsanguination and the liver NHC proteins prepared. Chromatin was extracted with 0.4 N sulfuric acid to remove the histones, the pellet was solubilized in sodium dodecyl sulfate and centrifuged to remove DNA, and the dialyzed supernatant used for analysis of NHC proteins (Elgin and Bonner, 1970). The activity of the various protein bands was determined by preparing sodium dodecyl sulfate gels, staining and destaining, cutting out the individual bands by hand with a razor blade, and counting according

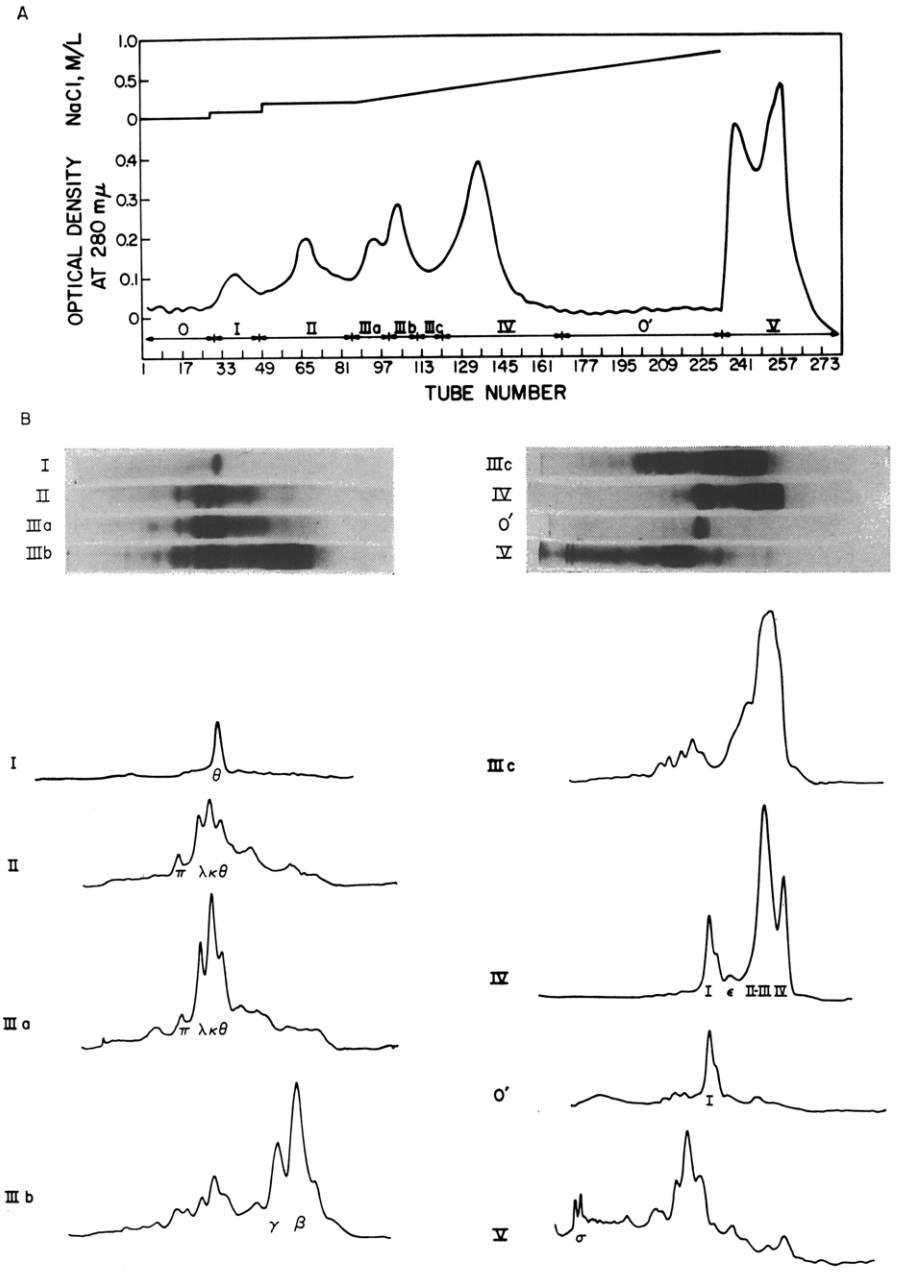

FIGURE 2: Chromatographic fractionation of the chromosomal proteins on Sephadex SE C-25. (A) Elution profile, 2-ml fractions. (B) Sodium dodecyl sulfate gel electrophoresis of pooled fractions. Gels run left to right.

to the methods of Ward *et al.* (1970). The incorporation of ^{32}P and ^{3}H counts, minus background, is presented as an indication of the synthesis and phosphorylation of the individual protein bands.

General Methods of Analysis: Molecular Weight Determinations. Molecular weights of the protein bands, both of the unfractionated NHC protein preparations and of the isolated fractions, were determined by sodium dodecyl sulfate gel electrophoresis (Shapiro *et al.*, 1967; Dunker and Rueckert, 1969; Weber and Osborn, 1969). Chymotrypsinogen was used as the standard protein for determining relative migration distances and myoglobin, pepsin, ovalbumin, albumin, and γ-globulin were used to establish the calibration curve. Standard plots of log molecular weight as a function of relative migration distance for 5 and 10% acrylamide gels gave straight lines by least-squares analysis with slopes of −1.29 and −0.662 and y intercepts of 504,500 and 121,000, respectively. The method was generally found to be accurate within 5% for the ranges used for a given per cent acrylamide gel. Standard proteins of known molecular weight were from Schwarz–Mann.

Isoelectric Focusing and Determination of pI. Isoelectric focusing in 3.7% polyacrylamide gels was carried out according to the method of Finlayson and Chrambach (1971) with the modification that the anolyte used was 0.01 M phosphoric acid and the catholyte was 0.02 M sodium hydroxide (Righetti and Drysdale, 1971). NHC protein samples were dissolved in 10 M urea and applied directly to the top of the polymerized gels with no prior electrophoresis. Insulin was run as a standard marker. The pH gradient achieved in duplicate gels is shown in Figure 7. A plateau effect in the region pH 5–6 is still observed, but the pH gradient is satisfactory. The isoelectric points indicated for the nonhistone chromosomal proteins are taken directly from the pH reading

TABLE I: Protein Content of SE-Column Fractions.

