Studies On
Non-Histone Chromatin Proteins

by

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In accordance with the Regulations of the University of Edinburgh, I hereby certify that the work described in this thesis is my own.
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SUMMARY

The non-histone chromatin proteins are the focus of intense research because of their putative roles both in the specific control of gene transcription and in the structure of chromatin. Studies on these molecules have been hampered by difficulties in the isolation and characterisation of this heterogeneous mixture of proteins.

A high resolution two-dimensional polyacrylamide gel electrophoresis system has been established for the analysis of the non-histone chromatin proteins (NHCP). This involves a separation on a charge basis in an isoelectric focusing rod gel followed by a separation on a molecular weight basis in a sodium dodecyl sulphate polyacrylamide slab gel. Using this system the NHCP of mouse liver have been resolved into above one hundred discrete protein spots in a reproducible manner. The NHCP of mouse cells in culture have also been examined by labelling of cells with $^{35}$S-methionine and subsequent fluorography of gels.

This two-dimensional analysis has been used in the examination of some current problems in NHCP research. The relationship between the NHCP and the nucleoplasmic proteins of the nucleus has been investigated. It is concluded that differences between these protein classes are quantitative in nature with few qualitative differences. Chromatin has been fractionated into transcriptionally active and inactive portions by a DNase II and salt-precipitation method. The NHCP of inactive chromatin resemble those of whole chromatin. Active chromatin is found to be enriched for specific NHCP and depleted in
histones. Finally a study of the proteins of nuclear ribonucleoprotein complexes has been carried out. While enriched for specific proteins, these complexes contain proteins also found in chromatin. The significance of these findings in relation to theories on the roles which NHCP play in chromatin is discussed.
CHAPTER I

INTRODUCTION
INTRODUCTION

Chromatin is defined as the interphase form of chromosomes; thus the terms chromatin and chromosomes define the same material at different stages of the cell cycle. While various methods of isolation yield chromatin of widely differing compositions, it is now generally accepted that the constituents of chromatin are DNA, its associated proteins - the histones and non-histone chromatin proteins, RNA and some lipid. In order to place the non-histone chromatin proteins in context, a brief description of the nature of the other components of chromatin is presented before a fuller discussion of these molecules.

DNA

The eukaryotic cell nucleus contains large amounts of DNA. The haploid mouse genome, for example, contains 2.8 picograms ($1.8 \times 10^{12}$ daltons) of DNA (Laird, 1971). Evidence from known rates of mutation and evolution suggest that this large amount of DNA is far in excess of that required for genes whose nucleotide sequences code for proteins (Ohta and Kimura, 1971; Ohno, 1972).

Much of this apparent large excess of DNA may be utilised in structural and regulatory functions. DNA may be required both for the packaging of chromatin into the nucleus and in the organisation of chromosomes; the role of chromatin proteins in these processes will be discussed in a later section on the structure of chromatin and chromosomes. Control sequences of DNA may mediate the specific control of eukaryotic gene transcription.
Although each cell of an organism contains the same genetic information, i.e. the same sequences of DNA, differentiated cells use different subsets of this genetic information by transcription of specific subsets of sequences of DNA. It is undoubtedly the possible involvement of the non-histone chromatin proteins in this process which has stimulated interest in these molecules. A later section of this introduction will discuss some of the evidence supporting the hypothesis that the non-histone chromatin proteins effect the specific control of gene transcription.

RNA

Chromatin has been known for many years to contain RNA as well as DNA and protein. The amount of RNA found in chromatin is dependent on both the source tissue or cell type and the chromatin preparation method. The ratio of RNA to DNA can vary from 0.04 - 0.18 depending on these factors (Busch et al., 1975).

Much of this RNA may reflect the products of transcription i.e. nascent RNA chains associated with the DNA template. When chromatin is prepared under conditions of low ionic strength and minimal shear, at least 80% of the newly synthesised RNA is associated with chromatin (Kimmel, Sessions and MacLeod, 1976). These workers present further evidence to suggest that this association is not the result of non-specific binding during chromatin preparation. The chromatin-associated RNA may include immediately post-transcriptional precursor ribosomal RNA and heterogeneous nuclear RNA. Such RNAs are themselves
associated with proteins but these will be discussed in
the Results and Discussion section (Chapter III, Section 3).

Lipid

The lipid content of chromatin also varies with the
preparation method used. Chromatin prepared from whole
cells contains more lipid than that prepared from isolated
nuclei, detergent treatment of nuclei reduces the lipid
content still further (Tata, Hamilton and Cole, 1972).
Although this lipid is commonly regarded as a membrane
contaminant of chromatin, some workers have presented
evidence suggesting a physical attachment of the DNA in
chromatin to the nuclear membrane (see Franke et al., 1973).

Chromatin Proteins

The proteins of chromatin have been divided into two
groups on the basis of their acid-solubility - the histones
and the non-histone chromatin proteins. The basic
nature of the histones renders these proteins easily
solubilised from chromatin by dilute mineral acids; the
proteins left behind have variously been termed the resi-
dual chromatin proteins, the acidic chromatin proteins or
the non-histone chromatin proteins. In this thesis the
term non-histone chromatin proteins is used to designate
all the proteins associated with chromatin which are not
histones.

Histones

The histones consist of five main classes of low
molecular weight proteins which are enriched in the basic
amino acids arginine and lysine. In general they are
found in all eukaryotic cells in a ratio of 1:1 by weight with DNA. The five histone classes show considerable homogeneity throughout species, histone H1 alone showing a moderate degree of tissue and species specificity. Thus suggestions that these molecules might serve as specific repressors of gene transcription would now seem unlikely to be correct. The high conservation of the amino acid sequences of the histones throughout evolution suggests that they may play a structural role in chromatin. Further discussion of this topic will be presented later in this introduction.

**Non-Histone Chromatin Proteins (NHCP)**

**Isolation**

The non-histone chromatin proteins (NHCP) are now most often isolated from chromatin which has been prepared from purified nuclei. The initial isolation of highly purified nuclei serves not only to remove cytoplasmic lysosomes with their high concentrations of degradative enzymes but is also important in reducing the problem of contamination of chromatin preparations with cytoplasmic elements. Up to 60% of the proteins associated with chromatin prepared from whole cells by repeated extraction with 0.14M NaCl are of cytoplasmic origin (Johns and Forrester, 1969). Using \( ^{35}S \)-labelled cytoplasmic preparations it has been estimated that 30% of the NHCP prepared from the chromatin of whole sea urchin embryos are derived from the cytoplasm (Hill, Poccia and Doty, 1971). This result was confirmed by immunological studies (Sevaljevic, 1973). Further experiments in which extraneous labelled cytoplasm was
added during chromatin preparation revealed that chromatin prepared from isolated nuclei contained under 5% contamination by cytoplasmic proteins (Bhorjee and Pederson, 1972; Karn et al., 1974). Thus it seems that the rupturing of nuclei in the presence of cytoplasm can lead to serious artefactual binding of cytoplasmic proteins to chromatin. Indeed experiments in which extraneous labelled cytoplasm is added during chromatin preparation may underestimate cytoplasmic contamination since homologous non-labelled cytoplasmic proteins may bind as contaminants to chromatin before extraneous labelled cytoplasmic proteins can do so.

In a comparative study it was found that chromatin from whole tissues is more contaminated with cytoplasmic membrane material and contains significantly higher levels of protease activity than chromatin prepared from purified nuclei. Purified nuclei washed with Triton X-100 are the least contaminated as judged both from phospholipid analysis and the activities of marker enzymes (Tata, Hamilton and Cole, 1972). It has been claimed that although Triton X-100 removes much of the nuclear membrane lipid, most of the nuclear membrane protein is not removed and is subsequently isolated with NHCP (Jackson, 1976). This work was carried out using avian erythrocyte chromatin which contains little NHCP thus making recognition of contamination more easy; the extent of possible membrane protein contamination in chromatins of tissues containing larger amounts of NHCP is difficult to estimate. Some workers (see Franke et al., 1973)
have argued that chromatin is intimately associated with the nuclear membrane and may share common elements with it - making the distinction between contamination and true in vivo association difficult. To compound the problem NHCP are thought to be synthesised in the cytoplasm prior to their transport to the nucleus (Stein and Baserga, 1971) while cytoplasmic proteins are known to migrate into the nucleus after hormone stimulation (O'Malley, Toft and Sherman, 1971). It is therefore probable that some NHCP will be common to both nucleus and cytoplasm.

MacGillivray et al. (1972) showed that the method of isolation of nuclei not only affects the chemical analysis of chromatin but also causes considerable variation in the composition of NHCP. Nuclei purified in citric acid retained more high molecular weight NHCP than nuclei prepared in sucrose. Treatment of nuclei prepared in sucrose with Triton X-100 removed only a few low molecular weight NHCP, while the double-detergent procedure of Penman (1966) yielded nuclei whose chromatin was generally depleted of protein.

Variation in nuclear isolation produces changes in NHCP composition as does variation in the method of chromatin preparation from the isolated nuclei. The different methods used in the preparation of chromatins from nuclei result in chromatins with characteristic chemical compositions (Busch et al., 1975) and template properties during transcription in vitro (de Pomera, Chesterton and Butterworth, 1974). Included in these
methods are saline-EDTA extraction (Zubay and Doty, 1959), tris-saline extraction (MacGillivray et al., 1972) or a combination of both (Rickwood and Birnie, 1976).

In turn NHCP may be isolated from chromatin by a variety of methods - most of which fall into one of two main classes. The first of these requires the initial removal of histones from chromatin by dilute acid such as 0.25M HCl or 0.2M H₂SO₄. After this step many methods have been used to separate the residual DNA and NHCP. Dehistonised chromatin has been dissolved in sodium dodecyl sulphate (SDS) solutions and the DNA removed by ultracentrifugation (Elgin and Bonner, 1970). Acid-extracted chromatin has been digested with DNase I and the NHCP precipitated with perchloric acid (Wilson and Spelsberg, 1973). Both these procedures yielded high recoveries of NHCP. Other workers have chosen to prepare selective fractions of the NHCP from dehistonised chromatin. Extraction by phenol may remove a variable amount of NHCP dependent on the exact method used (see MacGillivray, 1976), but is widely used in the preparation of phosphorylated NHCP. An important reservation to these techniques is that exposure of chromatin proteins to extremes of pH may cause extensive modifications to these proteins (Chen et al., 1974).

A second approach, which avoids extremes of pH, has been widely adopted in the preparation of NHCP from chromatin. This approach requires the initial solubilisation and dissociation of chromatin, which has been achieved in several ways. Among the first of these was the
dissociation of chromatin in solutions of high ionic strength (1 - 2M NaCl). The DNA and histones could then be co-precipitated by a reduction in the salt concentration to 0.14M (Wang, 1967). The components of sheared and salt-dissociated chromatin could also be separated by a combination of gel filtration and ion-exchange chromatography (Graziano and Huang, 1971). Similar separation procedures using guanidine hydrochloride to assist in the dissociation of chromatin have also been attempted (Hill, Poccio and Doty, 1971). 5M urea - 2M NaCl dissociated chromatin has been fractionated by a one-step hydroxyapatite column chromatography method to yield NHCP fractions (MacGillivray et al., 1972). Other groups have used various concentrations of salt (guanidine HCl as well as NaCl) and urea to dissociate chromatin followed by various combinations of methods (centrifugation, ion-exchange chromatography, gel filtration) to separate the chromatin components. An excellent account of such methods is given by MacGillivray (1976). A general drawback to many of these procedures is the lengthy chromatography centrifugation and dialysis steps involved during which the prevention of proteolysis is essential.

With the above discussion in mind, the criteria for an idealised NHCP isolation method can be designated. The ideal method should recover all those NHCP which are associated with chromatin in vivo and exclude any cytoplasmic protein contamination. No proteolysis or modification of the NHCP should take place during their
isolation. Unless a specific fraction of the NHCP is required, the final recovery of NHCP should be total in order that subsequent analysis would yield results representative of the total NHCP. Such an idealised procedure is probably unattainable in absolute terms but practical isolation methods should attempt, as far as possible, to satisfy these criteria.

**Characterisation**

Amino acid analyses of the NHCP isolated by the above techniques confirm the overall acidic nature of these molecules, the ratio of acidic to basic amino acid residues being in the range 1.2 - 1.9. Thus the NHCP are enriched in the amino acids aspartic and glutamic acid. The NHCP also contain tryptophan (Augenlicht and Baserga, 1973; Chaudhuri, 1973), an amino acid which is absent in histones. Other NHCP components show high amounts of both acidic and basic residues (Goodwin and Johns, 1973) while a NHCP which shows extensive sequence homology with histone 2A has recently been described (Goldknopf and Busch, 1975). This protein has been shown to be a conjugate protein containing an isopeptide linkage between non-histone and histone 2A polypeptides (Goldknopf and Busch, 1977).

The technique in universal use for the characterisation of NHCP is that of polyacrylamide gel electrophoresis. Initial separation attempts in non-detergent polyacrylamide gel electrophoresis systems utilised a variety of conditions including extremes of pH and the addition of urea (e.g. Hill, Poccia and Doty, 1971; Benjamin and Gellhorn, 1968).
However aggregation of NHCP led to a significant proportion of NHCP being deposited as it entered the gel. To overcome this difficulty most workers resorted to electrophoresis in the presence of the ionic detergent SDS. This technique separates proteins on a molecular weight basis since proteins bind similar amounts of negatively-charged detergent per unit mass. A wide variety of buffers, acrylamide concentrations, reducing and denaturing agents have been used for the analysis of NHCP in this system.

A few groups have also attempted to analyse the NHCP by isoelectric focusing (Sevaljevic and Stamenkovic, 1972; Elgin and Bonner, 1972; Arnold and Young, 1972). Proteins are separated on a charge basis by this technique. Under the influence of an applied current a pH gradient is established by small carrier ampholytes in a polyacrylamide gel, the proteins migrating through this gradient until reaching their isoelectric point.

One-dimensional polyacrylamide gel electrophoresis by these techniques, while greatly advancing the analysis of the NHCP, has not proved totally satisfactory. One protein band may represent several polypeptides of similar molecular weight or isoelectric point. Another drawback is often the high background underlying the separation of individual protein bands. This background may represent minor species of proteins as well as overlapping of protein bands. In order to overcome these problems some workers have advanced to electrophoresis in two dimensions.

Two-dimensional polyacrylamide gel electrophoresis is usually accomplished by the initial separation of the proteins in a rod gel followed by a second separation achieved by placing the rod gel on top of a slab gel. Different systems are used in each dimension to give
different separations. One approach has been to separate the NHCP in an acid-urea gel in the first dimension followed by electrophoresis in SDS in the second dimension (Yeoman et al., 1973). Both electrophoretic procedures separate the proteins largely on a molecular weight basis and hence the polypeptide spots are restricted to a diagonal area running across the slab gel. This method thus represents only a slight improvement on one-dimensional techniques. A similar approach has been the adaptation of the Kaltschmidt two-dimensional electrophoresis system (originally developed for ribosomal proteins) in the examination of DNA-binding proteins (Jost, Lennox and Harris, 1975).

A more useful method has been the separation of NHCP on a different basis in each dimension. This is most easily achieved by a first-dimension separation on a charge basis in an isoelectric focusing rod gel followed by a second dimension separation on a molecular weight basis in a SDS polyacrylamide slab gel. Several groups have used variations on this theme in the analysis of NHCP (MacGillivray and Rickwood, 1974; Barret and Gould, 1973; Jackowski, Suria and Liew, 1976).

The NHCP characterised by both one- and two-dimensional systems have been revealed as highly heterogeneous with a wide spectrum of molecular weights and isoelectric points. A range of molecular weight values within 5,000 - 200,000 is generally accepted (Elgin and Bonner, 1970; MacGillivray et al., 1972; Le Stourgeon and Rusch, 1973; Wu, Elgin and Hood, 1973). Several groups
consider the NHCP to have a range of isoelectric points from pH3 - pH10 (Sevaljevic and Stamenkovic, 1972; Elgin and Bonner, 1972; Arnold and Young, 1972; MacGillivray and Rickwood, 1974) while others consider the majority of these proteins to have pIs within the range of pH4 - pH8 (Barret and Gould, 1973; Lea, Koch and Morris, 1975; Bhorjee and Pederson, 1976a; Peterson and McConkey, 1976).

Polyacrylamide gel electrophoresis techniques have thus provided much valuable information on the NHCP and are still being refined to provide improved analyses of this heterogeneous mixture of proteins. The major species of NHCP are confined to relatively narrow molecular weight and isoelectric point ranges and hence two dimensional techniques, in which the proteins are separated by two different criteria, hold the greatest potential value. Essential to such techniques are high resolution (by which modifications in NHCP may be detected) and good reproducibility. Recently O'Farrell (1975) has described a two-dimensional (isoelectric focusing and SDS) polyacrylamide gel system capable of very high resolution of *Escherichia coli* proteins in a reproducible manner. By this system he was able to resolve 1,100 different protein components and to detect proteins differing in a single charge. The application of this system to the analysis of the NHCP will form a major part of the work described in this thesis.

**NHCP-Synthesis and Metabolism**

Research has been carried out into the synthesis and metabolism of chromatin proteins not only out of interest
in these processes per se but also in the expectation that such research would give further insight into the roles of these proteins in chromatin structure and gene transcription. It has been recognised for some time that the synthesis of histones is restricted to the S phase of the cell cycle and is coupled to the replication of DNA, being inhibited when DNA replication is inhibited (Spalding, Kajiwara and Mueller, 1966; Robbins and Borun, 1967).

