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Limited Heterogeneity of the Major Nonhistone Chromosomal Proteins*

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ABSTRACT: There has been much discussion but little detailed work on the chemistry and biology of nonhistone chromosomal protein. The principal hindrances in their study have been the tendency of the proteins to aggregate and the difficulty in dissociating them from histone and DNA. For this study purified chromatin was used as starting material. The histones were extracted with 0.4 N H_2SO_4, and the remaining nonhistone chromosomal proteins then solubilized by 1% sodium dodecyl sulfate (SDS) in 0.05 M Tris (pH 8).

DNA was next removed by ultracentrifugation. The nonhistone chromosomal proteins were then examined by SDS gel electrophoresis (molecular weight sieving). Our preparations of rat liver nonhistone chromosomal protein include 13 major polypeptide bands of molecular weight *ca.* 5000 to *ca.* 100,000. Homologous peptides are found in chicken liver nonhistone chromosomal protein, while an additional high molecular weight band is found in preparations from chicken erythrocyte. Rat kidney nonhistone chromosomal protein lacks two and possesses one additional band relative to the rat liver protein fractions. Pea bud nonhistone chromosomal proteins include half of these same bands. The striking similarity of the nonhistone chromosomal proteins of different organs and creatures suggests that some of them are common enzymes, such as those of nucleic acid metabolism, and/or common structural proteins (analogous to histones). Some of the apparent differences may be organ and species specific.

Isolated interphase chromatin is composed of DNA, RNA, histones, and nonhistone chromosomal proteins (NHC proteins).[1] Little is known about the latter; their isolation and fractionation have been severely hampered by the tendency of NHC proteins to aggregate with DNA, histones, and one another. Chromatin possesses several enzymatic activities which may be associated with NHC proteins. These include RNA polymerase (Weiss, 1960; Huang *et al.*, 1960) and a neutral protease that preferentially degrades deoxyribonucleohistone (Furlan and Jericijo, 1967; Furlan *et al.*, 1968). Several general findings suggest that the NHC proteins play some role in the regulation of template activity. Studies of chromatin of different pea tissues (Bonner *et al.*, 1968b), of different stages of the sea urchin embryo (Marushige and Ozaki, 1967), and of different stages of spermatogenesis in trout testis (Marushige and Dixon, 1969) indicate that the more template-active chromatins of a given organism contain more NHC protein than do less template-active chromatins. Teng and Hamilton (1969) have reported that one of the major events in the early action of estrogen in the uterus of the ovariectomized rat is an increased rate of synthesis and accumulation of NHC protein in the uterine chromatin. Interestingly, Sadgopal and Bonner (1970) have found a striking increase in the NHC proteins of HeLa metaphase chromosomes as compared to HeLa interphase chromatin. Histones turn over at a low rate and are conserved in cell division (Byvoet, 1966; Hancock, 1969); in contrast it appears that at least some of the NHC proteins turn over very rapidly (Holoubek and Crocker, 1968). The NHC protein fraction may also include nuclear membrane components. In eukaryotes DNA synthesis appears to be initiated at the nuclear membrane (Comings and Kakefuda, 1968); isolated crude

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[1] Abbreviations used are: NHC proteins, nonhistone chromosomal proteins; SDS, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; DOC, sodium deoxycholate.

TABLE I: Chemical Composition of Chromatin.

Source	No. of Prepns	DNA	RNA	Histone	NHC Protein
Rat liver	7	1	0.04 ± 0.01	1.15 ± 0.10	0.95 ± 0.11
Rat liver nuclei	2	1	0.06	1.40	1.19
Rat kidney	4	1	0.06 ± 0.03	0.95 ± 0.12	0.70 ± 0.15
Chicken liver	3	1	0.03 ± 0.01	1.17 ± 0.10	0.88 ± 0.16
Chicken erythrocyte	5	1	0.02 ± 0.01	1.08 ± .16	0.54 ± 0.14
Pea bud	2	1	0.05	1.10	0.41

chromatin has been found to include lipids (Jackson *et al.*, 1968). Thus at present it may be supposed that the NHC protein fraction includes DNA and RNA polymerases, nucleases, and other enzymes involved in the metabolism of chromatin; structural proteins perhaps analogous to, but more acidic than, histones; nuclear membrane components; and possibly specific repressor or activator proteins if they exist in eukaryotes.

For study of the structures and functions of the NHC proteins it is necessary to dissociate them from DNA and from each other. Ideally, one would wish to have a method that would (1) extract and solubilize all NHC proteins, and (2) maintain them in their native states. Unfortunately, none of the present methods (acid or base, salt, urea, or detergent extraction or a combination of these) achieves both goals. We have chosen, therefore, to examine initially only the chemistry of the NHC proteins. Acid-extracted (and therefore histone-free) chromatin is treated with 1% SDS, in which all NHC proteins are solubilized. We then examine the NHC protein fraction by SDS gel electrophoresis to determine its heterogeneity as well as the similarities and differences between the NHC proteins of selected tissues.