Fraction	% of Total Protein
I	3.6
II	10.1
IIIa	6.0
IIIb	7.7
IIIc	6.5
IV	50.2
O′	5.9
V	10.0

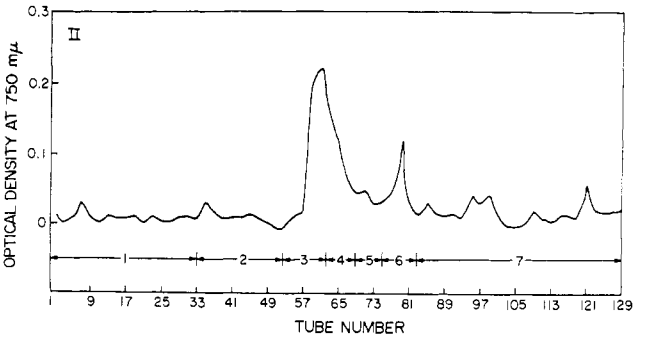

FIGURE 3: Chromatographic fractionation of II on Sephadex G-100, 1-ml fractions.

of a 0.01 M KCl extract of slices of the gel. Using this technique the reported p*I* of insulin, 5.3–5.4, was obtained for that protein (Young, 1963).

N-Terminal Analysis and Amino Acid Analysis. Qualitative N-terminal analysis was carried out by the method of W. Konigsberg (personal communication, 1971). Protein samples (0.1–1.5 mg) were dissolved in 0.1–0.2 ml of 0.5 M sodium bicarbonate (*ca.* pH 8) with 1% sodium dodecyl sulfate. The pH was adjusted to 7 with 1 N HCl and 0.5 volume of dansyl chloride in acetone (5 mg/ml) was added. The samples were then incubated at 37° for 30 min, and subsequently precipitated with 5 volumes of cold acetone and collected by centrifugation. The pellet was washed with 1–2 ml of 1 N HCl to remove soluble dansylic acid, re-collected and dried briefly. The protein was then hydrolyzed in 0.2 ml of constant-boiling HCl at 110° for 10 hr. Reaction products were taken up first in water-saturated ethyl acetate and subsequently in 50% pyridine and analyzed by thin-layer chromatography on polyamide sheets ("Chen-chin" polyamide layer sheets from Gallard-Schlesinger, N. Y.) in a series of appropriate solvents (Woods and Wang, 1967). The dansylamino acids were identified by their migration positions relative to the known pattern and to standards spotted on the back of the same polyamide sheet. Rough quantitation was provided by the degree of fluorescence of the various spots. Amino acid analyses were obtained on a Beckman Model 120B amino acid analyzer following hydrolysis of the samples in constant-boiling HCl, 25 μ of nitrogen, at 108° for 22 hr. No attempts were made to correct for serine or threonine losses.

Results

Dissociation of Chromatin. The techniques described above appear to dissociate the nucleic acid and protein components of chromatin completely. An outline of the isolation scheme is given in Figure 1. Approximately 85% of the recovered protein was obtained in the 90% supernatant fraction after centrifugation. Unfortunately, due to leakage in the centrifugation tubes, the total recovery of protein frequently approached only 80%. Sodium dodecyl sulfate gel electrophoresis of the chromatin, the dissociated chromatin before centrifugation, and the supernatant and pellet following centrifugation indicates that all the major protein species present in the chromatin are recovered in the centrifugation supernatant.

Fractionation of Chromosomal Proteins. The elution of proteins from the Sephadex SE C-25 column is shown in Figure 2. In general, all the protein applied to the column was recovered. The percentage protein in the various fractions for a typical experiment is given in Table I. Note that the optical density at 280 mμ is a somewhat misleading measure of protein: in particular, fractions I and V show more optical absorption than would be indicated by Lowry analysis for protein content. In the case of fraction I, spectral data indicate that this is due to an 8% contamination with residual nucleic acids. In the case of fraction V, it may indicate the presence of the breakdown products of the Sephadex column or of some nucleic acid (probably in a complex with protein and lipid; the presence of the high molecular weight bands in this fraction only suggests an undissociated membrane complex).

Sodium dodecyl sulfate gel electrophoresis of the various fractions obtained is shown in Figure 2. Fraction I is the NHC protein band previously named θ (Elgin and Bonner, 1970) as shown by molecular weight determinations and by split gel electrophoresis.[3] Fractions II and IIIa contain predominantly θ, κ, λ, and π. These fractions have been further purified by dialysis to LiCl–urea solvent and gel permeation chromatography on G-75 or G-100 Sephadex as described under Methods. A typical elution profile is shown for fraction II in Figure 3 and for fraction IIIa in Figure 4, including in the latter case sodium dodecyl sulfate gel electrophoresis of the protein present in each pooled chromatographic fraction. II3 and IIIa2 were identical by sodium dodecyl sulfate gel electrophoresis; since the parent fractions elute at the same ionic strength on the SE column (*ca.* 0.2 M NaCl) they were assumed to be the same and pooled for all analytical work.

Fraction IIIb is fairly heterogeneous but provides an opportunity to obtain bands β and γ by a second cycle of fractionation on the SE ion-exchange column. Fraction IIIb was diluted by the addition of 2 volumes of formic acid–urea solvent and rechromatographed on a 2.5 × 25 cm SE column with a gradient of 0.2–0.5 M sodium chloride. As shown in Figure 5, bands β and γ elute together as a very sharp peak at *ca.* 0.3 M sodium chloride. Amino acid analysis and analysis by gel electrophoresis indicate, however, that this peak still contains significant amounts of histones (up to 35–40%), most predominantly histone II. The lyophilized fraction IIIb3 was resuspended in 0.01 M Tris (pH 8) and acid extracted with 0.4 N H_2SO_4. The pellet was dissolved in 0.2 M NaOH, dialyzed against 0.01 M acetic acid, and lyophilized. This fraction is referred to as IIIb3′, while

[3] The NHC protein bands have been given arbitrary Greek letter names for purposes of discussion; see Elgin and Bonner (1970), or Figure 11.

