INVESTIGATIONS OF NONHISTONE CHROMOSOMAL PROTEINS

Thesis by

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To

Robert,

the most tolerant person I know.

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Abstract

The major nonhistone chromosomal proteins (NHC proteins) are a group of 14-20 acidic proteins associated with DNA in eukaryotic chromatin. In comparisons by SDS gel electrophoresis (molecular weight sieving) one observes a high degree of homology among the NHC protein fractions of different tissues from a given species. Tissue-specific protein bands are also observed. The appearance of a new NHC protein, A, in the NHC proteins of rat liver stimulated to divide by partial hepatectomy and of rat ascites cells suggests that this protein may play a role in preparing the cell for division. The NHC proteins of the same tissue from different species are also very similar. Quantitative but not qualitative changes in the NHC proteins of rat uterus are observed on stimulation (*in vivo*) with estrogen. These observations suggest that the major NHC proteins play a general role in chromatin structure and the regulation of genome expression; several may be enzymes of nucleic acid and histone metabolism and/or structural proteins analogous to histones. One such enzyme, a protease which readily and preferentially degrades histones, can be extracted from chromatin with 0.7 N NaCl.

Although the NHC proteins readily aggregate, they can be separated from histone and fractionated by ion exchange chromatography on Sephadex SE C-25 resin in 10 M urea-25% formic acid (pH 2.5). Following further purification, four fractions of NHC protein are obtained; two of these are single purified proteins, and the other two contain 4-6 and 4-7 different proteins. These 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. These isolated fractions appear more soluble and easier to work with than any whole NHC protein preparation.

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CHAPTER 1

INTRODUCTION TO NONHISTONE CHROMOSOMAL PROTEINS

The Reason Why

The chromatin of the eukaryotic cell is a complex of DNA, RNA histone, and nonhistone chromosomal protein (NHC protein). Chromatin can be isolated for *in vitro* study by chemically gentle techniques based on differential centrifugation (Bonner *et al.*, 1968a). That such isolated chromatin is a good model for chromatin as it exists *in vivo* has been established by experiments which show that its properties as a template for DNA-dependent RNA polymerase are not altered by isolation. To the extent that can be determined by present analytical techniques, the same regions of the genome are transcribed *in vitro* as *in vivo* (Marushige and Bonner, 1966; Paul and Gilmour, 1966, 1968; Bekhor *et al.*, 1969; Smith *et al.*,1969; Tan and Miyagi, 1970). The role of the various components of chromatin in directing the observed limited, tissue-specific transcription of the genome is a problem of central interest in biology today. The structure and function of the histones have been studied extensively. The relevant literature on this subject has been reviewed in Stellwagen and Cole (1969), Hearst and Botchan (1970), and Elgin *et al.*, (1971). The conclusion emerging from these studies is that the histones are structural proteins of chromatin as well as nonspecific repressors of template activity. There are only a small number of different histones, eight to twelve. They act in concert, no one species playing a key role in template repression, etc., although histone I has particular effects on chromatin solubility (Smart and Bonner, 1971). Experiments in which chromatin is dissociated and then reassociated by salt-urea dialysis indicate

that the tissue specificity of transcription, specificity in binding hormone receptor complexes, etc., is not a property of the histones but of the NHC protein component (which in these experiments also contains chromosomal RNA) (Gilmour and Paul, 1969, 1970; Bekhor *et al.*, 1969; Spelsberg *et al.*, 1971a,b).

In the past there has been very little work done on the NHC proteins, chiefly because of technical difficulties in their isolation and fractionation; they tend to aggregate with nucleic acid, histones, and one another. Because of the above results, however, it is apparent that we must learn more of the chemistry and biology of NHC proteins if we are to understand the mechanisms and specificity of controlled gene transcription on eukaryotes. For these reasons this thesis research was undertaken.

Possible Roles of the NHC Proteins

The nonhistone chromosomal proteins (NHC proteins) most probably include polymerases, nucleases and other enzymes involved in the metabolism of chromatin; specific repressor or activator molecules, both those analogous to RNA polymerase sigma factor and those analogous to the lac repressor (if such exist in eukaryotic cells); structural proteins analogous to histones; and some nuclear membrane components. That this protein fraction functions in part in a regulatory fashion has been inferred from findings that the quantity of NHC protein is related to the type and physiological state of the starting tissue (Dingman and Sporn, 1964; Bonner *et al.*, 1968b), from its relative abundance in euchromatin (Frenster, 1965) and

metaphase chromosomes (Sadgopal and Bonner, 1970), and from its high metabolic activity (Holoubek and Crocker, 1968). Recently, evidence has been presented for a sigma-like, species-specific enhancement of template activity by a nuclear acidic protein fraction (Teng et al., 1971). A regulatory protein factor in transcription of ribosomal genes in a eukaryotic system has also been reported (Crippa, 1970).

It seems possible that some NHC proteins will be found to be components of the nuclear membrane. A number of studies in prokaryotes, both electron microscope observations and biochemical experiments, have suggested that the bacterial chromosome is associated with the cell membrane; the DNA replicase is thought to be attached to the DNA, and may also be part of the membrane complex, although recent experiments with pol A^- cells suggest not (Stratling and Knippers, 1971). In several cases, it appears that viral DNA's must be associated with the host cell membrane for normal replication to occur (Knippers and Sinsheimer, 1968; Hallick et al., 1969). Similarly, there is considerable evidence from electron microscope observations and biochemical studies that the DNA of eukaryotes is firmly associated with the inner nuclear membrane, possible at the annuli (Comings and Okada, 1970a, b, c; Ormerod and Lehmann, 1971; Zentgraf et al., 1971), and that DNA synthesis occurs at the membrane (Comings and Kakefuda, 1968; Mizung et al., 1971; Yoshida et al., 1971). It is also known that isolated chromatin contains lipid, indicative of a membrane component (Jackson et al., 1968). Thus, although it may prove difficult to distinguish

contamination from biologically significant association, one must seriously consider the idea that some of the NHC proteins observed will prove to be inner nuclear membrane proteins.

There is some evidence concerning a structural role of NHC proteins. It seems likely that the protein elements of the synaptonemal complex, etc., will prove to be NHC proteins (Comings and Okada, 1971). Proteins analogous to the gene 32 protein of T4, necessary for the stabilization of single stranded DNA during replication and recombination, are now being isolated from eukaryotes. Since such proteins are required in stoicheometric rather than catalytic amounts, they might be observed by the techniques used in Chapter 2 (Alberts and Frey, 1970; Alberts et al., 1971). A residual NHC protein structure has also been observed in extracted preparations of metaphase chromosomes (Maio and Schildkraut, 1967).

In addition to problems of isolation, a further difficulty in the study of NHC proteins is in defining what is a chromosomal protein. Generally proteins prepared from nuclei, or preferably chromatin, which can be shown to coprecipitate with histones and DNA from low concentrations of NaCl are considered chromosomal. Johns and Forrester (1969) have shown that in 0.14 M NaCl, calf thymus deoxyribonucleoprotein adsorbs acidic protein from cytoplasm or nuclear sap; approximately two-thirds of such protein is apparently removed by 0.35 M NaCl. However, if chromatin prepared according to Bonner et al., (1968a) is extracted with 0.3 N NaCl, only 10% of the protein, principally nonhistone, is removed (Smart, 1970). The fraction of NHC protein removed is enriched in the higher molecular

weight polypeptides, but otherwise resembles the total standard preparations (Elgin, unpublished observation). Presumably all such NHC proteins are in dynamic equilibrium with the chromatin complex in the nucleus, so the question of the degree of association with DNA *in vivo* is somewhat academic as long as cytoplasmic contamination is ruled out.

Preparations

Several very different procedures have been used to isolate NHC proteins. Unfortunately, most involve conditions of extreme pH or high concentrations of denaturing reagents, made necessary by the tendency of NHC proteins to aggregate.

Benjamin and Gellhorn (1968) have described a preparation of nuclear acidic proteins from rat and mouse liver. Purified nuclei are extracted with 0.15 M NaCl - 0.01 M EDTA-Na_2 and with 0.1 M HCl to remove ribonucleoprotein and histones, respectively. The acidic nonhistone protein and DNA are then extracted with 4 M CsCl at pH 11.6 (lysine buffer) and separated by equilibrium density centrifugation. The recovered protein is dialyzed and concentrated in 0.01 M lysine-4M deionized urea-0.002 M EDTA-Na_2-0.005 M β-mercaptoethanol (pH 11.6). The resulting solution of acidic proteins can be examined for chemical characteristics and heterogeneity by gel electrophoresis. Some of the proteins appear to be phosphoproteins. At pH 11 the protein sediments as a single band of sedimentation coefficient 2.7S. Unfortunately, the high pH necessary to extract this protein fraction may alter it.

Holoubek and Crocker (1968) have prepared an acidic protein fraction from Ehrlich ascites cell nuclei and have studied the synthesis of this fraction relative to RNA synthesis. Nuclei are extracted to remove acid-soluble proteins and RNA, after which a 0.5 M hot perchloric acid extract removes the DNA together with closely associated acidic proteins. Some protein remains associated with this DNA on CsCl density gradient centrifugation. Labeling experiments with radioisotopes suggest that the acidic protein fraction represents a mixture of proteins labeled independently. This observation has been confirmed with another NHC protein preparation (see Chapter 4). The proteins are synthesized in the conventional fashion and appear to have a higher specific activity in situations wherein DNA-like RNA is being made in the cell.

Wang (1967) has done a considerable amount of work on the isolation and fractionation of "chromosomal" acidic proteins. Rat liver nuclei are isolated, washed with Mg-Tris buffer to remove soluble proteins, and the chromatin then solubilized with 1 M NaCl. The salt solution extract is then dialyzed to 0.14 M NaCl, precipitating the DNA together with such proteins as are again bound to it. The proteins left in the supernatant are considered to be chromatin acidic proteins. These proteins can be further fractionated by ammonium sulfate precipitation and by acid precipitation at pH 5.7 and pH 4.8. The protein fractions are very heterogeneous as indicated by starch gel electrophoresis; the ammonium sulfate fraction includes a protein with isoelectric point greater than pH 8.5. Acidic proteins prepared by this procedure sediment with DNA

in solutions of low salt concentration, and coprecipitate with histone. Each fraction can be further purified by DEAE cellulose chromatography at pH 8.2. In all cases there is a significant run-off peak (suggesting histone contamination), a protein peak which elutes at approximately 0.2-0.3 N NaCl, and a peak which elutes at 0.5-0.6 M NaCl (Wang and Johns, 1968). If chromatin (purified according to the method of Bonner et al., (1968a)) is precipitated from 0.15 N NaCl and centrifuged at ca. 12,000 g, the proteins of the supernatant include the whole population of nonhistone chromosomal protein and histone in small amounts; this is because centrifugation under these conditions does not pellet all of the chromatin. Wang's preparation no doubt includes these as well as other nuclear proteins. A phosphoprotein fraction can be prepared from "chromatin acidic proteins" by fractionation with calcium-phosphate gel (Langan, 1967; Kleinsmith and Allfrey, 1969). Unfortunately, these proteins rapidly aggregate and are difficult to handle (Gershey and Kleinsmith, 1969a).

A different means of preparing nonhistone chromosomal protein has been developed by Marushige _et al._ (1968). Chromatin isolated according to Bonner _et al._ (1968a) is acid extracted to remove histones and the residue solubilized in 1% sodium dodecyl sulfate (SDS)- 0.05 M Tris, pH 8. Following dialysis against 0.1% SDS-0.01 M Tris, the DNA is removed from the solution by centrifugation at 106,000 x g for 18 hours. The proteins in the supernatant are recovered by ammonium sulfate precipitation or used directly for chemical analyses and gel electrophoresis.

In the presence of 0.1% SDS proteins prepared by this procedure have an S value of 2.7 and an average molecular weight of 14,300. In the absence of detergent the protein aggregates to higher molecular weight forms; doubtless the standard protein preparation still has detergent associated with it. The NHC proteins generated by this isolation procedure can be examined directly by SDS gel electrophoresis (Shapiro et al., 1967). Thus the isolation procedure is a very useful one for determining the heterogeneity of the nonhistone chromosomal proteins and for comparing the NHC protein populations of various tissues. Although the acid extraction of chromatin may appear to be a somewhat drastic treatment, SDS gel electrophoresis of native chromatin in 0.1% SDS indicates the presence of the same population of NHC proteins as prepared by this method (see Chapter 2). Shirey and Huang (1969) have used SDS to prepare a total chromosomal protein fraction; however, solubility problems are again encountered as the detergent is removed.

A method of fractionating chromosomal proteins by selective coprecipitation with polyethylene sulfonate (PES) has been developed (Krauze et al., 1969). Chromatin is dissociated in 5 M urea-2 N NaCl and the DNA removed by ultracentrifugation. If a 20-fold dilution is made in the presence of PES, pH 7, a PES-histone complex precipitates. The nonhistone proteins are precipitated from the supernatant with PES at pH 4. This fraction appears similar to Wang's on disc gel electrophoresis (MacGillivray et al., 1969). Unfortunately the fractionation is incomplete and degradation and aggregation problems are apparent (Cameron et al., 1969).

It is also possible to fractionate chromosomal proteins by dissociating chromatin in 5 M urea-2 M NaCl, removing nucleic acid by centrifugation, and separating histones from NHC proteins by ion exchange chromatography, gel electrophoresis, etc. Unfortunately, the methods described to date are unsatisfactory; the yield of NHC protein is low, cross contamination with histones occurs, the proteins aggregate, etc. (Cameron et al., 1969; Elgin, unpublished observation). Shaw and Huang (1970) have described the dissociation of chromatin in 7 M urea-3 M NaCl; they also achieve a fairly good separation of chromosomal proteins and DNA by shearing chromatin in 3 M NaCl and fractionating it on a Bio-Gel A-50 column. To separate the NHC proteins from histones, these authors utilize either the method of Marushige et al. (1968) discussed above or disc gel electrophoresis at pH 2.7 (Panyim and Chalkley, 1969).

Separate fractions of histone and NHC protein can be obtained by eluting 2 M NaCl-5 M urea solubilized chromatin from hydroxyapatite with increasing phosphate. The NHC protein recovery is only ca. 50%, but the fraction appears representative of total NHC protein by SDS gel electrophoresis. One is forced to conclude, however, that NHC proteins prepared by this method tend to aggregate, since the next fractionation procedure employed by the authors in studying the NHC proteins is gel filtration in 0.1% SDS (MacGillivray et al. , 1971).

Allfrey's group has employed a phenol extraction procedure to obtain a nuclear acidic protein fraction that probably includes the NHC proteins as a subset. Purified nuclei are extracted with 0.14 M NaCl, 0.25 N HCl, chloroform-methanol, and ether to remove 0.14 M salt

soluble proteins, histones, and lipids, respectively. The nuclear acidic proteins are then extracted with an equal volume of phenol. These proteins can be dialyzed into 0.1 M Tris-HCl (pH 8.4) containing 8.6 M urea-0.01 M EDTA-0.14 M 2-mercaptoethanol without aggregation (Shelton and Allfrey, 1970, Teng et al., 1971). Although the authors report that this fraction looks like NHC proteins by SDS gel electrophoresis analysis, only about half the expected protein is recovered and the gels do not resemble those produced by direct analysis of chromatin by others (e.g., Elgin and Bonner, 1970).

Finally, a method for dissociating chromatin in 25% formic acid-8 M urea-0.2 M NaCl (pH 2.5) and fractionating the chromosomal proteins by ion exchange chromatography has been developed by Elgin and Bonner. Details of this procedure and its advantages and disadvantages are discussed in Chapter 4.

None of the present methods of preparing NHC proteins are ideal, particularly if one wishes to consider questions of biological activity. One must compromise between the necessity of using solvents to prevent aggregation and the desirability of using non-denaturing solvents. In further discussion, only acidic proteins prepared from chromatin will be referred to as NHC proteins; nuclear acidic proteins, such as the preparations of Wang and of Allfrey discussed above, probably include these as a subset.

Heterogeneity

There is remarkable agreement among different laboratories on the question of heterogeneity of the major NHC proteins. In a comparison of the NHC proteins of different tissues of a given

organism, or of homologous tissues of different vertebrates, one generally observes that the banding pattern in SDS gels (molecular weight sieving) is highly conserved. Apparent tissue-specific bands are also observed. This aspect of the study of NHC proteins is discussed in Chapter 2. Subsequent to the publication of Chapter 2 (Elgin and Bonner, 1970), several studies have appeared which confirm the conclusion of limited heterogeneity of the NHC proteins. MacGillivray _et al._ (1971) have compared the NHC proteins of mouse kidney, liver, and spleen, and the NHC proteins of kidney, liver, and brain of several organisms; in all cases they find very similar SDS electrophoresis patterns. Shaw and Huang (1970) observe similar patterns in urea gels, pH 2.7, for the NHC proteins of pig cerebellum and pig pituitary. Considerable overlap but somewhat greater tissue specificity has been described for nuclear acidic phosphoproteins (Platz _et al._, 1970) and for phenol-extracted nuclear acidic proteins (Teng _et al._, 1971; Shelton and Neelin, 1971). The results of these studies are consistent with the view that the nonhistone chromosomal protein fraction includes enzymes of chromatin metabolism and/or structural proteins common to all tissues as well as proteins that are tissue-specific. Further chemical and functional studies of these proteins are required to confirm this idea.

Interaction with Histones and DNA

One of the important reasons for interest in the NHC proteins is the possibility that they might be part of a mechanism which introduces DNA sequence specificity into histone-DNA interactions,

although such specificity could also be dependent on the associated chromosomal RNA (Bekhor *et al.*, 1969; Huang and Huang, 1969). A number of studies have suggested that nonhistone chromosomal proteins may be involved in gene activation. In order to demonstrate biologically significant derepression in an *in vitro* RNA synthesizing system, one must show that the observed effects cannot be explained by a simple coprecipitation of the histone; that the nonhistone protein is associated with the DNA; that it occurs at the normal salt concentrations of the nucleus, approximately 0.2 M (Langendorf *et al.*, 1961, 1966); that the effect is on the chromatin and not on exogenous RNA polymerase; that proteases have not been added; and that the derepression is specific. Such experiments have not been done. It has been observed that nonhistone chromosomal proteins can coprecipitate with histones (Marushige *et al.*, 1968; Wang and Johns, 1968) and so can reduce histone-nucleic acid association (Dastugue *et al.*, 1970). Such coprecipitation is most pronounced at low salt concentrations, such as those frequently used for RNA polymerase assay systems. A nonhistone phosphoprotein fraction has been isolated from rat liver nuclei by Langan (1967). This fraction also forms insoluble complexes with histones in low salt concentration.

Early studies indicated that if histones are selectively removed from chromatin leaving most of the NHC proteins, the resulting material has the template activity of DNA (Marushige and Bonner, 1966). When DNA and SDS-prepared nonhistone chromosomal proteins are mixed in solutions of high salt concentration and associated by dialysis to low salt concentrations, NHC proteins are bound to the

DNA and the reconstituted material is as effective a template for RNA synthesis as is deproteinized DNA (Marushige et al., 1968). Early experiments suggesting that NHC protein has some repressor capability were not interpretable since they involved acid-extraction of chromatin (Paul and Gilmour, 1968). Acid extraction, in addition to removing histones, can damage the remaining structure (probably by depurination of the DNA), resulting in changes in template activity and melting profile (Bannai and Terayama, 1969). However, Seligy and Neelin (1970) have removed histones from chicken erythrocyte chromatin with acid under conditions such that there is no significant damage to the DNA and find that the residual NHC proteins inhibit transcription by 20 to 25 percent (compared to deproteinized DNA). There are also reports that the chromatin of embryos shows restricted template activity not due to the presence of histones (e.g., Johnson and Hnilica,1970). These suggestions of repressor activity by NHC proteins stem from experiments with special systems.

