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CHROMOSOMAL NONHISTONE PROTEINS

Volume III
Biochemistry

Lubomir S. Hnilica

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Editor

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INTRODUCTION

The third volume of the *Chromosomal Nonhistone Proteins* treatise deals with the isolation and analysis of chromosomal proteins in general and with specific features of these proteins in particular. In addition to the high mobility group (HMG) proteins, postsynthetic modifications of chromosomal nonhistone proteins are discussed, together with some of their enzymatic properties and DNA binding.

As with all multiauthored volumes dealing with rapidly advancing and closely related aspects of a busy research area, the readers may see some overlaps between the individual chapters of this or the companion volumes. Unfortunately, this, despite the best intentions of all the contributing authors, is inevitable. Nevertheless, I hope that most will find this as well as the other volumes informative and helpful in their scientific endeavors.

THE EDITOR

Lubomir S. Hnilica is a professor of biochemistry and pathology at the Vanderbilt University School of Medicine, Nashville, Tennessee.

Dr. Hnilica received his degree in organic chemistry from Masaryk University, Brno, Czechoslovakia in 1952 and an advanced degree in biochemistry from the Czechoslovak Academy of Sciences. After postdoctoral training at the Czechoslovak National Cancer Institute in Bratislava, Dr. Hnilica continued his research as a WHO research fellow at the Chester Beatty Research Institute in London, England. After 2 years at the Baylor College of Medicine in Houston, Texas, Dr. Hnilica joined the staff of the University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute in Houston where he rose to the rank of Professor of Biochemistry and Chief of the Section of Biochemical Regulatory Mechanisms. In 1975 Dr. Hnilica joined the Vanderbilt University School of Medicine where he is Professor of Biochemistry and Pathology as well as director of the A. B. Hancock, Jr. Memorial Laboratory of the Vanderbilt University Cancer Center. His present research concerns proteins of the cell nucleus, their biochemistry and immunology, the interactions of nuclear proteins with DNA, and changes in gene expression during chemical carcinogenesis.

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Chapter 1

ISOLATION, FRACTIONATION, AND ANALYSIS OF NONHISTONE CHROMOSOMAL PROTEINS

Leokadia Klyszejko-Stefanowicz and Lubomir S. Hnilica

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Chromatin, the interphase chromosomal material of eukaryotic cells is a complex of DNA with a fixed level of histone proteins, variable amounts of nonhistone chromosomal proteins (NHCP), and small amounts of RNA. Of these principal chromatin components, the nonhistone proteins have received considerable attention as a class of macromolecules which may be associated with the regulation of genetic transcription.[1-4,25]

I. SOME CONSIDERATIONS OF NHCP ISOLATION AND FRACTIONATION

The resolution of NHCP by conventional methods of protein chemistry has proved particularly difficult because of (1) affinity of these proteins for DNA, and their tendency to form insoluble complexes with histones and themselves, (2) general insolubility in aqueous buffered solutions at physiological values of ionic strength and pH, and (3) proteolytic degradation during prolonged isolation and fractionation procedures.

The same considerations which apply for the isolation of nuclei[6] and chromatin[7] are operative in evaluating procedures for isolation and fractionation of NHCP (for review see Reference 5). Cytoplasmic contamination is important as the probable source of both proteolytic activity and cytoplasmic proteins in the nonhistone pool. Moreover, some special requirements in the NHCP isolation and fractionation are due to very difficult solubilization of the whole and especially, dehistonized chromatin. The greatest barrier lies in the potential ionic, hydrogen, and hydrophobic interactions between chromatin components, and often leads to the use of harsh conditions such as extremes of pH and organic solvents, e.g., sodium dodecyl sulfate (SDS), urea, guanidine hydrochloride (GdnCl), and/or 2-mercaptoethanol (2-ME), which may irreversibly affect the native structure of some nonhistone proteins.