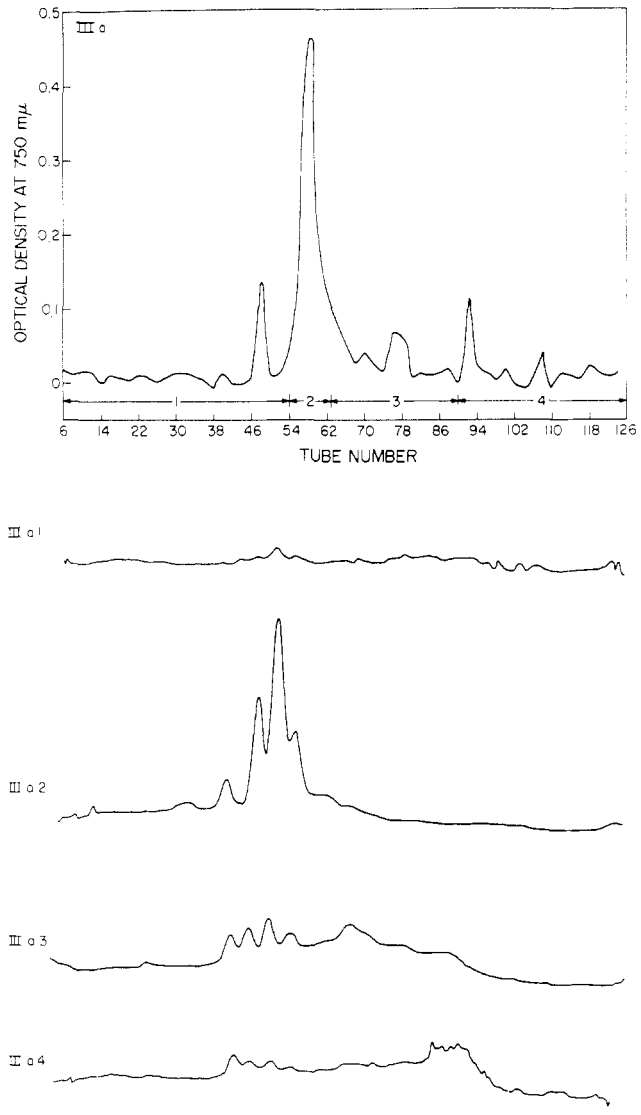

FIGURE 4: Chromatographic purification of IIIa on Sephadex G-75, 1-ml fractions. Scans below give sodium dodecyl sulfate gel analysis of column fractions. Gels run left to right. The purification of fractions II, IIIb, and IV was similarly monitored by sodium dodecyl sulfate gel electrophoresis.

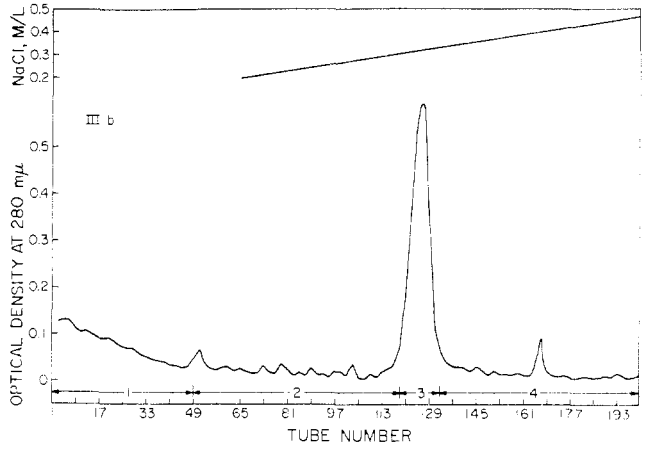

FIGURE 5: Chromatographic purification of IIIb on Sephadex SE C-25 in formic acid–urea solvent, NaCl gradient, 2-ml fractions.

TABLE II: Molecular Weights of NHC Proteins.[a]

Protein	5% Gel			10% Gel		
	m	σ	*n*	*m*	σ	*n*
α	12,500	2000	3	12,400	700	2
β	15,900	1700	4	15,100	300	3
γ	19,000	900	3	17,900	200	3
δ	25,000		3	25,000		2
ε	31,200	1200	3	33,400	300	2
η	37,800	200	3	38,400	700	2
θ	49,100	1100	5	46,900	800	4
κ	60,200	1200	5	56,400	1200	3
λ	71,400	2300	5			
μ	85,300	1700	3			
π	98,200	1200	5			
ρ	142,070	7400	3			

[a] *m* = mean, σ = standard deviation, *n* = number of determinations.

the histone supernatant is referred to as IV′. Fraction IIIc from the SE column was discarded, as it contains both histones and nonhistones. Fraction IV contains the bulk of the histone and at least one nonhistone, ε. It can be estimated from analysis by gel electrophoresis that *ca.* 95% of the protein in IV is histone (see Figures 2 and 9). However, ε could be separated from the histone by chromatography on phosphocellulose according to Ozaki *et al.* (1970) as described under Methods. Fraction O′ contains predominantly histone I. Fraction V, eluted with 100% formic acid, was discarded.

It should be noted that the proteins appear to elute from the SE column in order of increasing basicity. In particular, histone I tends to elute at the end of the histone peak and in fraction O′. All of the protein applied to the SE column was recovered in the fractions shown. The recovery of protein in fractions IV and O′ is 56% of the total, as expected from previous analysis of the percentage of histone and nonhistone protein in rat liver chromatin. Generally from 50 to 80% of the protein fractions used for further purification were recovered from the secondary columns as the peak of interest, depending on the separation achieved on the SE column (see Figures 3–5). The lyophilized fractions I, II3, IIIa2, IIIb3, IIIb3′, and ε were used for further chemical analysis. Sodium dodecyl sulfate gel analysis of these fractions, pooled from several preparations, is given in Figure 6. Sephadex SP C-25 did not adequately separate the histone and NHC proteins.

In addition to the formic acid–urea solvent, such purified NHC proteins appear to be soluble in all sodium dodecyl sulfate solutions, in LiCl–urea solvents, and in high concentrations of urea. Other solvents have not yet been explored.