In contrast, NHCP synthesis has been demonstrated to continue throughout the cell cycle (Borun and Stein, 1972). Inhibitors of DNA replication such as cytosine arabinoside and hydroxyurea do not reduce the rate of incorporation of labelled amino acids into NHCP during S phase (Stein and Borun, 1972). Variations in the rate of NHCP synthesis do occur throughout the cell cycle and, on the basis of a significant increase in the synthesis of NHCP during the prereplicative G1 phase of the cell cycle, it has been postulated that there is indeed a link between DNA replication and NHCP synthesis (Gerner, Meyn and Humphrey, 1976).

Increased rates of synthesis of NHCP during the G2 as well as the G1 phase of the cell cycle have also been observed (Borun and Stein, 1972).

It is possible that the synthesis of NHCP may, in part, be regulated at the translational level. In quiescent cells stimulated to proliferate, treatment with Actinomycin D, which completely blocks mRNA synthesis, has been found ineffective in reducing the increased rate of NHCP synthesis which occurs during G1, while synthesis of these proteins in later stages of the cell cycle is sensitive
to this treatment (Stein and Baserga, 1970). Thus it seems that the initial synthesis of NHCP in cells stimulated to proliferate occurs from preformed stable mRNA species.

The rate of turnover of the NHCP is more controversial. A high rate of turnover has been ascribed to these proteins by some workers (Borun and Stein, 1972; Tsanev, Djonjurov and Ivanova, 1974). On the other hand, Seale (1975) found the majority of the NHCP as metabolically stable as the histones and DNA, while not excluding the possibility of more rapid turnover of a subpopulation of NHCP. It is likely that differences in the protocols of the pulse-label and chase experiments of these workers may account for the differing conclusions reached.

The NHCP, in common with the histones, are subject to post-synthetic modifications. Many of the NHCP are known to be phosphorylated and evidence has been accumulated linking non-histone chromatin phosphoproteins with the control of gene transcription (see later and Kleinsmith, 1975). The extent of other modifications, such as methylation and acetylation, is largely undetermined.

The evidence on the synthesis of histones points clearly to a structural association of these proteins with DNA. No such intimation can be easily drawn from the accumulated evidence on the synthesis of NHCP - possibly reflecting multiple roles for this class of proteins in chromatin.

**NHCP and the Control of Gene Transcription**

In prokaryotes the topic of control of gene transcription has proved amenable to combinations of biochemical and
genetical analyses. It is well established that many genes in these organisms are clustered into operons to facilitate their co-ordinate transcription. In the lactose operon of *E. coli* a specific repressor protein can be isolated which regulates DNA transcription by binding to a specific site on the bacterial DNA (Gilbert and Mueller-Hill, 1966). Although a direct analogy is untenable because there is no evidence for clustering of genes in eukaryotic organisms (with the exception of histone genes) the eukaryotic model can provide a base from which research into eukaryotic gene transcription may be viewed. The DNA of eukaryotic chromatin is complexed with proteins - with the prokaryotic model in mind, the search for control elements in chromatin has been focused on these proteins - the histones and the NHCP.

The five major classes of histones are highly conserved throughout evolution, showing little tissue or species specificity. These molecules are therefore now considered unlikely to serve as the specific control elements in gene transcription (see Spelsberg, 1972 for a review). The histones, however, do play a fundamental role in the basic subunit structure of chromatin (see later) and have been shown to inhibit the ability of DNA to serve as a template for RNA synthesis *in vitro* (Huang and Bonner, 1962; Alifrey, Littau and Mirsky, 1963).

Experiments have also been carried out to determine the effect of the NHCP on transcription *in vitro*. The binding of rat liver chromatin acidic proteins to liver DNA resulted in a stimulation of template activity for
RNA polymerase as compared to DNA alone. (Teng, Teng and Alifrey, 1971; Shea and Kleinsmith, 1973). Loosely-bound NHCP (prepared by extraction of chromatin with 0.35M NaCl) from Ehrlich Ascites Tumour cells contain a fraction that specifically binds to Ehrlich Ascites Tumour DNA and exhibits a template- and RNA polymerase-specific enhancing effect on transcription from DNA (Kostraba, Montagna and Wang, 1975). In the same system the preparation of a NHCP which binds to DNA and inhibits transcription of DNA in vitro by the homologous RNA polymerase has been described (Kostraba and Wang, 1975). Thus both positive and negative effectors of in vitro transcription of DNA have been isolated.

The first experiments to suggest that the NHCP might effect the specific control of gene transcription were those of Paul and Gilmour (1968; Gilmour and Paul, 1969, 1970). Chromatins of rabbit organs were fractionated into DNA, histones and NHCP and reconstituted with homologous and heterologous NHCP components (Gilmour and Paul, 1970). The chromatin was then transcribed by E.coli RNA polymerase and the nucleotide sequences of the transcribed RNA examined by the hybridisation tests then available. By these techniques only those RNA sequences transcribed from highly re-iterated DNA could be examined; these RNA sequences do not appear to be present in the cytoplasm mRNA population of the cell in vivo. Nevertheless these experiments did show that a substantial proportion of the RNA sequences transcribed from highly-reiterated DNA in vitro is organ-specific and that the NHCP in some
way determine the specificity of transcription.

With the discovery of viral reverse transcriptase (RNA-dependent DNA polymerase) it became possible to synthesise a complementary DNA to a purified mRNA and to use this as an extremely sensitive probe to measure the concentration of sequences specific to that mRNA in chromatin transcripts. Chromatin isolated from erythropoietic tissues, but not from other tissues, was shown to be capable of serving as a template for the synthesis of globin-specific sequences in vitro (Gilmour and Paul, 1973; Axel, Cedar and Felsenfeld, 1973; Barret et al., 1974). Reconstitution experiments of the type described above were then repeated using cDNA as a probe for globin mRNA to detect transcription of the globin gene. By this method it was demonstrated that NHCP from erythropoietic tissues are specifically required for the expression of globin genes (Paul et al., 1973; Barret et al., 1974).

Similar experiments have been carried out in other systems. Histone mRNA is synthesised only in S phase of the cell cycle and using a cDNA probe to histone mRNA it was shown that only chromatin reconstituted with S-phase NHCP synthesised histone mRNA - chromatin reconstituted with G1 NHCP did not (Park et al., 1976). Tsai et al., (1976) using the ovalbumin gene system showed that the NHCP of chromatin prepared from chick oviduct stimulated by oestrogen are capable of specifically inducing the transcription of the ovalbumin gene in chromatin from chick oviduct which had been stimulated and then withdrawn from
hormone treatment.

While these demonstrations in different systems by several groups strongly indicate a role for the NHCP in the specific control of gene transcription, several technical details complicate the interpretation of the results of these experiments. The fidelity of transcription by exogeneous E.coli RNA polymerase is uncertain as is the fidelity of the reconstitution process itself. Contamination by endogenous mRNA sequences has proved a problem - in this respect the preparation of RNA-free NHCP is essential.

Further evidence linking the NHCP with the specific control of gene transcription is of a more correlative, less direct, nature. Changes in the polyacrylamide gel electrophoresis patterns of the NHCP have been observed in normal and abnormal growth (see Baserga, 1974 for a review of this topic); after virus transformation (Krause, Kleinsmith and Stein, 1975; Gonzalez and Rees, 1976) and hormone stimulation (Kadokama and Turkington, 1974; Cohen and Hamilton, 1975; Hemminki, 1976a); throughout embryonic development (Poccia and Winegardner, 1975) and differentiation of one cell type (Ruiz-Carrillo et al., 1974). It should be stressed, however, that it is important to distinguish between those changes in NHCP which may represent the cause of specific gene transcription (e.g. the synthesis of regulatory molecules) and those which may represent its effects. Thus changes in the polyacrylamide gel pattern of chromatin proteins after changes in gene transcription may reflect increased amounts
of those proteins which both bind to the nascent RNA and co-isolate with the NHCP (e.g. Pederson, 1974). In this respect characterisation of the proteins of nuclear ribonucleoprotein particles is essential. The actual changes in the population of NHCP effecting the specific control of gene transcription may also be below the level of resolution of the polyacrylamide gel systems at present used in the analysis of NHCP.

The specific control of gene transcription could also be effected by modifications to pre-existing NHCP rather than new or increased synthesis of NHCP. Several lines of evidence suggest that non-histone chromatin phosphoproteins may play an important role in the control of gene transcription. These proteins, like the total NHCP, are heterogeneous and are thought to be tissue-specific. They have been correlated with changes in gene activity in many instances (see Kleinsmith, 1975 for a summary). A purified NHCP phosphatase (showing no proteolytic activity) has been used to selectively dephosphorylate these proteins prior to chromatin reconstitution (Kleinsmith, Stein and Stein, 1975). Using proteins and DNA obtained from HeLa cell chromatin prepared from synchronised S-phase cells, it was found that dephosphorylation of the NHCP resulted in approximately a 50% reduction in the number of template sites available for the initiation of transcription. In addition, using a cDNA probe to histone mRNA, it was shown that dephosphorylation of NHCP specifically inhibited the ability of the histone genes to be transcribed. Thus phosphorylation of NHCP was directly implicated in the
control of gene transcription.

As a result of the accumulated mass of evidence of this nature, the highly heterogeneous mixture of proteins known as the NHCP are thought by many workers to contain elements which effect the specific control of gene transcription.

NHCP and Chromatin Structure

It is now well established from both nuclease digestion and electron microscopy studies that the basic subunit of chromatin is the nucleosome (for a review, see Elgin and Weintraub, 1975). The nucleosome consists of about 200 base pairs of DNA and 1 pair each of histone types H2A, H2B, H3 and H4. The eight histone molecules form an octamer, or possibly two tetramers, around which the DNA is wound. It seems that about 140 base pairs of DNA are closely associated with the histone core to form the core particle, while the remaining 60 base pairs are less closely associated, some of this DNA forming the 'string' which joins the 'beads' or nucleosomes. The remaining histone, histone H1, is thought to be associated with this internucleosomal DNA. The exact lengths of DNA in the core and string may vary from organism to organism as well as in different transcriptional states of the chromatin of any one organism.

With regard to this basic background of chromatin structure, very little evidence has been gathered as to the involvement of the NHCP. The isolated chromatin monomer (i.e. single nucleosomes) of chicken erythrocyte chromatin contains only histones (Oudet, Gross-Bellard and
Chambon, 1975) but this material, which is depleted of histone H1, initially contains little or no NHCP. Using chromatin from a human colon carcinoma cell line, a single distinct NHCP of high molecular weight was found (in addition to the usual histones) in the monomer fraction (Augenlicht and Lipkin, 1976).

Evidence of a more extensive association of NHCP with nucleosomes has been presented. Chromatin can be fragmented both by shearing and by micrococcal nuclease digestion into nucleosome-like bodies which are heterogeneous: one protein-rich class containing NHCP (Paul and Malcolm, 1976). Similarly characterisation of subfractions of purified monomeric nucleosomes from micrococcal nuclease digests of rat liver nuclei showed that, while containing the same size of DNA fragments, the subfractions differed in composition of histone H3 and several NHCP (Sanders and Hsu, 1977). It has also been claimed that different groups of NHCP are associated with nucleosomes and with the internucleosomal DNA (Liew and Chan, 1976). Goodwin, Woodhead and Johns (1976) have detected by polyacrylamide gel electrophoresis a NHCP known as HMGl (HMG - high mobility group) on isolated nucleosomes. HMGl is known to interact with histones and gives an example of the possible complications which may arise in the above experiments, i.e. that association of NHCP with isolated nucleosomes may reflect artefactual binding during the isolation procedure. The converse may also apply - NHCP may be lost from nucleosomes during the isolation procedure. The distribution of the enzyme
Poly(adenosine-diphosphate-ribose) polymerase after nuclease digestion suggests that nucleosomes may lose proteins during isolation (Mullins, Girl and Smulson, 1977). This enzyme is a tightly bound chromatin protein requiring 1M salt for dissociation. After digestion of chromatin much of the enzyme's activity is found in the non-sedimenting region of the sucrose gradient, the remainder being found in oligomers of nucleosomes. The eventual acceptors of the ADP-ribosylation are, however, the proteins of the nucleosome itself, implying an association of the enzyme with the nucleosome. It would seem, therefore, that the association of this enzyme with chromatin requires a higher order of structure than the nucleosome itself.

Evidence as to the possible involvement of NHCP in higher order structuring of chromatin is insubstantial. Indications that NHCP may be involved at this level of chromatin structure come mostly from physical studies. Changes in the circular dichroism (CD) spectra and thermal melting profiles of guinea pig liver DNA reassociated with histones and/or NHCP from cerebral or liver chromatin have been investigated (Tashiro and Kurokawa, 1975a). The characteristics of the CD spectrum of native chromatin are most satisfactorily reproduced in complexes containing NHCP as well as DNA and histones. In addition the DNA of these complexes is thermally stabilised to an extent comparable with the DNA of native chromatin. It is concluded that although the basic conformation of DNA in chromatin is largely determined by histones, the NHCP
also play an individual role. The pre-melting phenomenon in the CD melting curves of DNA and chromatin has also been examined (Xavier-Wilhelm, de Murcia and Daune, 1974). Chromatin does not show the characteristic pre-melting profile of DNA alone, but when the NHCP and the slightly-lysine-rich histones are dissociated pre-melting is observed. The absence of this phenomenon in chromatin is suggested to be a result of interaction of the NHCP and these histones.

Changes in NHCP content have been linked with changes in chromatin conformation. The chromatin of quiescent WI-38 cells when stimulated to proliferate shows a changed NHCP profile (Baserga, 1974) and undergoes a conformational change as judged by changes in CD spectra. The changes in CD spectra are abolished when chromatin is treated with 0.25M NaCl (removing mainly NHCP) but can be restored after reconstitution of salt-dissociated chromatin, again indicating a role for NHCP in chromatin conformation (Nicolini, Ng and Baserga, 1975).

Further evidence indicating a possible involvement of the NHCP in the higher order structuring of chromatin and chromosomes comes from cytogenetic observations. The N-banding technique has been used to define the location of the nucleolus organis in Xenopus laevis chromosomes (Matsui, 1974). Chemical extractions demonstrate that these N-bands show the typical biochemical properties of NHCP while comprising a small portion of the total nuclear protein. The N-bands show consistent morphology, number and size throughout interphase chromatin and mitotic
chromosomes and hence may represent structural NHCP of the ribosomal genes. A technique of great potential value in the analysis of NHCP and chromatin structure is that of Silver and Elgin (1976). Using immunofluorescent methods, these workers were able to band to the chromomere level the polytene chromosomes of Drosophila with antisera against NHCP. This method holds great promise for determining the in situ distribution of NHCP.

On the basis of dansyl-fluorescence of acid-extracted mouse metaphase chromosomes it has been suggested that particular NHCP are associated with the centromeric constitutive heterochromatin of mouse chromosomes (Matsukuma and Utajoki, 1977). When Drosophila somatic nuclei (with large amounts of heterochromatin) are compared to the polytene chromosomes of salivary glands (with little heterochromatin) two NHCP are found to be unique to constitutive heterochromatin (Elgin et al., 1974). In contrast Comings et al. (1977) have biochemically isolated the condensed chromatin of both mouse and Drosophila virilis and found no major NHCP unique to the satellite-rich heterochromatin fractions.

It is a general finding of studies attempting to fractionate chromatin into transcriptionally active and inactive fractions that the active fraction is enriched for NHCP (e.g. Benjamin and Gellhorn, 1968; Marushige and Dixon, 1969; Simpson and Reeck, 1973; Gottsfeld et al., 1974). There is some evidence which suggests active and inactive chromatin does show physical differences in vivo. Autoradiographic studies of $^{3}H$uridine-labelled
amphibian oocytes have shown that RNA synthesis is associated only with the extended fibrils of lampbrush chromosomes (Callan, 1969) and is confined to the puffs in the polytene chromosomes of insects (Berendes and Beerman, 1969). Electron microscopy of transcribing genes reveals that the length of a transcriptional unit (2 - 3µ) of ribosomal RNA genes is in close agreement with the precursor rRNA molecule synthesised in amphibians (Miller and Beatty, 1969). Thus the DNA of actively transcribing genes may not be packaged into nucleosomes as is the bulk of the DNA. Some doubt exists as to whether these differences are maintained in isolated chromatin. A fuller discussion of this topic will be presented in Chapter III.

Other Roles for NHCP in Chromatin

Contained within the class of nuclear non-histone proteins are many nuclear enzymes (see Busch et al., 1975 for a summary). As well as the aforementioned poly(adenosine-di-phosphate-ribose) polymerase, these include enzymes involved in DNA and RNA metabolism and others involved in the various modifications of nuclear proteins. Many of these enzymes may be integral components of chromatin, others may be only transiently associated with chromatin making their definition as NHCP more tentative. The relationship between the NHCP and the proteins of the nucleoplasm will be further discussed in the Results and Discussion section, Chapter III. Further research on the enzymic activities of the NHCP will require non-denaturing preparatory methods for these proteins which are at present
subjected to very often severe treatments during their purification and characterisation.

Aims of the Project

The initial aim of the project was to establish a high resolution polyacrylamide gel system for the analysis of the NHCP. In this respect it seemed that two-dimensional systems in which the proteins were separated by two different parameters held great promise. It was decided that the system of O'Farrel (1975), in which proteins are initially separated by charge in an isoelectric focusing gel and then by molecular weight in an SDS-polyacrylamide gel, should be investigated with a view to its use in the analysis of the NHCP.