A. Proteolysis

Since the late 1960s several investigators have reported that, apart from proteases of cytoplasmic origin associated with subcellular fractions as microsomes,[8] lysosomes,[9-11] mitochondria[12-14] or outer nuclear membrane,[15] there occurs a neutral protease of low molecular weight (e.g., 24,000,[18] 15,400,[19] or 25,000[20]), tightly associated with chromatin.[16,17] This acid-soluble enzyme was purified from calf thymus chromatin[19,21] and characterized as a serine-type protease.[22] It also appears to be the major diisopropylfluorophosphate (DIFP)-binding protein in the chromatin from nuclei of several rat tissues (liver, thymus, kidney, lung, testis, and Morris hepatoma 9121).[20]

Contrary to cytoplasmic proteases active at lower ionic strength, the activity of the chromatin-bound protease increases in solutions of high ionic strength, being optimal at pH 7.8[23] to 8.5.[19] This enzyme functions in a variety of denaturing solvents such as 5 *M* urea,[24] 0.2 *M* HCl, or H_2SO_4,[16] but not in SDS.[17] It is considered as chromatin nonhistone protein,[25] which attacks histones and nonhistones. It is extractable in acid with the lysine-rich histones,[18] more exactly with histone H2B,[26,27] and its proteolytic activity could not be separated from histone H2B.[26]

Although the danger of proteolytic degradation of chromatin was recognized long ago, the use of protease inhibitors such as $NaHSO_3$ (5 m*M*,[28] 50 m*M*,[29] or even 1 m*M*[30]), DIFP (0.5 m*M*[31] or 1 m*M*[29]), and 0.1 m*M* phenylmethanesulfonyl fluoride (PMSF)[32] inspired by experiments of Fahrney and Gold[33] on esterase inhibition, was not common initially. Sodium bisulfite, being an exception, has been reported to inhibit endogenous chromatin proteolytic activity at a concentration of 50 m*M*,[29,34] generally in solutions of low ionic strength. Proteolytic degradation of calf thymus nucleohistone could be totally inhibited over a period of several days at 2°, either in the presence of $NaHSO_3$ or simply by storing at very low ionic strength (5×10^{-4}).[35] Chae and Carter[24] showed that histones and nonhistone chromatin proteins are intensively degraded when chromatin is exposed to 2 *M* NaCl-5 *M* urea (most often used for its dissociation and reconstitution) or to 5 *M* urea (pH 6 to 8). The report from Busch's laboratory[36] that degradation of chromosomal proteins, observed in chromatin dissociaedt in 3 *M* NaCl-7 *M* urea, was not prevented by 50 m*M* $NaHSO_3$ but only by 1 m*M* PMSF, is also important. To this time, little attention has been given to the effects of proteolysis on the interpretation of chromatin dissociation or reassociation experiments as well as on polypeptide composition of NHCP in their electrophoretical patterns. Also, controls to check for possible proteolytic activity were often not carried out, although long dialysis and centrifugation steps were generally required.

More precise studies on chromatin-bound protease, active in 2 *M* NaCl-5 *M* urea or 5 *M* urea alone, came from Carter and Chae.[37] The proteolytic activity was demonstrated by these authors in chromatin of six rat tissues (liver, thymus, kidney, testis, brain, Ehrlich ascites), rabbit bone marrow (but not in chicken reticulocyte chromatin), and examined in the presence of seven different protease inhibitors at several concentrations. It was shown

that chromatin protease can be irreversibly and most efficiently inhibited by 1 m*M* concentration of two serine-specific inhibitors: water-soluble, but extremely toxic DIFP, and water-insoluble and less toxic PMSF. The protection of chromatin proteins was only partial when 1 m*M* PMSF was used without the presence of organic solvent (isopropyl alcohol, *p*-dioxan, or ethanol), in which it should be prepared as stock solution at 0.1 *M* concentration.[17] At 0.1 m*M* PMSF the protection reaches only 15% of complete proteolysis inhibition observed at final concentration of 1 m*M* PMSF-1% organic solvent.[17,37] Two principal protease inhibitors are used most frequently: PMSF at concentrations of 0.1 m*M*,[38-48] 0.2 m*M*,[49] 0.5 m*M*,[50-52] 1 m*M*,[53-60] 2 m*M*[61] or even 2.85 m*M*,[62] and DIFP at concentrations of 0.1 m*M*,[63] 0.5 m*M*,[64,65] or 1 m*M*.[66]

Among less-used protease inhibitors, the following should be mentioned: soybean trypsin inhibitor (1 μg/mℓ) without[44] or with 1 m*M* $NaHSO_3$;[67] *p*-chloromercuribenzoate at concentration of 2.5 m*M* (homogenate) and 1 m*M* (in other solutions),[68] or Trasylol.®[69] The $NaHSO_3$ at such relatively low concentrations as 1 m*M*,[70,71] 5 m*M*,[72] or 10 m*M*[40] alone or in combination with 1 m*M* PMSF[73] is also used in chromatin isolation. The use of 50 m*M* $NaHSO_3$ along with 40 m*M* NaF can also be quoted.[74] Some caveats should be observed with using protease inhibitors: PMSF may affect extraction of some proteins;[75] DIFP and $NaHSO_3$ may produce artifactual charge heterogeneity in electrophoretic patterns — DIFP by its nonspecific binding to proteins and $NaHSO_3$ by oxidizing conditions.[76]