Methods

Preparation of Chromatin. Chromatin from rat liver, rat kidney, and chicken liver was prepared essentially as previously described (Bonner *et al.*, 1968a). Frozen tissue was ground (Waring Blendor) in saline–EDTA (0.075 M NaCl plus 0.024 M EDTA, pH 8). The homogenate was filtered through two layers of Miracloth (Chicopee Mills, Inc.) and the pellet collected by centrifugation at 1500*g* for 10 min. The pellet was washed once in saline–EDTA and four times in 0.01 M Tris buffer (pH 8), being collected the last two times by centrifugation at 12,000*g* for 10 min. The gelatinous crude chromatin was further purified by centrifugation through 1.7 M sucrose (buffered with 0.01 M Tris, pH 8) for 2.5–4 hr at 50,000*g*. This purified chromatin was washed once, resuspended, and dialyzed overnight against 0.01 M Tris (pH 8). The chromatin was then sheared in a Virtis homogenizer at 30 V for 90 sec and centrifuged at 12,000*g* for 30 min; the supernatant, referred to as purified chromatin, or nucleohistone, was used as the starting material for the preparation of NHC protein.

Pea bud chromatin was prepared by the similar method described by Bonner *et al.* (1968a) with the following alterations: the grinding medium was 0.25 M sucrose, 0.05 M Tris buffer (pH 8), and 0.01 M $MgCl_2$. Crude chromatin was resuspended in 0.01 M Tris buffer (pH 8) and centrifuged through 1.7 M sucrose for 2.5 hr to yield purified chromatin, which was resuspended, dialyzed, and sheared as above.

Chicken erythrocyte chromatin was prepared as follows. Fresh chicken blood was centrifuged at 500*g* for 10 min and the supernatant and top layer of white cells were removed. The erythrocytes were washed three times in saline (0.85% NaCl) and then lysed by dilution in an equal volume of 0.01 M $CaCl_2$. The nuclei were then purified once by centrifugation at 750*g* through 0.33 M sucrose, 0.0033 M $CaCl_2$, and 0.005 M Tris buffer (pH 7.9). The pellet was washed once in saline–EDTA and then repeatedly with 0.01 M Tris (pH 8); the chromatin was purified and sheared as above.

That chromatin prepared by the method of Bonner *et al.* (1968a) is highly purified and free from gross contamination by ribonucleoprotein particles is shown by (a) the low RNA content of the preparations (see Table I) and (b) the absence of basic proteins other than histones, as shown by disc gel electrophoresis of acid extracts of chromatin (Bonner *et al.*, 1968a). Electron micrographs of typical pea bud chromatin show that it contains little granular matter (Griffith, 1970).

For several experiments rat liver or chicken liver chromatin was prepared from nuclei purified by the method of Dounce *et al.* (Dounce and Ickowicz, 1969; Umana and Dounce, 1964). Fresh rat livers were minced and homogenized in 0.44 M sucrose, pH adjusted to 5.8 with 0.1 N citric acid. The homogenate was filtered through gauze, rehomogenized, and diluted with one volume of 0.44 M sucrose. The pellet was collected by centrifugation at *ca.* 800*g* and washed twice with 0.44 M sucrose; it was then resuspended in 2.2 M sucrose and centrifuged at 58,500*g* for 90 min. The nuclei were resuspended in saline–EDTA. Such nuclei exhibited nearly complete morphological integrity with little debris under a phase-contrast microscope as described by Chauveau *et al.* (1956). The nuclei were next homogenized in 0.01 M Tris (pH 8), and the pellet collected by centrifugation at 12,000*g;* this step was repeated once. The final suspension was purified by centrifugation through 1.7 M sucrose and sheared to nucleohistone as detailed above.

Rat liver and rat kidney were from male Sprague-Dawley rats, approximately 200*g*. Frozen tissues were obtained from Pel-Freeze Biologicals, Rogers, Ark. Chicken liver and blood were from adult male White Leghorns.