Such a reproducible, high-resolution two-dimensional polyacrylamide gel system would allow a more detailed analysis of the NHCP thus yielding further information on the possible roles of these proteins in chromatin. The first question to be answered was on the identity of the NHCP and their relationship to other non-histone proteins present in the nucleus. An improved detection of both qualitative and quantitative differences in the NHCP complement of transcriptionally active and inactive chromatin could also be undertaken. This would provide valuable information on the nature of, and differences between, these fractions of chromatin. Finally the analysis of the proteins of the nuclear ribonucleoprotein complexes is of interest not only for its own sake but also in providing information as to the nature of the proteins associated with chromatin via RNA.
conjunction with the chromatin fractionation studies a comparison of the proteins associated with nuclear RNA and with actively transcribing chromatin could then be made.
CHAPTER II

MATERIALS AND METHODS
<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>DESCRIPTION</th>
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<tr>
<td>NHCP</td>
<td>non-histone chromatin proteins</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>nRNP</td>
<td>nuclear ribonucleoprotein complexes</td>
</tr>
<tr>
<td>DNase I</td>
<td>deoxyribonuclease I (E.C. No.3.1.4.5.)</td>
</tr>
<tr>
<td>DNase II</td>
<td>deoxyribonuclease II (E.C. No.3.1.4.6.)</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease A (from bovine pancreas)</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethylpropane-1,3-diol</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonidet P-40</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>bisacrylamide</td>
<td>NN' methylenebisacrylamide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid (tetra sodium salt)</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-Di-(2-(4-methyl-5 phenyloxazoly1))-benzene.</td>
</tr>
<tr>
<td>IF</td>
<td>isoelectric focusing</td>
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<tr>
<td>2D-PAGE</td>
<td>two-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>1D-PAGE</td>
<td>one-dimensional polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
MATERIALS

L-$^{35}$S\textsuperscript{7} methionine (272 mCi/mmol), L-$^{14}$C\textsuperscript{7}thymidine (456 mCi/mmol), L-$^{3}$H\textsuperscript{7}thymidine (26 Ci/mmol) and L-$^{3}$H\textsuperscript{7} uridine (3.3 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Acrylamide and bisacrylamide were supplied by BDH Chemicals Ltd. and were purified by recrystallisation from chloroform and acetone respectively.

SDS was supplied by BDH; DNase I, DNase II, RNase, sucrose (RNase-free) and PMSF were all supplied by Sigma. Ampholines (of various pH ranges) were obtained from L.K.B., Coomassie Brilliant Blue R from Searle, PPO and POPOP from Koch-Light. Kodak supplied RP Royal Xomat film, D19 developer and Kodafix. Calf thymus DNA was the gift of Dr. C.J. Bostock. All other chemicals were analytical reagent grade where available.
METHODS

Centrifugation

Low speed centrifugation was carried out in a 'Sorvall' RC2-B centrifuge. For large volumes, the 6x250ml angle rotor was used and for smaller amounts either the 8x50ml angle rotor or the 4x50ml swing-out rotor.

High speed centrifugation was carried out in the MSE '50' or '65' ultracentrifuges using the 10x10ml angle rotor or 3x23ml swing-out rotor.

Spectrophotometry

The optical density of samples was measured in the 'Unicam' SP 800 or Beckman 'DB-GT' spectrophotometers, using quartz glass cells with a 1cm. light path.

Cell Culture

Mouse A9 cells (Engel, McGee and Harris, 1969) were grown in monolayer culture in F10 medium (Flow Labs. Ltd.). Both 75cm.$^2$ plastic flasks (Falcon Plastics) and Roux Bottles were used in the course of the project. Radioactive labelling times are detailed in the report of each experiment. Cells were harvested in Dulbecco's salt solution with the aid of a rubber policeman and pelleted by low-speed centrifugation. A9 cells are a clonal sub-line of the murine L line (derived from normal subcutaneous and adipose tissue of a 100 day-old C3H/A mouse).

Isolation of nuclei

(1) From mouse liver

The sucrose-Triton X-100 method of MacGillivray et al. (1972) was used with the modification that PMSF was included in all solutions to prevent proteolysis (Ballal,
Goldberg and Busch, 1975). Throughout the course of the project mice of mixed sexes and various strains were killed by cervical dislocation; their livers were excised and immediately immersed in ice-cold isotonic saline. All subsequent operations were carried out at 4°C. The livers were homogenised in approximately five volumes 0.25M sucrose, 3mM CaCl₂, 0.1mM PMSF using a glass-Teflon homogeniser. After filtration through several layers of cheesecloth the homogenate was centrifuged at 1,000xg for 10 min. The pellet was then washed three times in the same sucrose solution by homogenisation and centrifugation as before. The final pellet was suspended by homogenisation in 2.2M sucrose, the suspension layered over 2.2M sucrose and centrifuged at 40,000xg for 1 hr. at 6°C. The 40,000xg pellet of purified nuclei was next homogenised in 0.25M sucrose, 3mM CaCl₂, 0.1mM PMSF and pelleted by centrifugation at 1,000xg for 10 min. This extraction was repeated except that the sucrose solution contained 1% Triton X-100 and was then followed by a similar wash using 0.25M sucrose, 3mM CaCl₂, 0.1mM PMSF alone. This procedure yielded pure nuclei free of cytoplasm as judged by phase contrast microscopy (figure la). Nuclei were stored at -20°C until use. (2) From cultured mouse cells.

The method of Stein and Burtner (1974) was used with some modifications. All operations were carried out at 4°C. With the aid of a Pasteur pipette cells were suspended and lysed with 80 volumes 80mM NaCl, 20mM EDTA, 0.1mM PMSF, 1% Triton X-100 (pH 7.2) and nuclei pelleted
Figure 1

Phase-contrast microscopy of nuclei, isolated as described in Materials and Methods.

a) mouse liver nuclei
b) mouse A9 cell nuclei

Bar in photographs represents 10 microns.
by centrifugation at 1,000xg for 4 min. Nuclei were resuspended in the above solution with the aid of a Pasteur pipette and pelleted by centrifugation as before. This procedure was repeated twice. The nuclei were then suspended with the aid of a Pasteur pipette in 0.15M NaCl, 0.01M Tris-HCl, 0.1mM PMSF (pH 7.0) and pelleted by centrifugation at 1,500xg for 3 min. This procedure yielded pure nuclei free of cytoplasm as judged by phase contrast microscopy (figure 1b). Nuclei were stored at -20°C until use.

Preparation of chromatin from isolated nuclei

The method of McGillivray (1976) was used. Purified nuclei were suspended by homogenisation in 0.14M NaCl, 0.05M Tris-HCl, 5mM EDTA, 0.1mM PMSF (pH 7.5) and chromatin pelleted by centrifugation at 1,000xg for 10 min. This procedure was repeated twice to yield the final chromatin pellet containing DNA, histones and NHCP. The combined supernatants constituted the saline-soluble nucleoplasmic fraction.

Fractionation of chromatin into active and inactive chromatin

The DNase II method of Gottesfeld and Bonner (1974) was used. Mouse liver chromatin prepared as described previously was suspended with the aid of a Pasteur pipette in 10mM Tris-HCl, 1mM PMSF (pH 8) and dialysed overnight against 200 volumes 25mM sodium acetate, 0.1mM PMSF (pH 6.6). The volume of the dialysed chromatin solution was adjusted with 25mM sodium acetate, 0.1mM PMSF (pH 6.6) to give an A$_{260}$ of 10. The solution was brought to 24°C and DNase II added to 100 units/ml. The reaction was terminated after 5 min. incubation by the addition of
50mM Tris-HCl (pH 11) to pH 7.5 and cooling on ice.

Undigested chromatin (P1) was removed by centrifugation at 27,500xg for 20 min. at 4°C. The supernatant was made 2mM in MgCl₂ by the dropwise addition of 0.2M MgCl₂ with stirring at 4°C. After 30 min. of additional stirring the suspension was centrifuged as above yielding a second pellet (P2) and supernatant (S2) fraction.

Preparation of nuclear ribonucleoprotein particles

The preparatory methods used were:

(1) Purified cell culture nuclei were suspended with the aid of a Pasteur pipette in 5 ml. 0.01M Tris (pH 8.0), 0.001M MgCl₂, 0.14M NaCl, 0.1mM PMSF and pelleted by centrifugation at 800xg for 10 min. This procedure was repeated and the supernatants containing the extracted ribonucleoprotein complexes pooled.

(2) a. Purified cell culture nuclei were suspended as above and sonicated for four 10 sec. bursts with cooling on ice in a MSE sonicator at 20 kcyc/sec. All nuclei were ruptured as monitored by phase contrast microscopy. The sonicate was layered on a linear 15 - 30% sucrose gradient (see Gradient formation - later in this section) and centrifuged at 60,000xg for 17 hr. 0.9 ml. fractions were collected and the fractions containing the nuclear ribonucleoprotein complexes pooled.

b. The chromatin pellets from the above sucrose gradients were suspended in 0.01M Tris (pH 7.4), 5mM MgCl₂, 0.1mM PMSF and digested with RNase at 100μg/ml for 5 min. at 37°C. The chromatin was pelleted by centrifugation at 1,500xg for 10 min. and the supernatant containing digested
ribonucleoprotein complexes retained.

**Preparation of $^{14}$C DNA**

$2 \times 10^8$ mouse A9 cells, labelled with $\text{U}^{14}$C thymidine for 36 hrs. (2μCi/ml. medium), were harvested and stored as described previously. For the preparation of $^{14}$C-labelled DNA, cells were thawed in 10 ml. 0.01M Tris-HCl, 0.01M EDTA, 0.01M NaCl (pH 8.0). An equal volume of the above buffer containing 1% sodium dodecyl sarcosinate and 100μg/ml. Protease K (Sigma Ltd.) was added. The resulting solution was incubated at 37°C overnight, dialysed against 0.01M Tris-HCl, 0.01M EDTA (pH 8.0) for five hours and the DNA sheared through a syringe. Solid CsCl was added to give an initial density of 1.700 g/cm$^3$ and the solution centrifuged at 80,000xg for 3 days at 25°C. 0.5ml. fractions were collected from the bottom of the gradient and an aliquot of each fraction used to determine DNA content by scintillation counting (fig. 2). Fractions containing $^{14}$C-radioactivity were pooled, diluted 5 with 0.01M Tris-HCl (pH 8.0) and centrifuged 18 hr. at 180,000xg. The supernatant was decanted, the tubes wiped and the pellet of DNA stored at -20°C.

**Sample radioactivity determination**

(1) Estimation of total radioactivity:

20 - 100μl samples were pipetted onto Whatman 3 mm. filter discs (2.4cm.). The discs were dried in hot air, added to vials containing 5ml. scintillation fluid (2.5 litres toluene - 12.5g PPO-0.75g POPOP) and counted in a Packard Tricarb (model 3330) liquid scintillation spectrophotometer.
CsCl density gradient centrifugation of DNA. DNA was isolated from cells labelled with $^{14}$C-thymidine (1μCi/ml medium) for 48 hr. and centrifuged as described in Materials and Methods. Fractions 16-22 were pooled and DNA further isolated (Materials and Methods).
(2) Estimation of TCA-precipitable radioactivity:
20 - 100μl samples were pipetted onto Whatman 3mm. filter discs (2.4cm.) and allowed to soak in. The discs were dropped into ice-cold 10% TCA and stood in this for a minimum of 10 min. The discs were then rinsed twice in ice-cold 5% TCA for 5 min. per rinse, soaked twice in ethanol/ether (1/1, v/v) at 37°C for 3-5 min. each time and further dried twice with ether at 37°C for 2-5 min. each time. After air drying the discs were added to vials containing scintillation fluid and counted as above.

Non-TCA-precipitable radioactivity was estimated by subtracting TCA-precipitable radioactivity from total radioactivity.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The method of O'Farrel (1975) was used with some modifications.

(1) Sample preparation.

Samples (chromatin, nRNP, etc.) were suspended in and/or dialysed against 0.01M Tris-HCl (pH 7.4), 1μM EDTA, 0.1mM PMSF overnight at 4°C. Samples of greater volume than 2 ml. were concentrated to that volume using polyethylene glycol. DNase I and RNase were added to 50 and 100μg/ml. respectively. The samples were then made 5mM in MgCl₂ and incubated for 5 min. at 37°C followed by immediate freezing to -70°C and lyophilisation. The samples were resuspended in loading buffer: 9.5M urea, 2% (w/v) NP40, 2% ampholines (1% pH 3.5-10, 1% pH 5-7), 5% β-mercaptoethanol to give a final non-histone protein...
concentration of approx. 5mg/ml.

(2) First dimension - isoelectric focusing

5.5 g. urea were added to a 250ml. side-arm flask then
1.33ml. 30% acrylamide stock: 28.38% acrylamide, 1.62%
bisacrylamide, (w/v) in H₂O, 2ml. stock NP-40: 10% (w/v)
NP-40 in H₂O, 1.97ml. H₂O plus 0.25ml. pH5-7 and 0.25ml.
pH3.5-10 amphotolines (40% w/v). The flask was swirled
until the urea had dissolved and 15μl 10% ammonium
persulphate: 10% (w/v) in H₂O added to the gel solution.
The gel solution was then degassed under vacuum for 1 min.,
10μl 10% TEMED: 10% (w/v) in H₂O added, and loaded into
the gel tubes to give 12cm. long gels. The gel solution
was then overlayed with 25μl. sample overlay solution:
8 M Urea, 1% amphotolines (0.5% pH3.5-10, 0.5% pH5-7).
This solution was removed after 2 hr. and replaced
with 25μl. loading buffer (as above) which was in turn
overlaid with 25μl. water. The gels were left to set for
a further 2 hrs. The Parafilm (Gallenkamp) sealing the
bottoms of the tubes was then replaced with dialysis tubing
held in place by a section of latex tubing. After
replacement of the loading buffer and water on top of the
gels with fresh loading buffer, the gel tubes were placed
in the electrophoresis chamber and filled with 0.02M NaOH.
The upper (cathodal) electrophoresis solution was 0.02M
NaOH (extensively degassed to remove CO₂), the lower
(anodal) electrophoresis solution was 0.01M H₃PO₄, 5M urea.
The gels were pre-run for 1/2 hour at 200V followed by 1/2 hour
each at 280V and 360V. The current was switched off and
the upper electrophoresis solution and loading buffer removed.
The sample (25μl. maximum) was loaded onto the top of the gel with the aid of an Eppendorf micropipette and overlaid with 25μl. sample overlay solution (as above) and the tubes filled with 0.02M NaOH. The upper electrophoresis buffer was replaced and the gels were run under constant voltage at 360V for 16 hrs., followed by 1 hr. at 420V. The gels were removed from the tubes by pressure from a syringe and equilibrated for 2 hr. in 5 ml. SDS sample buffer: 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS, 0.0625M Tris-HCl (pH 6.8). The gels were then frozen in this solution in an ethanol/dry ice bath and stored at -70°C.

(3) Second dimension - SDS polyacrylamide gel electrophoresis

The second dimension was based on the discontinuous gel system of Laemmli (1970). High and low concentration acrylamide solutions were mixed to yield a SDS slab gel with a linear polyacrylamide gradient of 7.5-20% (see later section - Gradient formation). The high concentration acrylamide solution contained 2.5 ml. 1.5M Tris-HCl (pH 8.8), 0.4% (w/v) SDS; 6.66ml. 30% acrylamide stock: 29.2% acrylamide, 0.8% bisacrylamide, (w/v) in H₂O; 0.73ml. glycerol; 50μl. ammonium persulphate: 10% (w/v) in H₂O; 50μl. TEMED: 10%(v/v) in H₂O. The low concentration acrylamide solution contained 2.5ml. Tris-SDS (as above); 2.125ml. acrylamide stock (as above); 5.175ml. H₂O and ammonium persulphate and TEMED (as above). When poured, the gradient gel solution was overlaid with 2ml. H₂O and allowed to set for 1hr. The water overlay was then removed and a 4.75% acrylamide stacking gel added: 2.5ml. 0.5M
Tris-HCl (pH 6.8), 0.4% SDS; 1.5 ml. 30% Acrylamide stock (as above); 5.9 ml. H₂O and 100 l. each of ammonium persulphate and TEMED (as above). The stacking gel was allowed to set for a further hour. An isoelectric focusing gel was then placed on top of the stacking gel and immersed in 2 ml. of melted 1% agarose in SDS sample buffer (as above). After 10 min. the entire slab gel was clamped to the electrophoresis tank and running buffer: 0.025 M Tris-base, 0.192 M glycine, 0.1% (w/v) SDS added. 0.04 ml. 0.1% bromophenol blue: 0.1% (w/v) in H₂O was added to the upper (cathodal) reservoir and the gel run at 20 mA constant current for 5 hr. The gel was stained in 0.1% (w/v) Coomassie Blue in 50% TCA for 20 min. Ampholines were removed and the gel destained by soaking the gel in 50% alcohol, 7% acetic acid, 0.005% (w/v) Coomassie Blue for 36 hr. followed by rehydration in 7% acetic acid, 0.005% (w/v) Coomassie Blue and storage in 7% acetic acid. Gels containing labelled proteins were fixed in 50% TCA for 20 min. and stored in 7% acetic acid before preparation for fluorography as described later.

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)

Samples were initially prepared as for 2D-PAGE. These were diluted 1:1 with H₂O, made 5% (w/v) min SDS and heated at 90°C for 3 min. The SDS slab running and stacking gels as described for 2D-PAGE were used with a slot-former in the stacking gel. Electrophoresis buffer was that used for 2D-PAGE and gels were run at 20 mA constant current for 3 hr. The gels were stained in 0.1% (w/v) Coomassie Blue in 50% TCA and destained in 7% acetic acid. Preparation of gels containing labelled proteins for fluorography is described later.
**DNA-agarose gel electrophoresis**

Samples of DNase II-digested chromatin (in 25mM Naacetate) were made 0.05% (w/v) in SDS and 1M in NaCl and the solution extracted twice with water-saturated phenol. The DNA was precipitated by the addition of 2 vols. absolute ethanol followed by storage overnight at 20°C. The DNA precipitates were collected by centrifugation and redissolved in 10mM Tris-10mM EDTA (pH 8.0). The DNA samples were then made 10% sucrose (w/v) and electrophoresed in 1.5% agarose slab gels for 16 hrs. at 10mA constant current. The electrophoresis buffer was 40mM Tris-20mM sodium acetate - 1mM EDTA (pH 8.2). The gels were stained with 0.5µg/ml ethidium bromide dissolved in electrophoresis buffer and fluorescence photographed under ultraviolet light with a yellow filter. Gels were subsequently prepared for fluorography as described later.