Chemical Characteristics of the Isolated NHC Proteins. The NHC proteins isolated range in molecular weight from *ca.* 15,000 to *ca.* 100,000. See Table II for the values determined. The large range includes the molecular weights of known structural chromosomal proteins, *i.e.*, histones, and those of many enzymes. Thus the distribution of molecular weights gives us no clues as to function. However, further separation based on this characteristic appears possible. Most of the analytical determinations were directed toward elucidating the complexity of each of the fractions isolated. Table III

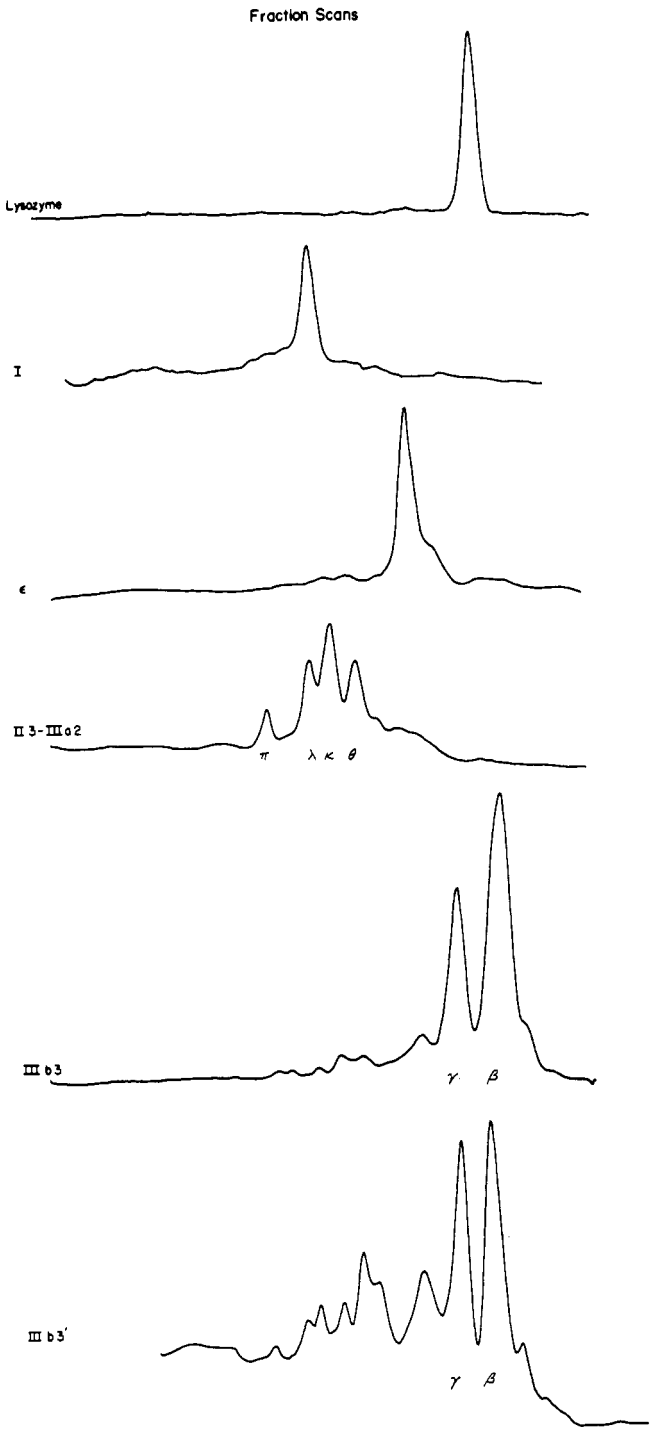

FIGURE 6: Sodium dodecyl sulfate gel electrophoresis of final protein fractions obtained. Gels run left to right.

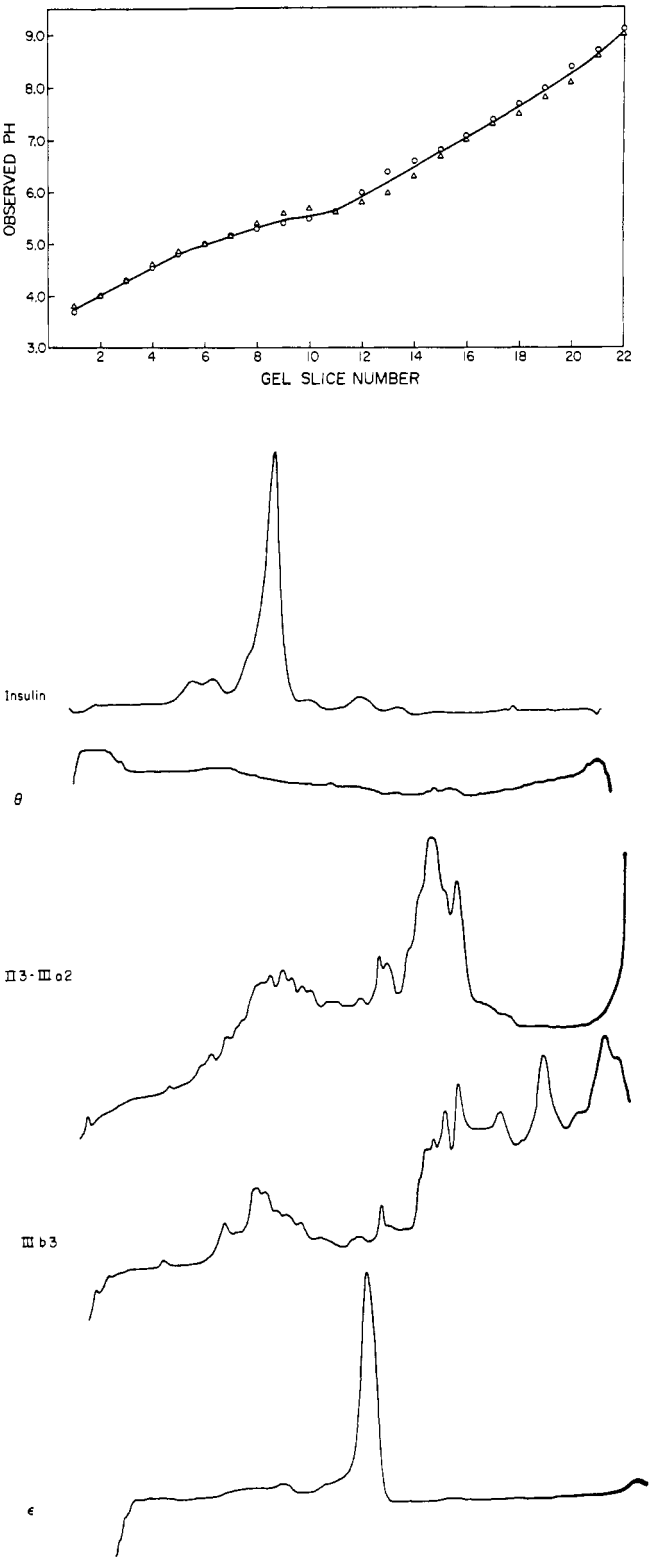

FIGURE 7: Isoelectric focusing: pH gradient obtained in polyacrylamide gels. Scans below show results with insulin standard and NHC proteins.