Gilmour and Paul (1969) have shown that a reconstituted template (salt-urea dialysis) consisting of DNA, histone, and NHC protein has the template activity of chromatin, while a reconstituted template consisting of DNA and histone has no template activity. The ability of acidic protein to inhibit the restriction of DNA template activity by histones is not a unique property of the NHC proteins; in the experiments of Gilmour and Paul (1969) bovine serum albumin will do as well or better. However, the NHC protein fraction appears necessary if the reconstitution is to be sequence

specific in terms of RNA sequences produced (hybridization-competition criteria) (Gilmour and Paul, 1970). Similar results have been obtained by Spelsberg et al., (1971a). It has been shown that such NHC protein fractions contain RNA (Cameron et al., 1969). This RNA may be identical with that shown to play a role in specific reconstitution of chromatin (Bekhor et al., 1969; Huang and Huang, 1969). Wang has shown that his acidic chromosomal protein fraction can significantly reduce the amount of inhibition by exogenous histone of an in vitro RNA synthesis system (DNA template). The degree of derepression depends upon the histone used for inhibition. Apparently the order of addition of components has no effect, but the other problems have not yet been investigated (Wang, 1968a). The preparation also reverses histone repression of DNA synthesis, apparently by competitive binding of the histone (Wang, 1969). Teng and Hamilton (1969) have demonstrated that the addition of non-histone protein to RNA synthesizing mixtures reverses inhibition by added histone (calf endometrium chromatin template). In this system the order of addition of protein components has a definite effect, but some mitigation of histone inhibition is achieved even if the histone is added prior to the NHC protein. Acidic nuclear proteins from other tissues are also effective. Complexes of nuclear phosphoprotein and histone cause some inhibition of DNA-dependent RNA synthesis in vitro, yet are not as effective as histones alone (Langan and Smith, 1966; Langan, 1967). Spelsberg and Hnilica (1969) have reported that acidic nuclear proteins, including the phosphoprotein preparation of Langan (1967), can prevent histone inhibition

of DNA-dependent RNA synthesis in vitro only if they interact directly prior to the addition of DNA template. They obtain similar results with a nuclear acidic protein fraction prepared according to Wang (1967). The observations are consistent with the ideas that NHC proteins do not in themselves repress template activity and that they do interact with histones and/or DNA to mitigate the repression by histones. To date neither a specific derepression or repression by NHC proteins per se has been demonstrated.

Enzyme Activities

A number of enzyme activities have been found in preparations of NHC proteins or acidic nuclear proteins. Several of these appear to be involved in the alteration or metabolism of histones. Among the best established is a neutral protease that preferentially attacks other histones; this enzyme is discussed in detail in Chapter 5. A protein phosphokinase in a phosphoprotein preparation from acidic nuclear proteins (analogous to Wang, 1967) has been demonstrated (Langan, 1968a; Kleinsmith and Allfrey, 1969). Unfortunately, it appears that histones tend to be good substrates for kinases (possibly because of their relatively open conformation), and that histone kinase activity is widely distributed in the cell. However, it has been shown that the histone kinase(s) phosphorylate histones at specific sites, and do not phosphorylate NHC proteins (Langan and Smith, 1967; Langan, 1968b). The extent of phosphorylation of histones changes in vivo in response to hormones (Langan 1968, 1969); histones already associated with DNA are phosphorylated

during spermatogenesis (Marushige, et al., 1969). Thus histone kinase must be a nuclear if not chromosomal enzyme. Kinases which phosphorylate nuclear phosphoproteins are also found in the nucleus and/or associated with chromatin (Gershey and Kleinsmith, 1969 b; Takeda et al., 1971). The work of Comb et al., (1966), Park and Kim (1970) and Burdon and Garven (1971) suggests that a methylase producing ε -N-methyl-lysine in histones is a chromosomal protein (see also Sekeris et al., 1967). Gallwitz (1968, 1970, 1971a) has isolated several histone-specific acetylases which are chromosomal enzymes, i.e., are tightly bound to chromatin in the cell. The distribution of these enzymes is organ specific (Gallwitz, 1971b). For a more complete discussion of histone metabolism and the possible significance of modifications such as phosphorylation, methylation, and acetylation, see Elgin et al., 1971.

A number of enzymes concerned with nucleic acid metabolism are associated with chromatin. The presence of aggregate RNA polymerase (EC 2.7.7.6) in chromatin is well established (Weiss, 1960; Huang et al., 1960). In addition, soluble RNA polymerase not associated with the chromatin has been prepared by several laboratories (Liao et al., 1968; Seifart and Sekeris, 1969). It has been suggested that only half of the total RNA polymerase is firmly associated with nuclear chromatin and/or participating in active RNA synthesis (Liao et al., 1968). It now appears that there are at least two, possibly more, different DNA-dependent RNA polymerase activities in the eukaryotic nucleus (Roeder and Rutter, 1970; Kedinger et al., 1970; Chesterton and Butterworth, 1971). DNA polymerase (EC 2.7.7.7) has

been purified from a Wang preparation of acidic chromosomal proteins of rat liver and calf thymus. The chromatin acidic proteins show a higher specific activity than do other fractions such as the nuclear sap (Patel, et al., 1967). The enzyme is active with templates from a variety of sources, but the greatest activity is shown with homologous rat liver nuclear DNA (Howk and Wang, 1969a). At low enzyme/DNA ratios the preferred template is native DNA; however, at higher enzyme/DNA ratios, the preferred template is heat denatured DNA. The alteration does not seem to be the consequence of endonuclease activity (Howk and Wang, 1970b). The preparation may contain two polymerases, one with a preference for each type of template (Howk and Wang, 1969b). The reaction product consists of small, heterogeneous DNA fragments (Howk and Wang, 1970a). Unfortunately, there is no evidence that these enzymes are chromosomal proteins in the strict definition, although Loeb (1970) and others have also reported DNA polymerase activity associated with chromatin. These preparations may also include some terminal transferase activity; such enzyme activity has been reported in a similar preparation from calf thymus (Wang, 1968b). Cytidine triphosphate polymerase activity has also been reported to be associated with isolated chromatin (Duda and Cherry, 1971). A neutral deoxyribonuclease (possibly EC 3.1.4.5) has been observed in the isolated nuclei of mammalian cells and remains associated with chromatin prepared by the method of Paul and Gilmour (1966), i.e., precipitated from 0.2 M sodium phosphate buffer. However, preparations of nucleohistone (soluble) by the method of Zubay and Doty (1959) were

free of the enzyme. The enzyme is of the deoxyribonuclease-1 type, the products being 3'-hydroxyl terminated fragments of DNA (Swingel *et al.*, 1967). O'Connor (1969) has isolated a deoxyribonuclease from rat liver acidic chromosomal proteins (Wang method) which functions predominantly as an endonuclease and may be identical with that reported above. A comparison of the template preference of DNA polymerase and deoxyribonuclease isolated from the acidic protein preparation and from the nuclear sap shows that enzymes with difference preferences for template are present in different amounts in the two fractions. Consequently, O'Connor (1969) has suggested that some of the enzymes under discussion may be chromosomal enzymes in the sense of being largely bound to chromatin *in vivo*, while others are not. There have also been numerous reports of NAD nucleosidase (3.2.2.5) activity associated with chromatin (e.g., Bock *et al.*, 1968).

Present research confirms the idea that enzymes concerned with nucleic acid metabolism are isolated with chromatin. These most likely function in repair and replication of the DNA and synthesis of RNA. It appears that the neutral protease may play a major role in changes of chromosome structure, such as the replacement of histones with protamines in spermatogenesis. Other enzymes modify histone structure (i.e., acetylases), but their role is unknown.

This introductory review of the methods of isolating NHC proteins and their possible biological role is in part taken from Chapter IV, "Nonhistone Chromosomal Proteins," of the review "The Biology and Chemistry of Chromosomal Proteins" by Sarah C.R. Elgin, Stanley C. Froehner, John E. Smart, and James Bonner, in Advances in Cell and Molecular Biology 1,1 (ed. by E.J. DuPraw, Academic Press, New York.) (1971).

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Chapter 2

LIMITED HETEROGENEITY OF THE MAJOR NONHISTONE CHROMOSOMAL PROTEINS

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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).

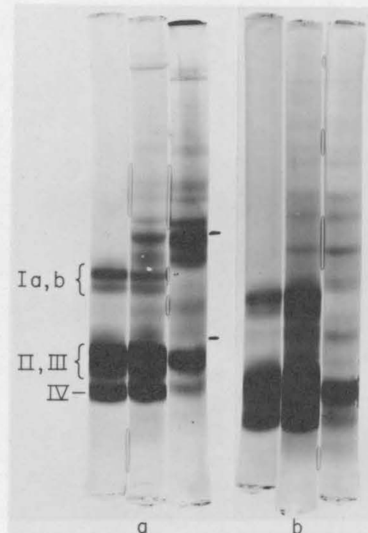

FIGURE 1: SDS gel electrophoresis of samples of histone, chromatin, and NHC protein (left to right). Samples prepared as given in Methods. Sample size, 10–50 μg. 5% acrylamide gels, pH 7.1, run top to bottom for 75 min at 48 V. Molecular weights in 1a indicate position of γ-globulin marker (heavy and 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 2). 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: cpm in Fraction	Radioactivity Estimate of Protein: % of Starting Material	Lowry Estimate of Protein: Color Units	Lowry Estimate of Protein: % 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-

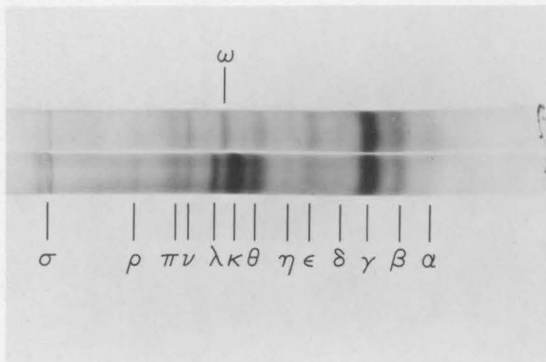

FIGURE 2: Top: rat kidney NHC protein. Bottom: rat liver NHC protein. SDS gels run left to right.

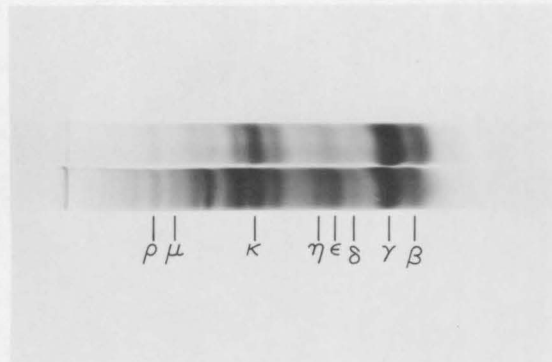

FIGURE 3: Top: rat liver NHC protein. Bottom: chicken liver NHC protein. SDS gels run left to right.

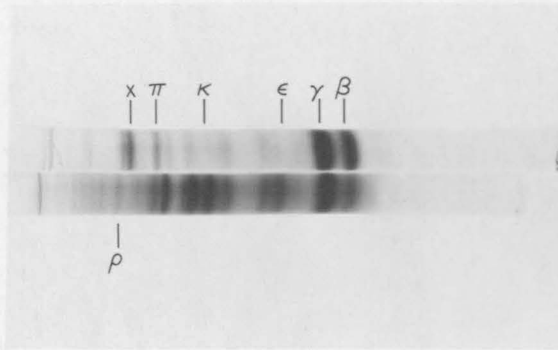

FIGURE 4: Top: chicken erythrocyte NHC protein. Bottom: chicken liver NHC protein. SDS gels run left to right.

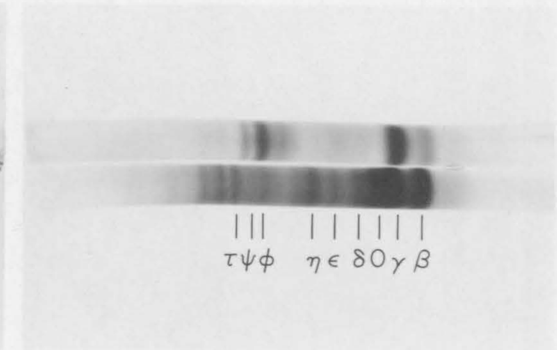

FIGURE 5: Top: rat liver NHC protein. Bottom: pea bud NHC protein. SDS gels run left to right.

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

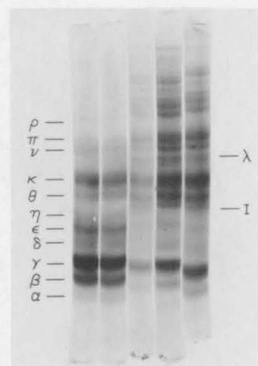

FIGURE 6: Rat liver NHC protein. SDS gels run top to bottom. Left to right: *ca.* 35 μg of NHC protein, regular chromatin preparation; *ca.* 35 μg of NHC protein, regular chromatin preparation incubated 22°, 2 hr; *ca.* 17 μg of NHC protein, nuclear chromatin preparation; *ca.* 35 μg of NHC protein, 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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Chapter 3

A NEW CHROMOSOMAL PROTEIN IN RAPIDLY DIVIDING RAT LIVER CELLS

Introduction

Isolated interphase chromatin is a complex of DNA, RNA, histone and nonhistone chromosomal proteins (NHC proteins). The DNA is, of course, the cell's hereditary matter. The histones appear to be involved in general maintainance of chromatin structure and general repression of gene activity. The role of the nonhistone chromosomal protein has not yet been established. The limited heterogenity observed in comparison of different tissues of the same organism suggest that the major proteins of this fraction may be common structural proteins or common enzymes of chromosomal metabolism (i.e., histone protease, histone acetylase, etc.). As such, one might expect that some of these proteins could play specific roles during chromatin replication. With this idea in mind we have investigated the alteration in the pattern of NHC proteins of rat liver during several states of the tissue. We have looked at normal adult rat liver, the liver of newborn rats, the remaining liver when two-thirds of the adult liver is removed by partial hepatectomy, and Novikoff ascities cells. (Ascites is a free floating tumor cell originally derived from rat liver but at the present time of a general epithelial cell type.) In the latter two cases a new nonhistone chromosomal protein has been observed.

Methods

Tissues

Normal rat liver was obtained frozen in dry ice from Pel-Freeze Biologicals, Rogers, Ark. The livers were those of adult male

Sprague-Dawley rats, approximate body weight 200 grams. Newborn rat liver was dissected from animals within 24 hours of birth, frozen in liquid nitrogen, and stored at -80° until used. Regenerating rat liver was obtained from animals on which a partial hepatectomy had been performed (removal of two of the major lobes of the liver) six hours previous. Animals were killed by decapitation and the livers quickly removed, frozen in liquid nitrogen, and stored at -80°. The six hour timepoint coincides with a wave of chromosomal RNA synthesis in the remaining lobe and precedes the major increase in chromosomal template activity (Mayfield and Bonner, 1971). A wave of DNA replication and cell division occurs in this tissue at 18-20 hours (Butler and Cohn, 1963; Orlova and Rodinov, 1970). The ascites cell line is maintained in rats (adult male, Sprague-Dawley, approximate 200 grams body weight) and harvested and transferred every 6 to 7 days. The ascites fluid taken from the intraperitoneal cavity of the animal is collected and added to one volume of TNKM buffer (0.05 M Tris (pH 6.7) -0.13 M NaCl-0.025 M KCl- 0.0025 M Mg Cl_2),filtered through Miracloth, washed in deionized water to lyse and remove erythrocytes, washed twice with TNKM buffer and then used for preparation of chromatin by the usual techniques. For purposes of comparison two mouse kidney tissues were also examined. Normal adult mouse kidneys were obtained from Pel-Freeze Biologicals. A corresponding cancer tissue, the RAG cell line (a mouse renal adenocarcinoma now in tissue culture) was used (Klebe _et al_., 1970). These cells were grown in flasks containing McCoy's 5A medium (Gibco) with 20% fetal calf serum and 100 ug/ml niamycin (Stubblefield and Klevitz, 1965). The cells

were harvested by washing from the culture bottles in saline-EDTA, pelleted, and used for chromatin preparation.

Preparation of chromatin.

Chromatin was generally prepared by the methods of Bonner *et al.* (1968a). The method as applied to frozen rat liver is also detailed in Elgin and Bonner (1970) (Chapter 2). In the case of ascites cells, purified nuclei were first obtained by breaking the cells with 0.5% Triton X-100. The nuclei were collected by centrifugation at 1500 g 10 min., washed in saline-EDTA, and used for the preparation of chromatin as above. A similar procedure was used for the RAG cells except that the detergent was Nonidet.

Preparation of nonhistone chromosomal protein and examination by gel electrophoresis.

NHC proteins were prepared in all cases by the method of Elgin and Bonner (1970). In brief, the chromatin was extracted with 0.4 N H_2SO_4 at 4° for 30 min. to remove histone; the pellet, collected by centrifugation, was washed once with 0.4 N H_2SO_4 and briefly with 0.01 M Tris (pH 8). The pellet was then solubilized in 1% SDS-0.05 M Tris (pH 8), and the DNA removed by ultracentrifugation at 50,000 RPM for 25 hours at 25° in a Spinco SW-50 rotor. The DNA forms a gelatinous pellet at the bottom of the tube; the NHC protein preparation is the supernatant. This preparation can be dialyzed against buffer III (0.1% SDS-0.1% β-mercaptoethanol-10% glycerol in 0.01 M sodium phosphate buffer, pH 7.1) and analyzed by SDS disc gel electrophoresis (Shapiro *et al.*, 1967). Chromatin and histone preparations were also dialyzed though

the buffer sequence as given in Elgin and Bonner (1970) and analyzed by SDS gel electrophoresis. The gels, stained with Coomassie brilliant blue R 250 (Schwarz/Mann), were photographed using an orange filter and/or scanned on a Gilford 2000 spectrophotometer at 600 mu. All gels directly compared were run at the same time.

General methods.

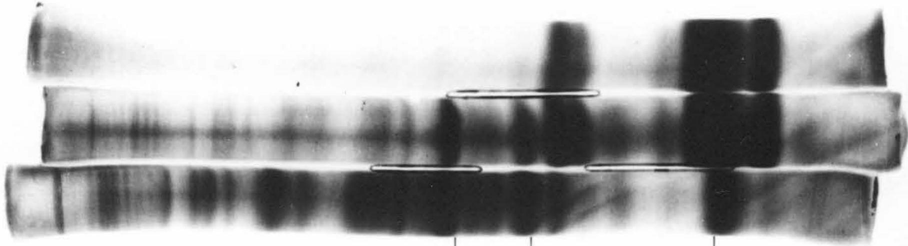
Chromatin samples were analyzed for chemical composition by the methods detailed in Elgin and Bonner (1970).

Results

SDS gel electrophoresis: analysis of the nonhistone chromosomal protein.

SDS gel elctrophoresis analyses of the preparation of ascites histone, ascites NHC protein, and ascites chromatin are shown in Figure 1. As can be seen, the separation of histone and NHC proteins is quite good. The sum of the components of the two gels represents all protein bands seen in the complete chromatin gel. Such an analysis was generated for all tissues examined with the same result. Figure 2 shows a comparison between the NHC proteins of adult rat liver and newborn rat liver. As one can see, there are significant differences in the relative quantities of the various NHC proteins. In particular the bands β, γ are increased in the newborn rat liver NHC proteins. Figure 3 presents the comparison of ascites NHC protein with normal adult rat liver NHC proteins. Several quantitative differences are apparent. In this case the most obvious is the relative decrease of the band κ in the ascites tissue. At this level of resolution it is

Figure 1: Top: rat ascites histone. Middle: rat ascites chromosomal proteins (total). Bottom: rat ascites NHC proteins. SDS gels run left to right.



ρ π ν λ κ θ A η ε δ γ β α

Figure 2: Top: newborn rat liver NHC proteins.
Bottom: adult rat liver NHC proteins. SDS gels run left to right.