**Hydroxylapatite Chromatography**

The method used was that of MacGillivray et al. (1972) with some modifications. Chromatin preparations (see earlier) were homogenised in approximately 10 vol. of 2M NaCl, 5M urea, 0.001M sodium phosphate (pH 6.8) using a Teflon-glass homogeniser. After centrifugation at 15,000xg for 15 min., the pellet was extracted in the same way. The two extracts were pooled and sonicated for four ten second periods at 24 kcyc/sec. in an MSE sonicator with cooling on ice.

Hydroxylapatite (Bio-Rad Labs.) was washed several times in 2M NaCl, 5M urea, 0.001M sodium phosphate (pH 6.8)
by suspension and decantation. The column was packed, equilibrated and run at room temperature in the above solution. A 25 ml. solution of sonicated chromatin (above; \( A_{260nm} = 10 \)) was allowed to come to room temperature and applied to a 20 cm. x 1.6 cm. column of hydroxylapatite. After elution of unretained material (histones; H1 of MacGillivray et al., 1972), the column was washed with 2 M NaCl, 5 M Urea, 0.05 M sodium phosphate (pH 6.8) to yield a non-histone chromatin protein fraction (H2 of MacGillivray et al., 1972)

Fluorography of gels

(1) Polyacrylamide gels

The method of Bonner and Laskey (1974) was used. Gels containing labelled proteins were soaked in ten volumes DMSO for 30 mins. This procedure was repeated twice with fresh DMSO each time. The gels were then soaked in 10 vols. DMSO containing 16% (v/v) PPO for 3 hrs. and, after removal of this solution, the PPO precipitated in the gels by soaking in water. The gels were then dried onto a piece of filter paper and placed in contact with pre-flashed Kodak Royal RP X-omat film (Laskey and Mills, 1975). The film was exposed to the gels at \(-70^\circ C\) for the required time and then developed in Kodak D19 developer and fixed in Kodafix.

(2) DNA-agarose gels

The above procedure was used except that ethoxyethanol replaced DMSO.

DNA and protein estimations

DNA estimation was by the diphenylamine method
(Giles and Myers, 1965) using calf thymus DNA as a standard.

Protein estimation was by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Histone and non-histone protein contents were determined using the method of Spelsberg et al. (1973). Chromatin solutions were extracted for 30 min. at 4°C with H\textsubscript{2}SO\textsubscript{4} (to final concentration of 0.2M) followed by centrifugation at 1,200xg for 10 min. The supernatant was neutralised with NaOH and histone content estimated by the Lowry method; the pellet was resuspended and the non-histone protein content estimated by the Lowry method.

Gradient formation

(1) For sucrose density gradient analysis:

Two solutions were prepared, the first containing sucrose at the concentration required at the top of the gradient and the second containing sucrose at a concentration 5% greater than required at the bottom of the gradient. The sucrose solutions were prepared by dissolving sucrose in distilled water and adjusting to the desired buffer concentration. Each gradient was prepared by placing the lighter solution in a mixing tube, pumping in the heavier solution with constant stirring and pumping out the mixture (at twice the rate that the heavier solution was pumped in) to the bottom of a centrifuge tube. The volume of lighter sucrose solution used (V) was determined by the
following formula:

\[ V = \frac{1}{2} \frac{V_g(C_3-C_1)}{(C_2-C_1)} \]

where \( C_1 \) = concentration at the top of the gradient (15%)
\( C_2 \) = desired concentration at the bottom of the gradient (30%)
\( C_3 \) = heavier concentration prepared (31.66%)

\( V_g \) = final volume of gradient (18ml.)
\( V \) = volume of lower concentration sucrose placed in mixing tube (10ml.)

(2) For polyacrylamide gel electrophoresis

The above equation was used but high concentration acrylamide solution placed in the mixing tube and low concentration acrylamide solution pumped in at half the rate the mixture was pumped out. The initially high concentration acrylamide mixture was pumped to the bottom of the slab and overlaid with successively lower concentrations of mixture so that the top of the gradient gel had the desired low concentration of acrylamide.

Thus, in this case:

\( V \) = volume high concentration to be added to mixing chamber (6.88ml.)
\( V_g \) = final volume gradient (15ml.)
\( C_1 = 100 \) - concentration at the bottom of the gradient (20%)
\( C_2 = 100 \) - lower concentration prepared (6.375%)
\( C_3 = 100 \) - lower concentration at the top of the gradient (7.5%)
CHAPTER III

RESULTS AND DISCUSSION
SECTION 1

Isolation of Chromatin

In view of the number of different chromatin isolation methods discussed in the introduction it is felt that some comment on the chromatin isolation methods used and the reasons for the choice of these methods is warranted. Two main considerations were taken into account in the choice of chromatin isolation methods. The first of these was the purity of the final chromatin preparation, the second its functional state. With respect to the first consideration the prior isolation of highly purified nuclei was considered essential. The procedure chosen for the preparation of nuclei from mouse liver cells was the sucrose-Triton method of MacGillivray et al. (1972) (see Materials and Methods). Isolation of nuclei in sucrose is well established as a useful method in obtaining good yields of pure nuclei, the addition of Triton removing contaminating cytoplasmic and nuclear membrane material without any major loss of protein (MacGillivray et al., 1972). Thus, after homogenisation of the cells, nuclei were purified by centrifugation through isotonic sucrose containing Ca\(^{++}\) ions to prevent damage to the nuclei. The nuclei were then further purified by centrifugation through hypertonic sucrose in a modification of the Chauveau procedure (Chauveau et al., 1956). The purified nuclei were subsequently washed with isotonic sucrose containing Triton X-100 to remove contaminating membrane material. Attempts to obtain pure nuclei from cultured mouse A9
cells by this technique proved unsuccessful. The method adopted for the isolation of nuclei in this case was that of Stein and Burtner (1974). This method employed hypotonic shock to rupture the cells and subsequent removal of cytoplasmic and membrane material adhering to the nuclei with 0.14M NaCl and Triton X-100. All operations during the isolation of nuclei were carried out at 4°C and PMSF was included in all solutions to prevent proteolysis. By these techniques good yields of highly purified nuclei were obtained.

Chromatin was prepared from isolated nuclei by the method of MacGillivray (1976). The second main consideration mentioned previously was the functional state of the prepared chromatin - chromatin prepared by this and similar methods has proved capable of being transcribed in vitro to yield RNA sequences similar to those synthesised in vivo by the homologous chromatin (Gilmour and Paul, 1973). This ability, in the absence of any definitive criterion for the biological activity of chromatin, was a major factor leading to the choice of this chromatin preparation method.

The above preparation of chromatin from nuclei involves homogenisation steps which may expose the chromatin to shear forces. Recently concern has been expressed as to possible damaging effects of shear on the native structure of chromatin. When chromatin is prepared by a limited nuclease digestion method and then further nuclease digested its DNA shows a characteristic pattern at 200 base pair intervals upon subsequent gel electrophoresis.
This pattern is indicative of the nucleosome structure (discussed in Introduction) thought to represent the basic structure of chromatin in vivo. Chromatin subjected to extensive physical shearing before nuclease digestion gives no band pattern but only a smear of DNA indicating disruption of this level of chromatin organisation (Noll, Thomas and Kornberg, 1975). Chromatin sheared by sonication shows changes in CD spectra (Tashiro and Kurokawa, 1975b). However proteolytic digestion of histones indicates that the nucleosomes of nuclei and of sheared chromatin are similar (Marks and Keller, 1977) and shear does not appear to affect the specific transcription of globin genes from chromatin templates isolated from tissues actively synthesising globin mRNA—quantitatively similar results have been obtained with both sheared and unsheared chromatin preparations (Axel et al., 1973; Gilmour and Paul, 1973; Barret et al., 1974).

The method and extent of shear imposed on chromatin during preparation may well have differential effects on its native structure. The method of chromatin preparation described earlier in this thesis involves three homogenisation steps using a loose-fitting glass-Teflon homogeniser at low speeds and hence probably represents minimal shear compared to the extensive shearing which produced the damaging effects on chromatin structure described in the examples cited above. Later studies on chromatin fractionation were repeated with intact nuclei in order that any artefactual effects
due to exposure of chromatin to shear forces might be ruled out.

The chemical compositions of the chromatin yielded by the above methods are given in Table 1. In agreement with the results of many workers the ratio of histone to DNA was found to be close to 1. The ratio of non-histone protein to DNA was lower (0.78 for mouse liver, 0.62 for mouse A9 cells). Since chromatin of different chemical compositions are given by different preparation procedures even in the same tissue, the comparison of results between different methods is difficult. Using a sucrose isolation procedure the protein/DNA ratio of mouse liver chromatin was found to be 1.7 by MacGillivray et al. (1972) - giving a NHCP/DNA ratio of 0.7 (assuming the histone/DNA ratio is 1). The ratio of NHCP/DNA in rat liver chromatin may vary from 0.65 - 0.95 (Gottesfeld et al., 1974; Elgin and Bonner, 1970) while chromatin from several sources (isolated by a variety of methods) were listed as having NHCP/DNA ratios of 0.48-1.37 (Busch et al., 1975). The chromatin compositions reported in this work lie well within the range previously reported by other workers.

Polyacrylamide Gel Electrophoresis of Non-Histone Proteins

The initial goal of the project was to improve upon existing techniques for the polyacrylamide gel electrophoretic analysis of the non-histone chromatin proteins. After a survey of existing techniques it became apparent that this objective might best be achieved by the application of a high-resolution two-dimensional polyacrylamide gel
TABLE I

LEGEND: Chemical composition of chromatins prepared as described in Materials and Methods. DNA and protein estimations as described in Materials and Methods; figures given are in g/gDNA.

<table>
<thead>
<tr>
<th>CHROMATIN SOURCE</th>
<th>DNA</th>
<th>TOTAL PROTEIN</th>
<th>HISTONE PROTEIN</th>
<th>NON-HISTONE PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Liver</td>
<td>1</td>
<td>1.88</td>
<td>1.02</td>
<td>0.78</td>
</tr>
<tr>
<td>Mouse A9 Cells</td>
<td>1</td>
<td>1.65</td>
<td>1.05</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Figures given are averages of four determinations.
The final results of 1D-PAGE and 2D-PAGE separations of non-histone proteins are presented first and are followed by a section discussing in greater detail the work carried out in the establishment of the 2D-PAGE system. This order of presentation places much of this work out of chronological order (e.g. the final established system is presented before the work leading to it) but enables the results to be discussed in a more comprehensible manner.

**1D-PAGE of Proteins**

The 1D-PAGE system used for the analysis of proteins was the SDS-polyacrylamide system of Laemmli (1970) (see Materials and Methods). This system was that used for the second dimension of the 2D-PAGE procedure and has the advantage that samples prepared for 2D-PAGE could also be utilised for 1D-PAGE (see Materials and Methods). Such samples contain DNase and RNase (see later discussion on sample preparation for 2D-PAGE). The position of these enzymes in the one-dimensional gels could easily be determined by the inclusion, in one of the sample slots of the slab gel, of a sample containing DNase and RNase alone. The enzymes also served as useful internal standards for molecular weight determination. Samples prepared for 2D-PAGE also contain ampholines (necessary for the isoelectric focusing dimension). The ampholines, being low molecular weight carrier ampholytes, bind SDS and run as a discrete band immediately behind the marker dye (Bromophenol Blue) front and were usually run off the gel.

Figure 3A shows photographs of resulting SDS-polyacrylamide
Figure 3

1D-PAGE analysis of chromatin proteins. Samples were prepared and run in 1D-PAGE as described in Materials and Methods:

a) chromatin proteins (sample load - 120µg)
b) chromatin proteins (sample load - 100µg)
c) DNase and RNase (sample load - 10µg)
d) histones (sample load - 40µg), prepared by the hydroxylapatite method of MacGillivray et al (1972).

Figures quoted are molecular weight x10^-3

Samples are from mouse liver chromatin.
slab gels containing samples of total chromatin proteins, histones, DNase and RNase. The two total chromatin protein samples analysed were from similar preparations of chromatin. The pattern of protein bands between these chromatin samples is very similar, with slight variations at very high molecular weight. The position of the DNase in the protein samples can be determined by comparison with the sample containing DNase and RNase alone. The total chromatin protein samples also contain histones. The positions of the histone bands can be assigned by comparison with the sample containing histones purified by the hydroxylapatite method of MacGillivray et al. (1972). RNase co-migrates with the low molecular weight histones and cannot be clearly distinguished.

By direct visualisation of the gels presented in this figure upwards of fifty protein bands can be assigned to the NHCP. The NHCP can be seen to constitute a heterogeneous mixture of proteins with molecular weights ranging from 10,000 to over 100,000 (the molecular weights given in figure 3 were determined from the position of the marker enzymes and bovine serum albumin in parallel gel runs). Results obtained by the various methods used by other workers reveal the NHCP as a complex mixture of proteins, there being 12-18 major SDS-protein components in most tissues, covering a molecular weight range of 5,000 to about 200,000 (Elgin and Bonner, 1970; MacGillivray et al., 1972; Le Stourgeon and Rusch, 1973; Wu, Elgin and Hood, 1973). Estimates of the number of major protein species contributing to the NHCP of rodent tissues range
from 10-15 for rat-liver chromatin (Elgin and Bonner, 1972) to over twice that number for mouse-liver chromatin (MacGillivray and Rickwood, 1974). The gels presented in figure 3 support the latter estimate but the differences in the number of major protein species observed probably reflect differences in chromatin preparation. The procedure of Elgin and Bonner, which involves extensive washing of chromatin with dilute buffers, may remove much of the loosely-bound NHCP, these being retained when the chromatin preparation procedure of MacGillivray (1976) is used (as in this thesis). The number of NHCP components observed will also be dependent on the resolution of the gel system used.

The 1D-PAGE system described in this thesis does not, and was not intended to, give a complete analysis of the NHCP. The inclusion of DNase and RNase in the sample obscures any NHCP of similar molecular weight. Similarly no attempt was made to remove histones from the sample (this was found unnecessary for satisfactory 2D-PAGE) and hence NHCP of molecular weights similar to those of the histones are obscured. The method does allow, however, a simultaneous one-dimensional separation of several samples prepared for 2D-PAGE. Thus an initial low-resolution examination of samples to be compared by 2D-PAGE can be achieved by this method with substantial saving in time. A further advantage lies in that both histones and non-histones are resolved by this 1D-PAGE procedure allowing histone analysis of samples prepared for 2D-PAGE (which does not resolve histones - see later).
The major disadvantage common to all 1D-PAGE procedures is the low resolution attained. It is becoming increasingly apparent as the complexity of the NHCP is revealed by increasingly refined separation methods that their resolution by a procedure which separates them only by differences in molecular weight is limited. Thus many bands resolved by 1D-PAGE may be constituted by several polypeptides of similar molecular weight. A common feature to many analyses of NHCP by 1D-PAGE systems is the high background of stain throughout the gel, which may reflect unresolved minor species of polypeptides of intermediate molecular weight between the major species which constitute the bands as well as overlapping of bands. For these reasons, research into methods of 2D-PAGE analysis of NHCP is now becoming increasingly widespread.

2D-PAGE of Non-Histone Proteins

Several two-dimensional polyacrylamide gel electrophoretic systems have recently been applied to the analysis of the NHCP (these will be discussed later). It was felt that none of these systems realised the full potential that 2D-PAGE analysis had in the analysis of NHCP. Other systems, not previously utilised in the analysis of NHCP, were reviewed for potential use in the analysis of this complex mixture of proteins. In this respect the procedure of O'Farrel (1975) seemed outstanding. By this technique proteins are separated according to isoelectric point by isoelectric focusing in the first dimension and according to molecular weight by SDS-PAGE in the second dimension. Since isoelectric point and molecular weight are unrelated
It is possible to resolve proteins which differ in only one of these factors. This technique resolved 1,100 different protein components from *E. coli*, while the technique is theoretically capable of resolving a maximum of 5,000 proteins. Although the complexity of the NHCP is probably well below this number, the relatively narrow ranges of molecular weight and isoelectric point of the major species of NHCP made the application of this system to their analysis seem attractive.

Anticipated difficulties in the application of this system to NHCP were the low solubility of these proteins, their DNA-binding properties and their susceptibility to proteolysis. A fuller discussion of control experiments regarding the procedure will be presented in a following section. For the moment the discussion will be limited to an analysis of the final separation attained.

Figure 4 presents a two-dimensional separation of mouse liver nuclear non-histone proteins. The proteins were separated in the first dimension in an isoelectric focusing rod gel and in the second dimension in a SDS-polyacrylamide slab gel. The non-histone proteins are visualised as spots distributed throughout the slab gel. Over 50 major protein species can be easily identified from the photograph; by direct visualisation of the gel over 100 distinct protein spots can be identified.

The horizontal scale at the top of the photograph shows the pH gradient of the isoelectric focusing gel (see legend, Figure 5). The vertical scale down the side of the photograph indicates the molecular weight range of the gel.
2D-PAGE analysis of nuclear non-histone proteins

Sample prepared for electrophoresis as described in Materials and Methods. Sample load — 200μg total protein. 2D-PAGE as described in Materials and Methods. Scales for this and subsequent 2D-PAGE analyses:—

Horizontal — isoelectric focusing (IEF); figures quoted are pH x10.

Vertical — sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS); figures quoted are molecular weight x10^3

Arrowed spots are DNase I, see text for details.

Sample obtained from mouse liver nuclei.