Preparation of NHC Protein. The NHC proteins were prepared following the procedure of Marushige *et al.* (1968) with alterations as noted. Histones were extracted from the

nucleohistone with 0.4 N H_2SO_4 at 4° for 30 min. The pellet was washed once with 0.4 N H_2SO_4 and briefly with 0.01 M Tris (pH 8). Over 95% of the acid-soluble protein is removed by this treatment (Fambrough and Bonner, 1966). The pellet was dissolved by gentle homogenization in 1% SDS–0.05 M Tris (pH 8), stirred overnight at 37°, and dialyzed to 0.1% SDS–0.01 M Tris (pH 8) at 37°. The DNA was removed by centrifugation at 36,000 rpm for 18 hr at 25° in a Spinco SW-50 rotor. The top two-thirds of the supernatant were taken as the NHC protein preparation and analyzed by SDS gel electrophoresis following dialysis against buffer III (see below). This buffer dissociates most proteins into their individual polypeptide chains (Shapiro *et al.*, 1967).

Preparation of Labeled NHC Proteins. To obtain labeled NHC proteins, a rat was given intraperitoneally 0.055 mg of algal protein hydrolysate-^{14}C (0.1 mCi, uniformly labeled, New England Nuclear Corp.) 24 hr before killing. The liver was frozen in Dry Ice and processed, and NHC protein was prepared from the sheared chromatin as detailed above.

Disc Gel Electrophoresis. SDS disc gel electrophoresis was carried out according to the method of Shapiro *et al.* (1967) (final gel composition is 5% acrylamide, 0.13% *N,N'*-bismethyleneacrylamide, 0.1% SDS, 0.1 M sodium phosphate buffer (pH 7.1), 0.05% *N,N,N',N'*-tetramethylenediamine, and 0.075% ammonium persulfate). Purified acrylamide (Bio-Rad Laboratories) was used. The gels were 6 cm in length and were run at 47 V for 75 min. Gels were routinely stained in 0.25% coomassie brilliant blue R-250 (Mann Research Laboratories) in 5:5:1 water–methanol–acetic acid and destained sideways electrophoretically in 17:1:2 water–methanol–acetic acid. Gels were photographed using an orange filter with Kodak TriX-10 4 × 5 film; the pictures were printed on Dupont Varilour-VL-RW-SW paper. All gels photographed together were run at the same time. Human γ-globulin (Mann Research Laboratories) was used as a molecular weight marker.

In order to detect low molecular weight proteins, the method of Laico *et al.* (1970) was occasionally employed. In this case 11-cm long disc gels (same gel composition as above) are run at 40 V for approximately 6 hr. The gels are fixed in 20% sulfosalicylic acid (three changes, 24-hr total), stained for 5 hr in 0.25% coomassie brilliant blue, and photographed after 4-hr destaining in 10% acetic acid.

Samples other than NHC proteins were prepared for SDS gel electrophoresis by dialysis against buffer I, 12 hr, room temperature; buffer I, 12 hr, 37°; buffer II, 12 hr, room temperature; buffer III, 4–12 hr, room temperature. (Buffer I is 1% SDS–1% β-mercaptoethanol in 0.01 M sodium phosphate buffer, pH 7.1; buffer II is 0.1% SDS–0.1% β-mercaptoethanol in 0.01 M sodium phosphate buffer, pH 7.1; buffer III is 0.1% SDS–0.1% β-mercaptoethanol–10% glycerol in 0.01 M sodium phosphate buffer, pH 7.1.)

Acrylamide disc gel electrophoresis of acid-extracted histones dialyzed against 8 M urea–0.01 M Tris (pH 8) was also performed by the method of Bonner *et al.* (1968a) at pH 4.3 in the presence of urea (15% acrylamide gel).

To obtain NHC protein fractions for amino acid analysis, identical samples were electrophoresed in SDS in an eight-slot, vertical slab gel electrophoresis unit (E-C Apparatus Corp., Model EC470) by the usual method. Samples were run at 110–150 V for *ca.* 2.5 hr or until a bromophenol blue marker (Matheson, Coleman & Bell) had traveled 9 cm. One strip was removed, stained, and destained as usual (first paragraph, this section) and the desired bands were cut out using this guide. The gel was broken up (by forcing it through a fine stainless steel mesh) and put in a short column; the protein was eluted with one column volume of running buffer (0.1% SDS in 0.1 M sodium phosphate buffer, pH 7.1). This protein solution was dialyzed extensively against water at 37°, lyophilized, and analyzed for amino acid composition with a Beckman Model 120B instrument. A gel blank had to be subtracted from these values. Cytochrome *c* (horse heart, Mann Research Laboratories) was analyzed by this procedure as a control; the mole per cent amino acid composition after gel electrophoresis of cytochrome *c* differed by less than 2% from the standard before gel electrophoresis. Portions of protein samples used for amino acid analysis were dialyzed to buffer III and reelectrophoresed. Some higher molecular weight material, presumably aggregates, was observed.