Some authors failed to demonstrate autolytic activity of chromatin or of NHCP by differences in electrophoretic patterns of NHCP isolated with or without the use of serine-specific inhibitors.[40-50,77-79] Proteolytic degradation of NHCP has not been observed by Elgin,[79] who used SDS to dissolve dehistonized chromatin[80] or salt-formic acid-urea in the case of whole chromatin.[81] The experiments of Boffa and Allfrey[40] have pointed out the usefulness of [^{3}H] catalase, added as a marker of proteolysis to the nuclear extracts during isolation from dehistonized chromatin of phenol-soluble and urea-GdnCl-2-ME-Na_2HPO_4-soluble NHCP.

A neutral protease of high molecular weight (200,000) was isolated from rat liver chromatin and characterized by Bonner's group.[34,82] Most active at pH 7.0, sensitive to sulfhydryl and chelating reagents, inactive at salt concentrations higher than 1 *M* NaCl, this enzyme can be considered as an extranuclear protease,[37] inhibited by 50 m*M* $NaHSO_3$ and also by DIFP and PMSF.[82]

Another high molecular weight protease (103,000) was purified over 800-fold from chromatin isolated from fresh rat liver homogenate.[83] This enzyme has a similar substrate specificity as the protease isolated from rat liver mitochondria,[84] but it shows marked differences in molecular weight, in the effects of monovalent and divalent ions, and different sensitivity to inhibitors. For example, the soybean trypsin inhibitor, a strong inhibitor of the mitochondrial enzyme, is almost totally ineffective with this protease (17% of inhibition).[83] With other inhibitors tested, the following percentages of inhibition were obtained: 0.1% SDS (88%), 1 m*M* EDTA (54%), 50 m*M* $NaHSO_3$ (53%).

Three types of proteases have been described by Miyazaki et al.[66] in nuclei from various normal rat tissues and Rhodamine sarcoma, with the pH optima at 4.5, 7.5, and 10.0, all significantly inhibited by 1 m*M* DIFP.

In addition to neutral proteases, Hagiwara et al.[85] found an alkaline protease associated with chromatins of various normal and tumorous tissues of rats. This enzyme was not observed in other subcellular fractions. Perhaps it is functional in the hydrolysis of NHCP and H1 histones. Inhibited by DIFP, soybean trypsin inhibitor, and chymostatin, it is a chymotrypsin-like protease, with an approximate molecular weight of 18,000. Its activity was found high in rapidly proliferating cells: Yoshida sarcoma > Rhodamine sarcoma > AH130 > thymus ⩾ spleen ⩾ kidney > liver ⩾ brain.

B. Cytoplasmic and Membrane Contamination

Since the experiments of Dounce and Lan[86] who used saponin (heterogeneous mixture of sapogenic glycosides) to isolate chicken erythrocyte nuclei, many efforts have been exerted to eliminate cytoplasmic and membrane contamination in nuclear preparations used as starting material for the isolation of chromatin and chromosomal proteins. To remove the outer nuclear membrane and the attached traces of endoplasmic reticulum, treatment with detergents is required. For this aim the use of SDS (0.4%)[87] and the following nonionic detergents should be noted: Tween® 80, i.e., polyoxyethylene sorbitan monooleate (0.1%);[88] Cemulsol NPT6 (0.15%) and Cemulsol® NPT12 (0.15%);[89,90] a mixture of sodium deoxycholate (0.43%) with Tween® 40 (0.86%) in Penman's[91] double detergent technique[50,92] or with Tween® 80;[93] Nonidet® P-40 (0.2%);[42,54,56,94-96] a mixture of Nonidet® P-40 (0.5%) and Triton® X-100 (0.5%);[97,98] Triton® N-101 (0.3%).[45,99] However, the most widely accepted is the use of Triton® X-100 alone at different concentrations, e.g., 0.05%,[100] 0.1%,[40] 0.2%,[101] 0.25%,[65,102] 0.5%;[46,55,72,97,103-107] 1%,[57,61,73,108-114] or even 2%.[115]