Vertical streaking seen in this and subsequent 2D-PAGE analyses is variously due to incomplete saturation of proteins in the isoelectric focusing gels with SDS, staining artefacts or displaced material from the top of the isoelectric focusing gel.
This was determined from the position of standard proteins in parallel gels - the standards used were bovine serum albumin (m.wt. 68,000), DNase I (m.wt. 31,000) and RNase (m.wt. 14,700) (Weber and Osborn, 1969). A grid system can be set up using these two scales to denote the position of any one spot. The notation system used will give the pH value of the spot in two figures followed after an oblique by the molecular weight value of the spot given in two figures (i.e. pH/m.wt.). Thus the two arrowed spots in figure 4 at pH 5.6 and 5.5 and molecular weight 31,000 are assigned numbers 56/31 and 55/31 respectively. These two spots represent DNase I, which was present in the sample (see sample preparation and later). RNase A, also present in the sample, has a high isoelectric point (above pH 9) and hence does not enter the isoelectric focusing gel and is not present in the final two-dimensional analysis.

Both the pH and molecular weight values quoted can only be regarded as approximate. The pH is estimated as described in the legend to figure 5 by soaking the gel slices in H₂O to leech out the ampholines. The urea in the gel slices is also removed by this process and interferes with accurate pH determination (Bull et al., 1964). The molecular weight values given were determined in an identical gel run under the same conditions, but small variations in the acrylamide gradients of gels may occur. However, in addition to the grid system described above, spots may also be identified by their relative positions - hence comparison of spots between gels is not difficult.

Figure 4 shows that a complex mixture of non-histone
Figure 5

pH gradient of isoelectric focusing gels.

Isoelectric focusing gels were prepared and run as described in Materials and Methods. One gel of each set (8) contained no sample and after electrophoresis was extruded onto parafilm, cut into 5 mm. sections and placed in a vial containing 3 ml. degassed H₂O. After 4 hr. (with occasional shaking) the pH of the resulting solution was measured.
proteins can be separated and individually identified by this procedure (indeed the system proved capable of handling samples of far greater complexity - see figure 17a later). Several problems were encountered in initial attempts to separate NHCP by this technique. When crude chromatin was prepared for 2D-PAGE of its proteins without the DNase I and RNase digestion step (see sample preparation, Materials and Methods) the resulting two-dimensional analysis showed extensive streaking in the isoelectric focusing dimension (Figure 6). Streaking in this dimension has also been encountered by other workers (O'Farrel, 1975; Peterson and McConkey, 1976; MacGillivray, 1976). and has been attributed to the effects of polynucleotide material in the sample. During the separation of E. coli proteins by 2D-PAGE, O'Farrel (1975) found this streaking effect "not severe". In the case of chromatin proteins where no attempt is made to remove polynucleotide material the effect very obviously is severe.

Initial attempts to circumvent this problem were directed towards the fractionation of chromatin into its constituent DNA, histone and non-histone protein fractions by the hydroxylapatite method of MacGillivray et al. (1972). The NHCP fraction obtained by this method still showed streaking in the isoelectric focusing dimension and hence may still contain some polynucleotide material (figure 7). MacGillivray (1976) has also noted that polynucleotide material is incompletely removed by this method and has recently added a CsCl-urea centrifugation step to his preparation of the NHCP fraction to further remove polynucleotide
Figure 6

2D-PAGE analysis of mouse liver NHCP.

Sample (150μg total protein) was prepared for electrophoresis minus the nuclease digestion step (Materials and Methods). 2D-PAGE was as described in Materials and Methods.
Figure 7

2D-PAGE analysis of mouse liver NHCP. NHCP were prepared by the hydroxylapatite method of MacGillivray et al. (1972) (see Materials and Methods). Sample load - 75μg total protein. Sample prepared for electrophoresis minus nuclease digestion step; 2D-PAGE as described in Materials and Methods.
material. This latter procedure is lengthy, involving extended chromatography, centrifugation and dialysis steps with concomitant losses of protein and opportunities for the action of proteases. An alternative method for the preparation of NHCP for 2D-PAGE was therefore sought. O'Farrel (1975) has suggested that samples containing polynucleotide material can be digested with DNase I and RNase, after which treatment streaking in the isoelectric focusing dimension is reduced. The adoption of such a procedure, as opposed to a complete purification of a NHCP fraction, seemed reasonable since it is essential only that the polynucleotide material causing the streaking effect in the isoelectric focusing dimension be removed. Prior separation of the histones from the NHCP is not essential since the histones have high isoelectric points (above pH 9) and therefore do not enter the isoelectric focusing gel (Peterson and McConkey, 1976). Such a digestion procedure has previously been applied to the separation of NHCP from DNA (Wilson and Spelsberg, 1973). These workers digested acid-extracted chromatin with DNase I and then precipitated the non-histone chromatin proteins with perchloric acid. When a similar method (using TCA) was applied to the preparation of a non-histone protein sample for 2D-PAGE by the system described in this thesis, the pH gradient was found to be decreased in size, probably as a result of residual TCA in the sample.

As an alternative to this procedure the sample was immediately frozen to -70°C and lyophilysed after digestion with DNase I and RNase. The optimal time of digestion was
determined by following the time course of the digestion of \( ^{14} \text{C} \) thymidine-labelled DNA in chromatin (Figure 8). After 5 min. digestion 90-95% of the \( ^{14} \text{C} \) thymidine-labelled DNA in the sample was converted to a TCA-soluble form. 5-10% of the \( ^{14} \text{C} \) thymidine-labelled DNA in chromatin remains undigested even after extended periods of digestion. This undigested fraction of DNA may exist in intimate, possibly covalent, association with protein thus being protected from digestion. Both rat liver soluble nuclear and cytoplasmic proteins with high affinity to polynucleotides have been described (Schweiger and Mazur, 1975). These proteins are released from poly(A)- and poly(U)-Sepharose columns only by treatment with 50% formamide. It has also been suggested that up to 60% of NHCP contain small amounts of tightly bound nucleic acid - separated proteins in polyacrylamide gels require incubation in 5% TCA for 30 min. at \( 90^\circ \text{C} \) to remove this nucleic acid (Bhorjee and Pederson, 1976b). The distribution of \( ^{14} \text{C} \) thymidine-labelled DNA of DNase- and RNase-digested chromatin throughout an isoelectric focusing gel is shown in figure 9. Of the DNA which enters the gel the majority remains at the top of the isoelectric focusing gel. This result is in agreement with the observation of O'Farrel (1975) that high molecular weight \( ^{32} \text{P} \)-labelled nucleic acid forms a smear at the top of the isoelectric focusing gel. The DNA deposited at the top of the isoelectric focusing gel, upon subsequent second dimension electrophoresis, forms a streak down the two-dimensional gel as can be seen from figure 10. This shows an autoradiograph of a 2D-PAGE
Digestion of $^{14}$C-DNA in chromatin with DNase I

Chromatin was prepared from mouse A9 cells (Materials and Methods) grown in the presence of $^{14}$C-thymidine (2μCi/ml medium for 48 hr.). Chromatin was suspended in 0.01M Tris-HCl (pH 7.4), 5mM MgCl$_2$, 0.1mM PMSF (1.5 x 10$^5$cpm $^{14}$C/ml) and digested with DNase I (50μg/ml) and RNase (100μg/ml) at 37°C. 25μl samples were removed at varying times of digestion and $^{14}$C radioactivity counted as described in Materials and Methods.

- total $^{14}$C radioactivity
- TCA-precipitable radioactivity
Chromatin was prepared from mouse A9 cells (Materials and Methods) grown in the presence of $^{14}$C-thymidine (2μCi/ml medium for 48 hr.). Chromatin (10,000 cpm $^{14}$C) was prepared for electrophoresis and isoelectric focusing gels run as described in Materials and Methods. Isoelectric focusing gels were extruded onto Parafilm and cut into 5mM sections. Each 5mm. section was dried, under vacuum, onto a Whatman 3mm. filter disc and radioactivity determined as described in Materials and Methods.

0 mm. corresponds to the top (alkaline) end of isoelectric focusing gel.
Figure 10

Fluorography of 2D-PAGE analysis of chromatin containing $^{14}\text{C}$-DNA.

Chromatin was prepared from mouse A9 cells (Materials and Methods) grown in the presence of $^{14}\text{C}$-thymidine (2μCi/ml medium for 48 hr.). Chromatin samples (10,000 cpm $^{14}\text{C}$) were prepared and run in the 2D-PAGE system as described in Materials and Methods. The resulting slab gel was prepared for fluorography as described in Materials and Methods. Exposure time was 30 days.
separation of chromatin containing $^{14}$C thymidine-labelled DNA. The nature of this material as DNA was confirmed by heating an identical gel to 90$^\circ$C for 30 min. in 5% TCA (Bhorjee and Pederson, 1976b) - no radioactivity was observed upon subsequent fluorography indicating the destruction of DNA. Smearing throughout the two-dimensional gel due to DNA was also observed in gels containing both $^{35}$S-labelled protein and $^{3}$H-DNA (5x10$^5$ cpm loaded) (see figure 22 later). The streak of DNA at the top of the gel in figure 10 corresponds to the protein streak seen in the two-dimensional separation of proteins at the top of the pH gradient (e.g. figure 4 stars; O'Farrel, 1975 - figure 13; Peterson and McConkey - figure 1). As well as protein which does not enter the isoelectric focusing gel because of its isoelectric point, this protein may reflect that fraction previously described with high affinity to polynucleotides (since it protects this fraction of DNA from digestion and is not dissociated from polynucleotides under the effect of the 9.5M urea and 2% NP-40 in which the sample is loaded) and has been estimated at 20% of the total NHCP (Peterson and McConkey, 1976).

A possible source of artefactual heterogeneity lies in the occurrence of proteolysis during the digestion of chromatin samples with nucleases. This could result from the action of endogenous proteases in chromatin or contaminating proteases in the nuclease enzymes. Gross proteolytic activity does not occur - figure 11 shows that when chromatin containing radioactively-labelled proteins
Figure 11

Effect of sample nuclease digestion on $^{35}$S-labelled proteins.

Chromatin was prepared from mouse A9 cells (Materials and Methods) grown in the presence of $^{35}$S-methionine (1μCi/ml medium for 48 hr.). Chromatin was suspended in 0.01M Tris-HCl (pH 7.4), 5μM EDTA, 0.1mM PMSF (1 x 10$^5$ cpm $^{35}$S/ml), brought to 37°C and DNase I (50μg/ml) and RNase (100μg/ml) added. 25μl samples were removed - corresponding to zero time samples. MgCl$_2$ was added to 5mM and 25μl samples removed at varying times of digestion. After 30 min. incubation at 37°C Protease K (100μg/ml) was added and the chromatin sample incubated a further 5 min. prior to the removal of further 25μl samples (+P). $^{35}$S radioactivity was counted as described in Materials and Methods.

- Total $^{35}$S radioactivity
- TCA-precipitable $^{35}$S radioactivity
is incubated in the presence of DNase and RNase, even for lengthy time periods, the amount of TCA-precipitable radioactivity closely parallels the total radioactivity of the sample. The general reduction seen in both total and TCA-precipitable radioactivity after zero time is due to a Mg$^{++}$-induced insolubility effect on the chromatin sample, being abolished when the chromatin sample is treated with Protease K. $^3$H ovalbumin was added to a chromatin sample by Wilson and Spelsberg (1973) in their 30 min. digestion of chromatin with DNase I; upon subsequent electrophoresis of the extracted proteins, no changes in the number of counts in, or the relative mobility of, the $^3$H-ovalbumin band were observed.

Figure 12 shows 2D-PAGE separations of NHCP after various times of sample digestion with DNase I and RNase. At zero time extensive streaking is observed - the effect of polynucleotide material in the sample as described previously. After 5 min. digestion the streaking is minimised and the pattern of spots is characteristic of that observed for the NHCP (see figure 14 later). With longer periods of digestion this pattern becomes less well-defined, the individual spots being more diffuse and minor spots absent. Thus some indication of proteolysis is observed even in the presence of the protease inhibitor PMSF which is included in the digestion buffer (see Materials and Methods). Evidence that proteolysis is not a problem for samples which are digested for only 5 min. can be seen from a later figure (figure 14) in which 2D-PAGE separations of two different samples of NHCP
2D-PAGE analysis of NHCP at various times of digestion with nucleases.

Chromatin was prepared from mouse liver as described in Materials and Methods, suspended in 0.01M Tris-HCl (pH 7.4), 5mM MgCl₂, 0.1mM PMSF and digested for various times (see below) with DNase I (50μg/ml) and RNase (100μg/ml) before immediate freezing to -70°C.

Sample loads - 160μg total protein.

a) 0 min. digestion
b) 5 " "
c) 10 " "
d) 15 " "
e) 30 " "
f) 60 " "

2D-PAGE was as described in Materials and Methods.
prepared from chromatin digested for 5 min. are shown - the high reproducibility observed in the separation of these two samples indicates that no serious artefactual heterogeneity due to proteolysis has been induced.

The final step in the preparation of samples involved their lyophilisation and resolubilisation in sample loading buffer to a suitable protein concentration. While this method was quicker than other concentration methods some concern was felt about the resolubilisation of the non-histone proteins which are notoriously difficult to solubilise. A comparison of saline-soluble nucleoplasmic non-histone proteins concentrated by this method and those concentrated by dialysis against polyethylene glycol (by which method the proteins remain in solution) is shown in figure 13. The saline-soluble nucleoplasmic proteins and NHCP will later be shown to be very similar - this experiment was not carried out with NHCP because these proteins could not be satisfactorily concentrated to the extent required for 2D-PAGE by the polyethylene glycol method. Quantitative differences between the two-dimensional separations reflect the ease with which samples of suitable protein concentration may be prepared by the lyophilisation procedure and the difficulty in handling small volumes of protein solutions when concentrating against polyethylene glycol and dialysing against sample loading buffer. In addition some qualitative differences may be seen - the lyophilysed sample has one major spot (closed triangle) and several minor spots (around spot indicated by open triangle) which are absent from the sample concentrated
Figure 13

2D-PAGE analysis of saline-soluble nucleoplasmic proteins
concentrated against polyethylene glycol or by lyophilisation.

Saline-soluble nucleoplasmic proteins were dialysed against
0.01M Tris-HCl (pH 7.4), 5mM MgCl₂, 0.1mM PMSF and
digested with DNase I (50µg/ml) and RNase (100µg/ml)
for 5 min. at 37°C. Samples were concentrated either
a) against polyethylene glycol with subsequent dialysis
   against sample loading buffer.
b) by lyophilisation followed by resuspension in sample
   loading buffer.

2D-PAGE analysis as described in Materials and Methods.
Sample protein loads: a) 100µg
                     b) 150µg
against polyethylene glycol. The absence of these proteins in the sample concentrated against polyethylene glycol may be a result of proteolysis during the extended dialyses steps involved in this procedure.

The 2D-PAGE separations obtained by the above methods must be shown to be reproducible before any meaningful interpretation of the results can be undertaken. 2D-PAGE analyses of two different preparations of mouse liver NHCP are shown in figure 14 (a,b). The pattern of NHCP spots between the two-dimensional analyses can be seen to be highly reproducible. Minor variations do occur however. These are largely as a result of slight differences in the pH gradients of the isoelectric focusing dimension. These differences occur at high pH values, this end of the pH gradient being found more variable. Attempts to increase the pH range of the isoelectric focusing gels above pH 7.5 by using different proportions of various pH range ampholines were unsuccessful - although higher pH values could be obtained the pH gradient became more variable and reproducibility between different gel runs was poor. The NHCP routinely separated were those which focused in the isoelectric focusing dimension between pH 4.5 and 7.5. Previously reported data of other workers suggests that this pH range includes the majority of the NHCP. NHCP from HeLa cells analysed by Peterson and McConkey (1976) focused in the isoelectric focusing dimension within the pH range 5.9 - 7.45; few additional proteins were found when the pH gradient was expanded to pH 5.0 - 7.8. The 2D-PAGE system of Barret and Gould (1973)
Figure 14

2D-PAGE analysis of NHCP of different chromatin preparations from mouse liver.

Two different chromatin preparations from mouse liver were analysed for NHCP content as described in Materials and Methods.

Sample loads:  

a) 140μg total protein  
b) 125μg total protein
revealed that the majority of the NHCP of both rat and chicken tissues were to be found in the pH range 5 - 7. Isoelectric focusing studies have also shown that the bulk of the NHCP from rat liver have isoelectric points in the pH range 5 - 7 (Elgin and Bonner, 1972) while the majority of the nuclear proteins of rat hepatomas are also found in this pH range (Lea, Koch and Morris, 1975). A small number of NHCP components may, however, have isoelectric points outwith this range (Elgin and Bonner, 1972; MacGillivray and Rickwood, 1974).

In the second dimension the NHCP of mouse liver can be seen from figure 14 to range in molecular weight from 10,000 - 100,000. These molecular weight values correspond to those found by many other workers (see Introduction for references). The major species of NHCP are found within the molecular weight range 30,000 - 70,000. Many proteins of similar molecular weight within this range are well separated from each other in the isoelectric focusing dimension - a major advantage of this system over one-dimensional techniques. Several multiple spots are also seen (arrowed in figure 14a, b) which may reflect both groups of similar proteins and modifications to a single protein. The high reproducibility of these multiple spots indicates that they are unlikely to have been artefactually induced.

The analysis of NHCP by 2D-PAGE can also be visualised by radioactive labelling of protein and subsequent fluorography of two-dimensional gels. A stained gel of mouse A9 cell NHCP is presented in figure 15a. Extensive
2D-PAGE analysis of NHCP of mouse A9 cells.

a) Chromatin was prepared from mouse A9 cells and NHCP content analysed by 2D-PAGE and Coomassie Blue staining as described in Materials and Methods.
Sample load - 125μg total protein.

b) Chromatin was prepared from mouse A9 cells grown in the presence of 35S methionine (1μCi/ml medium for 48 hr.) and NHCP content analysed by 2D-PAGE and subsequent fluorography of the 2D gel as described in Materials and Methods.
Sample load - 1.5 x 10^5 cpm total protein
Exposure time 32 days.