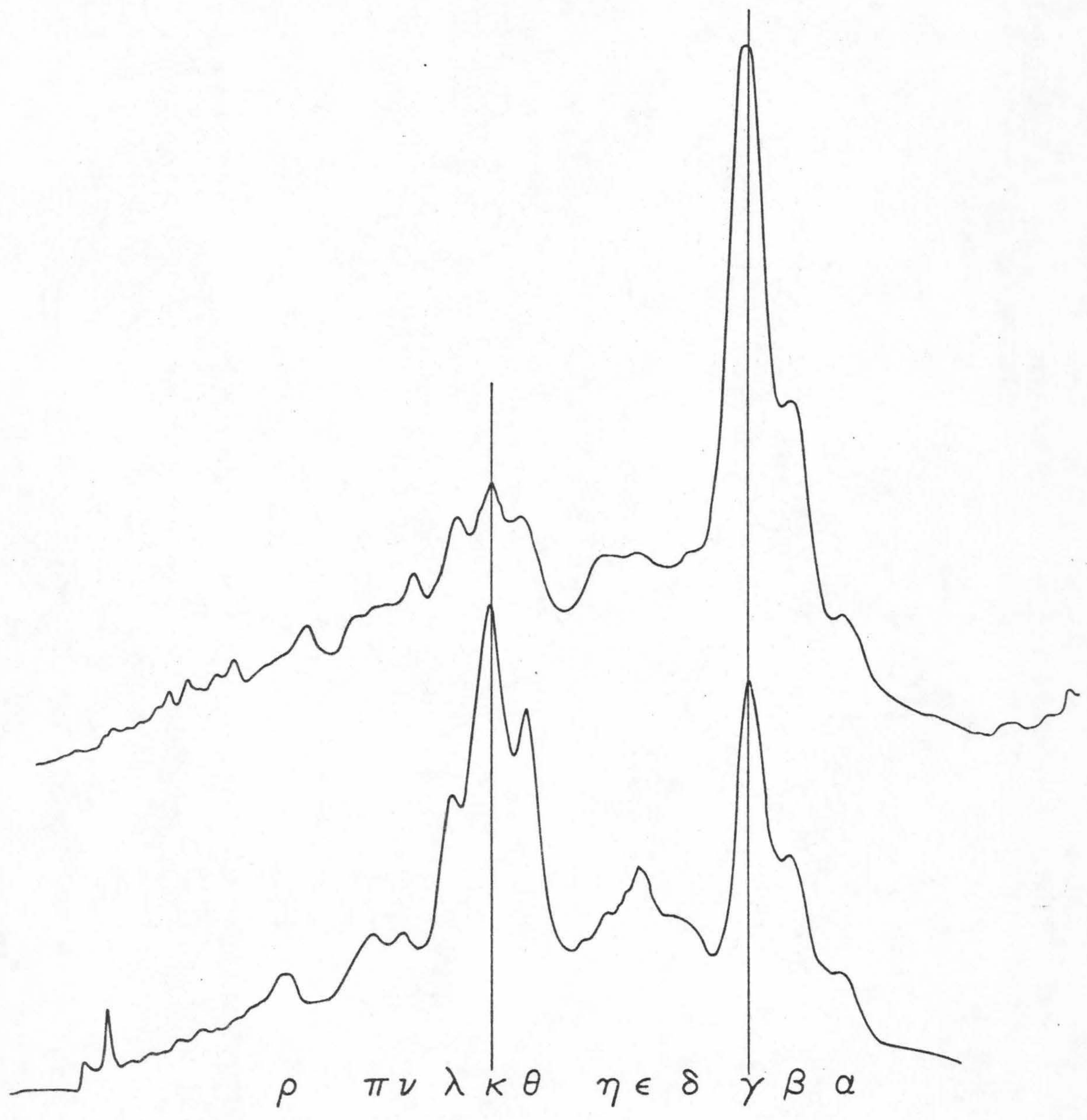
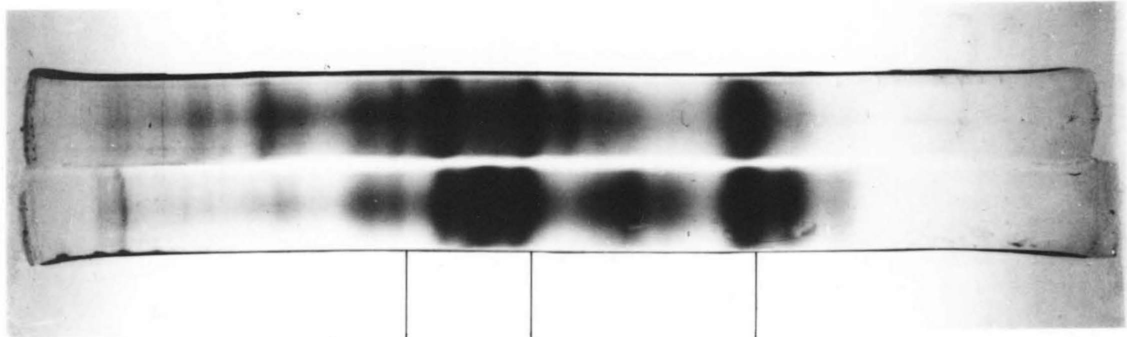

ρ π ν λ κ θ η ε δ γ β α

Figure 3: Top: rat ascites NHC protein. Bottom: normal rat liver NHC protein. SDS gels run left to right.



A

ρ πν λκθ ηεδ γβα

possible to discern that κ now is a doublet in the ascites pattern. Of greater interest is the observation that a new band, labeled A has appeared in the ascites case. This band has an estimated molecular weight of 43,000 daltons. Although it runs close to the position of histone I, there is no reason to think that the acid extraction of the tissue was incomplete. In addition, A elutes with Fraction II (an acidic fraction) on SE chromatography (see Chapter 4). Figure 4 is a comparison of the regenerating rat liver NHC proteins with normal adult NHC proteins. This tissue also contains the new NHC protein A. In addition, several striking quantitative changes are observed, i.e., an an increase in γ, and a decrease in the high molecular weight doublet σ (see Table I).

In order to examine the possibility that A might occur in all cancerous or rapidly dividing tissues and not in normal adult tissues we have made a comparison of the NHC protein of normal adult mouse kidney with that of RAG cells. This comparison is given in Figure 5. A band at the position of A is observed in the RAG cell preparation. A similar band is observed in the normal adult kidney preparation. There are, however, quantitative differences; band A is much more pronounced in the RAG cell preparation, as would be anticipated if a new protein of this molecular weight appeared in the RAG cells in addition to a normal band at this position. It is of interest to note that the NHC protein patterns of the two cancer tissues in Figure 5 are more homologous than those of the two mouse tissues in the high molecular weight region.

Chemical composition of chromatin.

The chromatin samples used in the preparation of NHC proteins were analyzed for composition. The results are given in Table II in the form

Figure 4: Top: normal rat liver NHC protein. Bottom: regenerating rat liver NHC protein. SDS gels run left to right.

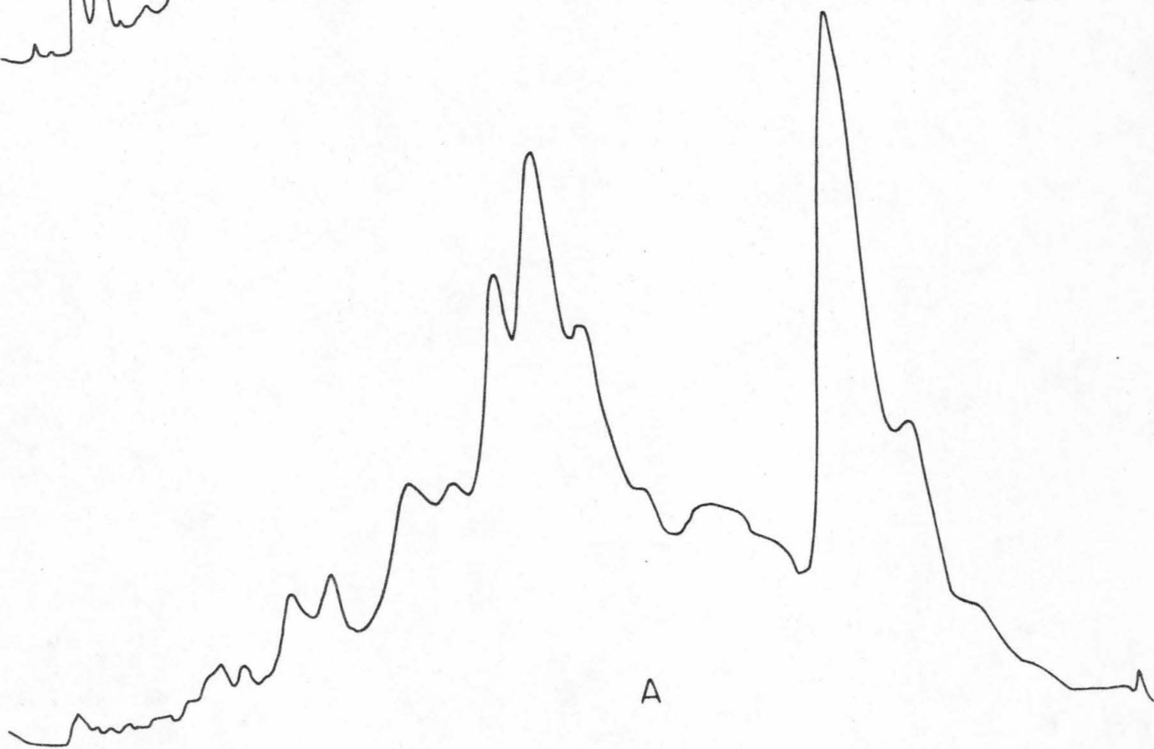
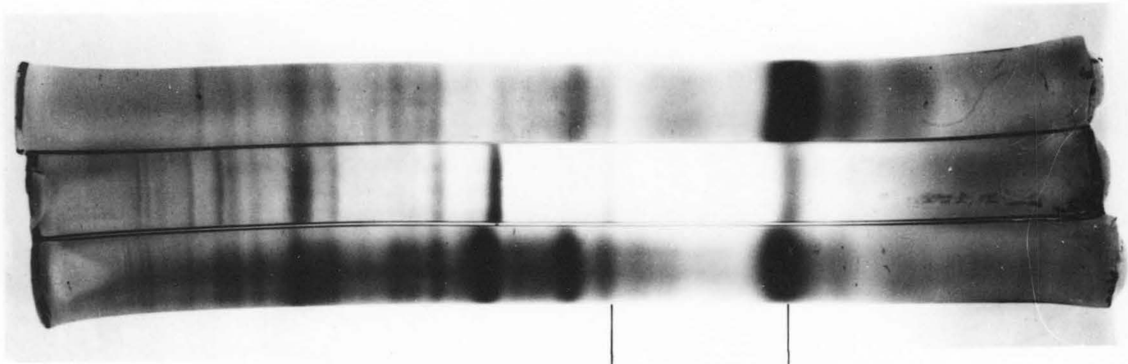

A

Table I. Quantitative Changes in NHC Proteins of Regenerating Rat Liver

Band	Per cent of total NHC protein[1]	
	Normal rat liver	Regenerating rat liver
α	3.6	2.4
β	5.6	6.2
γ	15.4	21.4
δ	4.9	3.6
ε	7.8	5.3
η	3.6	3.8
A	0.7	3.2
θ	9.9	8.5
κ	14.5	14.4
λ	8.2	9.8
ν	4.2	3.9
π	8.3	8.0
ρ	3.5	2.9
σ	2.5	0.5

[1]Estimated from gel scans. See Chapter 5, Part II.

Figure 5: Top: mouse kidney NHC proteins. Middle: mouse RAG cell NHC proteins. Bottom: rat ascites NHC proteins. SDS gels run left to right.



ρ π ν λ κ θ A η ε δ γ β α

Table II. Chemical Composition of Chromatin

Source	No. of Prepns.	DNA	RNA	Histone	NHC Protein
Rat Liver	7	1	0.04	1.15	0.95
Newborn rat liver	3	1	0.05	1.07	0.63
Regenerating rat liver	2	1	0.03	0.97	0.82
Rat Novikoff ascites[1]	3	1	0.13	1.16	1.00
Mouse kidney	2	1	0.02	1.11	0.93

[1] Data from Bonner *et al.*, 1968b.

of mass ratios of RNA, histone and NHC protein to DNA. The ratio of NHC protein to DNA shows much greater variability than the ratio of histone to DNA. There is no apparent correlation between the NHC protein/DNA ratio and the appearance of band A.

Discussion

Of the rat tissues examined, two, Novikoff ascites and regenerating rat liver, have cell cycle times of 18-22 hours with most of the cells dividing; one, new-born rat liver, has a comparable cell cycle time but only ca. 30% of the cells are dividing; and one, adult rat liver, shows essentially no cell division (Post and Hoffman, 1964; Cameron, 1971). The observation that NHC protein A occurs only in the former two tissues suggests that it may play a role in cell division (This hypothesis implies that A is present, but not observed, due to the relatively smaller amount, in the new born rat liver) Significant changes in the relative amounts of the NHC proteins are also observed in the comparison of ascites and normal rat liver. The present observations stand in contrast to those on the histones of normal and cancer tissues. There is considerable evidence to the effect that the histones of cancer cells are the same as those of normal cells in type and primary structure (Hnilica <u>et al</u>., 1963; Laurence <u>et al</u>., 1963; Hnilica, 1966; Desai, <u>et al</u>., 1969; Boulanger <u>et al</u>., 1969; Johns <u>et al</u>., 1970; Sadgopal and Bonner, 1970; Wilhelm and McCarty, 1970), although quantitative differences, particularly in histone I, have been reported (Vescia <u>et al</u>., 1967; Kinkade, 1968). Panyim and Chalkley (1969) have reported that a unique histone I subfraction occurs in

slowly dividing tissues (brain, lung, liver, kidney) and is absent in rapidly dividing tissues (thymus, mouse Ehrlich ascites tumor). We have not yet examined the NHC proteins of naturally rapidly dividing cells for the presence of A.

A recent paper by Sheppard (1971) delineates a system in which it would be possible to test the hypothesis that A is necessary for cell division. It has been found that spontaneously transformed and virally transformed mouse cells are restored to "contact inhibited" growth by the addition of dibutyryl cyclic AMP and theophylline (an inhibitor of the relevant phosphodiesterase) to the culture medium; removal of these agents leads to release from growth control, although the subsequent doubling time of the cells is slightly lengthened (25-30%, to 28 $\pm$ 1 hour). Examination of the NHC proteins of "contact-inhibited" and "released" cells for the presence of A would appear to be a very good test of the correlation suggested above. It is to be hoped, however, that the experiments could be done with rat cells, as mouse cells appear to have a second NHC protein of this molecular weight present in non-dividing cells.

Acknowledgments

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Chapter 4

PARTIAL FRACTIONATION AND CHEMICAL CHARACTERIZATION OF THE MAJOR NONHISTONE CHROMOSOMAL PROTEINS

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 free of DNA and of histones in a soluble form. It has recently been shown by comparative SDS 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 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. Several roles have been proposed for the nonhistone chromosomal proteins. The limited heterogeneity mentioned suggests that these proteins are common structural elements 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). It has also been suggested that the nonhistone chromosomal proteins may

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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-(6-phenyloxazolyl)] benzene.

be specific determinants in selective gene activation (Paul and Gilmour, 1968; Gilmour and Paul, 1970). Definitive evidence on the function of the major NHC proteins is lacking, although there have recently been some demonstrations of enzyme activity associated with chromatin (histone acetyltransferase, Gallwitz, 1971; histone protease, Garrels et al., 1971). Information on the chemistry of the NHC proteins has not been available previously.

The method which we have developed permits the separation of the nonhistone chromosomal proteins from DNA and histone, and initially fractionates these proteins into four groups. The method is such as to allow for further fractionation of each group and is suitable for chemical studies of the nonhistone chromosomal proteins. The pH conditions used would not, however, allow one to assume that the isolated proteins could subsequently be used for biological 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 SDS bands observed earlier. Our results continue to support the idea of a limited heterogeneity of the NHC protein fraction.

Methods

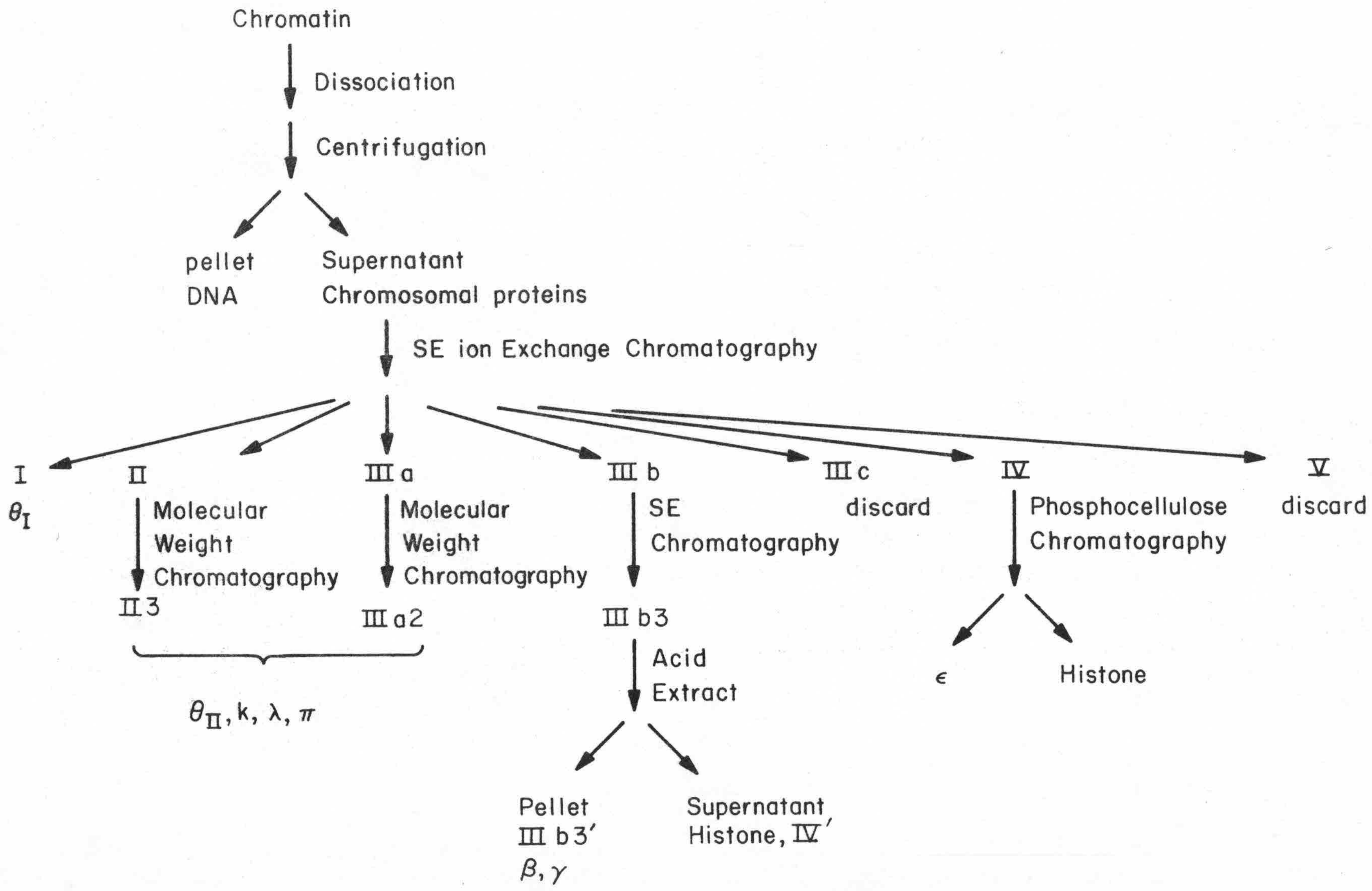
Preparation of Chromatin. All studies were carried out on rat liver chromatin prepared according to methods previously described (Elgin and Bonner, 1970). Chromatin was prepared, dialyzed against 0.01 M Tris (pH 8) at 0° for 4 hr, sheared, and dissociated without delay to avoid inherent problems of degradation (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 then stirred slowly for one-half hour. 5 M sodium chloride 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 the DNA; the top 10 ml of each tube was removed by pipette and taken as the supernatant solution.