General Methods. Chromatin samples were analyzed as follows. Histones were extracted with 0.4 N H_2SO_4 and their concentration was determined by ultraviolet absorption at 230 mμ using $\epsilon = 4.15$ (l./cm g) (R. H. Jensen, 1966, unpublished data). The pellet was dissolved in 1.0 N NaOH and the NHC protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as a standard. Using fresh chromatin samples RNA was separated from DNA by the modified Schmidt–Tannhauser procedure of Ts'o and Sato (1959). RNA was determined by the orcinol method (Dische and Schwarz, 1955) after hydrolysis in 0.3 M KOH; yeast RNA (Sigma) was used as a standard. DNA was determined from the ultraviolet spectrum of the nucleohistone, assuming that all absorption at 260 mμ is due to nucleic acids and making an empirical correction for scattering (Marushige and Bonner, 1966). The absorptivity of DNA contained in chromatin is 22 (l./cm g) at 260 mμ (Tuan, 1967); for RNA contained in chromatin it is assumed to be 25 (l./cm g) at 260 mμ. Radioactivity of samples was determined as follows: aliquots were dried on Bac-T-Flex membrane filters (Schleicher & Schuell Co.) using a vacuum oven. This technique minimizes quenching differences between the samples due to different solvent systems. Samples were counted on a Beckman liquid scintillation system LS-200B in toluene scintillation fluid (22.6 g of PPO plus 0.75 *g* of POPOP in eight pints of toluene; fluors from New England Nuclear Corp.). Deoxyribonuclease I was obtained from Worthington Biochemicals; ribonuclease A from bovine pancreas was from Sigma.

Results

Chemical Composition of Chromatin. All the chromatins used in the preparation of NHC proteins were analyzed for composition by the methods described. The results, given in Table I, are in approximate agreement with values in the literature (Dingman and Sporn, 1964; Bonner *et al.*, 1968a; Smart, 1970). The preparations are reasonably reproducible as shown by their standard deviations. The larger protein content of rat liver chromatin prepared from purified nuclei as compared to that prepared from a crude nuclear pellet possibly results from the reduced exposure of the chromatin to cytoplasmic proteases, such as that with a preference for basic proteins (Paik and Lee, 1970). Alternatively, the increase in protein could result from increased nonspecific protein

TABLE II: Amino Acid Compositions in Mole Per Cent.

Amino Acid	Nonhistone $\beta + \gamma$[a] (Rat Liver)	Histone IIb[b] (Calf Thymus)	Histone III + IV[b] (Calf Thymus)
Lysine	4.8	13.5	9.7
Arginine	5.9	7.9	11.9
Histidine	3.9	2.8	1.9
Aspartic acid	8.4	5.6	5.0
Glutamic acid	10.9	8.7	10.4
Serine	9.9	7.0	4.6
Threonine	4.3	5.2	6.7
Phenylalanine	3.3	1.3	2.5
Tyrosine	2.6	3.0	2.2
Tryptophan	ND[c]		
Alanine	8.3	11.5	11.6
Valine	5.2	6.7	5.9
Isoleucine	3.7	4.5	5.3
Leucine	7.7	8.6	8.9
Methionine	1.4	0.8	1.3
Proline	4.4	4.7	4.2
Glycine	14.7	8.2	8.6
Cystine + cysteic acid	1.1		Present[d]

[a] No corrections made for any loss of serine or threonine during acid hydrolysis. Average of two experiments. [b] Values from Rasmussen *et al.* (1962). [c] ND = not determined. [d] Fambrough and Bonner (1968).