The two analyses presented are from similar preparations of chromatin.
effort is required in the production of the quantity of cells needed to yield suitable amounts of NHCP for their analysis by staining in two-dimensional gels. However the two-dimensional analysis of these proteins shows one major protein (56/60) and several intermediate protein species (59/50, 59/75, 64/55) which may correspond to those in similar positions in the two-dimensional analysis of mouse liver NHCP (figure 14). Figure 15b shows a photograph of a fluorogram of a similar sample which contained $^{35}$S-methionine-labelled proteins. Several minor spots, present on the stained gel are absent from this analysis (e.g. those at 65/85 and 68/80; also 56/31 and 55/31 which are DNase I). These may be proteins which are lacking in methionine or which are extremely stable - hence not being labelled. Considerably finer detail in the gel containing labelled protein (b) can, however, be seen in the spots around 53/55. From this figure it can be seen that the NHCP of mouse A9 cells show similarities to those of mouse liver, ranging in molecular weight from 10,000-100,000, the major protein species being in the 30,000-80,000 molecular weight range.

The two-dimensional analyses obtained with the above adaption of the O'Farrel (1975) system correspond closely to those obtained by Peterson and McConkey (1976), who independently have adapted this system to the analysis of the non-histone chromosomal proteins of HeLa cells. Both sets of results reveal the NHCP as highly complex (in the order of hundreds of components) with a wide range of isoelectric points and molecular weights.
Other two-dimensional systems using isoelectric focusing in the first dimension and SDS polyacrylamide gel electrophoresis in the second dimension have been applied to the analysis of the NHCP (Barret and Gould, 1973; MacGillivray and Rickwood, 1974; Liew and Chan, 1976) and DNA-binding proteins (Jost, Lennox and Harris, 1975). When compared to these systems the method described in this thesis can be seen to offer increased resolution in the analysis of the complex mixture of proteins which constitute the NHCP. The analysis described here also improves upon the two-dimensional system of Busch's group (Yeoman et al., 1973) in which the NHCP, separated by one parameter (i.e. molecular weight) are crowded into a diagonal area running across the two-dimensional gel. In common with other two-dimensional systems the resolution attained is greatly improved upon that obtained by 1D-PAGE.

2D-PAGE has been shown both necessary for, and successful in, the analysis of the complex NHCP. Future research will undoubtedly lead to a further refinement in 2D-PAGE techniques for the analysis of these proteins. In this respect the finding that normal isoelectric focusing in the presence of SDS can be achieved in polyacrylamide gels containing high concentrations of urea (P. Coffino, personal communication) may well be utilised, since the solubility of the NHCP remains a problem.
The Identity of the Non-Histone Chromatin Proteins

The first question to which high-resolution 2D-PAGE analysis was applied was that of the identity of the NHCP i.e. are the NHCP unique to chromatin or do they represent a class of nuclear proteins which are more closely associated with chromatin but are also found in the intra-nuclear milieu (nucleoplasm)? This question could be directly answered by comparing the non-histone protein complement of nuclei, nucleoplasm and chromatin.

Samples for 2D-PAGE were prepared from nuclei, nucleoplasm (the saline-soluble fraction in the preparation of chromatin from nuclei - see Materials and Methods) and chromatin. The resulting 2D-PAGE analyses are presented in figure 16 (figure 4 for nuclei). These three samples show many common non-histone protein components. Many of these common components are present in different proportions in the chromatin and nucleoplasmic samples (e.g. those indicated by open arrows), showing that quantitative differences occur between these samples. Very few components are present in the chromatin sample which may be absent from the nucleoplasmic sample (e.g. those enclosed in circles). However several proteins are unique to the nucleoplasmic sample (e.g. those indicated by closed arrows) which contains more individual protein species (a reflection of non-histone protein sample loads). The overall impression gained from these gels is the considerable similarity between chromatin and nucleoplasmic non-histone proteins - almost
Figure 16

2D-PAGE analysis of NHCP and saline-soluble nucleoplasmic proteins.

a) NHCP (see legend to figure 14a).

b) saline soluble nucleoplasmic proteins (see legend to figure 13b).
all of the NHCP being represented in the nucleoplasm.

A similar analysis to the above is shown in figure 17. Figure 17a and b shows the proteins of mouse A9 cell cytoplasm and nuclei respectively. The two-dimensional separation of cytoplasmic proteins shows a highly complex array of protein spots throughout the gel (no attempt has been made to quantify the number of protein species present). The non-histone proteins of mouse A9 cell nuclei are more limited in number (figure 17b), several spots being greatly enriched in this sample. Figure 17c and d present the non-histone proteins of mouse A9 cell saline-soluble nucleoplasm and chromatin respectively. Very similar results are obtained to those in figure 16 - extensive homology is shown between the two separations, with quantitative differences in homologous proteins (open arrows); the nucleoplasmic sample is, however, more complex, showing many protein species not seen in the chromatin sample (e.g. closed arrows). Neither of these samples show the large spots around position 55/55 seen in the 2D analysis of nuclei (figure 17b) because of a differing nuclei preparation (Stein and Burtner, 1974 for figure 17c and d; modified as in Materials and Methods for figure 17b).

These results may be directly compared with those of MacGillivray and Rickwood (1974) who used identical procedures for the preparation of nucleoplasm and chromatin, but a different 2D-PAGE system for the analysis of non-histone proteins. These workers found that the two-dimensional gel patterns of soluble nuclear
Figure 17

2D-PAGE analysis of mouse A9 cell proteins.

Cells were labelled with $^{35}$S-methionine at 1μCi/ml medium for 48 hrs. Proteins prepared for and run in 2D-PAGE as described in Materials and Methods.

a) cytoplasmic proteins:– 80mM NaCl, 20mM EDTA, 1% TRITON (pH 7.2) and 0.15M NaCl, 0.01M Tris-HCl (pH 7.0) extracts of cells combined (see Materials and Methods).

Sample load - $5 \times 10^5$ cpm Exposure time - 32 days

b) nuclear proteins:– nuclei prepared as described in Materials and Methods

Sample load - $2 \times 10^5$ cpm Exposure time - 32 days

c) nucleoplasmic proteins:– saline-soluble extract of nuclei (Materials and Methods) prepared by method of Stein and Burtner (1974)

Sample load - $1.5 \times 10^5$ cpm Exposure time - 32 days

d) Chromatin proteins:– chromatin prepared from nuclei (Materials and Methods) prepared as in (c) above.

Sample load - $1.5 \times 10^5$ cpm Exposure time - 32 days
(nucleoplasmic) proteins differed markedly from the non-histone proteins of mouse liver chromatin. Some polypeptides were found to be of similar molecular weight and isoelectric point but they suggested that further analysis was required to confirm this identity. The 2D-PAGE system described here offers such an improved high resolution analysis, thus confirming the identity of many non-histone proteins in both nucleoplasmic and chromatin fractions, and suggesting considerable homogeneity between these fractions.

Further evidence in support of this view comes from the extensive studies of Comings and co-workers (Comings and Tack, 1973; Comings and Harris, 1975; Comings and Harris, 1976). Initially, in studies employing 1D-PAGE in the analysis of nucleoplasmic and chromatin non-histone proteins of mouse liver, these workers found many similar protein bands. Later studies, using higher resolution 1D-PAGE techniques revealed that all the prominent non-histone proteins which remain bound to DNA are also present in similar proportions in saline-EDTA and Tris washes of nuclei-supporting the conclusion that there is no clear differences between many nucleoplasmic and chromatin-bound non-histone proteins. Inspection of the chromatin and nucleoplasmic non-histone protein separations by 2D-PAGE of Peterson and McConkey (1976) reveals considerable identity of these samples. Several workers have compared the nucleoplasmic proteins with those non-histone proteins which can be extracted from
chromatin with 0.35M NaCl. The general conclusion
drawn by these workers is also that considerable
similarity is found in the electrophoretic patterns of
these fractions (Kostraba, Montagna and Wang, 1975;
Banks-Schlegel et al., 1976; Umansky et al., 1976).

The identity of nucleoplastic and chromatin
non-histone proteins indicates that the non-histone
proteins of the nucleus contain species with varying
affinity to chromatin. The results reported here
support the view of a fluid exchange of proteins
between chromatin and nucleoplasm. This is not
inconsistent with the possible role of the NHCP in the
control of gene transcription — regulatory proteins
in eukaryotes may exhibit a spectrum of DNA-binding
properties. Thus future searches for genetic
regulatory elements may need to take into account, as
well as the NHCP, the non-histone proteins present
in the nucleoplasm i.e. the total non-histone
protein complement of the nucleus.
SECTION II
Proteins of Fractionated Chromatin

A discussion of some differences between active and inactive chromatin in vivo was presented earlier and will not be re-iterated here. The active fraction of chromatin is defined as that portion containing DNA sequences which are in the process of being transcribed. The extent of transcription of the non-repetitive DNA sequences of mouse liver has been estimated at around 5% (Grouse, Chilton and McCarthy, 1972). Measurements of the homology between total cell RNA and DNA by DNA-RNA hybridisation indicate that around 10% of the base sequences of eukaryotic cell DNA are represented in the population of RNA sequences of the eukaryotic cell (Davidson and Britten, 1973). Therefore studies on isolated whole chromatin necessarily represent studies on the properties of the inactive (>90%) portion of the genome. A prerequisite of studies on the active fraction of chromatin is its separation from the majority of the chromatin, allowing more detailed studies into the possible roles of the NHCP in the structure of active chromatin and the control of gene transcription. An analysis of the NHCP content of fractionated chromatin is presented later in this section after a discussion of chromatin fractionation techniques.

A wide variety of methods have been used to fractionate chromatin into active and inactive portions. The general approach has been to fragment chromatin to a size smaller than the average transcriptional unit,
since active and inactive fractions are thought to be interspersed (Frenster, 1965). To achieve this chromatin has often been fragmented by random shearing, either by sonication, extensive homogenisation or passage through a small orifice at high pressure. This method is random and may be non-selective and therefore chromatin must be extensively sheared before fragments containing predominantly active chromatin are released. Fractions of chromatin, putatively active and inactive, have subsequently been separated on a physical basis by a number of techniques - differential centrifugation (e.g. Frenster, Alifrey and Mirsky, 1963; Yasmineh and Yunis, 1969; Duerksen and McCarthy, 1971), sucrose or glycerol density gradient centrifugation (e.g. Duerksen and McCarthy, 1971; Rodriguez and Becker, 1976a), isopycnic centrifugation (Rickwood et al., 1974), gel exclusion chromatography (Janowski, Nasser and McCarthy, 1972), ion-exchange chromatography (e.g. Reeck, Simpson and Sober, 1972), salt precipitation (Marushige and Bonner, 1971) and partition in two phase aqueous polymer systems (Turner and Hancock, 1974).

It is not possible here to review individually the evidence presented bearing on the validity of the fractionations obtained by workers using the above techniques. Several criteria for valid fractionations have been presented, each of which has drawbacks. Active chromatin would be expected to be enriched for both endogenous RNA polymerase and nascent RNA. However RNA polymerase is present only in small quantities
in chromatin and is readily detached during the manipulation of chromatin as is a large proportion of nascent RNA. Redistribution of these molecules during chromatin preparation and/or fractionation might therefore easily take place; hence lack of these molecules cannot be regarded as lack of activity in transcription. The increased ability of a chromatin fraction to be transcribed with exogenous bacterial RNA polymerase has been taken as indicative of active chromatin. However deproteinised DNA is an excellent template for bacterial RNA polymerase and therefore, if the chromatin preparation and fractionation procedure had caused any loss of protein from DNA, template activity measurements would be misleading. Loss of protein might also lower the melting temperature of a chromatin fraction - a lower melting temperature is often assumed to be an indication of less condensed (active) chromatin. Depletion in satellite DNA content has been quoted as characteristic of active chromatin. Satellite DNA has long been associated with heterochromatin which is thought to be transcriptionally inactive (Pardue and Gall, 1970). Failure to detect satellite DNA in a chromatin fraction may merely reflect the absence of heterochromatin containing satellite DNA from that fraction. No information can be gathered about the bulk of the heterochromatin which does not contain satellite DNA.

A good example of the difficulties of establishing criteria for the purity of active chromatin is given by Krieg and Wells (1976). These workers fractionated
chromatin (sheared in a French pressure cell) from purified populations of chick erythroid cells by gel exclusion chromatography into two fractions which showed many of the properties of active and inactive chromatin. Thus the "active" fraction showed a lower melting profile and preferential association of nascent RNA. Krieg and Wells synthesised cDNA probes to the DNA sequences of globin genes which are active in erythroid cells and to keratin genes which are inactive in these cells. They then hybridised these probes to "active" and "inactive" chromatin DNAs and found only a slight enrichment for globin gene sequences relative to the DNA content of the "active" fraction. An equal distribution of keratin gene sequences between "active" and "inactive" fractions was found. The presence of globin gene sequences in "inactive" chromatin may be explained by the supposition of multiple forms of globin genes, not all of which are active. The finding that keratin gene sequences are present in the "active" fraction of chromatin, however, questions the validity of the fractionation of chromatin into active and inactive material.

A second approach which avoids the problem of shear forces is the limited digestion of chromatin with nucleolytic enzymes. The rationale behind this approach is that active chromatin is thought to exist in a more extended form, being more accessible to endogenous RNA polymerase and also more accessible to nucleases than inactive chromatin. Thus limited digestion with nucleases should preferentially excise active chromatin which may
then be separated from the bulk of the chromatin. The effects of micrococcal nuclease digestion on chromatin have already been described (see Introduction). Two fractions of chromatin can be prepared by digestion with Staphylococcal nuclease and metrizamide density gradient centrifugation (Paul and Malcolm, 1976). The base sequence complexity of the DNA from these fractions is not distinguishable from that of total DNA and there is no evidence of any concentration of DNA sequences complementary to polysomal polyadenylated RNA molecules. This suggests that no fractionation into active and inactive chromatin is obtained. On the other hand there is now evidence that transcriptionally active chromatin can be selectively digested using DNase I. Limited digestion of the DNA of isolated nuclei with DNase I preferentially removes globin DNA sequences from nuclei obtained from chick red blood cells but not from nuclei obtained from fibroblasts, brain or red blood cell precursors (Weintraub and Groudine, 1976). Similarly treatment of oviduct nuclei from the laying hen with pancreatic DNase I results in the preferential digestion of over 70% of the ovalbumin gene sequences when only 10% of the total nuclear DNA has been solubilised (Garel and Axel, 1976). In both cases no specific digestion of transcriptionally active chromatin was observed in similar digestions with micrococcal nuclease. At present, however, the nature of the digestion products of the above DNase I digestions is unknown. A major disadvantage of the application of these findings to
chromatin fractionation studies may be that the DNA of
only one fraction (i.e. the nuclease-resistant one)
is obtained, that of the nuclease-susceptible transcrip
tionally-active fraction being destroyed during
the separation.

Endogenous nucleolytic activities in chromatin have
also been used in attempts to prepare active and inactive
chromatin fractions. Thus mouse TLT hepatoma chromatin
incubated in the presence of added MgCl₂ and CaCl₂, but
in the absence of added exogenous nucleases, can be
fractionated by glycerol density gradient centrifugation
into putatively inactive and active chromatin (Paul and
Duerksen, 1976a, b). This method yields chromatin
fractions which retain their native beaded structure as
determined by electron microscopy, but the evidence for
an active/inactive chromatin fractionation is based upon
satellite DNA content and nascent RNA distribution and
hence cannot be regarded as rigorous for the reasons
expressed previously.

A further nuclease digestion procedure applied to
the fractionation of chromatin is that of Gottesfeld et al.
(1974). Rat liver chromatin is selectively sheared
by limited digestion with DNase II, the released portion
being further fractionated on the basis of its solubility
in 2mM MgCl₂. Three fractions are obtained by this
procedure - P₁, undigested chromatin, containing 85% of
the total DNA; P₂, digested MgCl₂-insoluble chromatin,
containing 4% of the total DNA; S₂, digested MgCl₂-soluble
chromatin, containing 11% of the total DNA. Convincing
evidence now exists that fraction S2 represents the transcriptionally active portion of chromatin. This fraction exhibits many of the previously discussed properties of active chromatin such as enrichment for nascent RNA and high template activity. DNA renaturation studies show the fraction S2 is composed of a specific subset of whole genomical DNA sequences. In addition, DNA-RNA hybridisation studies indicate that almost 60% of the non-repetitive DNA sequences of this fraction hybridise to total liver RNA (assuming asymmetrical transcription), an enrichment of 5-6 fold over the total DNA of the genome.

More recently Gottesfeld, Murphy and Bonner (1975) have shown that both transcriptionally inactive (P1) and active (S2) fractions of chromatin contain nuclease-resistant structures. Those of inactive chromatin are DNA-histone complexes; however, those of active chromatin are complexes of DNA, RNA, histone and non-histone protein. Thermal denaturation and circular dichroism studies also suggest that active chromatin is in a more extended DNA-like conformation than inactive chromatin (Gottesfeld et al., 1975). These results indicate that there is indeed some difference in the structural organisation of active and inactive chromatin.

The DNase II digestion method was adopted for the fractionation of chromatin. The rationale for the following studies in this thesis is based on the assumption that differences in chromatin structure would be reflected in chromatin proteins, particularly the NHCP. Thus,
after fractionation of chromatin by the above DNase II method, the proteins of putatively active and inactive chromatin were analysed by 1D-PAGE and 2D-PAGE techniques.