Triton® X-100, extensively used for the disruption and solubilization of cytoplasmic components, is a member of the octylphenoxyethanol series of the nonionic detergents, in which X indicates the average length of the hydrophobic polyoxyethylene chain. For Triton® X-100, X has the value of 9 to 10.[116] Rapid and satisfactory preparation of nuclei with the application of Triton® X-100 was first performed by Hymer and Kuff.[116] Similar products having an additional $-CH_3$ group on the hydrophobic end of the detergent molecules, i.e., Tritons® of nonylphenoxyethanol series[116] (Triton® N-101), are also effective in removing contaminating membranes. Usually concentration of Triton® X-100 (most often 0.25 to 1%) depends on the fragility of nuclei and must be individually determined for the particular tissue.[16] Nuclei treated with Triton® X-100 appear to have lost cytoplasmic and outer nuclear membranes as determined by electron microscopy, without fragmentations of the nuclei which has been observed by Blobel and Potter[117] in the case of the Penman's (DOC and Tween® 40) procedure.[91]

Both the amount and distribution of phospholipids, which were taken to indicate the presence of membrane (microsomal and nuclear) fragments by Tata et al.,[118] were extremely reduced in chromatin prepared from nuclei pretreated with Triton® X-100, as compared with chromatin preparations isolated from whole tissue[119] or from nuclei purified without the Triton® X-100 wash.[120]

Following reports indicating that Triton® X-100 washing of rat liver[121] or chicken erythrocyte nuclei[105] does not remove the outer nuclear membrane completely, Jackson[122] has shown that nonionic detergents (0.1 and 1% Triton® X-100, 0.1% Nonidet® P-40) are capable of extracting a majority of the membrane phospholipids but fail to remove membrane proteins to the same degree as with the Waring® blender procedure.[123] These data[122] suggest that the bulk of contaminating membranes in chromatin prepared from whole tissue is microsomal[118] while in chromatin prepared from the isolated nuclei, even pretreated with detergents, nuclear membrane is probably the major contaminant. Thus, the fraction of membrane proteins solubilized or contaminating the nonhistone protein preparations undoubtedly varies, depending upon the source of the membranes and the exact conditions of the extraction procedure.[124] It should be emphasized, however, that in the experiments of Jackson[122] the nuclear preparations pretreated with Triton® X-100 were not further purified by centrifugation through dense sucrose solution,[6,55] which is extremely helpful in removing the residual cytoplasmic and outer nuclear membranes.

The inclusion in the extraction buffers of divalent cations, required for the maintenance of morphologically intact nuclei, has been reported to be especially detrimental to complete membrane solubilization.[125] This may explain why the detergent extraction procedures, commonly employed in the preparation of detergent nuclei, remove so little membrane protein.

While considering the efficiency of Triton® X-100 in the removal of cytoplasmic contaminants from isolated cell nuclei, the experiments of Weiss and Braun[126] should be mentioned. According to these authors, the use of 1% Triton® X-100 for purifying nuclei from the cellular slime mold *Dictyostelium discoideum* resulted in a dramatic enrichment of two polypeptides: 44,000 mol wt (actin-like) and 180,000 mol wt (myosin-like), as compared with the preparation of nuclei purified without this detergent. This was presumably caused by artificial adsorption of an actin-like protein to chromatin. The authors suggested that studies demonstrating a large amount of actin and other contractile proteins as chromatin constituents deal with cytoplasmic contaminations since they all employ nonionic detergents for lysing cells and purifying nuclei.

C. Use of Organic Solvents

Many media used to solubilize chromatin, especially dehistonized chromatin, involve some organic solvents, among them strong denaturants such as: SDS, urea, GdnCl, and 2-ME.

1. Sodium Dodecyl Sulfate

Of all the synthetic detergents which may be classified as anionic, cationic, or nonionic, the SDS (principally the salt of alkyl sulfate half esters containing straight 12-hydrocarbon tail and a polar head on the same molecule) has found most application in the studies of NHCP. The dispersal effects of this amphiphile are produced by its two kinds of association with proteins: primarily through the salt-like interactions between the charged groups of protein and detergent, and secondly, by less specific weak forces between the nonpolar side chains of the proteins and hydrophobic moiety of the detergent.[127] The latter stabilize the ionic bonds and are responsible for the secondary nonpolar attraction of the additional detergent to that already electrostatically bound with proteins (Figure 1).