summarizes these data and gives the number of bands observed in each fraction following sodium dodecyl sulfate gel electrophoresis and urea gel electrophoresis at pH 4.3 and 8.9. Scans of the pertinent urea gels are given in Figures 8 and 9. The N-terminal analyses, although qualitative, are also critical in this regard. Fraction I has an N terminal of glutamic acid. Small amounts of serine and threonine were also observed. A control of lysozyme carried through with exposure to the same formic acid–urea solvent for equivalent times gave a strong lysine N-terminal as expected and also showed small amounts of glycine and serine. This indicates a small amount of acid hydrolysis at susceptible peptide bonds. II3–IIIa2 gave strong glycine and alanine N-terminals with fainter spots for valine, isoleucine, leucine, and threonine and variable amounts of glutamic acid. These results suggest that although four bands were observed on sodium dodecyl sulfate gel electrophoresis, six polypeptide chains may be

TABLE III: Complexity of NHC Fractions.

Fraction	No. of Bands Separated by Gel Electrophoresis[a]			No. of N-Terminals	p*I*	No. of Proteins
	Sodium Dodecyl Sulfate	Urea, pH 4.3	Urea, pH 8.9			
I(θ_I)	1	Ag + 1	*1* + 1	1	<3.7	1
II3-IIIa2	4	5	Ag + 4	*2*–4	5.4, 6.0, 6.6	4–6
IIIb3	*2*–8	*4*–8	Ag + *4* + 3	*6*–3	6.4–7.0, 7.0–8.0	6–12
IIIb3′	*2*–8	*4*–3	No change from IIIb3	Nd (assume *5* + 2)	No change from IIIb3	4–7
ϵ	1	*1*–2	*1*–1	1	5.6	1

[a] Ag indicates aggregate on top of gel. Notation *4* + 2 means 4 major polypeptides + 2 minor polypeptides observed. Nd indicates not determined.

present in this fraction. They also suggest that θ as observed in fraction I is not the same as θ observed in fraction II. In discussion hereafter these will be referred to as θ_I and θ_{II}. The fraction ϵ gave one predominant N terminal, glycine. The fraction IIIb3 yielded as N terminals threonine, isoleucine, leucine, valine, proline, methionine, and an unidentified spot, possibly a dipeptide. Fainter spots for aspartic acid, alanine, and, occasionally, serine were also observed. Proline and alanine may be considered as deriving from the contaminating histone, but most histone N terminals are blocked and do not interfere. Even allowing the strong possibilities of cross-contamination with II contributing toward valine, isoleucine, and leucine, it is apparent that fraction IIIb3 is much more heterogeneous than indicated by sodium dodecyl sulfate gel electrophoresis, where only two dominant bands are observed. The above results were obtained from a water-saturated ethyl acetate extraction of the dansylation hydrolysate. Such extraction does not yield the basic amino acid derivatives. Further extraction with 50% pyridine indicated the presence of ϵ-dansyllysine, in all fractions, but no N-terminal basic amino acids.

The results of the isoelectric focusing experiments are given in Figure 7. No bands were observed for fraction I, suggesting that the isoelectric point of θ_I is less than 3.7. The early elution position on the SE column and high acid: base ratio on amino acid analysis are consistent with this idea (see Table IV). Fraction II3–IIIa2, although revealing many bands on isoelectric focusing, shows principal groupings at p*I* 5.4 and 6.6 with an additional sharp band at 6.0. One should be reminded that almost all proteins which are

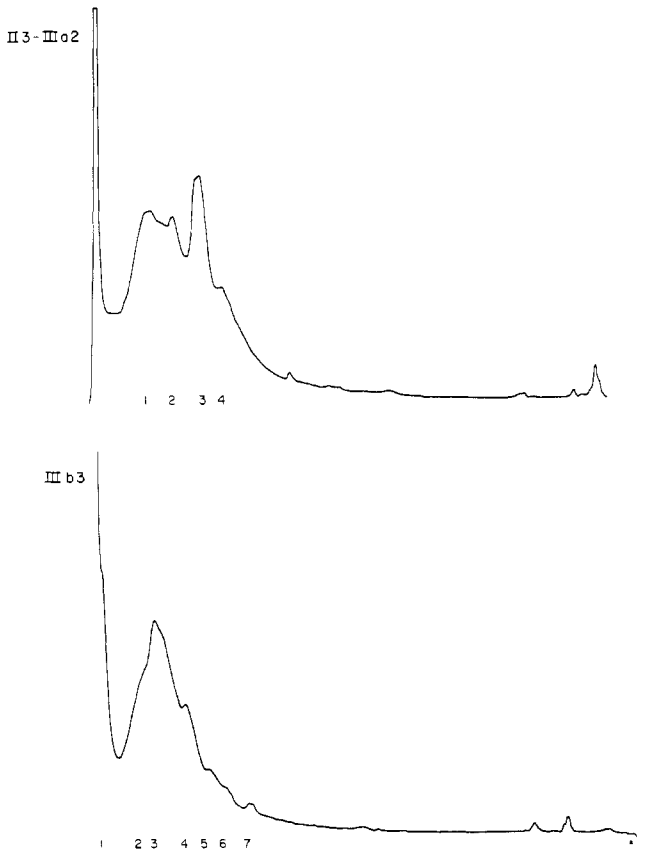

FIGURE 8: Polyacrylamide gel electrophoresis at pH 8.9 of NHC protein samples.

TABLE IV: Amino Acid Composition of NHC Proteins and Fractions.