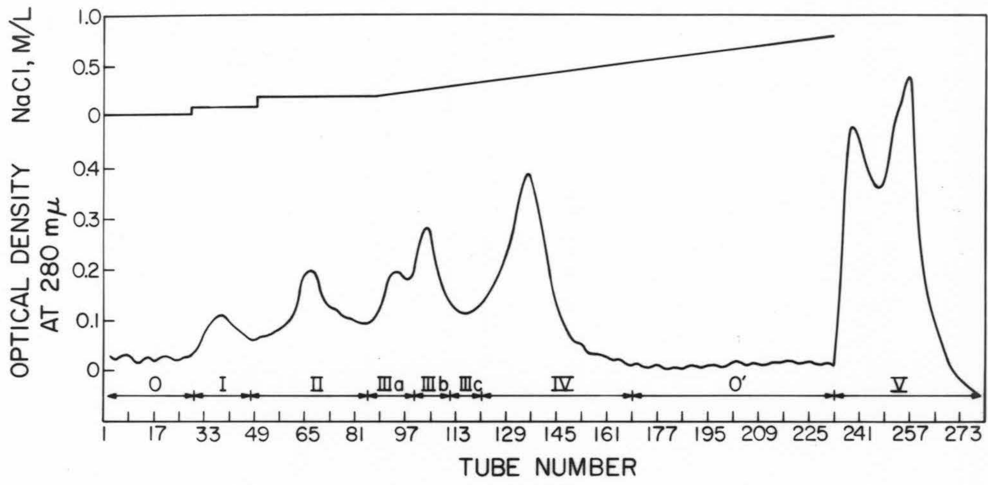
Ion Exchange Column Chromatography. Sephadex resin SE-25 was prepared by soaking overnight in formic acid-urea solution (25% formic acid-10 M urea). The resin was then combined with the above supernatant and the mixture diluted with a 10-fold excess of formic acid-urea solution with rapid stirring. This 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 X 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. The flow rate was generally maintained at 15 ml/hr. The column was assayed by absorbance of fractions at 280 mu. Fractions were pooled as indicated in Figure 2 and either diluted and 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

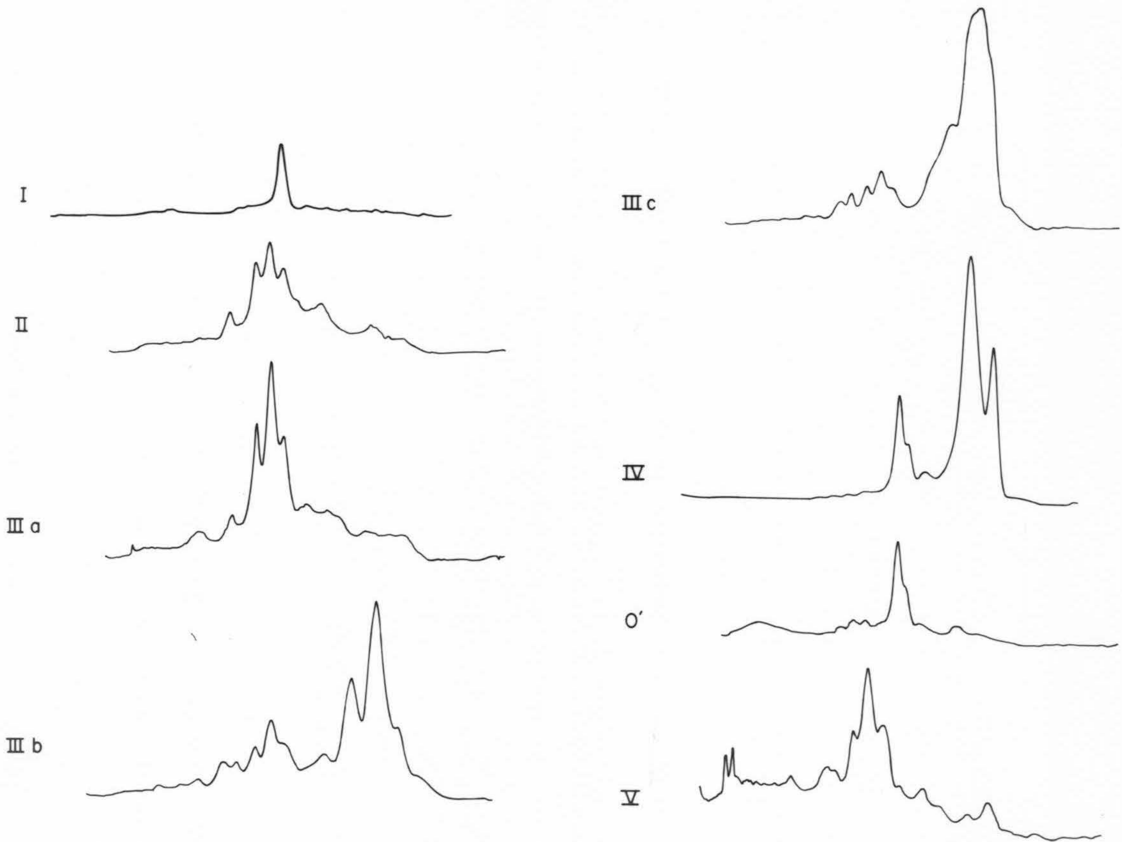
Figure 1: Summary of fractionation procedure.



Chromatin
Dissociation
Centrifugation
pellet
DNA
Supernatant
Chromosomal proteins
SE ion Exchange Chromatography
I
θ_I
II
Molecular
Weight
Chromatography
II 3
III a
Molecular
Weight
Chromatography
III a2
θ_{II}, k, λ, π
III b
SE
Chromatography
III b3
Acid
Extract
Pellet
III b3′
β, γ
Supernatant
Histone, IV′
III c
discard
IV
Phosphocellulose
Chromatography
ϵ
Histone
V
discard

Figure 2: Chromatographic fractionation of the chromosomal proteins on Sephadex SE C-25. a. Elution profile. 2 ml fractions. b. SDS gel electrophoresis of pooled fractions. Gels run left to right.


NaCl, M/L
1.0
0.5
0
OPTICAL DENSITY
AT 280 mμ
0.4
0.3
0.2
0.1
0
O
I
II
IIIa
IIIb
IIIc
IV
O'
V
1
17
33
49
65
81
97
113
129
145
161
177
195
209
225
241
257
273
TUBE NUMBER


I
II
III a
III b
III c
IV
O'
V

the formic acid on Sephadex, such a column can be used only once before discarding.[2]) This column was developed from the method of E. Ruoslahti as used by Smart <u>et al</u>. (1971).

<u>General Methods of Chromatography</u>. Fractions II, IIIa and IV from the SE column were dialyzed against two changes of Nomura solvent (Ozaki <u>et al</u>., 1970), concentrated by ultrafiltration over a PM-10 membrane (Amicon, Inc.) and further dialyzed against Nomura 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 x 10^{-3} M β -mercaptoethanol-0.1 M lithium chloride. Fractions II and IIIa were further purified on a 1.5 x 90 cm. column of Sephadex G-75 or G-100. Columns were run at ambient temperature; flow rates on the order of ca. 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 <u>et al</u>., (1970). Unfortunately, the relative elution positions of the proteins varied depending upon the batch of phosphocellulose. Generally, a 1.2 X 62 cm. column running at ca. 5-10 ml/hr was most satisfactory. In all the above purifications, column fractions were analyzed for protein by the method of Lowry <u>et al</u>.(1951).

[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.

Disc gel electrophoresis. Samples were generally analyzed by SDS disc gel electrophoresis as described in Elgin and Bonner (1970). All SDS gels were stained with Coomassie brilliant blue R-250 (Mann/Schwartz). Split SDS gel elctrophoresis according to the methods of Spiegel et al. (1970) was used to confirm the identification of isolated bands. 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 amidoschwarz 10 B (Matheson, Coleman and Bell) and the latter with Coomassie brilliant blue. Purified gel reagents (Bio-Rad Laboratories) were used throughout. Gels were photographed using an orange filter and/or scanned at 600 mu 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 7 and 10.

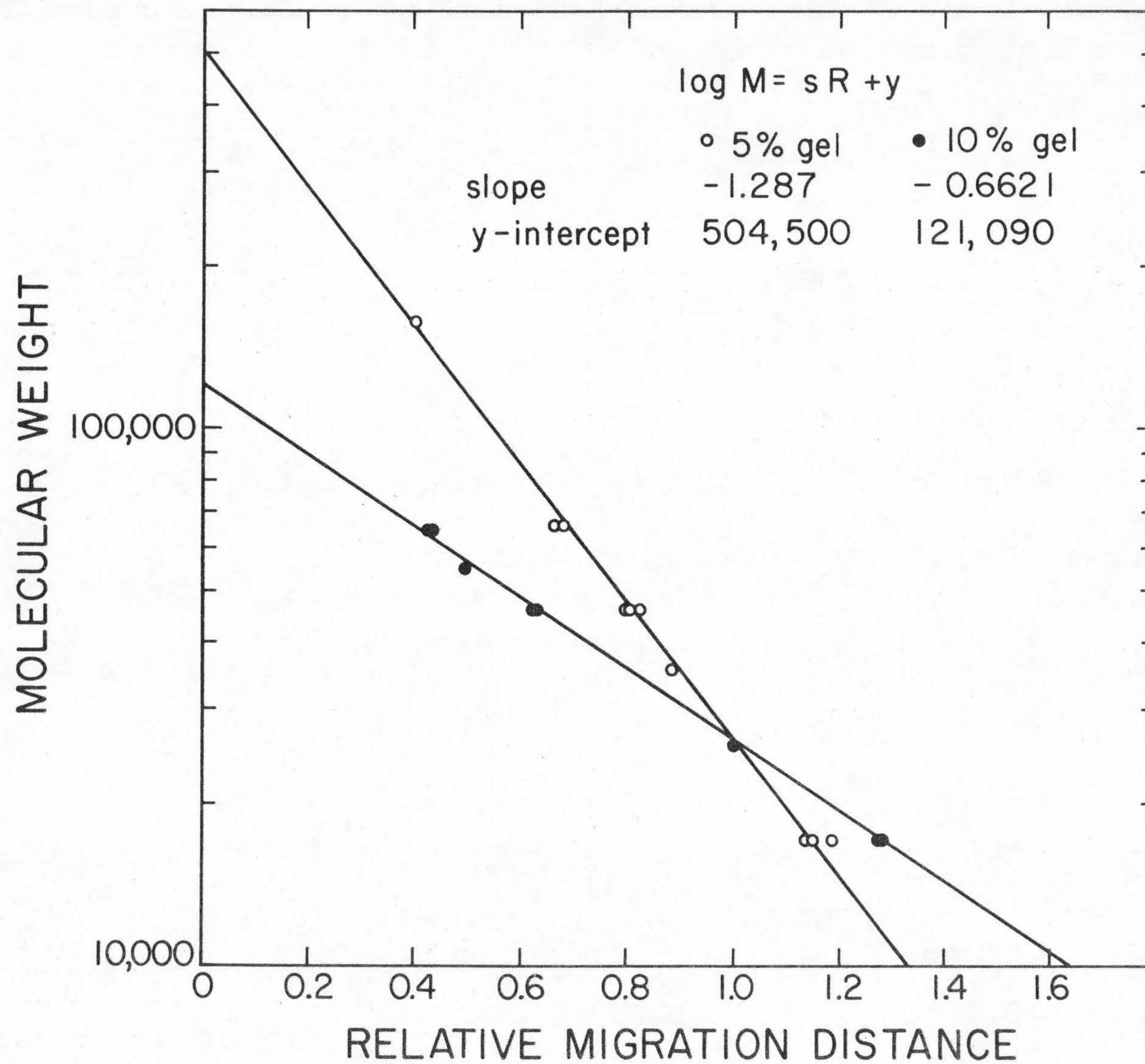
Labeling and counting of nonhistone chromosomal proteins. Labeled preparations of nonhistone chromosomal proteins were obtained by injecting rats (male albino Sprague-Dawley, ca. 200 g body weight) interperitoneally (under ether anesthesia) with tritiated leucine ca. 50 C/mmole, 1.0-1.5 mC/rat, 24 hr prior to sacrifice; and with inorganic phosphate, carrier-free, 2-6 mC/rat, 3 hr prior to sacrifice. The animals were sacrificed by exsanguination (no anesthesia) and the liver NHC proteins prepared. Chromatin was extracted with 0.4 N sulfuric acid to remove the histones, the pellet was solubilized in SDS 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 SDS gels, staining and destaining, cutting out the individual bands by hand with a razor blade and counting according to the methods of Ward <u>et al</u>. (1970). The incorporation of ^{32}P and ^{3}H counts, minus background, is presented as an indication of the turnover and phosphorylation of the individual protein bands.

<u>General Methods of Analysis: Molecular weight determinations</u>. Molecular weights of the protein bands both of the unfractionated NHC protein preparations and of the isolated fractions were determined by SDS gel electrophoresis (Shapiro <u>et al</u>., 1967; Dunker and Rueckert, 1969; Weber and Osborn, 1969). Chymotrypsinogen was used as the standard protein for determining relative migration distances. Standard plots of log molecular weight as a function of relative migration distance for 5% and 10% acrylamide gels are given in Figure 3. The experimental points were fitted to straight lines by least squares analysis. The method was generally found to be accurate within 5% for the ranges used for a given percent acrylamide gel. Standard proteins of known molecular weight were from Schwartz/Mann.

<u>Isoelectric focussing and determination of pI</u>. Isoelectric focussing 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 8. A plateau effect in the region pH 5 to pH 6 is still

Figure 3: Semi-log plot of molecular weight M as a function of relative distance of migration R. Reference protein, chymotrypsinogen (25,700); data points myoglobin (17,200), pepsin (35,500), ovalbumin (46,000), gamma globulin heavy chain (55,000), albumin (66,000), gamma globulin (160,000).


log M= s R +y
○ 5% gel
● 10% gel
slope
-1.287
- 0.6621
y-intercept
504,500
121,090
MOLECULAR WEIGHT
100,000
10,000
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
RELATIVE MIGRATION DISTANCE

observed, but the pH gradient is satisfactory. The isoelectric points indicated for the nonhistone chromosomal proteins are taken directly from the pH reading of a 0.01 M KCl extract of slices of the gel. Probably a small negative correction is warranted due to the urea content (Ui, 1971).

N-Terminal Analysis. Qualitative N-terminal analysis was carried out by the method of Konigsberg (1971). Protein samples, 0.1 to 1.5 mg, were dissolved in 0.1 to 0.2 ml of 0.5 M sodium bicarbonate, ca. pH 8, with 1% SDS. The pH was adjusted to 7 with 1 N HCl and 1/2 volume of dansylchloride 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, recollected 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 dansyl amino 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.

General Methods. The yields of protein from the SE Sephadex column were determined by dialyzing aliquots of each fraction twice versus 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. 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. Amino acid analyses were obtained on a Beckman Model 120 B amino acid analyzer following hydrolysis of the samples in constant boiling HCl at 108° for 22 hr. No attempts were made to correct for serine or threonine losses. Where used, acid extraction of histone from samples was carried out as described in Elgin and Bonner (1970). All urea used was either obtained in highly purified form (Schwartz/Mann, ultra pure) or was purified by passage of a 10 M solution through a mixed bed ion exchange column (Barnstead D0803). SDS (Sipon WD) was obtained from Alcolac Chemical Corp. (Baltimore, Md.) and was recrystallized once from 80% ethanol before use.

Results

Dissociation of Chromatin. The techniques used 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 supernatant fraction after centrifugation. Unfortunately, due to leakage in the tubes, the total recovery of protein frequently approached only 80%. SDS gel electrophoresis of the chromatin, unspun dissociated solution, spun supernatant and spun pellet indicates that all the protein species present in the chromatin are recovered in the spun supernatant. 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 dye is proportional to the protein content of a band only over the range of 1-20 ug. Different proteins bind dye to different extents (Elgin, unpublished observations).

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 mu is somewhat misleading as concerns the presence 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, this appears to be due to a 10% 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 comples with protein). (High tryptophan content could also contribute to these peaks; this parameter has not yet been determined.)

SDS 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 the bands θ, κ, λ and π. These fractions have been further purified by dialysis to Nomura solvent and gel permeation chromatography on G-75 or G-100 Sephadex as described under Methods

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

Table I: Content of SE-Column Fractions

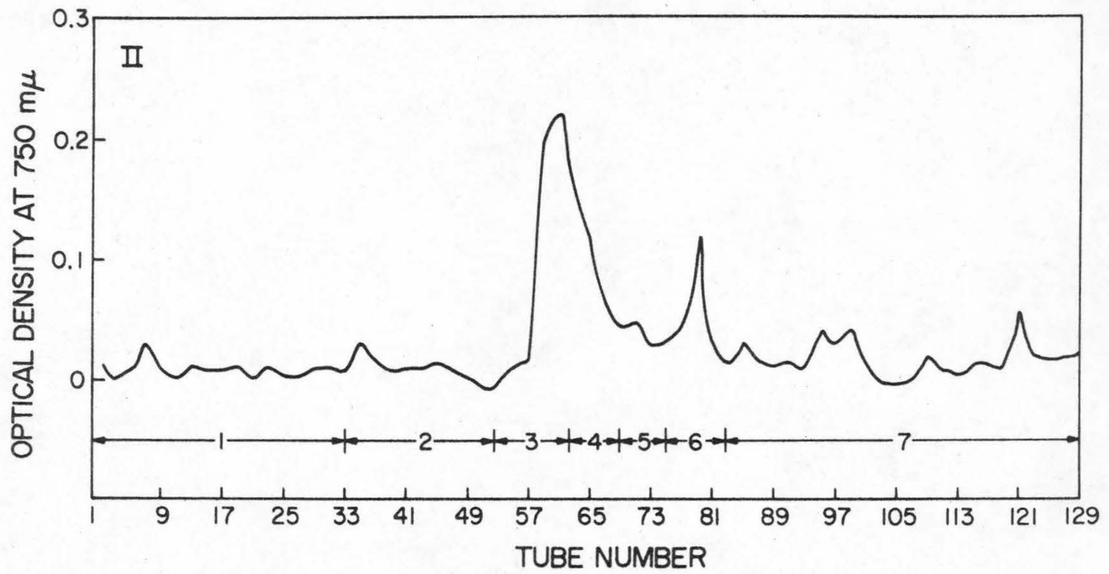
Fraction	% of Total Protein
I	3.6
II	10.1
IIIa	6.0
IIIb	7.7
IIIc	6.5
IV	50.2
O'	5.9
V	10.0

A typical elution profile is shown for fraction II in Figure 4 and for fraction IIIa in Figure 5, including SDS gel electrophoresis of the protein bands present in each pooled chromatographic fraction II3 and IIIa2 were identical by SDS gel electrophoresis and elute at the same ionic strength on the SE column: they were therefore assumed to be the same and pooled for all analytical work.

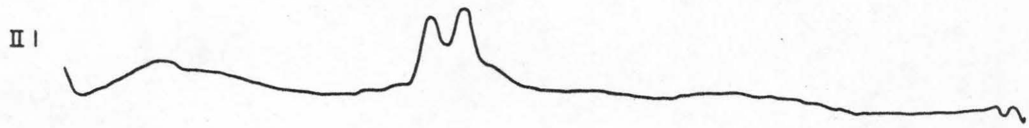
Fraction IIIb is fairly heterogeneous but provides an opportunity to obtain bands β and γ by a second cycle of fractionation on the SE ion exchange column. Fraction IIIb was diluted by the addition of 2 volumes of formic acid-urea solvent and rechromatographed on a 2.5 X 25 cm. SE column with a gradient of 0.2-0.5 M sodium chloride. As shown in Figure 6, bands β and γ elute together as a very sharp peak at ca. 0.3 M sodium chloride. Analysis indicates, however, that this peak still contains significant amounts of histone (up to 35-40%), most probably predominantly histone II. The lyophilized fraction was resuspended in 0.01 M Tris (pH 8) and acid-extracted as given in General Methods. 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 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 . ε could be separated from the histone by chromatography on phosphocellulose according to Ozaki et al. (1970) as described under Methods. Fraction V, eluted with 100% formic acid, was discarded.

It should be noted that the proteins appear to elute from the SE

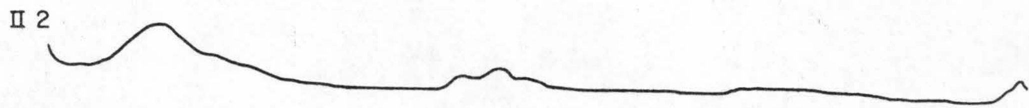
Figure 4: Chromatographic fractionation of II on Sephadex G-100. 1 ml fractions.