The percentages of total DNA and protein in the various fractions of mouse liver and mouse A9 cell chromatin digested with DNase II (Materials and Methods) are presented in table 2a and b. The total amount of DNA digested \((P2 + S2)\) in mouse liver chromatin was found to be approximately 25%, 10% of which was soluble in 2mM MgCl\(_2\). The comparable figures for mouse A9 cell chromatin were 58% and 7% respectively. Thus while the amount of DNA in the S2 fraction was comparable to that found by Gottesfeld et al. (1974), the total amount of DNA digested \((P2 + S2)\) was greater. Digestion of the chromatin of Friend Erythroleukaemia cells with DNase II yielded a 15% digestion of total DNA, 5% of which was found in the S2 fraction (Lau and Ruddon, 1977). Lysed hen oviduct nuclei digested with DNase II by a modified procedure gave a 48% digestion of DNA, 5% of which was again found in the S2 fraction (Hemminki, 1975b). It is apparent that while fairly large variations do occur in the amount of total DNA digested, the results presented here and those of other workers show that the amount of DNA in the MgCl\(_2\)-soluble digested fraction of chromatin is fairly constant (5-11% of the total DNA).

The variation in the total amount of DNA digested may well result from small variations in experimental procedure. Gottesfeld et al. (1975) have shown that,
Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml. medium) and $^3$H-thymidine (1μCi/ml. medium) for 48 hr. Cells were harvested, chromatin prepared and digested with DNase II as described in Materials and Methods. Aliquots of the resulting fractions were used to determine $^{35}$S- and $^3$H- radioactivity by double-label scintillation counting with correction for spillover. Control values signify those for chromatin fractions obtained in the absence of DNase II.

<table>
<thead>
<tr>
<th>CHROMATIN FRACTION</th>
<th>% TOTAL DNA</th>
<th>% TOTAL PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>41.6</td>
<td>44.2</td>
</tr>
<tr>
<td>P2</td>
<td>51.5</td>
<td>32.7</td>
</tr>
<tr>
<td>S2</td>
<td>6.7</td>
<td>22.0</td>
</tr>
<tr>
<td>CONTROL P1</td>
<td>99.1</td>
<td>85.5</td>
</tr>
<tr>
<td>CONTROL P2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CONTROL S2</td>
<td>0.3</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Figures given are averages of two determinations.
for a given ratio of enzyme concentration to DNA, the extent of DNA digestion is highly time-dependent, the amount of DNA in fraction P2 after 7 min. digestion being 4 times that after 5 min. digestion. Alternatively the extent of digestion may vary with the state of chromatin prepared by various techniques. In the present study chromatin was subjected to an identical treatment as that for DNase II digestion but without the addition of the enzyme. In this control experiment 15% of the total DNA of mouse liver chromatin was solubilised, 5% of the total DNA being found in the S2 fraction. In a similar control experiment with mouse A9 cell chromatin no such solubilisation of DNA was found. The cause of this solubilisation of mouse liver chromatin DNA is undetermined and no similar control experiments have been reported by other workers. It is possible that this effect is a result of the preparation method of mouse liver chromatin (see Materials and Methods) which includes steps in which 3mM CaCl₂ is present. Upon subsequent incubation of chromatin prepared by this method, endogenous Ca²⁺/Mg²⁺-induced endonucleases may be activated leading to autodigestion of chromatin. Similar phenomena have been reported by other workers (Paul and Duerksen, 1976a,b; Urbanczyk and Studzinski, 1974). Since the chromatin of mouse A9 cells is not exposed to Ca²⁺ or Mg²⁺ during preparation no similar phenomenon would be expected.

The protein content of the various fractions of chromatin obtained by DNase II digestion was analysed.
Figure 18 shows a 1D-PAGE analysis of mouse liver total chromatin proteins, fractions P1, P2 and S2 proteins and their respective controls done in the absence of DNase II. Fraction P1 and its control show identical protein banding patterns to the total chromatin protein sample both with respect to histone and non-histone proteins. This is to be expected since these fractions constitute some 75% and 85% of the total chromatin respectively. Fraction P2 is depleted in NHCP, but shows the standard complement of histones. The control fraction P2 is also depleted in NHCP, shows no histone H1, but may include the lower molecular weight histones (the presence of RNase in the sample obscures these). A similar situation is found in fraction S2 and its control i.e. these samples appear to be deficient in histone H1, but may include some of the lower molecular weight histones. The presence of histones in the control samples P2 and S2 suggest that some autodigestion of chromatin as discussed previously has taken place. The control fraction S2, when compared to total chromatin, shows an altered banding pattern from that of total chromatin. Fraction S2 shows a double band of high molecular weight (closed arrow) which is absent from all other fractions and the total chromatin protein sample. This represents the DNase II used in the digestion - in this and all subsequent analyses of DNase II-digested mouse liver chromatin and nuclei, DNase II was found to remain in the supernatant fractions after centrifugation and hence was
Figure 18

1D-PAGE analysis of fractions of DNase II-digested chromatin.

Mouse liver chromatin was prepared, digested with DNase II and the resulting fractions prepared for 1D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

Sample slots:

a) total chromatin proteins (70μg)
b) fraction P1 proteins (80μg)
c) control fraction P1 proteins (75μg)
d) fraction P2 proteins (45μg)
e) control fraction P2 proteins (20μg)
f) fraction S2 proteins (50μg)
g) control fraction S2 proteins (40μg)

Closed arrow is DNase II

Figures given are molecular weight x 10^-3
DNase I and RNase are present as a result of their use in sample preparation.
found in the S2 fraction.

The major finding in this analysis is that of two prominent bands (open arrow) of molecular weight approximately 45,000 in the S2 fraction. These bands are enriched in this fraction over both the control S2 and P1 fractions. Thus specific NHCP components can be demonstrated to be enriched in the S2 fraction.

The chromatin fractions analysed above were also subjected to 2D-PAGE. Fraction P2 and its control fraction were not analysed however since these fractions could be seen from 1D-PAGE to be deficient in NHCP. Figure 19 presents 2D-PAGE analyses of fraction P1 and the control fraction P1. The analyses of fraction P1 and control fraction P1 can be seen to be similar and correspond closely to those of total chromatin presented in figure 14. Some differences were observed between these fractions. Fraction P1 contains protein spots of high isoelectric point which are absent from control fraction P1 (e.g. 66-67/50-55). One intermediate spot (55/80) is possibly present in control fraction P1 but absent from fraction P1. Differences may also exist at low molecular weight values (e.g. control P1 may be enriched for spots around 63/25) but these are more difficult to determine because of the poorer quality of the gel containing the control P1 sample in this region.

Fractions S2 and control S2 contain only 10% and 5% of the total chromatin proteins respectively. Part of these figures is represented by histones - it thus
2D-PAGE analyses of fractions of DNase II-digested chromatin.

Mouse liver chromatin was prepared, digested with DNase II and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

Samples:

a) P1; sample load 200μg total protein

b) control P1 (minus enzyme); sample load 180μg total protein

Arrowed spots are DNase I.
proved difficult to obtain sufficient of these samples for analysis by 2D-PAGE. However analyses of these samples are shown in figure 20. Fraction S2 shows significant enrichment of several spots (closed arrows) over the analysis of fraction P1 (fig. 19a). Most of the protein spots enriched in S2 can be seen to be absent from control S2 (e.g. at 63/35, 55/29, 54/55). Thus again enrichment for specific NHCP components in the S2 fraction of DNase-II digested chromatin has been demonstrated. The spots indicated by the open arrows are DNase II (determined by an analysis of a sample containing DNase II alone).

The above analyses were repeated with chromatin prepared from mouse A9 cells grown in the presence of $^{35}$S-methionine ($1\mu$Ci/ml medium) and $^3$H-thymidine ($1\mu$Ci/ml medium) for 48 hrs. This was necessary since later studies of the proteins of nuclear ribonucleoprotein complexes were carried out using these cells (no success was achieved in attempts to prepare these complexes from mouse liver - see later). $^3$H-thymidine was included in the chromatin fractionation studies to facilitate measurement of the DNA in chromatin fractions.

A 1D-PAGE analysis of the fractions of mouse A9 cell chromatin digested with DNase II is presented in figure 21. Fractions P1 and control P1 are again similar and show considerable homogeneity with fraction P2 (in the absence of DNase II no P2, i.e. control P2, fraction was obtained). Histones are present in all
Figure 20

2D-PAGE analyses of fractions of DNase II-digested chromatin. Mouse liver chromatin was prepared, digested with DNase II and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

Samples:

a) S2; sample load 100\mu g total protein

b) control S2; sample load 80\mu g total protein

Open arrows are DNase II
Closed arrows are those proteins enriched in the S2 fraction.
Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml medium) and $^3$H-thymidine (1μCi/ml medium) for 48 hr. Chromatin was prepared, digested with DNase II and the resulting fractions prepared for 1D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

Sample slots:-

a) and b) P1; approximate sample loads 1 and 1.5 x $10^4$ cpm $^{35}$S respectively.

c) P2; approximate sample load 5.5 x $10^3$ cpm $^{35}$S.

d) and e) S2; approximate sample loads 5 and 7.5 x $10^3$ cpm $^{35}$S respectively.

f) control P1; approximate sample load 1.5 x $10^4$ cpm $^{35}$S

g) control S2; approximate sample load 1 x $10^4$ cpm $^{35}$S

Exposure time 21 days. Fluorography as described in Materials and Methods.
three of these fractions. Fraction S2 and the control fraction S2 are also very similar. These fractions can be seen to be depleted in histones and show some differences in NHCP complement at high molecular weight. A major feature of comparison of these samples is quantitative differences in NHCP bands in the molecular weight range around 50 - 60,000 (arrowed). In general differences in NHCP complement are not as clear as those which can be seen for mouse liver chromatin in figure 18, however fraction S2 does seem to be enriched over fractions P1 and control S2 for specific NHCP bands in the above molecular weight range.

Analyses by 2D-PAGE of fractions P1 and P2 of mouse A9 cell chromatin after DNase II digestion are presented in figure 22. Fraction P1 is similar to that of total mouse A9 cell chromatin presented earlier (figure 15b), as was its control fraction P1. Fraction P2 shows quantitative differences from P1. Thus spots at 60 and 58/70 may be depleted as may be those around position 54/55. In the absence of DNase II no control P2 fraction was obtained. Fraction P2 may thus represent a defined portion of chromatin digested by DNase II and depleted in NHCP.

A dark shading effect can be seen at the higher end of the pH gradient and in the lower molecular weight range of the SDS dimension. This material is thought to be $^3$H-DNA and its breakdown products (see section 1). All gels containing $^{35}$S-labelled proteins were impregnated with PPO before fluorography, this method being ten times
Figure 22

2D-PAGE analyses of fractions of DNase II-digested chromatin.

Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml medium) and $^3$H-thymidine (1μCi/ml medium) for 48 hr. Chromatin was prepared, digested with DNase II and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods.

a) P1; sample load $4 \times 10^4$ cpm $^{35}$S. 40 day exposure

b) P2; sample load $2.5 \times 10^4$ cpm $^{35}$S. 40 day exposure

Fluorography as described in Materials and Methods.
more sensitive than conventional autoradiography (Bonner and Laskey, 1974). The method increases the sensitivity of detection of $^3$H, however, some 500-fold and makes the detection of $^3$H almost as efficient as the detection of $^{35}$S by conventional autoradiography. The shading of the films shown in figure 22 does not seriously interfere with the analysis of $^{35}$S-labelled proteins.

Figure 23 presents 2D-PAGE analyses of the S2 fraction of DNase II-digested mouse A9 cell chromatin and its corresponding minus enzyme control fraction. When compared with fraction P1, fraction S2 is enriched for NHCP in the region 52-56/45-60. Comparison with the control S2 (minus DNase II) fraction indicates that this enrichment is also seen for some spots in the control fraction. A significant difference between the S2 fraction and the control S2 fraction is an enrichment for three NHCP spots in the 50-60,000 molecular weight range (arrowed). This corresponds to an enrichment for proteins of this molecular weight in the S2 fraction of mouse A9 cell chromatin as analysed by 1D-PAGE (figure 21).

From this series of analyses it can be seen that enrichment for particular NHCP species in fraction S2 of DNase II-digested chromatin over fraction P1 is observed (e.g. bands at molecular weight 40,000 and 55,000 in figures 18 and 21, arrowed spots in figures 20 and 23). The situation is complicated, however, when the results of the minus enzyme control experiments are
2D-PAGE analysis of fractions of DNase II-digested chromatin.

Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml medium) and $^{3}$H-thymidine (1μCi/ml medium) for 48 hr. Chromatin was prepared, digested with DNase II and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods.

Samples:

a) S2; sample load $2.25 \times 10^4$ cpm. Exposure time 40 days.

b) control S2 (minus enzyme) $4 \times 10^4$ cpm. Exposure time 40 days.

Fluorography as described in Materials and Methods. No DNase II is seen in this analysis, since only labelled proteins are revealed by fluorography.

Closed arrows indicate those proteins enriched in the S2 fraction.
taken into account. In mouse liver chromatin, some autodigestion of chromatin may have taken place and the results for the DNase II digested chromatin must be viewed against a background of material (DNA and protein) released into the S2 fraction in the absence of DNase II. The results of other workers suggest that the chromatin released during autodigestion is enriched for active chromatin (Paul and Duerksen 1976a,b) and hence such autodigestion, if occurring, might reinforce the separation obtained by DNase II. In the analysis of mouse A9 cell chromatin a considerable portion of protein is released into the minus enzyme control S2 fraction without any release of DNA. Possible reasons for this finding will be discussed in section III of this chapter. Despite these complications specific NHCP components can be demonstrated to be enriched in the S2 fraction.

In the study of the proteins of fractionated chromatin, the possibility of protein redistribution during the fractionation procedure must be taken into account. The use of shear forces has been found to induce movement of histones in chromatin (e.g. Doenecke and McCarthy, 1976). Under some conditions of nuclease digestion of chromatin released proteins may aggregate onto undigested DNA or even degraded DNA (Itzhaki, 1974). Although the DNase II digestions described here were carried out under ionic conditions reported to cause minimal redistribution of proteins (Gottesfeld et al., 1974; Billing and Bonner, 1972)
it was felt important to attempt to determine whether any non-specific release or re-distribution of proteins could be detected during the digestion procedure.

The first experiment designed to detect redistribution of proteins during DNase II digestion of chromatin involved the addition of extraneous labelled DNA to mouse liver chromatin during digestion. The rationale behind this experiment lay in the supposition that protein redistribution might involve proteins being released during digestion from chromatin and re-bound at another site. Such proteins could bind to extraneous, labelled, protein-free DNA and protect that DNA from digestion by DNase. The full experimental procedure of this experiment is included in the legend to figure 24. Briefly, \(^{14}\)C-labelled DNA from mouse A9 cells was added to mouse liver chromatin samples during DNase II digestion. A minus enzyme control, digestion of mouse liver chromatin and mouse A9 cell DNA alone and their minus enzyme controls were also included. Immediately after digestion DNA was prepared from these samples for DNA-agarose gel electrophoresis. Figure 24A presents an ethidium bromide-stained gel of these samples. Slot a represents the pattern produced by mouse liver chromatin digested with DNase II. The minus enzyme control sample is presented in slot b. The characteristic banding pattern produced by the action of nucleases on chromatin can be seen in slot a. In the absence of DNase II most of the DNA remains at the top of the gel and this is of high molecular weight. Slots c and d present the identical
DNA-agarose gels of DNase II digestion of chromatin and $^{14}$C-DNA

Mouse liver chromatin and $^{14}$C-labelled DNA were prepared as described in Materials and Methods. Chromatin was suspended and dialysed overnight as for DNase II digestion (Materials and Methods) and subsequently adjusted to a concentration of 20 $A_{260}$nm with 25mM NaAcetate, pH 6.6. Chromatin was further diluted to 10 $A_{260}$nm with 25mM NaAcetate alone or containing $^{14}$C-DNA. Similarly $^{14}$C-DNA in 25mM NaAcetate was diluted $x_{\frac{1}{2}}$ with 25mM NaAcetate. These three solutions were then digested with DNase II as described in Materials and Methods; samples were also treated in an identical fashion but minus enzyme. DNA was isolated from these samples and run in DNA-agarose gels as described in Materials and Methods.

Samples:

a) DNase II digested chromatin (5µg DNA)
b) minus enzyme control chromatin (4µg DNA)
c) DNase II digested chromatin and $^{14}$C DNA (5µg DNA, 270 cpm $^{14}$C)
d) minus enzyme control chromatin and $^{14}$C DNA (5µg DNA, 400 cpm $^{14}$C)
e) DNase II digested $^{14}$C-DNA (250 cpm $^{14}$C)
f) minus enzyme control DNA (600 cpm $^{14}$C)

A) Photograph under UV illumination.
B) Fluorograph of above. Fluorography as described in Materials and Methods. Exposure time 30 days. Specific activity $^{14}$C-DNA was 400 cpm/µg.
samples to slots a and b respectively but mouse A9 cell DNA was included in these samples. Similar results are found — mouse liver chromatin and mouse A9 cell DNA digested with DNase II shows a range of DNA of varying molecular weight; in the absence of DNase II most of the DNA remains of high molecular weight. Slots e and f show DNase II-digested and undigested mouse A9 cell chromatin respectively — again the high molecular weight DNA in the undigested sample is absent from that treated with DNase II. After photography under UV illumination this gel was processed for subsequent fluorography. Figure 24B shows the resulting fluorogram. Slots a and b contained no 14C-labelled DNA and hence are absent from the fluorogram. Slots d and f represent undigested 14C-thymidine DNA in the presence of mouse liver chromatin and on its own respectively. Slots c and e contained 14C-thymidine DNA digested with DNase II in the presence and absence of mouse liver chromatin respectively. In both cases the high molecular weight DNA present in the control slots (d and f) is absent, indicating the digestion of DNA. Hence even in the presence of mouse liver chromatin, extraneous DNA is completely destroyed suggesting that no protein has been released from the chromatin and bound to the extraneous labelled DNA during the digestion procedure.