Amino Acid	θ_I	II3-IIIa	IIIb3′	ϵ	IV
Aspartic acid	9.0	10.3	8.6	10.6	5.0
Glutamic acid	12.8	13.3	13.5	17.2	8.6
Lysine	4.2	6.4	8.3	13.3	16.0
Arginine	2.7	5.6	7.6	5.3	9.3
Histidine	1.3	1.6	2.5	1.3	1.9
Serine	13.3	7.9	7.8	9.1	6.2
Threonine	5.5	5.8	5.4	3.7	6.5
Phenylalanine	3.9	3.3	3.2	2.9	2.4
Tyrosine	2.6	3.1	2.6	2.8	2.8
Alanine	9.0	7.8	8.1	8.2	13.0
Valine	4.2	5.2	4.9	2.5	5.5
Isoleucine	2.7	4.6	4.1	2.8	4.2
Leucine	4.8	9.4	9.4	4.5	7.7
Methionine	1.5	2.7	1.9	1.4	1.2
Proline	6.1	4.3	3.7	4.8	3.7
Glycine	16.4	8.6	8.4	9.5	6.0
Cystine + cysteic acid	0	0.1	0	0	>0
(Asp + Glu)/(Lys + Arg + His)	2.7	1.7	1.2	1.4	0.54

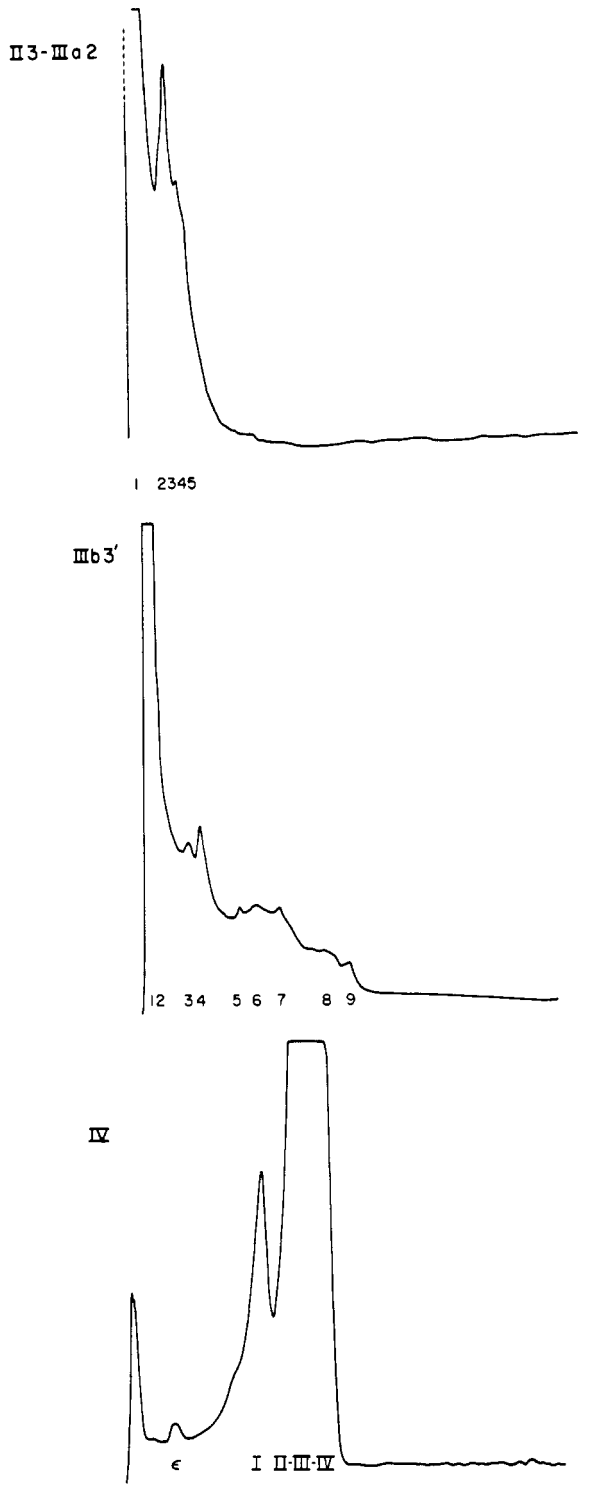

FIGURE 9: Polyacrylamide gel electrophoresis at pH 4.3 of NHC protein samples.

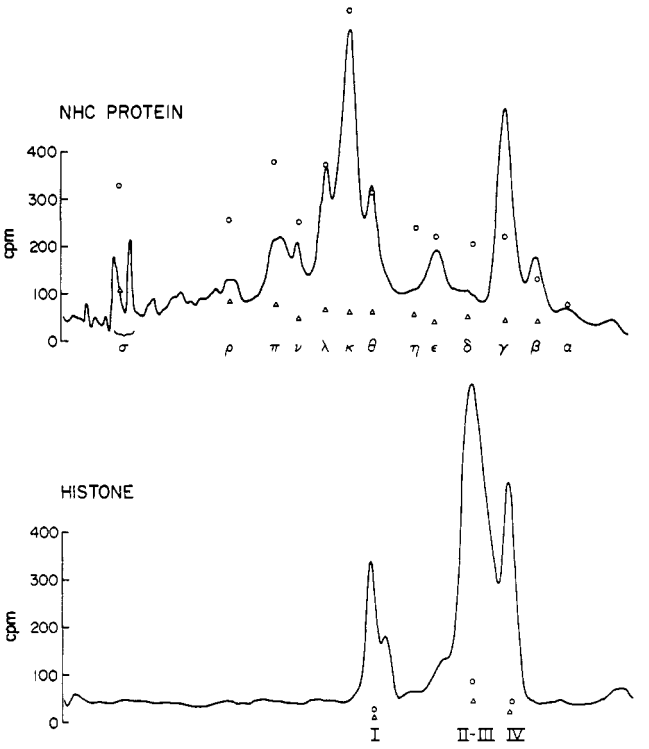

FIGURE 10: Labeling of the chromosomal proteins *in vivo* with [^{3}H]leucine and carrier-free inorganic phosphate. Data plotted on sodium dodecyl sulfate gel scan, electrophoresis left to right: (○) ^{3}H counts; (△) ^{32}P counts.

regarded as pure show multiple bands on isoelectric focusing; minor alterations such as acetylation, phosphorylation, etc., can change the isoelectric focusing point of a protein significantly. Fraction IIIb3′ shows essentially the isoelectric focusing pattern of IIIb3 (Figure 7), as histones do not focus below pH 8. The bands at isoelectric point *ca.* 5.2 may be assumed to be cross-contamination from fraction II3–III2a. In addition there is a different banding pattern in the region 6.4–7.0 and a series of four prominent bands from pH 7 to pH 8. The purified protein ε is astounding in that it focuses in one major band at isoelectric point 5.6. This is a very strong indication of the homogeneity of this protein. An isoelectric point of 5.6 is lower than might be anticipated from the amino acid composition (Table IV), implying less than the usual amount of amidation.