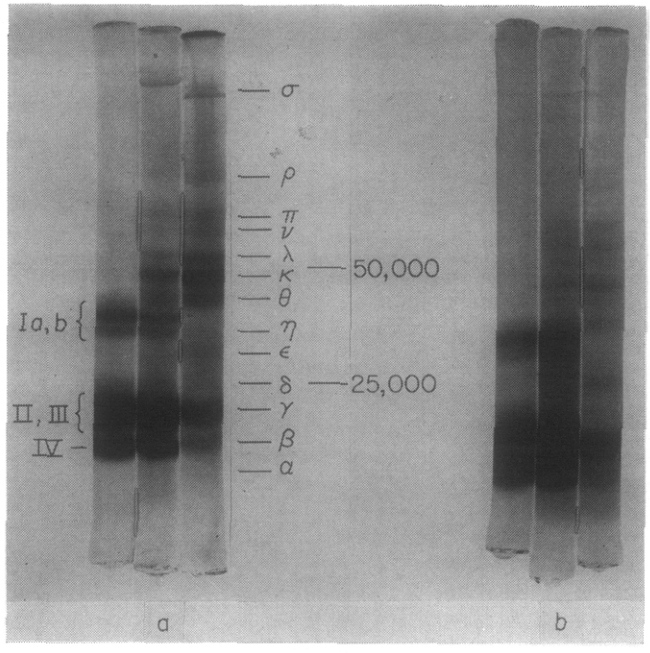

FIGURE 1: SDS gel electrophoresis of samples of histone, chromatin, and NHC protein (left to right). Samples prepared as given in *Methods*. Sample size, 10–50 μg. 5% acrylamide gels, pH 7.1, run top to bottom for 75 min at 48 V. Molecular weights in 1a indicate position of γ-globulin marker (heavy and light polypeptide chains) run simultaneously; note that the log of the distance of migration is directly proportional to the molecular weight of the polypeptide chain (Shapiro *et al.*, 1967). (a) Rat liver and (b) rat kidney.

binding in the nucleus, particularly of aggregate structures (see below). All preparations were free of gross contamination by ribonucleoprotein particles, etc., as shown by the absence of ribosomal protein bands on urea disc gel electrophoresis of the acid-extracted histones.

Fractionation and Recovery of Protein. The fact that all the protein bands on an SDS polyacrylamide gel of chromatin are found in either the histone or NHC fraction (Figure 1) shows that the present methods recover and separate all the major chromosomal proteins. This is true for all tissues examined. The separation of histones from NHC proteins appears to be essentially complete. Specifically, no histone I is found on the NHC protein SDS gels; no high molecular weight NHC proteins appear on the histone SDS gels or on urea gels (pH 4.3) of the acid extract (latter not shown).

The histone bands on SDS gels have been tentatively identified on the basis of correlation of known bands on urea gels with bands on SDS gels for the same sample and by molecular weights (Figure 1a). The NHC proteins have been assigned arbitrary identification letters for purposes of discussion (Figure 1). The histone fraction frequently contained a trace of ε; this contaminant has also been noted in DOC extraction of pea bud histones from chromatin (Smart, 1970). The NHC protein fraction includes bands β and γ at the same position on SDS gels as histones IV and II–III. However, amino acid analysis of the β–γ protein shows that these bands contain primarily acidic proteins; the ratio (Glu + Asp)/(Arg + Lys + His) is 1.32, ruling out the possibility of major histone contamination (Table II).

The total recovery of protein from chromatin in all fractions is 90–95% as shown by recovery of amino acid-^{14}C from *in vivo* labeled chromatin. Lowry determinations of NHC proteins in SDS also indicate that the recovery of protein during the separation from DNA is 90% (Table III). To ensure that the radioactivity observed represented ^{14}C label in the protein only, fresh chromatin samples were incubated with deoxyribonuclease I (1.0 mg/ml) and ribonuclease A (0.01 mg/ml) for 90 min at 37° and precipitated with 10% trichloroacetic acid. No radioactivity was released into the supernatant by this treatment. Incorporation of 10% or more of the radioactivity into nucleic acids would have been detected by this method. Thus essentially all protein is accounted for in the present procedure. Only about half of the NHC protein is recovered in the top two-thirds supernatant of the 36,000-rpm spin, the material used for the SDS gels of NHC protein shown. In all cases the DNA pellet was rehomogenized in the remaining supernatant and SDS gels of this solution were run. These gels show the same bands as those of Figure 1, but exhibit higher background and some streaking, due presumably to the high concentration of nucleic acid and consequent re-formation of complexes in the sample.

Limited Heterogeneity of NHC Proteins. As is apparent from Figures 2–5, the NHC protein fractions of different tissues are very similar. Comparison of NHC proteins of rat kidney with those of rat liver by SDS gel electrophoresis (Figure 2) shows that the fractions are identical except that

TABLE III: Recovery of Protein at Successive Stages in the Purification of NHC Proteins of *in Vivo* Amino Acid-^{14}C Labeled Chromatin.

Process	Fraction	Radioactivity Estimate of Protein		Lowry Estimate of Protein	
		cpm in Fraction	% of Starting Material	Color Units	% of Starting Material
Chromatin preparation	Nucleohistone	15,710	100		
Acid extraction	Acid extract + wash	4,173	26.6		
	Pellet solubilized in SDS	11,640*	74.1	2.95	100
			100.7		
SDS dialysis	SDS protein solution	11,070	70.5	2.74	93
Centrifugation	Supernatant two-thirds	4,448	28.3	1.22	41
	Pellet one-third	6,682	42.5	1.46	49
			70.8[a]		90

[a] 96% of *.