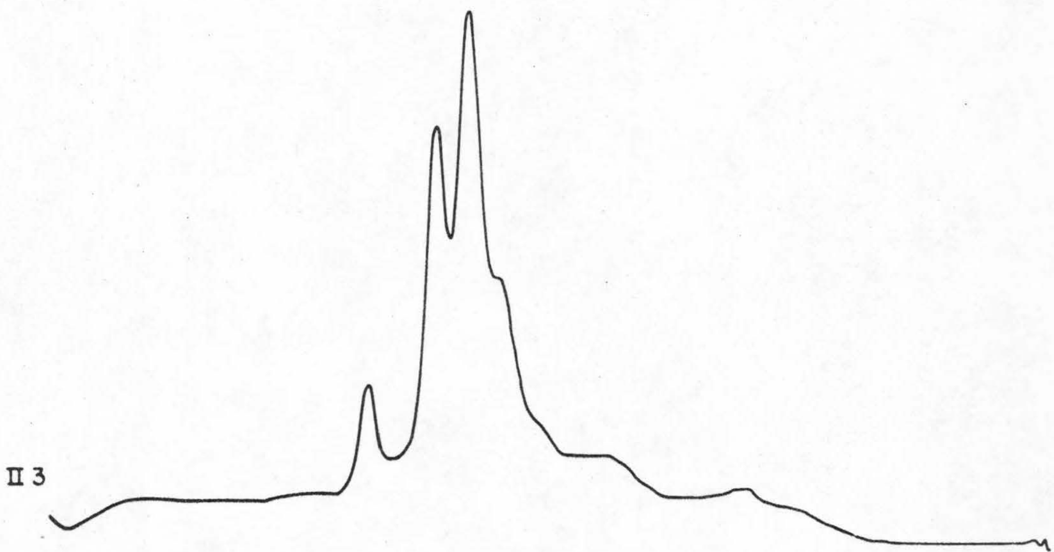
OPTICAL DENSITY AT 750 mμ
0.3
0.2
0.1
0
II
1
2
3
4
5
6
7
1
9
17
25
33
41
49
57
65
73
81
89
97
105
113
121
129
TUBE NUMBER



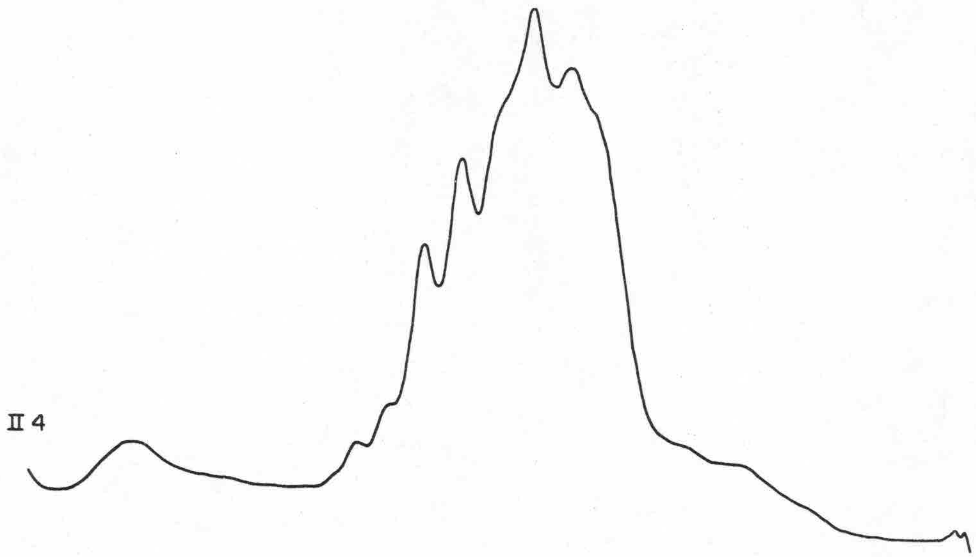
II 1

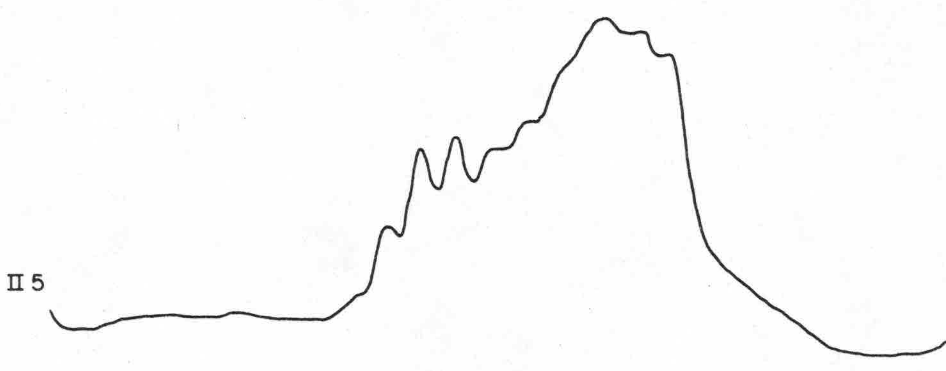


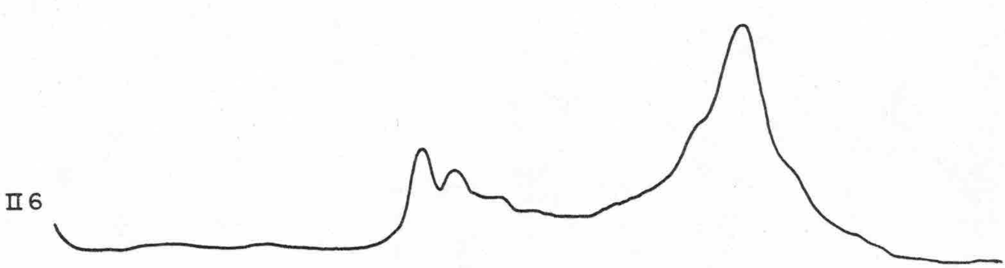
II 2



II 3


II 4


II 5


II 6

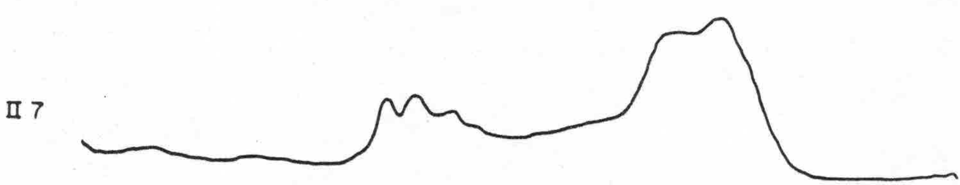
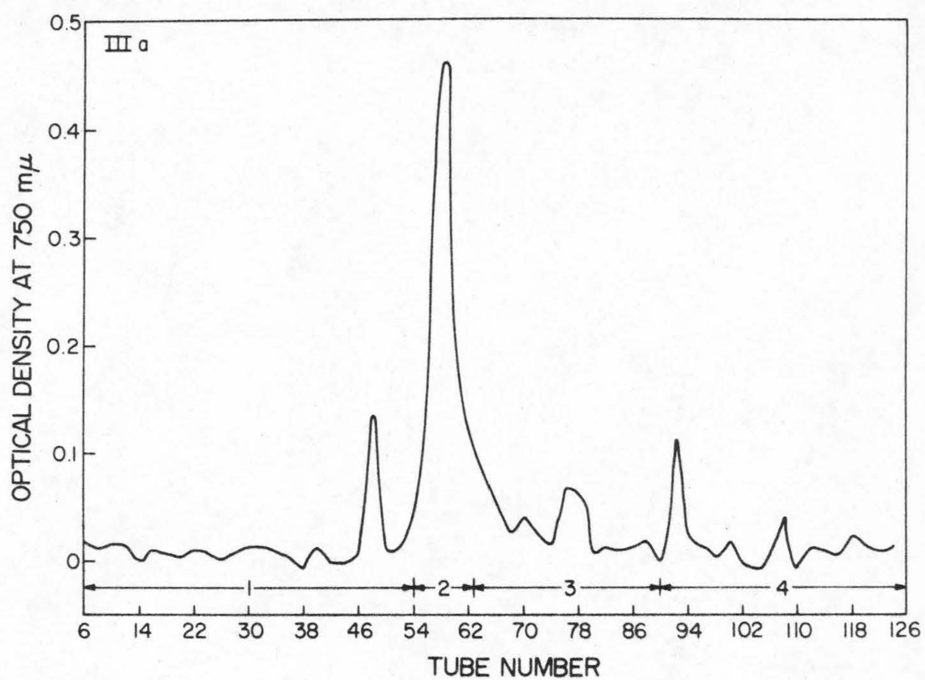
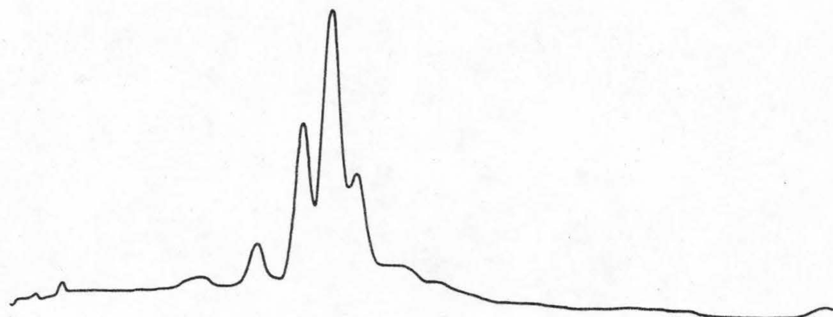

II 7

Figure 5: Chromatographic purification of IIIa on Sephadex G-75. 1 ml fractions.

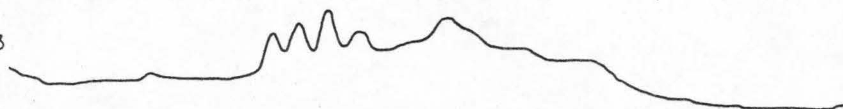


III a 1

III a 2



III a 3



III a 4

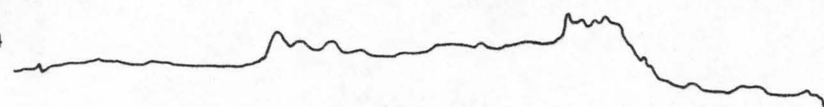
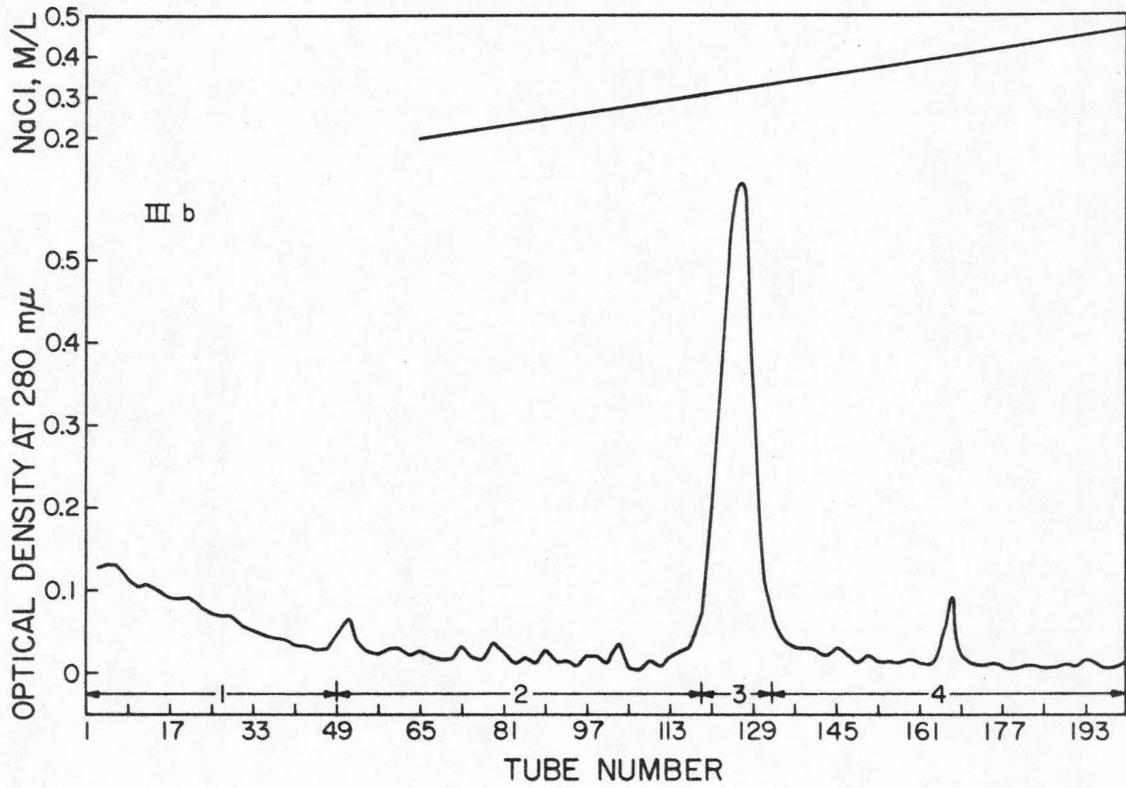
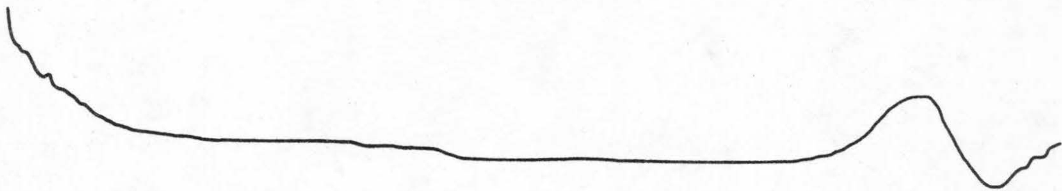


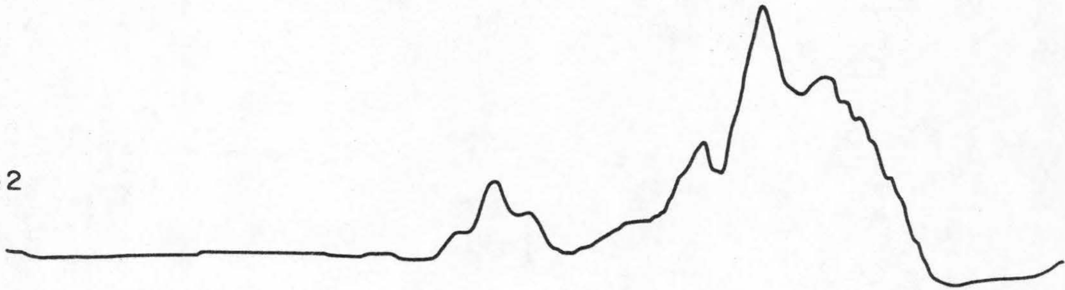
Figure 6: Chromatographic purification of IIIb on Sephadex SE C-25 in formic acid-urea solvent, NaCl gradient. 2 ml fractions.



III b I



III b2



III b3

III b4

column in order of increasing basicity or lysine content. 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 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 4-6). The lyophilized fractions I, II3, IIIa2, IIIb3' and ε were used for further chemical analysis. SDS gel analysis of these fractions, pooled from several preparations,is given in Figure 7.

In addition to the formic acid-urea solvent, such purified NHC proteins appear to be soluble in all SDS solutions, in Nomura solvent, and in high concentrations of urea. Other solvents have not yet been explored.

Chemical Characteristics of the Isolated NHC Proteins. The NHC proteins further analyzed range in molecular weight from ca. 10,000 to ca. 100,000. See Table II for the values determined. This 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 summarizes these data and gives the number of bands observed in each

Figure 7: SDS gel electrophoresis of final protein fractions obtained. Gels run left to right.

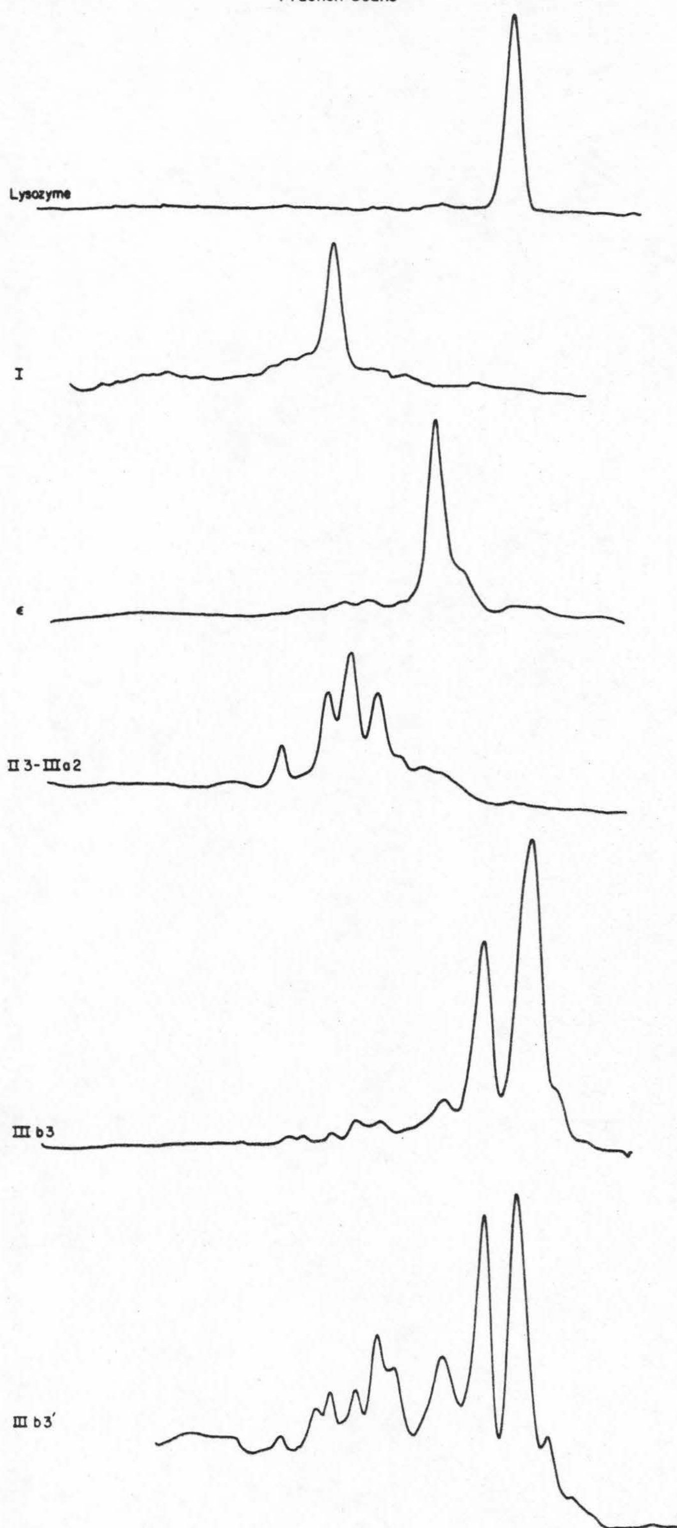

Fraction Scans
Lysozyme
I
ε
II 3-IIIa2
III b3
III b3′

Table II: Molecular Weights of NHC Proteins

Protein	5% Gel			10% Gel		
	m	σ	n	m	σ	n
α	12,530	2000	3	12,400	670	2
β	15,940	1740	4	15,080	260	3
γ	19,020	870	3	17,920	160	3
δ	25,000		3	25,000		2
ε	31,210	1160	3	33,390	290	2
η	37,830	230	3	38,390	700	2
θ	49,120	1060	5	46,920	810	4
κ	60,230	1220	5	56,400	1230	3
λ	71,370	2270	5			
μ	85,340	1670	3			
π	98,190	1190	5			
ρ	142,070	7400	3			

m = mean, σ = standard deviation, n = number of determinations

Table III: Complexity of NHC Fraction

Fraction	No. of bands separated by gel electrophoresis[1]			No. of		Conclusion:
	SDS	urea, pH 4.3	urea, pH 8.9	N-terminals	pI	Number of Proteins
I(θ_I)	1	ag + 1	$\underline{1}$ + 1	1	<3.7	1
II3-IIIa2	4	5	ag + 4	$\underline{2}$-4	5.4, 6.0, 6.6	4-6
IIIb3	$\underline{2}$-8	$\underline{4}$-8	ag + $\underline{4}$ + 3	$\underline{6}$-3	6.4-7.0, 7.0-8.0	6-12
IIIb3'	$\underline{2}$-8	$\underline{4}$-3	no change from IIIb3	n.d. (assume $\underline{5}$ + 2)	no change from IIIb3	4-7
ε	1	$\underline{1}$-2	$\underline{1}$-1	1	5.6	1

[1]ag indicates aggregate on top of gel. Notation $\underline{4}$ + 2 means 4 major polypeptides + 2 minor polypeptides observed.

n.d. indicates not determined.