Each fraction was further analyzed by urea–polyacrylamide gel electrophoresis at pH 4 and 9 (see Figures 8 and 9 for relevant scans). The number and position of the major bands confirm the previous data in that one major band is indicated for fractions θ_I and ε (although ε is observed to be a broad band and in both systems), 4–5 bands are indicated for fraction II3–IIIa2, and 4–7 major bands are indicated for fraction IIIb3′.

The results of the amino acid and phosphate-labeling experiment are given in Figure 10. The data indicate differences both in the rate and amount of synthesis of the different NHC proteins and in the extent of phosphorylation. The NHC proteins show considerably higher incorporation of [^{3}H]leucine than do the histones, in agreement with previous reports that NHC proteins turn over more rapidly (Holoubek and Crocker, 1968).

The amino acid analyses of the fractions θ_I, II3–IIIa2, IIIb3′, ε, and IV are given in Table IV. These data suggest that the chromosomal proteins elute from the SE column in order of increasing lysine content; other interpretations are not consistent with the elution of ε with the histones in fraction IV. (ε contains *ca.* twice as much lysine as do the other NHC proteins.) The cysteine values given are no doubt a minimum estimate and probably significantly low since no reagent was used to prevent cysteine oxidation during hydrolysis.

Discussion

The above results establish more firmly the idea that a small number of NHC proteins make up a significant amount of that chromatin component. The role of these major NHC proteins is still obscure. The limited heterogeneity of the major NHC proteins has led to the suggestion that these proteins are common structural proteins or common enzymes of chromosomal metabolism. Recent studies which show the close association between chromosomal DNA and nuclear membranes suggest that some of these proteins may be nuclear membrane components (Comings and Okada, 1970a,b). No work has yet been done—no assay is as yet available—to explore the possible structural role of NHC proteins. Several enzyme activities are known to be associated with the isolated chromatin, *e.g.*, histone protease (Furlan and Jericijo, 1967; Garrels *et al.*, 1972) and histone acetyltransferase (Gallwitz, 1971); see Elgin *et al.* (1971) for a more detailed discussion of this aspect. It should be noted that if present estimates of amounts of several important enzymes (including RNA polymerase) are correct, they are present in chromatin at levels too low to be observed by the techniques used here (significantly less than 1% of the NHC protein) (S. C. Froehner, personal communication 1971). Tissue-specific major NHC proteins are also observed. The NHC proteins have been implicated in mechanisms of hormone response because of their tissue specificity, their selective synthetic increase observed at early times after hormone administration (Teng and Hamilton, 1970; Shelton and Allfrey, 1970), and their role in target-selective binding of hormones to chromatin (Spelsberg *et al.*, 1971b). It has been demonstrated that the NHC proteins can counter the repressive effect of histones in template activity assays, but rigorous proof of specific gene activation is lacking (Wang, 1968; Gilmour and Paul, 1969, 1970; Teng and Hamilton, 1969; Spelsberg *et al.*, 1971a). Recent experiments by Teng *et al.* (1971) have led them to suggest that a NHC protein fraction can play a σ-like, organism-specific role in stimulating RNA synthesis from DNA templates. The weight of present evidence suggests only a general role (but including tissue-specific response, *e.g.*, hormone response) for the major NHC proteins in the structure of chromatin and in the regulation of gene activity. This analysis does not include minor NHC proteins, present in very small amounts, which may yet be found to play a role in specific gene responses.

The chemical characteristics of the NHC protein fractions are not only of intrinsic interest, but also aid in speculation on their role, and in devising further fractionation procedures. Although it is difficult to make detailed comparisons to the work of other authors because of the variety of tissues and species used and the variety of techniques used to isolate chromatin and chromosomal proteins, certain generalities concerning NHC proteins are beginning to emerge. There appears to be little doubt that such proteins exist and are not artifacts of acid treatment of histones (as suggested by Sonnenbichler and Nobis, 1970) since (a) similar sodium dodecyl sulfate gel electrophoresis patterns, including many bands at positions other than those of histones, are observed on extraction of chromatin by a variety of solvents, some at neutral pH (S. C. R. Elgin, unpublished observations and Elgin and Bonner, 1970), and (b) NHC proteins with (glutamic + aspartic acids)/(lysine + histidine + arginine) = $a/b > 1$ have been isolated by a variety of techniques such that the proteins are not exposed to pH values below 2.5 (Wang, 1967; Shaw and Huang, 1970; MacGillivray *et al.*, 1971). Acid hydrolysis in the presence of DNA does not alter the ratio of acidic to basic amino acids of histones significantly (S. C. R. Elgin, unpublished observation). The amino acid compositions of whole NHC protein preparations and fractions given in the literature are frequently quite similar, but of such a general sort as to make comparisons meaningless. The ratio a/b of different NHC protein fractions ranges from *ca.* 1.2 to 2.7 (θ_1, this paper), calculated in all cases without consideration of possible amides (Wang, 1967; Marushige *et al.*, 1968; Benjamin and Gellhorn, 1968; Shirey and Huang, 1969; Shaw and Huang, 1970; Shelton and Allfrey, 1970; Teng *et al.*, 1971; MacGillivray *et al.*, 1971). This is in agreement with the observed range of isoelectric points (see Figure 7). Several of the proteins show a low level of phosphate incorporation (Figure 10; Platz *et al.*, 1970; Teng *et al.*, 1971); turnover appears to be more rapid than that of the histones (Figure 10; Holoubek and Crocker, 1968). Comparisons of the NHC proteins of different tissues of the same organism show many similarities, although tissue-specific proteins are also found (Elgin and Bonner, 1970; Shaw and Huang, 1970; Platz *et al.*, 1970; MacGillivray *et al.*, 1971; Teng *et al.*, 1971).