Apparently, SDS was first used by Sreenivasaya and Pirie[128] to disintegrate crystalline plant virus nucleoprotein and later by Mirsky and Pollister[129] to dissociate DNA from the isolated chromosomes of animal tissue. Since the experiments of Marco and Butler[130] and Kay et al.,[131] SDS solution (0.41%)[131] has been used as extracting and deproteinizing agent, improving the preparation of highly purified and polymerized DNA.[132] The high protein-denaturing potency of synthetic detergents, and their ability to keep the denatured protein in solution, was discovered by Anson[133] and in the experiments of Bonner and associates,[80,81,134] SDS has been introduced to isolate and also to fractionate NHCP, i.e., using polyacrylamide gel electrophoresis (PAGE).

Like urea and GdnCl, SDS brings about, in addition to unfolding of the native protein molecule which assumes a more random orientation, the dissociation of oligomer structures. Profound studies by Tanford[137] and Tanford and Reynolds[135,136] on interactions of proteins with amphiphiles have shown that a variety of proteins bind ionically and hydrophobically identical amounts of SDS on a weight-to-weight basis with the eventual formation of a micelle with a high ratio of dodecyl sulfate to protein (1.5 to 2.3 g dodecyl sulfate per gram protein).[135,138] Saturated complex with a stoichiometry of 0.4 g of SDS per gram of protein is formed between 0.5 and 0.8 m*M* SDS monomer, and a second complex, which is saturated at 1.4 g of SDS per gram of protein, is observed above the 0.8 m*M* SDS monomer.[136] The binding of large amounts of SDS to protein is primarily hydrophobic in nature and independent of ionic strength; only the monomeric form of the amphiphile binds to protein, not the micelar form.[135,137] From hydrodynamic studies[124,136] the protein-SDS complex is believed to be a highly ordered, rod-like particle, the length of which varies uniquely with the molecular weight of the protein moiety. This explains the empirical observations that proteins dissolved in aqueous solutions containing high concentrations of SDS lose all their specificity

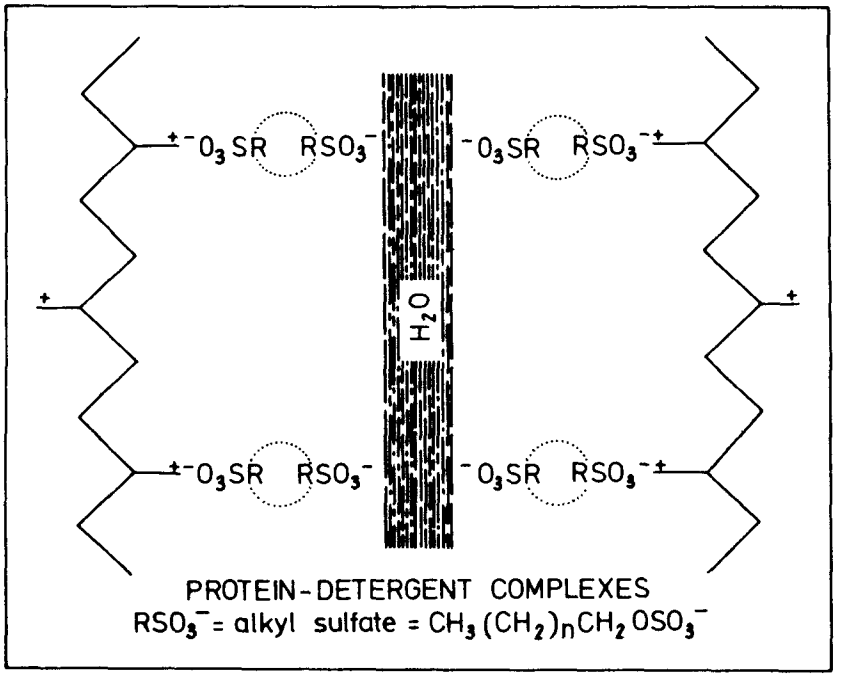

FIGURE 1. Proposed structure of protein-detergent complexes. Dotted lines indicate that extra bound detergent is attached by weak nonpolar forces to detergent stoichiometrically bound by electrostatic forces. This gives rise to distribution of similarly charged groups along the peptide, which repel similarly changed groups of an adjacent chain. (From Putnam, F. W., *Advances in Protein Chemistry*, Vol. 4, Anson, M. L. and Edsall, J. T., Eds., Academic Press, New York, 1948, 79. With permission.)

and have electrophoretic mobilities in polyacrylamide gels which are the function of their molecular weight.[139,140]