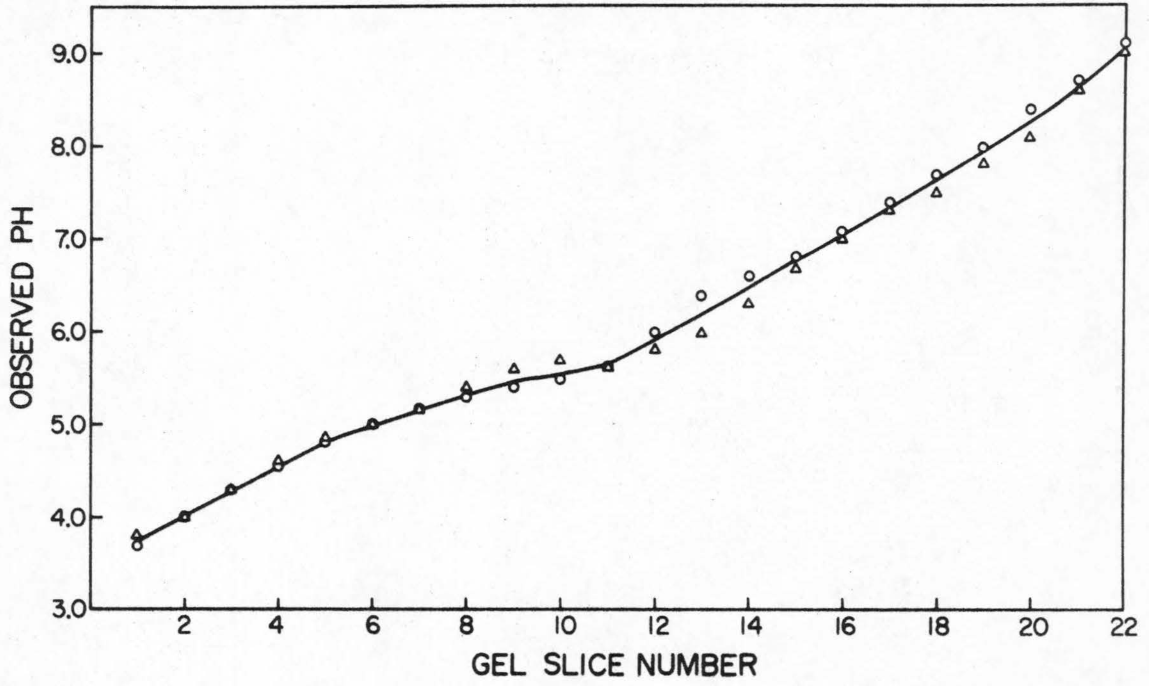
fraction for SDS gel electrophoresis and urea gel electrophoresis at two pHs. Scans of the pertinent urea gels are given in Figure 9 and 10. The N-terminal analyses, although qualitative, are also critical in this regard. Fraction I has an N terminal of glutamic acid. Small amounts of serine and threonine 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 SDS gel electrophoresis, six polypeptide chains may be 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 isoleucine and leucine, it is apparent that fraction IIIb3 is much more heterogeneous than indicated by SDS 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-lysine in all fractions, but no N-terminal basic amino acids.

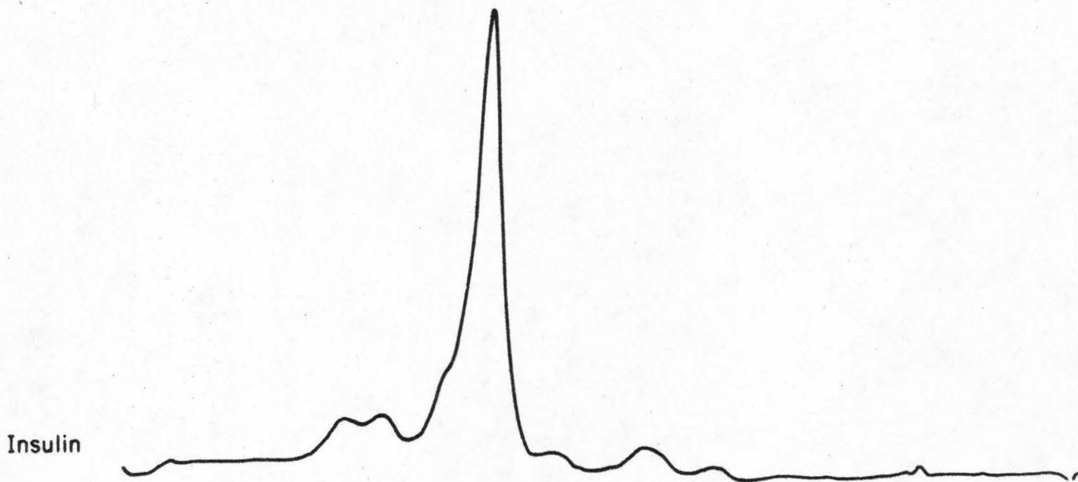
The results of the isoelectric focussing experiments are given in Figure 8. No bands were observed for fraction I, suggesting that the isoelectric point of θ_I is less than 3.7. The early elution position on the SE column and high acid:base ratio on amino acid analysis strengthen this idea. Fraction II3-IIIa2, although revealing many bands on isoelectric focussing, shows principal groupings at pI 5.4 and 6.6 with an additional sharp band at 6.0. One should be reminded that almost all proteins which are regarded as pure show multiple bands on isoelectric focussing, since the alteration of an acid to an amide, etc. can change the isoelectric focussing point significantly. Fraction IIIb3' shows essentially the isoelectric focussing patter of IIIb3 (Figure 8), 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 absolutely astounding in that it focusses in one major band at isoelectric point 5.6. This is a very strong indication of the homogeneity of this protein.

Scans of the gels of each fraction in urea at pH's 4 and 9 are given in Figures 9 and 10. The number and position of the major bands

Figure 8: Isoelectric focussing: pH gradient obtained in polyacrylamide gels. Scans below show results with NHC proteins.

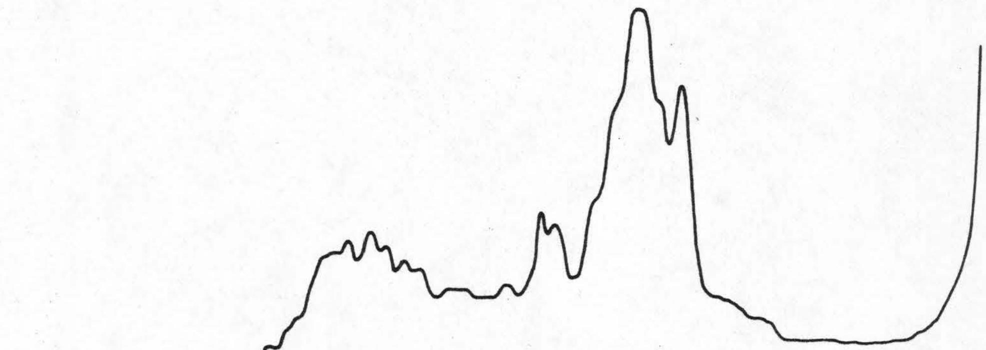


OBSERVED PH
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2
4
6
8
10
12
14
16
18
20
22
GEL SLICE NUMBER

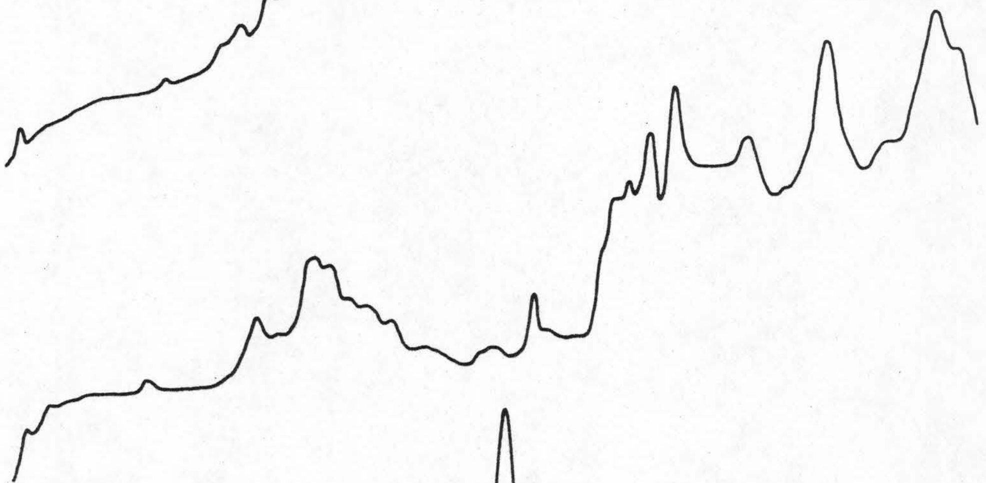


Insulin

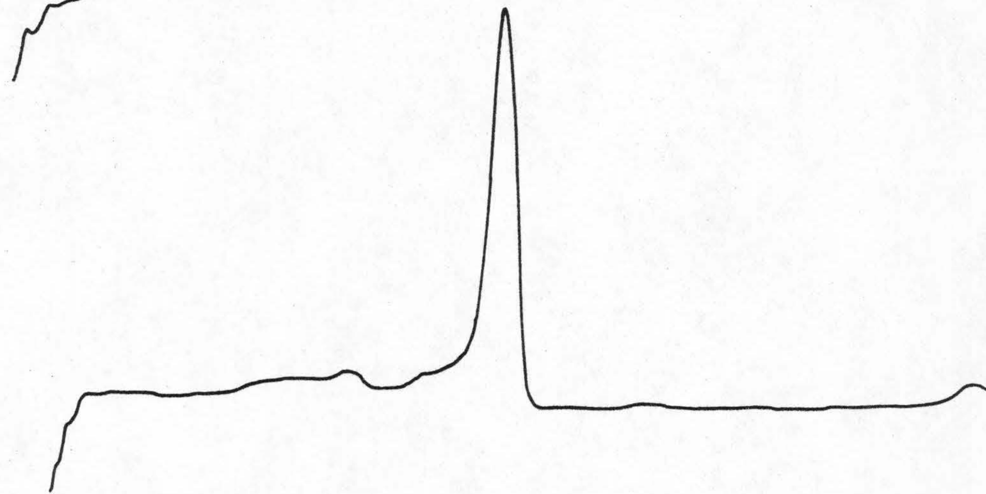
θ



II3-IIIa2

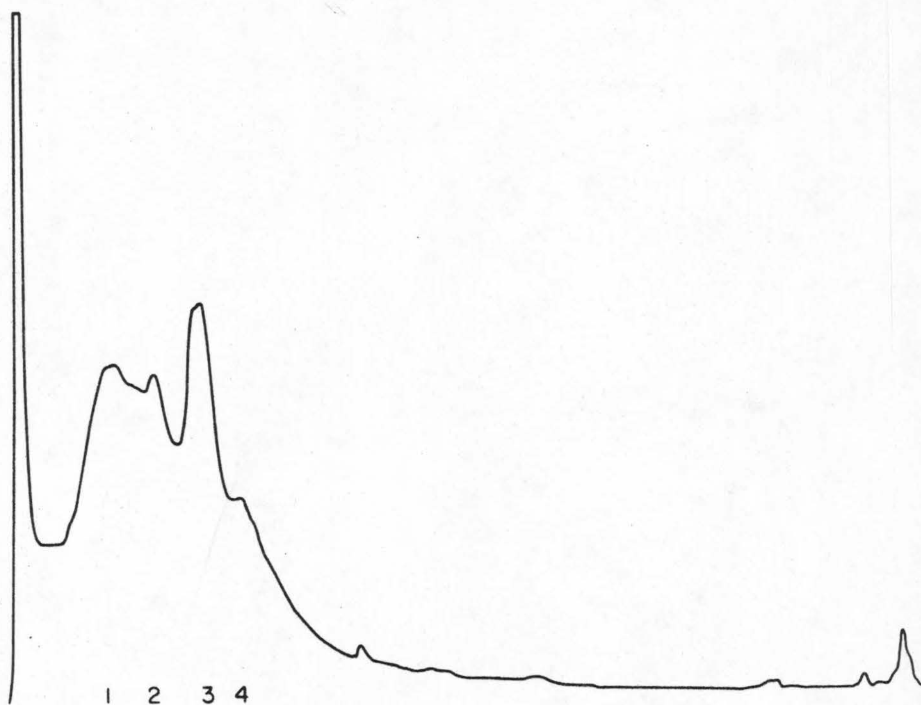


IIIb3



ε

Figure 9: Polyacrylamide gel electrophoresis at pH 8.9 of NHC protein samples.


II 3-III a2
1 2 3 4

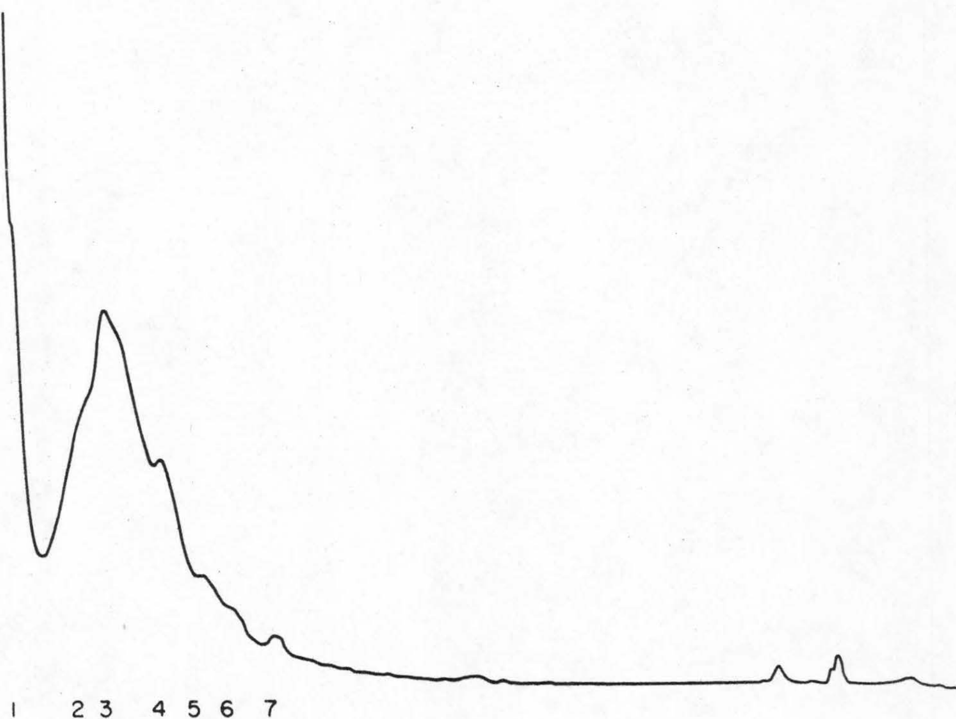
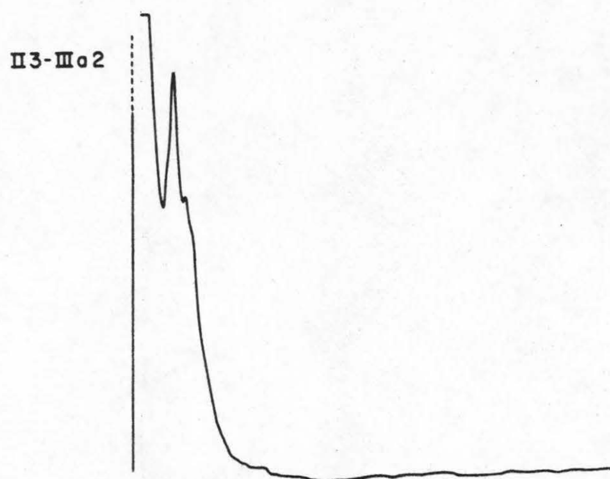
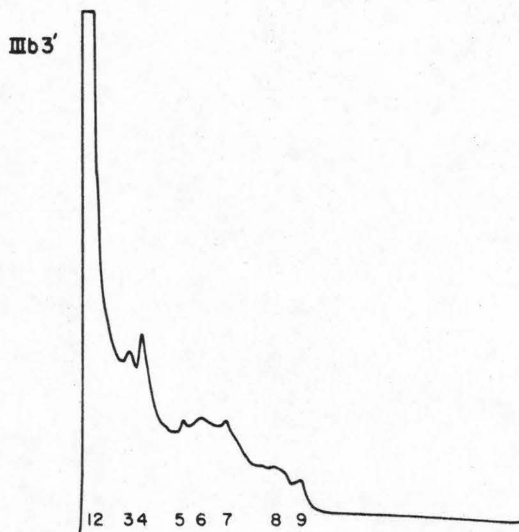

III b3
1 2 3 4 5 6 7

Figure 10: Polyacrylamide gel electrophoresis at pH 4.3 of NHC protein samples.


II3-IIIa2
1 2345


IIIb3'
12 34 5 6 7 8 9

IV

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-IIIa3, and 4-7 major bands are indicated for fraction IIIb3'.

The results of the amino acid and phosphate labeling experiment are given in Figure 11. The data indicate differences both in the rate and amount of synthesis of the different NHC proteins and in the extent of phosphorylation. It is apparent that the NHC proteins turn over more rapidly than do the histones, as has previously been observed (Holoubek and Crocker, 1968).

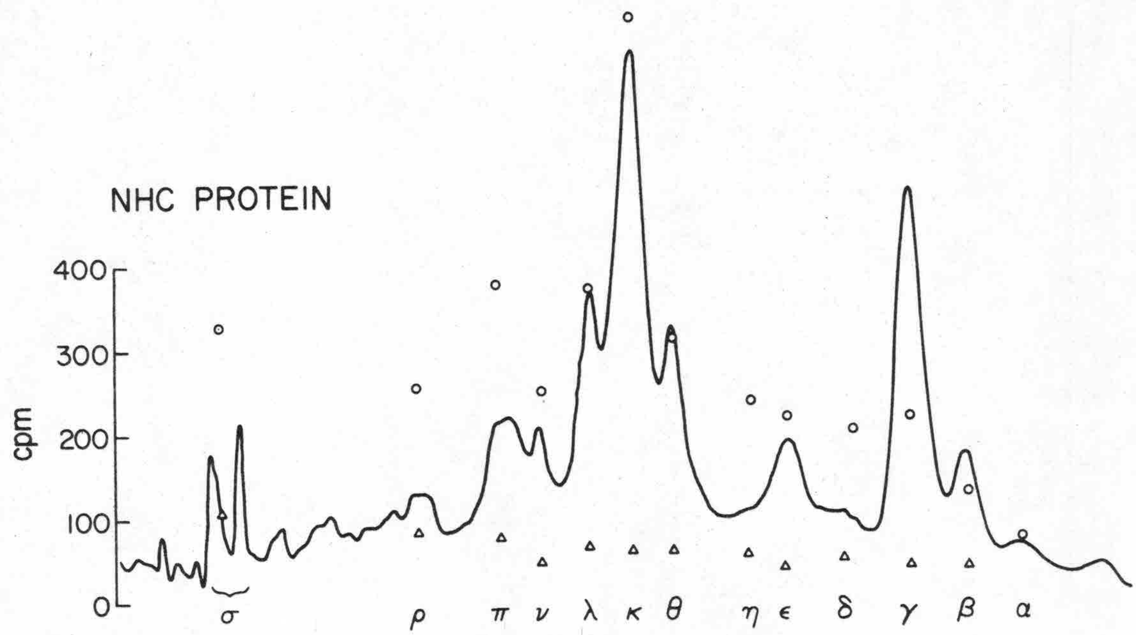
The amino acid analyses of the fractions θ_I, II3-IIIa2, IIIb3', ε, and IV are given in Table IV. The cysteine values given are no doubt low since a) no reagent was used to prevent cysteine oxidation during hydrolysis, and b) prior to hydrolysis the proteins were stored lyophilized, at -20°, conditions which do not prevent disappearance of cysteine in Los Angeles county air (Pierce, 1971).

Discussion

The limited heterogeneity of the NHC proteins has led to the suggestion that these proteins are common structural proteins or common enzymes of chromosomal metabolism. The NHC proteins have been implicated in mechanisms of hormone response because their selective synthetic increase has been observed at early times after hormone administration (Teng and Hamilton, 1970; Shelton and Allfrey, 1970). It has been demonstrated that the NHC proteins can counter the repressive effect of histones in template activity assays, but

Figure 11: Labeling of the NHC proteins *in vivo* with ^{3}H-leucine and carrier-free inorganic phosphate. Data plotted on SDS gel scan, electrophoresis left to right.

o ^{3}H counts; Δ ^{32}P counts.