The method developed and presented here allows the separation of NHC proteins free of histone and DNA in a form suitable for further fractionation. The solvents used and the analytical results obtained indicate that we are fractionating the single complete polypeptide chains rather than aggregates or complex molecules. Unfortunately, the pH of the formic acid–urea solvent is sufficiently low (pH 2.5) so that one cannot *assume* that the isolated proteins will return to their native conformations under suitable conditions. However, many enzymes are known to renature after acid denaturation and this may prove true for NHC proteins also. In any event, these proteins can certainly be used for chemical characterization. The advantages of the method over those already in the literature are (1) good separation of some of the NHC proteins and histones from each other, (2) recovery of NHC protein fractions amenable to further fractionation techniques (*i.e.*, no detergent bound to them), (3) ability to process large amounts of material (*i.e.*, no need to rely on preparative gel electrophoresis), (4) ability to use isolated purified chromatin as a starting material, and (5) avoidance of extreme conditions [*i.e.*, pH 2.5 is not too gentle, but pH 11.6 (Benjamin and Gellhorn, 1968) or 0.5 M hot perchloric acid (Holoubek and Crocker, 1968) is worse!]. (See also Wang, 1967; Marushige *et al.*, 1968; Gershey and Kleinsmith, 1969; Shaw and Huang, 1970; MacGillivray *et al.*, 1971.)

Following the initial fractionation of the chromosomal proteins on Sephadex SE C-25 ion-exchange resin and secondary fractionations by gel permeation or phosphocellulose chromatography, four fractions of NHC proteins have been obtained. Two of these, I (or θ_1) and ϵ, are each probably single polypeptide chains. Two are heterogeneous; II3–IIIa2 probably contains 4–6 proteins, and IIIb3′ probably contains 4–7 proteins. Criteria examined include number of bands on sodium dodecyl sulfate and urea gel electrophoresis, number of bands on analytical isoelectric focusing, and N-terminal analysis. It can be estimated from a quantitative scan of stained sodium dodecyl sulfate gels of whole NHC protein that the bands analyzed here (β, γ, ϵ, κ, θ, λ, π) account for a minimum of *ca.* 70% of the protein (see Figure 10) assuming the isolated fractions to be representative of the protein in these molecular weight bands. The analysis therefore suggests once again that a relatively small number (10–15) of proteins make up the bulk of the NHC protein fraction.

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References

Benjamin, W., and Gellhorn, A. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 262.

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

Comings, D. E., and Okada, T. A. (1970a), *Exp. Cell Res. 62*, 293.

Comings, D. E., and Okada, T. A. (1970b), *Exp. Cell Res. 63*, 62.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244*, 5074.

Elgin, S. C. R. (1971), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Elgin, S. C. R., and Bonner, J. (1970), *Biochemistry 9*, 4440.

Elgin, S. C. R., Froehner, S. C., Smart, J. E., and Bonner, J. (1971), *Advan. Cell Mol. Biol. 1*, 1.

Fairbanks, G. (1969), Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass.

Finlayson, G. R., and Chrambach, A. (1971), *Anal. Biochem. 40*, 292.

Furlan, M., and Jericijo, M. (1967), *Biochim. Biophys. Acta 147*, 145.

Gallwitz, D. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1083.

Garrels, J. I., Elgin, S. C. R., and Bonner, J. (1972), *Biochem. Biophys. Res. Commun.* (in press).

Gershey, E. L., and Kleinsmith, L. J. (1969), *Biochim. Biophys. Acta 194*, 331.

Gilmour, R. S., and Paul, J. (1969), *J. Mol. Biol. 40*, 137.

Gilmour, R. S., and Paul, J. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9*, 242.

Holoubek, V., and Crocker, T. T. (1968), *Biochim. Biophys. Acta 157*, 352.

Loeb, J. E., and Creuzet, C. (1970), *Bull. Soc. Chim. Biol. 52*, 1007.

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

MacGillivray, A. J., Carroll, D., and Paul, J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 204.

Marushige, K., Brutlag, D., and Bonner, J. (1968), *Biochemistry 7*, 3149.

Ozaki, M., Mizushima, S., and Nomura, M. (1970), *Nature (London) 222*, 333.

Panyim, S., Jensen, R. H., and Chalkley, R. (1968), *Biochim. Biophys. Acta 160*, 252.

Platz, R. D., Kish, V. M., and Kleinsmith, L. J. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12*, 38.

Righetti, P., and Drysdale, J. W. (1971), *Biochim. Biophys. Acta 236*, 17.

Shapiro, A. L., Vinuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.

Shaw, L. M., and Huang, R. C. (1970), *Biochemistry 9*, 4530.

Shelton, K. R., and Allfrey, V. G. (1970), *Nature (London) 228*, 132.

Shirey, T., and Huang, R. C. (1969), *Biochemistry 8*, 4138.

Smart, J. E., Newman, R. S., and Dreyer, W. J. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1119.

Sonnenbichler, J., and Nobis, P. (1970), *Eur. J. Biochem. 16*, 60.

Spelsberg, T. C., Hnilica, L. S., and Anseven, A. T. (1971a), *Biochim. Biophys. Acta 228*, 550.

Spelsberg, T. C., Steggles, A. W., and O'Malley, B. W. (1971b), *J. Biol. Chem. 246*, 4188.

Spiegel, M., Spiegel, E. S., and Meltzer, P. S. (1970), *Develop. Biol. 21*, 73.

Teng, C. S., and Hamilton, T. H. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 465.

Teng, C. S., and Hamilton, T. H. (1970), *Biochem. Biophys. Res. Commun. 40*, 123.

Teng, C. S., Teng, C. T., and Allfrey, V. G. (1971), *J. Biol. Chem. 246*, 3597.

Toevs, L. A., and Brackenbury, R. W. (1969), *Comp. Biochem. Physiol. 29*, 207.

Wang, T. Y. (1967), *J. Biol. Chem. 242*, 1220.

Wang, T. Y. (1968), *Exp. Cell Res. 53*, 288.

Ward, S., Wilson, D. L., and Gilliam, J. J. (1970), *Anal. Biochem. 38*, 90.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133*, 369.

Young, E. G. (1963), *Comp. Biochem. 7*, 23.