kidney NHC protein lacks λ and κ and possesses a band, ω, in the same molecular weight region. (Only bands of the molecular weight of ρ (*ca.* 100,000) and below are considered in this paper; it seems probable that the fine, higher molecular weight bands above this position are undissociated aggregates.) Similarly, there is a high degree of homology between rat liver NHC proteins and those of chicken liver (Figure 3). In this case the only difference is an additional band, μ, in the chicken liver preparations. A very faint band at this position is, however, observed in some preparations of rat liver NHC proteins. Bands δ and η are not readily apparent in the gel of rat liver NHC protein shown in Figure 3. However, reference to Figure 2 shows that bands δ and η are apparent in gels which are more heavily loaded with rat liver NHC protein. The similarity of liver NHC proteins of different organisms is perhaps not surprising considering the similarity in structure and function of rat and chicken liver. Interestingly, the NHC protein fraction of chicken erythrocyte shows most of the NHC protein bands of chicken liver, indeed all except ρ, and contains also an additional high molecular weight band χ (Figure 4). However, the distribution of amounts of protein is strikingly different. With the exception of β and γ, the chicken erythrocyte preparation shows less low molecular weight protein than does the liver preparation, and more high molecular weight protein species, particularly π and χ. If one prepares chicken liver chromatin from purified nuclei, the NHC protein appears to contain proportionally more π and μ; the band χ, however, is not observed.

The only nonvertebrate NHC protein fraction examined is that of pea bud chromatin. As is clear in Figure 5, this material is quite different from the vertebrate NHC protein frac-

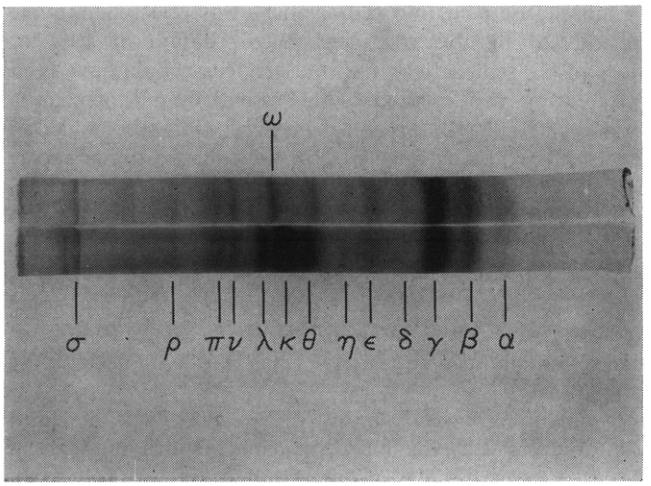

FIGURE 2: Top: rat kidney NHC protein. Bottom: rat liver NHC protein. SDS gels run left to right.

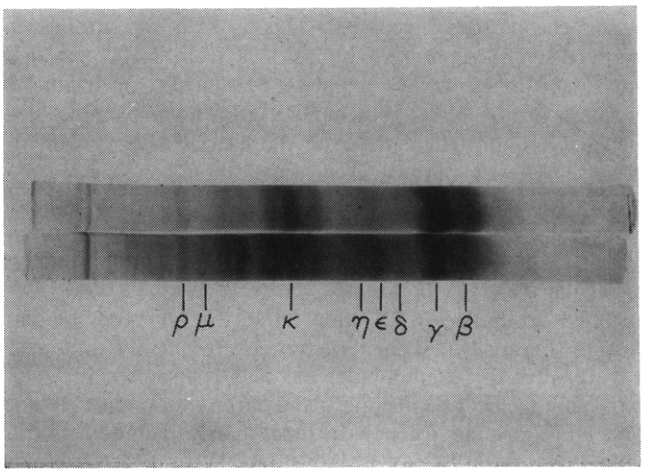

FIGURE 3: Top: rat liver NHC protein. Bottom: chicken liver NHC protein. SDS gels run left to right.

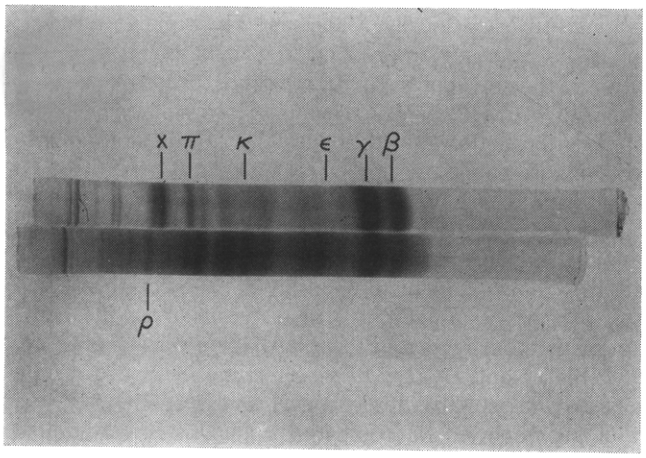

FIGURE 4: Top: chicken erythrocyte NHC protein. Bottom: chicken liver NHC protein. SDS gels run left to right.