NHC PROTEIN
400
300
200
100
0
cpm
σ
ρ
π
ν
λ
κ
θ
η
ε
δ
γ
β
α

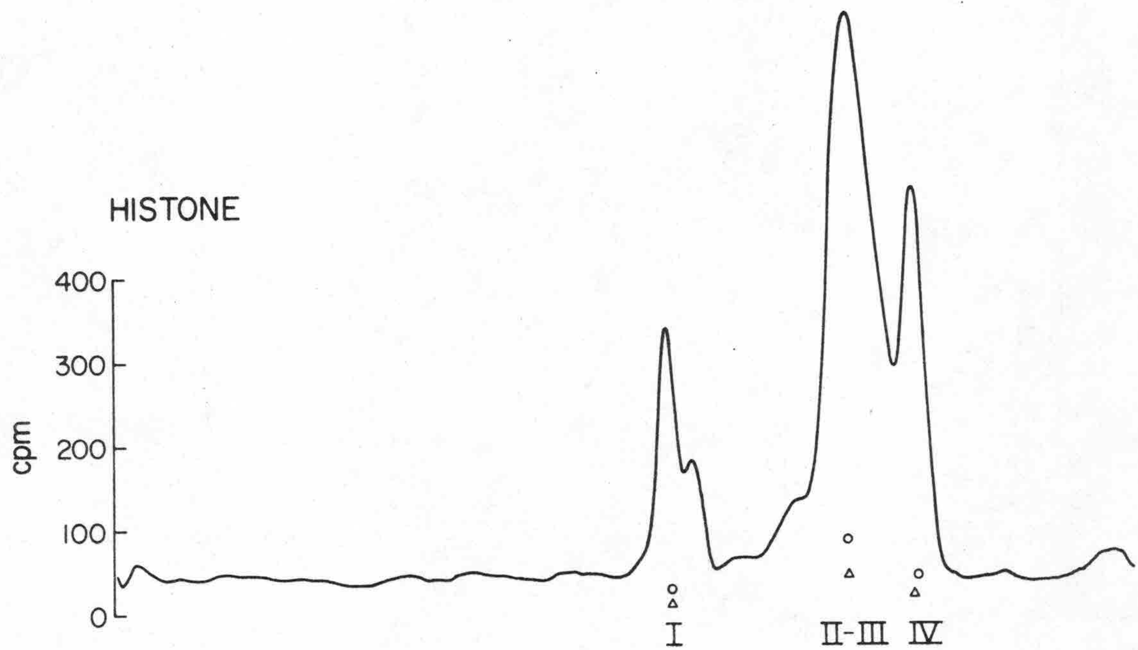

HISTONE
400
300
200
100
0
cpm
I
II-III
IV

Table IV: Amino Acid Composition of NHC Proteins and Fractions

Amino Acid	θ_I	II3-IIIa2	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
$\frac{\text{asp + glu}}{\text{lys + arg + his}}$	2.7	1.7	1.2	1.4	.54

rigorous proof of specific gene activation is lacking (Wang, 1968 ; Gilmour and Paul, 1969; Teng and Hamilton, 1969; Spelsberg et al., 1971). Recent experiments have suggested that a NHC protein fraction can play a σ -like, organism-specific role in stimulating RNA synthesis from DNA templates (Teng et al., 1971). Several enzyme activities are known to be associated with isolated chromatin, e.g., histone protease (Furlan and Jericijo, 1967, Garrels et al., 1971), and histone acetylase (Gallwitz, 1971); see Elgin et al., (1971) for a more detailed discussion of this aspect. No work has yet been done-- no assay is as yet available-- considering a structural role for NHC proteins. The weight of present evidence suggests only a general role (but including tissue-specific response, i.e., hormone response) for the major NHC proteins in possibly the structure of chromatin and in regulation of gene activity. [Of course, 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 (analogous to the lac repressor).]

The chemical characteristics of the NHC protein fractions are of interest in themselves, in speculation on their role, and for 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 SDS 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 (Elgin, unpublished observations), and b) NHC proteins with

$$\frac{\text{glutamic + aspartic acids}}{\text{lysine + histidine + arginine}} = a/b > 1$$

have been isolated by a variety of techniques such that the proteins are not exposed to pHs below 2.5 (Wang, 1967; Shaw and Huang, 1970; MacGillivray _et al._, 1971; Elgin and Bonner, this manuscript). Acid hydrolysis in the presence of DNA does not alter the amino acid composition of histones significantly (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 ranges from ca. 1.3 to 2.7 (θ_I, this paper), calculated in all cases without consideration of possible amides (Wang, 1967; 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 4). Several of the proteins show a low level of phosphate incorporation (Figure 11; Platz _et al._, 1970; Teng _et al._, 1971); turnover appears to be more rapid than that of the histones (Figure 11; 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. 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 necessarily return to their native conformations under suitable conditions. Nonetheless, 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-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 SDS and urea gel electrophoresis, number of bands on analytical isoelectric

focussing, and N-terminal analysis. It can be estimated from a quantitative scan of stained SDS gels of whole NHC protein that the bands analyzed here (β , γ , ϵ , θ , κ , λ , π) account for a minimum of ca. 70% of the protein (see Figure 11). The analysis therefore suggests once again that a relatively small number (10-15) of proteins make up the bulk of the NHC protein fraction.

Acknowledgments

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Chapter 5

COLLABORATIVE STUDIES ON THE BIOLOGICAL FUNTION OF THE NONHISTONE CHROMOSOMAL PROTEINS

Chapter 5

Part I

HISTONE PROTEASE: A CHROMOSOMAL ENZYME

INTRODUCTION

It has been observed for some time that chromatin prepared by the method of Bonner et al. (1968a) is not stable over long periods of refrigerator storage. This is particularly true of chromatin from certain tissues, such as calf thymus. 20% degradation of calf thymus chromatin within one day has been observed under conditions of 0°, 0.01 M Tris, pH 8, which are the conditions one frequently uses at the end of the preparation (Panyim et al., 1968). The degradation of chromatin has been shown to be due to the degradation of the various histones rather than of DNA (Panyim et al., 1968). Earlier work of Phillip and Johns (1959) indicates the presence of a histone protease which is isolat d with histones, while Dounce and Umana (1962) have reported on significant protease activity in rat liver nuclei. The above authors report that the proteolysis can be substantially inhibited by 1 mM dissopropylfluorophosphate, suggesting the presence of a serine residue at the active center.

Furlan and Jericijo (1967 a,b; Furlan et al., 1968) have studied a protease from calf thymus, which is isolated from a high salt concentration (1.0 N - 2.5 N) extract of nuclei. The purified protease, molecular weight approximately 24,000, has a pH optimum of 7.8 and maximum activity in 0.1 M NaCl or 1 M NaCl. It is inhibited by p-chloromercuribenzoate and by diisopropylfluorophosphate, again suggesting a serine residue at the active site. Deoxyribonucleoprotein is 6-7 times more susceptible as substrate than are other proteins, histone I being most rapidly degraded. That the protein is normally a constituent of chromatin is indicated by the fact that it

coprecipitates with DNA and histones at NaCl concentrations between 0.1 and 0.3 N. As shown by reconstitution and acid extraction experiments, the interaction between the protease and DNA appears to be quite similar to that between DNA and histone I (Furlan and Jericijo, 1967 a,b; Furlan et al., 1968). The characteristics of this enzyme suggest that it may be responsible for part or all of the histone degradation observed by Panyim et al. (1968) and by others working in this field. Recently Bartley and Chalkley (1970) have reported that in calf thymus nucleohistone, histones I and III are attacked most rapidly by the associated protease, while if free histones (not associated with nucleic acid) are the substrate only histone I is resistent. They also find that the enzyme has maximal activity at pH 8 and is relatively inactive below pH 7.

There seems little doubt, then, that a histone protease is a normal constituent of chromatin. The biological role of such an enzyme has been a topic of speculation. It has been observed that tissues with a high cell turnover exhibit a greater rate of proteolysis of their isolated nucleohistone than do other tissues. This observation has led Bartley and Chalkley (1970) to suggest that the histone protease is merely required for cell autolysis and is not involved in mechanisms of chromosomal control. However, recent work from Dixon's group has indicated that histones may be removed from DNA in the transformation of spermatogenesis (wherein the histones are replaced by protamines in the trout) by selective enzymic degradation. High specificity of substrate and/or the presence of other selection clues is implied by the fact that such a protease would have to

degrade the histones without affecting the protamine which is present to replace them. It has, in fact, been shown that the histones are phosphorylated at specific sites before they disappear from the chromatin complex. This phosphorylation occurs to histone molecules which are bound to DNA and is not concomitent with histone synthesis (Dixon et al., 1969; Marushige et al., 1969). Small heterogeneous, acid-soluble protein fragments have been observed in trout testis chromatin late in the transformation from nucleohistone to nucleoprotamine; these could be breakdown products of histones II, III and IV, which are rapidly disappearing at this time (Marushige and Dixon, 1971). The sum of evidence from Dixon's group is consistent with the hypothesis that, in spermatogenesis, histones are removed from DNA by proteolytic degradation following phosphorylation or acetylation (Sung and Dixon, 1970), the minor modifications being necessary to render the histones available and/or susceptible to the protease. Should a similar mechanism be operative to remove histones during gene depression, the histone protease would be an enzyme of considerable biological significance.

There is considerable evidence to the effect that histones play a role of general repression in chromatin, i.e., if histones are bound to a given sequence of DNA that portion of the genome is not transcribed (Bonner et al., 1968b; Smart and Bonner, 1971). However, it has not been conclusively shown that RNA polymerase cannot "read through" a section of histone-bound DNA provided proper initiation occurs, and bearing in mind that histones can occur in several states. To test this question it will be necessary to do the following

experiment: bind RNA polymerase to a specific, well characterized initiation site and add one or two nucleotide triphosphates so that initiation occurs (So and Downey, 1970). Subsequently bind histones to the free DNA by careful dialysis techniques and determine the template activity and transcription characteristics of the resulting complex. The possible outcomes of the experiment are as follows: 1) RNA polymerase is not inhibited by histones bound to DNA at other than the initiation site, 2) RNA polymerase is stopped by the association of any histone with its template DNA, 3) RNA polymerase can "read through" histone-DNA complexes only if the histone is modified by phosphorylation, acetylation, etc. If either of the first two possibilities are correct, then histones must be removed from DNA during selective gene depression. Specific proteolytic degradation remains the most attractive hypothesis as to how this could be accomplished. In vitro, histones may be removed from DNA by acid extraction or by treatment with high concentrations of salt, both of which are totally unsuitable mechanisms for the in vivo situation. Further, the phosphorylation of histones during spermatogenesis does not affect their dissociation from DNA by salt or sodium deoxycholate (Marushige, et.al., 1969). Histones can be replaced on DNA by competition with more basic molecules; however, only histone I can be replaced at physiological concentrations of, for example, protamine (Evans et al., 1970). It has been suggested that histones may be induced to leave DNA by the presence of an equally negatively charged molecule, for example, an RNA. However studies indicate that only histone I is free to move from one nucleic acid molecule to another

under the salt conditions one would anticipate for the nucleus (Jensen and Chalkley, 1968). In certain instances of hormone stimulation, the effect does not occur unless the hormones are present during a round of cell division (e.g., the mammary system; see Stockdale and Topper, 1966). This suggests that histones could be "removed" by not allowing them to complex with newly-synthesized DNA in the special cases. Discarding these alternatives for the general case leaves one again with the proposition that histones may be removed from DNA as necessary by their proteolytic degradation. Accordingly, to investigate the question we have isolated a histone protease from rat liver chromatin and are investigating its substrate specificity.

METHODS

Enzyme preparation.

Histone protease is prepared from purified chromatin by the following method: the chromatin, prepared according to the methods of Bonner *et al.* (1968a), is extracted with 0.7 M NaCl. The DNA and undissociated proteins are removed by centrifugation at 36,000 RPM for 4 hours in a Spinco #40 rotor. The supernatant is concentrated by ultrafiltration over a PM 10 (Amicon) membrane to a small volume and applied to a Sephadex G-75 or Sepharose G-200 column. Residual nucleic acid is eluted in the runoff volume. A peak of protease activity follows. All columns must be run at 0.7 M NaCl to avoid aggregation. Early experiments were performed using either the 0.7 M NaCl extract or the enzyme as purified by one pass through a molecular sieve column.

Assay.

Histone protease is assayed by the following method: an incubation mixture (0.2 ml) containing enzyme, exogenous histones (either whole purified rat liver histones or chromatin), salts, and buffer is incubated for 4 hours at 37°. At the end of this time 0.5 ml of ninhydrin reagent is added to the tube. The assay tube is then capped, put in a boiling water bath for 4 min, and then cooled in ice; subsequently the sample is read at the maximum, 506 mu. Zero incubation time samples are used as controls to subtract the original ninhydrin positive response. As shown by standard curves utilizing amino acids, the assay is linear over a considerable range (0 -1 mM), although the extinction coefficient varies by a factor of two (or less) depending on the amino acid (excluding proline) (see Figure 1). The ninhydrin reagent is that of Tsarichenko (1966) (i.e., 0.4 gm ninhydrin in 80 ml 95% ethanol mixed with 1.25 gm $CdCl_2 \cdot 2\ 1/2\ H_2O$ in 20 ml water and added to 10 ml acetic acid). This assay appears superior to others used for the investigation of protease activity in that it measures both the liberation of amino acids and the creation of small or large peptides. It also involves a minimum of manipulative operations, thus reducing overall error.

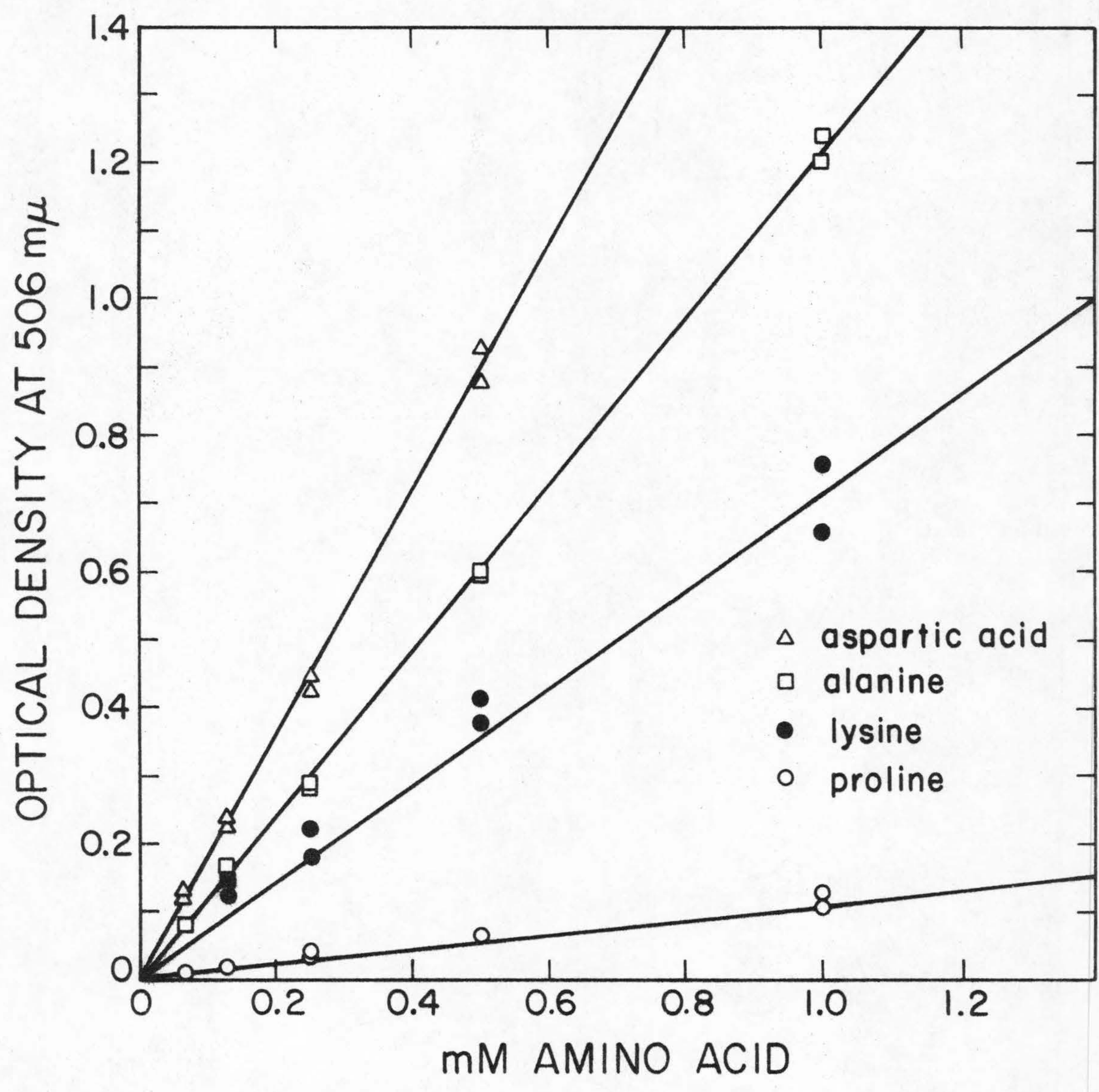
General methods.

Whole rat liver histones were prepared for substrate by acid extraction of chromatin (Bonner *et al.*, 1968a). Other substrate proteins used were from Calbiochem (cytochrome c, myoglobin) or from Sigma (lysozyme, ribonuclease, bovine serum albumin).

Figure 1: Standard curves, ninhydrin assay.

Reaction carried out as described under Methods.

Samples in 0.01 M Tris, pH 8.


OPTICAL DENSITY AT 506 mμ
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
1.2
mM AMINO ACID
aspartic acid
alanine
lysine
proline

RESULTS

Histone protease activity is readily obtained from rat liver chromatin and is extracted by relatively low salt concentrations (see Table I). The enzyme shows high specificity in the degradation of exogenous proteins; it is clearly most active with whole histone as a substrate (Table II). Chromatin is also a very successful substrate, although it contains the enzyme and shows high self activity. Preliminary results indicate that the enzyme has low activity on exogenous histone I. The pH optimum of the enzyme appears to be ca. 8, while there is a drastic reduction in activity below ca. 6.8. In these characteristics the enzyme appears similar to the activities in calf thymus previously reported (see Introduction, this chapter); however, we do not observe the salt dependence observed by Furlan and Jericijo (1967a) and the behavior of the enzyme during Sephadex chromatography suggests a larger molecular weight. The enzyme is stable for at least a month (when stored on ice); it is inactivated by treating at 100°C.

DISCUSSION

It is of interest to consider the basis of specificity of histone protease. The low activity with lysozyme or cytochrome c as substrates eliminates the idea that the protease attacks basic proteins in general. Specificity could not be directed towards a specific bond since virtually all amino acid bonds are present in the vast majority of proteins. It is possible that a small section of protein, such as the classical sequence of four basic amino acid

Table I. Protease Extraction

Salt Concentration	Relative Specific Activity[1]	
	Pellet	Supernatant
0.3 M	34	43
0.4 M	26	71
0.7 M	0	100

[1] Assay carried out in 0.1 N NaCl, 0.033 M sodium citrate (pH 7.8); calf thymus histone (Calbiochem) used as substrate.

Table II. Histone Protease

Substrate	Relative Specific Activity[1]	pI[2]
Whole histone (rat liver)	100%	>9
Lysozyme	1%	11.0, 11.2
Myoglobin	0%	6.99
Bovine serum albumin	0%	4.7, 4.9
Cytochrome c	1%	9.8, 10.1
Ribonuclease	9%	
Protamine	8%	11.7, 12.1

[1] Reaction carried out in 0.3N NaCl, 0.01 M Tris (pH 8)

[2] Young, 1963.

residues followed by one aliphatic residue, which occurs in all the histones, is the signal for specificity. However, it should be noted that this sequence also occurs frequently in the protamines. Our enzyme shows ca. 10% activity with protamine as the substrate relative to histone. Preliminary reports from Dixon's group indicate that they have isolated, by similar techniques, a crude enzyme preparation from trout sperm chromatin which is specific for histone and does not degrade protamine (Marushige and Dixon, 1971). The two enzymes appear to be similar; thus this sequence could not be the key to specificity. Alternatively, the specificity observed could be the consequence of the relatively open structure of histones as compared to globular proteins. These possibilities are being explored by the use of small peptides, protamines, synthetic poly amino acids, and heat denatured protein for substrates. The relative activities of the enzyme with histone substrate associated with DNA or not, and phosphorylated or not, are also of great interest (see Introduction, this Chapter). Regardless of the outcome of these experiments the facts that a) chromatin contains a protease, b) the histones of chromatin are susceptible to this enzyme, require further consideration of the probability of histone removal in processes of gene regulation.