The principal disadvantages of using SDS are protein denaturation caused by this detergent, which is known to avidly bind to proteins[135-137] and to markedly influence their physical state, and the difficulty of removing SDS from the NHCP without rendering them insoluble.[134,141] The majority of SDS can be removed from chromosomal protein preparations by the urea dialysis-$BaCl_2$ precipitation procedure described by Marushige et al.[134] and by Shirey and Huang.[141]

Knowing that the removal of denaturing agents like urea or GdnCl, which transform proteins into random coil conformation,[142] is generally accompanied by recovery of the native structure,[143] Weber and Kuter[144] have described a procedure for successful reactivation of several oligomeric enzymes after denaturation by exposure to SDS. To avoid disulfide formation, all solutions were made in 2-ME (10 m*M*). Proteins in SDS solutions were first incubated in concentrated urea (6 *M*) and then freed of the detergent by an anion exchange resin (Dowex® AG 1-X2, 200 to 400 mesh). The resulting dodecyl sulfate-free proteins could be renatured from the urea solution by standard procedures. This method has also been used for successful recovery by enzymatic[144] and immunological activity[43,48] of proteins after their electrophoresis on polyacrylamide gels in the presence of SDS.

2. *Urea*

For over two decades, multimolar concentrations of urea[145,146] have been used to solubilize

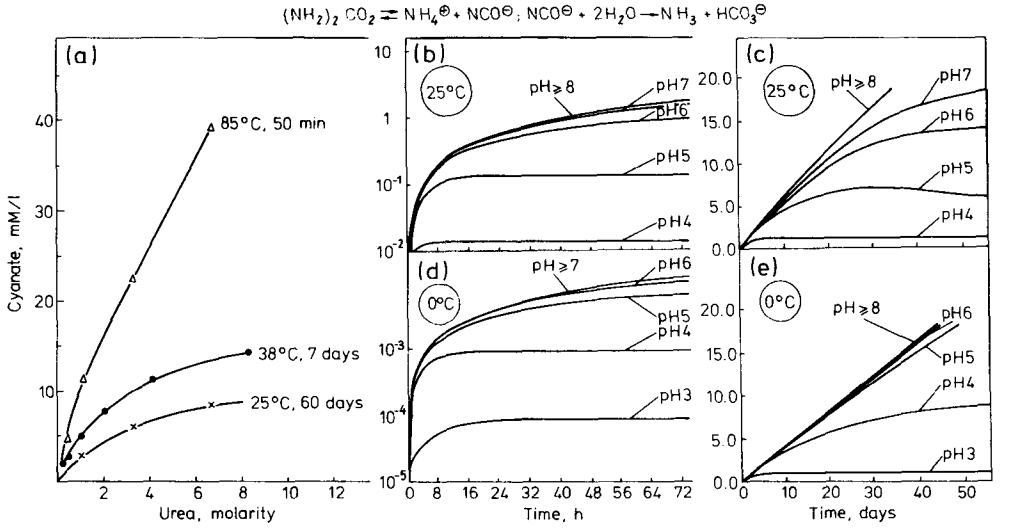

FIGURE 2. Effect of temperature and urea concentration on cyanate accumulation during equilibration of aqueous urea solutions. (a) Accumulation of cyanate ions in an aqueous solution of 8 *M* urea at indicated pH (b-e) during the first 3 days (b,d) and over a period of 2 months (c,e) at 25°C (b,c) and at 0°C (d,e). (Figure (a) from Marier, J. R. and Rose, D., *Anal. Biochem.*, 7, 304, 1964; Figures (b-e) from Hagel, P., Gerding, J. J. T., Fleggen, W., and Bloemenal, H., *Biochem. Biophys. Acta*, 243, 366, 1971. With permission.)

and dissociate chromosomal proteins from DNA as well as to isolate and fractionate NHCP on a preparative and analytical scale (for review see Reference 5). Unfortunately, the use of this reagent is not free from drawbacks. High concentrations of urea (5 to 9 *M*) destroy noncovalent and nonionic interactions in proteins and may cause complete protein denaturation. However, experiments on reconstitution of chromatin (for review see Reference 147), and on dissociation and reassociation of chromosomal proteins from DNA indicate that the effect of urea is not irreversible and under suitable conditions many of the proteins return to their native conformation.[148]

Urea is often assumed to be a reagent which brings about physical rather than chemical changes in protein molecules. This assumption is valid only if the urea is completely free of cyanate.[149] It is known[149-154] that the dissociation of proteins into subunits by the action of urea may be accompanied by the formation of ammonia, cyanate, and carbonate ions (Figure 2). The amount of cyanate formed in aqueous solutions of urea depends on urea concentration, duration of storage, and temperature and pH.[149,151,155] In the range from 1 to 9 *M* urea (the most widely used in protein chemistry) there exists a linear relationship between the cyanate and urea concentrations, especially evident at higher temperatures (Figure 2a). At a given urea concentration, the maximum levels of cyanate attainable by direct equilibration at 85, 38, and 25°C can be expressed by the proportions 4.3: 1.7: 1.0, respectively.[151] In other words, whereas only 50 min are required to attain a maximum cyanate level at 85°C, approximately 7 days are required at 38°C and 60 days are needed at 25°C.