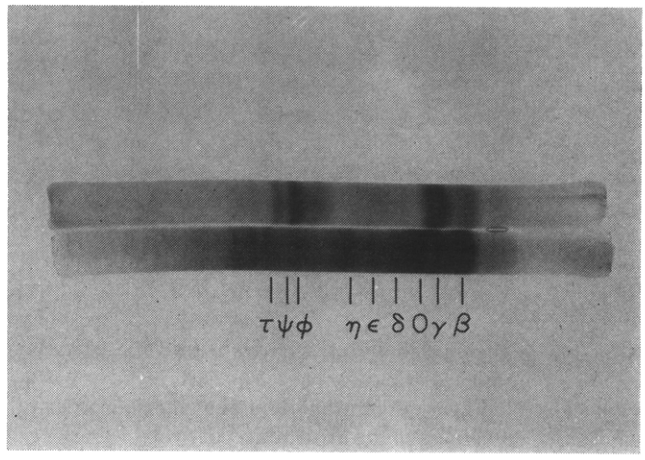

FIGURE 5: Top: rat liver NHC protein. Bottom: pea bud NHC protein. SDS gels run left to right.

tions in the high molecular weight regions, although there is good homology in the β–η region. Bands 0, ϕ, ψ, and τ are apparently present only in pea bud.

In addition to the protein bands shown, all chromatin and NHC protein preparations exhibit a band M, which runs in the same position as insulin when analyzed on 11-cm SDS gels according to the method of Laico *et al.* (1970). This band behaves in a manner analogous to their miniprotein which is a major component of biological membranes. It is apparently not fixed in the gel by our usual techniques, and is significantly smaller in molecular weight than any of the bands shown.

Isolation of Nuclei Prior to Chromatin Preparation. It has been suggested many times that chromatin may absorb proteins from the cytoplasm during isolation (Johns and Forrester, 1969). On the other hand, legitimate chromosomal proteins may be degraded or dissociated during the isolation procedure. To explore this question we have prepared chromatin from purified rat liver nuclei and subsequently prepared NHC proteins from this material. Gels of the NHC protein from chromatin prepared in this way are compared to those of our standard preparations in Figure 6. Although there are obvious quantitative differences between the preparations, for the most part the same protein bands below ρ are present. NHC protein preparations made from chromatin as usually prepared in this laboratory contain less of the higher molecular weight bands ρ, π, ν, and in particular can be deficient in λ. The band α does not appear in NHC proteins from nuclear chromatin. Unfortunately, chromatin preparations from purified nuclei are more difficult to fractionate into histone and NHC protein by acid extraction, as the acid precipitate is very fine and loose. The resulting NHC protein gels contain some histone I. The histone gels also contain the NHC proteins δ, ϵ, θ, κ, λ, ν, π, and other high molecular weight material, all in small amount. The increase in very high molecular weight material in NHC protein preparations *via* purified nuclei could be due to increased nonspecific absorption of material from the nucleoplasm onto the chromatin.

Figure 6 also shows NHC protein gels which result when (a) chromatin, prepared by usual techniques, is allowed to stand at room temperature for 2 hr prior to acid extraction; (b) the purified nuclei prepared for chromatin and NHC protein extraction are allowed to stand at room temperature for 2 hr in 2.2 M sucrose. Degradation appears to be minor. Quantitatively, a 5–10% decrease in amount of protein appears to take place during the 2-hr incubation.

There appear to be no alterations in NHC proteins during storage of frozen liver at −80°; chromatin prepared from rat livers within 24 hr of excision and freezing is identical with that prepared by our usual procedures both in chemical composition and in NHC protein gel pattern.

Discussion

The most striking finding of this study is the high degree of homology between the NHC proteins of different origin as compared by SDS gel electrophoresis (molecular weight sieving). This homology holds for a given organ in different vertebrates (rat liver and chicken liver) as well as for different organs of a given species (rat liver and rat kidney; chicken

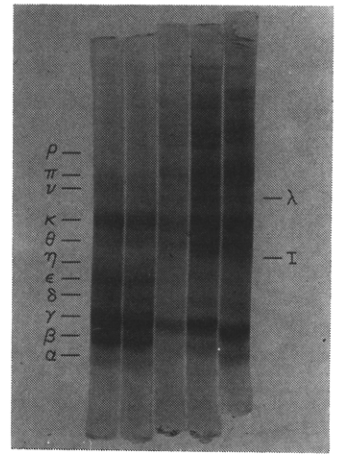

FIGURE 6: Rat liver NHC protein. SDS gels run top to bottom. Left to right: *ca.* 35 μg of NHC protein, regular chromatin preparation; *ca.* 35 μg of NHC protein, regular chromatin preparation incubated 22°, 2 hr; *ca.* 17 μg of NHC protein, nuclear chromatin preparation; *ca.* 35 μg of NHC protein, nuclear chromatin preparation incubated 22°, 2 hr; *ca.* 35 μg of NHC protein, nuclear chromatin preparation.