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Chapter 5

Part II

QUANTITATIVE CHANGES IN RAT UTERINE NONHISTONE CHROMOSOMAL PROTEINS ON STIMULATION WITH ESTROGEN

Introduction

It is now well accepted that one of the relatively early and necessary events in the response of a target organ to hormonal stimulation is an alteration in the amount and kind of RNA transcribed from the chromatin. An increase in RNA synthesis *in vivo* and/or an increase in chromatin template activity *in vitro* have been demonstrated for induction of the immature rabbit uterus with estrogen (Cohen *et al.*, 1964), induction of rat liver with hydrocortisone (Dahmus and Bonner, 1965), and induction of tadpole liver by thyroxin (Kim and Cohen, 1966). Although the changes in chromatin composition which might be anticipated (such as a decrease in the histone/DNA ratio with increasing template activity) are frequently too small to be detected accurately by present analytical methods, the changes in the transcription program are clearly related to the chromatin protein complement; DNA isolated from induced and control chromatins are equally efficient as templates (Dahmus and Bonner, 1965; Kim and Cohen, 1966). Experiments with chromatin to date have implicated the nonhistone chromosomal proteins (NHC proteins) and chromosomal RNA rather than histones as the critical elements in directing specificity of transcription (Bekhor, *et al.*, 1969; Gilmour and Paul, 1969). It is therefore of interest to examine the NHC proteins of a hormone target tissue in the normal and induced state in some detail to look for specific characteristic changes. We have chosen to look at the effect of estradiol stimulation on rat uterine NHC proteins since this system is one of the best characterised.

Methods

Preparation of chromatin and NHC protein

Chromatin was prepared according to the methods given in Teng and Hamilton (1968) (by S. Barth at the University of Texas) from the uterine tissue of normal adult female rats (sample N), ovariectomized female rats (sample O), and ovariectomized female rats which had been given estradiol (20 micrograms per rat) 12 hours previously (sample E). The chromatins were then acid extracted and the pellets solubilized in 1% SDS - 0.05 M Tris, pH 8, and sent to Caltech for processing and gel analysis by the methods in Elgin and Bonner (1970) (see Chapter 2). That such preparations of NHC proteins are quantitatively reproducible is shown by the duplicate analyses of rat liver NHC proteins in Figure 1.

Quantitative SDS gel electrophoresis

Experiments with standard proteins (i.e., cytochrome c and other nonenzymic molecular weight markers (Schwarz/Mann) were used to determine the binding of Coomassie brilliant blue R250 (Schwarz/Mann) to the protein bands. The stained and destained gels were scanned on a Gilford 2000 spectrophotometer at 600 millimicrons using a 0.05 mm slit, and the scan peaks cut out and weighed to obtain the relative peak area. A plot of peak area versus amount of protein is given in Figure 2. It shows that the response is linear in the range of 1-20 micrograms of protein. Beyond this range the curve flattens out to give an overall S shape. This result, however, does allow one to make limited statements about the quantitative changes in relative amounts of given NHC proteins. The amount of dye bound per milligram protein

Figure 1: Duplicate preparations of rat liver NHC proteins analyzed by SDS gel electrophoresis. Gels run left to right.

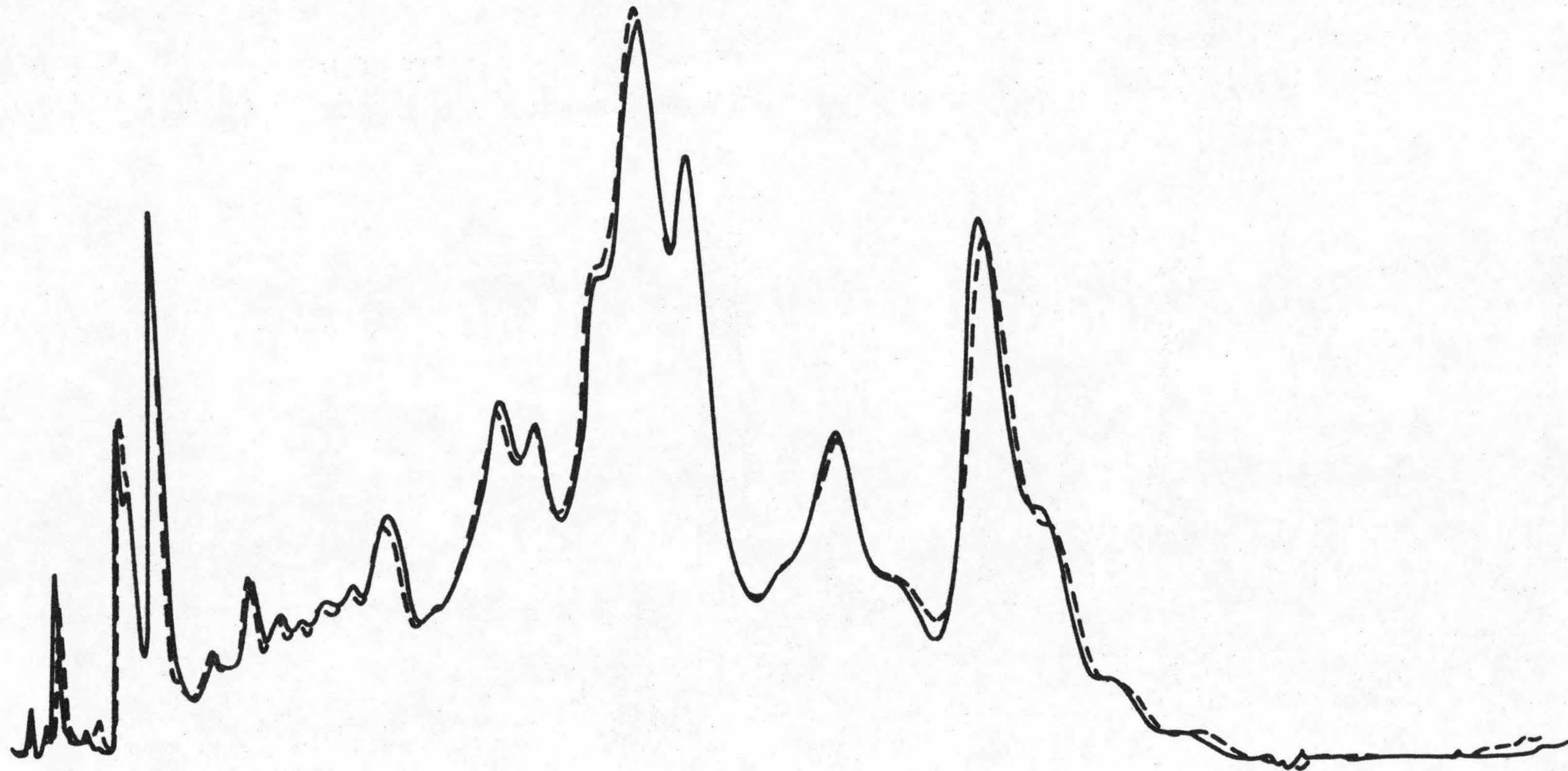
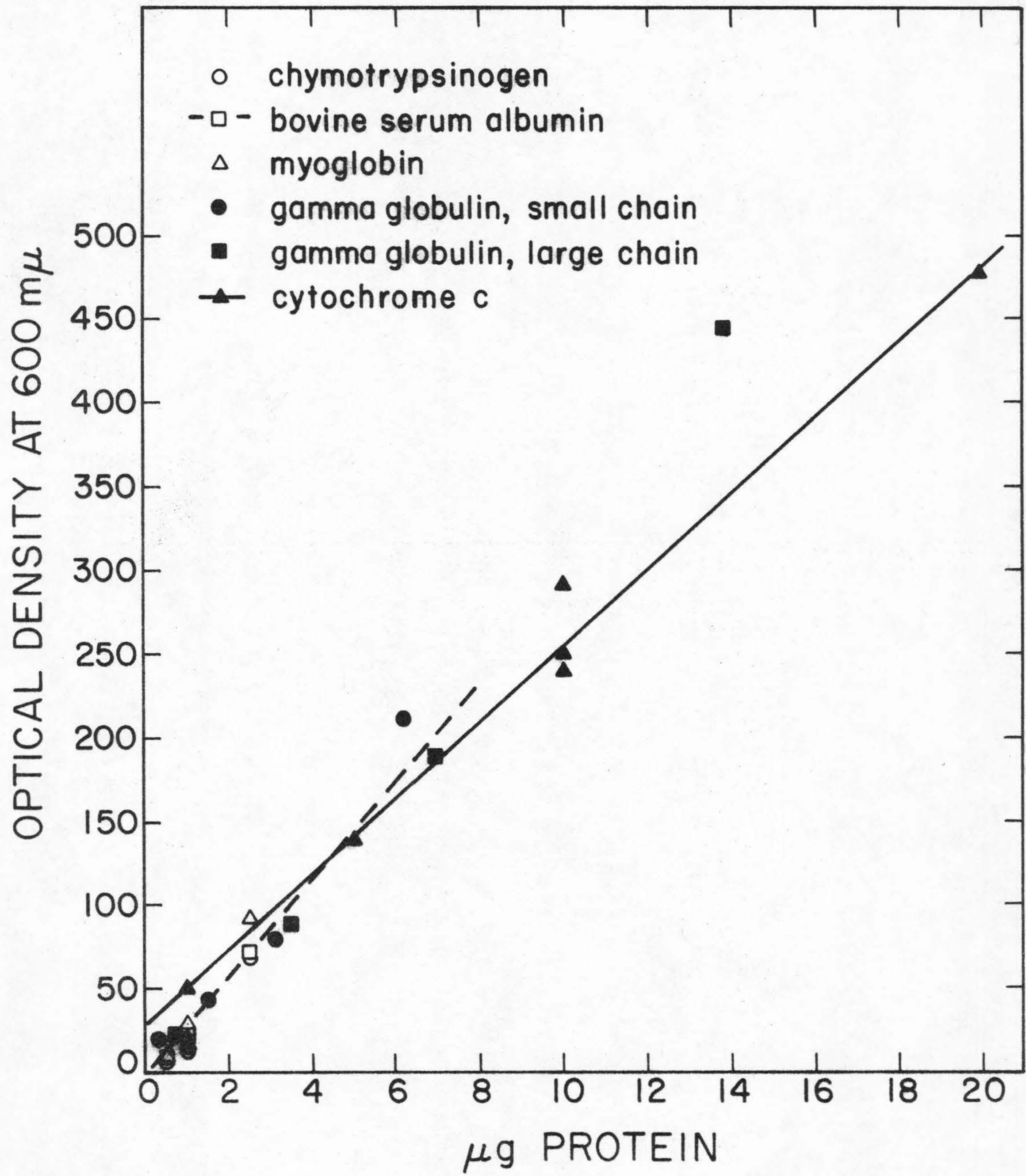


Figure 2: Peak area (arbitrary units) as a function of the amount of protein in scans of SDS gels stained with Coomassie brilliant blue R-250.



chymotrypsinogen
bovine serum albumin
myoglobin
gamma globulin, small chain
gamma globulin, large chain
cytochrome c
OPTICAL DENSITY AT 600 mμ
0
50
100
150
200
250
300
350
400
450
500
0
2
4
6
8
10
12
14
16
18
20
μg PROTEIN

appears to vary by less than a factor of 2 for the proteins examined; however one cannot make any absolute statement comparing relative amounts of different NHC proteins.

Results

Figure 3 presents a comparison of gel scans of the NHC proteins of each of the three uterine tissues described. The following quantitative shifts are observed. The protein ϵ appears to be decreased in sample O and increased in sample E, both in proportion to the band β and as compared to sample N. The band π appears to exhibit a relative increase in the sample O and is again decreased in the sample E compared to sample N. No qualitative changes, i.e., appearance of new bands or disappearance of old, are seen. The reason for the increased amount of lower molecular weight protein indicated by the increased background in sample E is not known.

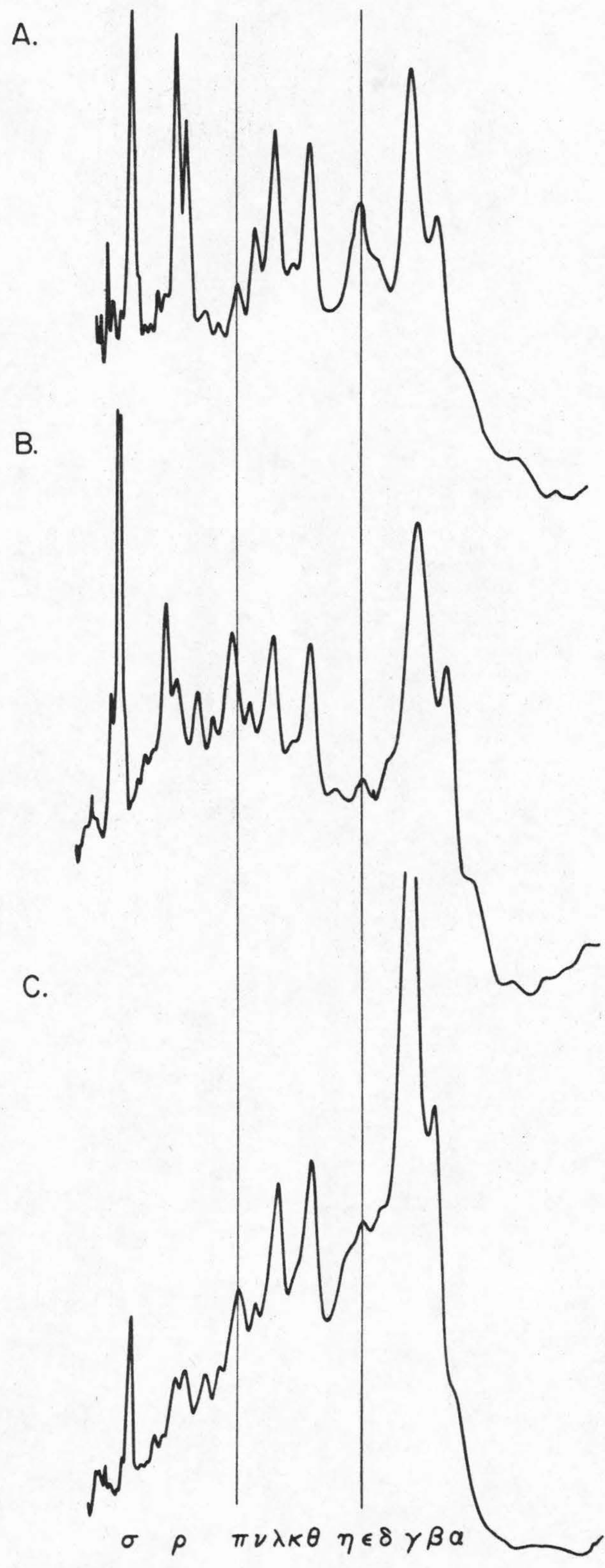
In order to properly evaluate observed relative changes in the amounts of various nonhisone chromosomal proteins it would be necessary to carry out a double-label experiment, i.e., one in which protein is labelled with one isotope in the case of the normal rat and with a second isotope in the case of the ovariectomized rat or ovariectomized rat estrogen-treated. One would also wish to observe the changes over time, since estrogen treatment of the ovariectomized rat is known to elicit a long series of changes in cellular metabolism stretching over at least 24 hours. A necessary control would be to check whether the estrogen treatment elicits any changes in a tissue not considered a target tissue for this hormone, e.g., lung.

Figure 3: A Top: Normal rat uterine NHC proteins

B Middle: Ovarietomized rat uterine NHC proteins.

C Bottom: Ovariectomized, estrogen-treated rat uterine NHC proteins.

SDS gels run left to right.


A.
B.
C.
σ ρ π ν λ κ θ η ε δ γ β α

Discussion

It is difficult to evaluate and fit together all the experimental information on the activation of uterine chromatin by estradiol because of the different animals and different techniques (in administering the hormone, isolating the chromatin, etc.) used by different investigators. The following is a model sequence of events that appears consistent with most of the data: estradiol - 17 β binds to a protein in the cytoplasm of the target tissue, uterus, forming a complex which sediments at 8S. This interaction and subsequent interactions with chromatin do not involve chemical transformation of the hormone. The bound hormone is transferred to the nucleus by a temperature-dependent process; there it is found in a complex, sedimenting at 5 S, which becomes associated with the chromatin fraction. The bound estradiol-receptor complex can be released from chromatin by DNase treatment. (Toft and Gorski, 1966; Maurer and Chalkley, 1967; Jensen et al., 1969; Harris, 1971). This uptake and binding occurs very soon, on the order of 0.5 to 1.0 hour after hormone treatment. On a similar time scale one observes induction of synthesis of at least one soluble uterine protein (Barnea and Gorski, 1970; Mayol and Thayer, 1970), and alterations in chromosomal proteins (reduced amount of arginine-rich histone and increased specific activity of an associated NHC protein) (Barker, 1971). It has been suggested that one or more of these nonhistone proteins may be an estrogen-induced depressor of RNA synthesis. Church and McCarthy (1970), working with rabbits, report an increase in uterine chromatin template activity by two hours with little subsequent increase.

They find that the estradiol-induced RNA synthesis is at least partly organ specific (hybridization competition experiments). They further suggest that regulation is also effected by selective transport of RNA from the nucleus to the cytoplasm, since estrogen-stimulated uteri show a larger amount of rapidly labeled, small size (4 S) RNA restricted to the nucleus. These data might also be interpreted as suggesting a control function in subsequent gene activation for such small, rapidly-labeled nucleus-restricted RNA (see Mayfield and Bonner, 1971, for discussion of an analogous system of gene activation, regenerating rat liver in which this appears to be the case). Teng and Hamilton (1968, 1969), working with rats, observe maximal estradiol binding and maximal _in vitro_ template activity of uterine chromatin at 8-12 hours after hormone treatment. They also observe a general increase in the NHC protein/DNA ratio and an increased specific activity of a specific NHC protein (Teng and Hamilton, 1970). Whether or not two bursts of tissue-specific RNA synthesis occur, one at 1-2 hours and one at 8-12 hours following hormone induction, or whether the time differences for increased RNA synthesis noted are the consequence of differences of animals and techniques is a question that must be answered by more careful experiments. At least part of the new RNA synthesized in response to the hormone passes to the cytoplasm and presumably directs the synthesis of new proteins, thus causing subsequent tissue changes (Means _et al._, 1971). There is also evidence that estradiol causes an enhanced rate of ribosome formation in the uterus (Church and McCarthy, 1970).

Several roles for the NHC proteins are suggested by the above model.

The nuclear hormone-receptor protein could be considered an NHC protein itself; however, present estimates of the number of binding sites for the hormone-receptor complex per nucleus indicate that there is not enough of this protein to observe by the gross analytical techniques applied here. It has been shown in the case of the progesterone-receptor complex that its specificity of binding to chromatin (ca. a 10-fold enhancement) is dictated by the NHC proteins as opposed to the histones (Steggles et al., 1971; Spelsberg et al., 1971). Thus the tissue-specific NHC proteins observed are good candidates to be involved in such interactions (see Chapter 2). To date, no tissue-specific NHC proteins have been identified as such with certainty in rat uterine chromatin, partly because of the complexity of the patter (see Figure 3) and partly because not enough tissues have as yet been examined. Finally, the limited heterogeneity of the rat NHC proteins has led previously to the suggestion that these proteins are structural proteins and/or enzymes of chromosomal metabolism. The present observation of changes in the relative amounts of the NHC proteins of the target tissue does indicate that they play a biologically significant role in hormone induction. (Similar results have been obtained for histone I subfractions in the mouse mammary gland system (Stellwagen and Cole, 1969; Hohmann and Cole, 1971)). Fairly specific stimulation of synthesis of a NHC protein of the target tissue in response to hormone has been observed by Teng and Hamilton (1970) (this system) and by Shelton and Allfrey (1970) (cortisol effect on rat liver). If we either obtain further information on the events of chromatin induction in response to hormones, or learn more about the function of the NHC proteins (particularaly ε and π), the

information will obviously help solve both problems. At present, no more can be concluded.

Acknowledgments

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