Figure 2 (b to e) summarizes graphic representations of the accumulation of cyanate in 8 *M* urea solutions of different pH (3 to 8) at two temperatures (0°C [d,e] and 25°C [b,c]) over the period of 2 months (c,e) and in the range from 0 to 72 hr (b,d). Hence, it is clear that the accumulation of cyanate ions is most pronounced at a pH higher than 4 and increases with the duration of storage, especially at higher temperatures. The formation of the cyanate ions in urea solutions is extremely slow at low temperatures.[155]

It has been shown by comparative electrophoresis in polyacrylamide gels that a long contact of proteins (α-crystalline[155] and very low density lipoproteins[156]) with concentrated urea (e.g., 6 *M*), especially at room temperature and at higher pH (8.9 to 9.4), gives rise to a significant cyanate accumulation and, in consequence, to a high degree of protein carbamylation[149,154-156] at the α- and ϵ-amino as well as hydroxyl and –SH groups. Especially important is carbamylation of the ϵ-amino groups, which converts positively charged lysine to neutral homocitruline,[156] leading to changes in electrophoretic mobility (greater anodal migration) of the affected proteins. This can produce artifactual polymorphism and erroneous conclusions about the number of individual subunits or protein species.[154-156] The carbamylation of tyrosine or cysteine would not change the molecular charge at the given pH; moreover *O*-carbamyltyrosine and *S*-carbamylcysteine are decomposed in a slightly alkaline medium.[149,154]

The potential problems caused by the formation of cyanate and ammonium ions can be minimized or avoided by simple precautions during the preparation of urea buffers, e.g., according to Herbert et al.[156] urea should be prepared in a stock solution (10 *M*) using high-quality crystalline reagent,[76] without heating (at temperatures not exceeding 25°C), and stored at 5°C in stoppered glass bottles.[151] Immediately before the preparation of buffer, the urea solution should be deionized by passing through a column of mixed-bed resin (e.g., AG® 501-X8,[157,158] Rexyn® I-300,[156,159] or Amberlite® MB-3[160]). Fresh buffer should be rapidly cooled, if necessary, and used immediately after preparation. The urea buffers must be used within two days of preparation,[161] even in the case of deionized urea solutions.[157] To compete for the reaction of isocyanate with protein, lysine should be included in all urea buffers.[76] The addition of 2 m*M* TrisCl to buffers for chromatography on hydroxylapatite[162] has also been reported to protect the proteins against urea cyanate degradation.[155,156]

3. Guanidine Hydrochloride and 2-Mercaptoethanol

Interactions between DNA, RNA, histones, and nonhistones in chromatin involve ionic, hydrophobic, and hydrogen bonds. A few covalent disulfide linkages can also exist between chromatin proteins.

Compared with others salts, GdnCl can be considered to be an effective denaturant which brings about complete unfolding of globular proteins.[163] In a series of papers, Tanford et al.[164-167] presented the results of measurements of some hydrodynamic properties which are especially sensitive to conformation, e.g., intrinsic viscosity, sedimentation coefficients, optical rotatory dispersion, osmotic pressure, and titration studies of globular proteins in concentrated GdnCl solutions. Such proteins lose their characteristic native structure and appear to be devoid of any residual noncovalent structures, either in the presence or absence of disulfide bonds. In the presence of a reducing agent, e.g., 2-ME, they behave like randomly coiled simple linear organic polymers.