liver and chicken erythrocyte). This finding supports the idea that many NHC proteins will prove to be common enzymes of nucleic acid and histone metabolism, and perhaps common structural proteins. It is interesting that although chicken erythrocyte chromatin contains only half as much NHC protein as does liver, the bands found in chicken liver NHC protein are all represented. No doubt there are cell type specific NHC proteins, and the unique electrophoretic bands found (kidney ω, erythrocyte χ) may prove to be such. Most probably specific effectors would be present in quantities too small to be observed by the present technique. Only about half of the electrophoretic bands of pea bud preparations are homologous to those of vertebrate NHC protein. In all cases, there are significant and reproducible relative quantitative differences in the distribution of protein.

Our results are in agreement with previous observations. Benjamin and Gellhorn (1968) have studied an acidic nuclear protein fraction, which probably includes the NHC proteins. Gel electrophoresis of rat and mouse liver preparations yield the same protein band pattern. Wang (1967) has done extensive work on "chromatin acidic proteins" (CA proteins) prepared by extracting washed nuclei with 2.0 M NaCl and reprecipitating chromatin with 0.15 M NaCl. The supernatant is taken as the CA protein fraction; it probably includes NHC protein. Comparative electrophoresis of such fractions has been carried out by Loeb and Creuzet (1969). They find homologous as well as different bands in comparisons of preparations from the same organ (liver) of different vertebrates or of different organs in a given vertebrate. They observe two dominant bands in chicken erythrocyte CA proteins which may correspond to our bands γ and χ.

The methods reported here appear to be the best available for extraction and examination of NHC proteins. That the protein bands observed actually represent *chromosomal* proteins is confirmed by the following considerations. (1) Chromatin is a chemical complex which can be reproducibly prepared from a variety of tissues; its properties as a template for DNA-dependent RNA polymerase are the same *in vitro* as they are *in vivo* (Bonner *et al.*, 1963; Marushige and Bonner 1966; Paul and Gilmour, 1966, 1968; Bekhor *et al.*, 1969; Smith *et al.*, 1969). (2) All the protein bands observed in the chromatin SDS gels are represented in histone or NHC protein gels. Recovery of protein is better than 90%. Thus no proteins are lost or degraded to any large extent during the procedure. (3) NHC proteins prepared from chromatin extracted from highly purified nuclei are essentially the same as those from the standard chromatin preparation. Thus little or no protein is adsorbed by chromatin from the cytoplasm in our isolation procedure. Extraction of rat liver chromatin with 0.15 or 0.30 M NaCl removes 10–15% of the NHC protein (Smart, 1970). The extracted protein includes the normal population of NHC proteins and is enriched in the higher molecular weight bands. Thus it seems unlikely that there are any significant nonspecifically adsorbed proteins, dissociable by low salt concentrations, in the preparation, although it is known that repeated extraction with salt will gradually remove some enzyme activities such as ribonuclease (Smart, 1970). (4) Comparison of NHC protein preparations made from purified nuclei chromatin to those made from our standard rat liver chromatin suggests that there is some slight degradation in the course of the latter procedure. Chemical composition of the chromatins substantiates this. Specifically, λ appears to be rapidly degraded; α may be a degradation product. In general, however, degradation does not appear to be a serious problem. (5) The similarity of NHC protein fractions from different tissues supports the notion that these proteins are integral components of chromatin.

The present technique allows for successful separation of soluble NHC proteins from histones and DNA. Unfortunately, the NHC proteins of acid-extracted chromatin are extremely difficult to dissolve in anything other than SDS. The detergent is also difficult to dissociate from the NHC proteins without rendering them insoluble (Marushige *et al.*, 1968; Shirey and Huang, 1969). Thus the method can only yield proteins suitable for chemical analyses. The SDS gel technique employed for comparative analysis is also limited in that the proteins are sieved only by molecular weight. The present bands may be shown to contain several polypeptide species when they are fractionated by methods dependent on isoelectric point, etc. In addition, the technique of gel electrophoresis is limited in that one arbitrarily selects conditions suitable for visualization of the major bands; minor bands are not visualized because of the required total protein load limit and the limit of dye sensitivity. On the other hand, all proteins, regardless of isoelectric point, do run on these gels. The technique has made possible the present comparative survey of the NHC protein fraction and serves as a basis for our future work.

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