In addition to urea and GdnCl, some authors introduce moderate concentrations of a reducing agent, 2-ME, to break disulfide bonds and to prevent their formation when they are not present in the native proteins. The rupture of disulfide inter- and intra-chain cross-links facilitates protein unfolding[168] and was found to diminish aggregation between histone and nonhistone proteins, thereby facilitating the subsequent separation of these two protein groups.[169]

II. ISOLATION OF CHROMOSOMAL NONHISTONE PROTEINS

A. General Remarks

Since the discovery of nonhistone proteins in deoxyribonucleoprotein (DNP) complex,[170] numerous attempts have been made to isolate these proteins and to characterize their chemical and biological properties. For a long time, drastic procedures such as strong acid, alkaline

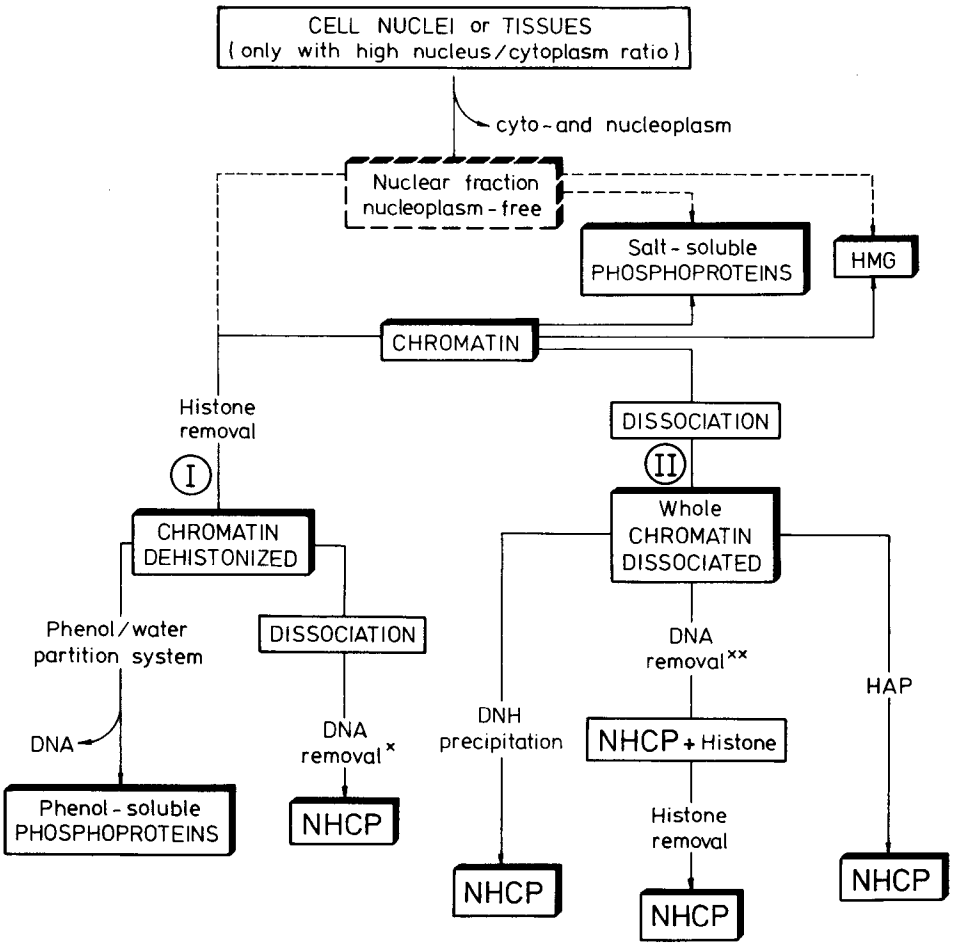

FIGURE 3. Scheme for principal procedures of NHCP isolation.[457]

or thioglycolate treatment,[171-173] extraction by ionic detergent,[80] urea,[174] GdnCl,[163] or phenol[175] have been used for NHCP isolation or solubilization. To study the biological phenomena involving NHCP it is essential to avoid denaturing conditions. Unfortunately, milder extraction procedures[176-178] frequently lead to only partial recovery of these proteins.

The isolation of NHCP generally follows one of two basic schemes. For isolation from dissociated dehistonized or whole chromatin the starting point is most frequently purified and nonsheared chromatin (for review of isolation methods see Reference 7) obtained from purified cytoplasm-free nuclei (for review of isolation methods see Reference 6) or even from total cell homogenate. This second procedure, described by Bonner et al.,[179] and based on the method of Zubay and Doty[180] may be applied only to tissues with a high ratio of nucleus to cytoplasm, such as thymus.[16] There are some NHCP groups which may be isolated both from chromatin and cell nuclei, e.g., nuclear phosphoproteins (for review see Reference 181 and Chapters 3 and 4) or HMG (High Mobility Group) proteins (for review see Reference 182 and Chapter 2). As can be seen in Figure 3, there are at least two principal approaches to the isolation of nonhistone proteins.