An alternative method to detect redistribution of protein during DNase II digestion was also attempted. This was considered necessary since protein redistribution
could be caused by the "sliding" of proteins along chromatin (without their complete removal and re-binding). Such a phenomenon might lead to these proteins binding to a site in chromatin with a different affinity than they would exhibit at their original site. These proteins might therefore be detected by the ionic strength with which they were dissociated from digested chromatin as opposed to undigested chromatin. An experiment, based on binding affinities, was designed to detect such differences.

The proteins released from the P1 fraction of DNase II-digested mouse liver chromatin and from undigested mouse liver chromatin at various concentrations of NaCl were analysed by 1D-PAGE (figure 25). Slots marked P1 represent DNase II-digested mouse liver chromatin fraction P1, those marked C mark untreated chromatin. The total protein content of these samples has already been shown to be identical under 1D-PAGE (figure 18). Slots A represent the 0.15M NaCl wash of these samples, slots B and C the 0.30M and 0.50M NaCl washes respectively. Slots D represent the protein content of the residual material after these washes. The proteins released from both these samples at the various salt concentrations can be seen to be identical; the differences observed between the samples in slots D represent quantitative differences in sample load.

Some comments on the release of proteins from these samples might be made. The amount of protein released at each stage from digested P1 and undigested chromatin is given in Table 3. Some NHCP are released at low salt.
### TABLE 3

**NaCl dissociation experiment**

For full experimental procedure see legend to figure 25.
Figures quoted are standard deviations around a mean (of three determinations).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Protein released</th>
<th>Undigested chromatin</th>
<th>Chromatin fraction P1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15M NaCl wash</td>
<td>19.1 ± 4.2</td>
<td></td>
<td>23.2 ± 6.8</td>
</tr>
<tr>
<td>0.30M NaCl wash</td>
<td>25.9 ± 7.1</td>
<td></td>
<td>23.9 ± 1.0</td>
</tr>
<tr>
<td>0.50M NaCl wash</td>
<td>30.9 ± 2.6</td>
<td></td>
<td>33.1 ± 8.5</td>
</tr>
<tr>
<td>chromatin residue</td>
<td>25.1 ± 4.0</td>
<td></td>
<td>20.5 ± 0.4</td>
</tr>
</tbody>
</table>
Figure 25

NaCl dissociation of DNase II-treated chromatin fraction P1 and untreated chromatin.

Mouse liver chromatin was prepared and digested with DNase II as described in Materials and Methods. Untreated chromatin and fraction P1 of DNase II-digested chromatin were suspended by homogenisation in 0.15M NaCl, 0.01M Tris-HCl (pH 7.5), 5mM MgCl₂, 0.1mM PMSF. The solutions were centrifuged at 1,000xg for 10 min. The supernatants were retained and the pellets resuspended in the above solution at 0.30M NaCl. After centrifugation this procedure was repeated with 0.50M NaCl. The resulting NaCl washes and the residual pellets were prepared for analysis by 1D-PAGE.

Samples:

P1 A - 0.15M NaCl wash of P1 fraction (65μg total protein)
P1 B - 0.30M " " " " (55μg " " )
P1 C - 0.50M " " " " (65μg " " )
P1 D - residual material after NaCl washes of P1 fraction (50μg total protein)
CA - 0.15M NaCl wash of untreated chromatin (50μg total protein)
CB - 0.30M " " " " (55μg " " )
CC - 0.50M " " " " (50μg " " )
CD - residual material after salt washes of chromatin (45μg total protein)

Sample preparation was as described in Materials and Methods, including DNase I (closed arrow) and RNase (open arrow) digestion step.
concentrations - it is well known that continued washing of chromatin removes NHCP. The majority of different NHCP species, as can be seen from figure 25, are washed from chromatin only by 0.5M NaCl or remain attached to chromatin after this treatment indicating a strong association of these proteins with chromatin. The first evidence of histones being removed occurs in the 0.5M NaCl wash (although DNase and RNase obscure the presence of these proteins histone H1 and the lower molecular weight histones can be seen). Most of histone H1 is removed from these samples by 0.50M NaCl, a large amount of the other histones remains attached even after these salt treatments. The similarity between the proteins (histones and NHCP) released by the various salt treatments of the PI fraction of DNase II-digested and untreated mouse liver chromatin suggests that no protein redistribution leading to proteins binding with differing affinities to sites in chromatin other than their original site has taken place.

As tested by the above methods no protein redistribution during the digestion of chromatin could be detected. These experiments do not rule out the possibility of redistribution of protein during the step immediately prior to the digestion of chromatin i.e. the preparation of chromatin from nuclei. As a check for this chromatin was digested in situ in nuclei and the proteins of the resulting chromatin fractions examined to determine if these showed similar enrichments for specific NHCP.

The DNA and protein contents of the various fractions
of mouse liver and mouse A9 cell nuclei obtained by
digestion with DNase II for 5 min. are shown in Table 4a
and b. The total amount of DNA digested (P2 + S2) in
mouse liver nuclei was approximately 15%, in mouse A9
cell nuclei the comparable figure was 10%. These figures
are lower than the figures for the respective chromatins
(table 2a, b) - this may reflect the lower accessibility
of chromatin in nuclei to DNase II. The amounts of
DNA in the S2 fractions were also decreased (3% for
mouse liver nuclei, 0.5% for mouse A9 cell nuclei).

Longer digestion times were not attempted because of concern over proteolysis during prolonged digestion.

In the case of the minus enzyme control experiments very little solubilisation of DNA was observed. Both S2 and control S2 fractions show increased amounts of protein over their corresponding fractions in chromatin (Table 2) - these fractions contain the proteins of the nucleoplasm, which are normally removed from the chromatin preparations.

The protein contents of the various fractions of
DNase II-digested mouse liver nuclei were analysed by
1D-PAGE (figure 26). In the case of the DNase II digestion
of intact nuclei the presence of nucleoplasmic proteins
not tightly bound to chromatin must be taken into account. Many of these would be expected to be released into the S2 fraction simply as a result of the processing of nuclei for DNase II digestion. Hence differences between the S2 and P1 fractions cannot be taken as differences between different fractions of chromatin. In these studies only differences observed between the nuclear fractions and
Mouse liver nuclei (Materials and Methods) were digested as for chromatin with DNase II and the DNA and protein contents of the various fractions determined as described in Materials and Methods. The figure in fraction S2 was corrected for the presence of DNase II. Control values signify those for nuclear fractions obtained in the absence of DNase II.

Figures given are averages of three determinations.

<table>
<thead>
<tr>
<th>NUCLEAR FRACTION</th>
<th>% TOTAL DNA</th>
<th>% TOTAL PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>85.3</td>
<td>46.1</td>
</tr>
<tr>
<td>P2</td>
<td>12.1</td>
<td>14.7</td>
</tr>
<tr>
<td>S2</td>
<td>3.3</td>
<td>40.0</td>
</tr>
</tbody>
</table>

| CONTROL P1        | 98.6        | 60.2            |
| CONTROL P2        | 1.2         | 4.2             |
| CONTROL S2        | -           | 35.7            |
Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1µCi/ml. medium) and $^3$H-thymidine (1µCi/ml. medium) for 48 hr. Cells were harvested, nuclei prepared and digested (as for chromatin) with DNase II as described in Materials and Methods. An aliquot of each fraction was used to determine $^{35}$S and $^3$H radioactivity by double-label scintillation counting with correction for spillover. Control values signify those for chromatin fractions obtained in the absence of DNase II.

Figures given are averages of two determinations.

<table>
<thead>
<tr>
<th>NUCLEAR FRACTION</th>
<th>% TOTAL DNA</th>
<th>% TOTAL PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>89.6</td>
<td>58.6</td>
</tr>
<tr>
<td>P2</td>
<td>9.9</td>
<td>7.8</td>
</tr>
<tr>
<td>S2</td>
<td>0.5</td>
<td>33.7</td>
</tr>
<tr>
<td>CONTROL P1</td>
<td>99.0</td>
<td>68.1</td>
</tr>
<tr>
<td>CONTROL P2</td>
<td>0.9</td>
<td>6.3</td>
</tr>
<tr>
<td>CONTROL S2</td>
<td>-</td>
<td>25.5</td>
</tr>
</tbody>
</table>
Figure 26

1D-PAGE analysis of fractions of DNase II-digested nuclei

Mouse liver nuclei were prepared and digested with DNase II (as for chromatin) and the resulting fractions prepared for 1D-PAGE as described in Materials and Methods.

Control fractions are those obtained in the absence of DNase II.

Sample slots:

N, total nuclear proteins (200 μg)
NPI, fraction P1 proteins (150 μg)
NPI (cont), control fraction P1 proteins (140 μg)
NP2, fraction P2 proteins (175 μg)
NP2 (cont), control fraction P2 proteins (175 μg)
NS2, fraction S2 proteins (160 μg)
NS2 (cont), control fraction S2 proteins (175 μg)
their respective control fractions prepared in the absence of DNase II can be considered as evidence for the fractionation of the chromatin in nuclei on a protein basis. Inspection of the gel presented in figure 26 reveals no such differences. These samples were further analysed by 2D-PAGE. The P1 fraction obtained by DNase II digestion of nuclei and its corresponding control (minus enzyme) P1 fraction are shown in figure 27. The NHCP protein patterns of these two samples can be seen to be identical. Some doubling and trebling of spots can be seen and this effect is highly reproducible between these samples.

Analyses of the S2 fraction of DNase II-digested nuclei and its control (minus enzyme) S2 fraction are presented in figure 28. The NHCP protein pattern differs markedly from those of the P1 fractions shown in figure 27. Much of this difference may lie with the proteins of the nucleoplasm as discussed previously. When the S2 fraction of DNase II-digested nuclei is compared to its control (minus enzyme) S2 fraction an elongated triple spot can be seen (55-54/75-65) which represents DNase II. A major difference is the presence of a NHCP spot at 63/35 (arrowed) - this protein was also found to be enriched in the S2 fraction of mouse liver chromatin (see figure 20). A further NHCP component which enriched in the S2 fraction of both DNase II - digested mouse liver chromatin and nuclei over their respective control fractions is that at position 56/28 (arrowed) (see also figure 20). Thus specific
Figure 27

2D-PAGE analysis of fractions of DNase II-digested nuclei.

Mouse liver nuclei were prepared and digested with DNase II (as for chromatin) and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

Samples:

a) P1, sample load 135µg
b) control P1, sample load 180µg
Figure 28

2D-PAGE analysis of fractions of DNase II-digested nuclei.

Mouse liver nuclei were prepared and digested with DNase II (as for chromatin) and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

Samples:

a) $S_2$, sample load $160\mu g$ total protein

b) control $S_2$ (minus enzyme), sample load $110\mu g$ total protein

Closed arrows indicate proteins enriched in the $S_2$ fraction. Open arrow indicates DNase II.
NHCP components, enriched in the S2 fraction of chromatin, are also enriched in the S2 fraction of nuclei over both P1 and control S2 fractions.

The above analyses of the fractions obtained by DNase II digestion of mouse liver nuclei were repeated with mouse A9 cell nuclei. Figure 29 presents a 1D-PAGE analysis of the fractions of DNase II-digested nuclei of mouse A9 cells grown in the presence of $^{35}$S-methionine and $^3$H-thymidine. Again no comparison can be made between the P1 and S2 fractions since the latter contains many nucleoplasmic proteins. Comparison of each fraction with its minus enzyme control shows no differences - an identical result to that found for mouse liver nuclei (see figure 26). These samples, obtained by DNase II-digestion of mouse A9 cell nuclei, were further analysed by 2D-PAGE. The NCHP complements of mouse A9 cell nuclei and fraction P1 of DNase II-digested nuclei are shown in figure 30. Here fraction P1 can be seen to contain most of the non-histone protein population of nuclei being enriched for some components (e.g. around 60/60, 64/60). In this experiment sufficient material was obtained to allow an analysis of the non-histone proteins of fraction P2 of DNase II-digested nuclei and the control (minus enzyme) fraction P2. These fractions, shown in figure 31, can be seen to be similar although fraction P2 is enriched for several spots of high isoelectric point and for other specific spots (e.g. 61/58, 55-56/60, 62/70). Finally analyses of the NHCP of fraction S2 of DNase II-digested mouse
Figure 29

1D-PAGE analysis of fractions of DNase II-digested nuclei.

Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1 µCi/ml medium) and $^3$H-thymidine (1 µCi/ml medium) for 48 hr. Nuclei were prepared, digested with DNase II (as for chromatin) and the resulting fractions prepared for 1D-PAGE as described in Materials and Methods.

Control fractions are those obtained in the absence of DNase II.

Sample slots:-

a) total nuclei; sample load $8 \times 10^4$ cpm $^{35}$S
b) P1; " $8 \times 10^4$ cpm $^{35}$S
c) control P1; " $7 \times 10^4$ cpm $^{35}$S
d) P2; " $3 \times 10^4$ cpm $^{35}$S
e) control P2; " $6 \times 10^3$ cpm $^{35}$S
f) S2; " $1 \times 10^5$ cpm $^{35}$S
g) control S2; " $1.2 \times 10^5$ cpm $^{35}$S

Exposure time 3 days. Fluorography as described in Materials and Methods.
Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml medium) and $^3$H-thymidine (1μCi/ml medium) for 48 hr. Nuclei were prepared and digested with DNase II (as for chromatin) as described in Materials and Methods. Nuclei and the resulting fraction P1 were prepared for 2D-PAGE as described in Materials and Methods.

a) nuclei; sample load $2 \times 10^5$ cpm $^{35}$S. 21 day exposure.

b) P1; sample load $2 \times 10^5$ cpm $^{35}$S. 21 day exposure.
Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1µCi/ml medium) and $^3$H-thymidine (1µCi/ml medium) for 48 hr. Nuclei were prepared, digested with DNase II (as for chromatin) and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

a) P2, sample load $7.5 \times 10^4$ cpm $^{35}$S. 80 day exposure.

b) control P2 (-enzyme), sample load $1.5 \times 10^4$ cpm $^{35}$S. 80 day exposure.
A9 cell nuclei and its respective control (minus enzyme) S2 fraction are presented in figure 32. Although the quality of the gel containing fraction S2 is somewhat reduced several differences can be detected between these samples. Fraction S2 of DNase II-digested chromatin is enriched for several spots in the range 58-64/40 and below. There is also an apparent increase in the amount of radioactivity in the 52-57/50-60 area (arrowed), which corresponds to a similar increase seen in this region in the S2 fraction of DNase II-digested mouse A9 cell chromatin both by 1D- and 2D-PAGE. The S2 fraction of DNase II-digested nuclei may also be depleted of NHCP spots at 64/65, 63/65 and 57/85.

Several general conclusions may be drawn from this series of experiments. An enrichment for specific NHCP components has been demonstrated both by 1D-PAGE and 2D-PAGE in the S2 fraction of DNase II-digested chromatin. This enrichment has been shown to be relative both to the amount of these proteins in the P1 fraction of these chromatin and to the amount of these proteins released from chromatin in the absence of enzyme treatment. No redistribution of proteins could be detected during the digestion procedure suggesting that these differences in NHCP are not artefactual. Furthermore these differences could also be detected in the DNase-II digestion of intact nuclei indicating that the results are not an artefact produced when chromatin is isolated from nuclei. It is unlikely that these results are due to differential proteolysis because...
Figure 32

2D-PAGE analyses of fractions of DNase II-digested nuclei.

Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml medium) and $^3$H-thymidine (1μCi/ml medium) for 48 hr. Nuclei were prepared, digested with DNase II and the resulting fractions prepared for 2D-PAGE as described in Materials and Methods. Control fractions are those obtained in the absence of DNase II.

a) S2, sample load 1 x $10^5$ cpm $^{35}$S. 21 day exposure.

b) control S2 (-enzyme), sample load 1.2 x $10^5$ cpm $^{35}$S. 21 day exposure.

Closed arrow indicates proteins enriched in the S2 fraction.
a) the high reproducibility of the two-dimensional gels, b) the digestions took place under ionic conditions under which chromatin proteases have been reported minimally active (Bartley and Chalkley, 1970; Carter and Chae, 1976) c) the protease inhibitor PMSF was included in the digestion solution and in subsequent dialyses in the preparation of samples for electrophoresis and d) chromatin and fractions of chromatin were maintained at 4°C, except for the 5 min. during which the digestion took place at 24°C. Thus the S2 fraction of DNase II-digested chromatin which corresponds to that fraction shown by Gottesfeld et al. (1974) to be enriched for unique DNA sequences coding for proteins is enriched for specific non-histone chromatin proteins.

While, to my knowledge, no similar 2D-PAGE analyses of the NHCP of fractionated chromatin have been reported, these results may be directly compared with those of others who have used DNase II to fractionate chromatin. In this study fraction S2 of mouse liver chromatin was found by 1D-PAGE to show increased amounts of specific NHCP components of molecular weight range 45-50,000. In the 2D-PAGE analyses of mouse liver chromatin specific NHCP spots of molecular weight 29,000, 35,000 and 55,000 are enriched in the S2 fraction. The above molecular weight bands seen in the 1D-PAGE analysis are not seen in the 2D-PAGE analysis - these proteins may have isoelectric points outwith the range of the pH gradient of the isoelectric focusing gels. Those NHCP found enriched in the 2D-PAGE analysis of fractionated chromatin
were also found enriched in the 2D-PAGE analysis of fractionated nuclei.

In mouse A9 cell chromatin digested with DNase II, NHCP of molecular weight range 50-60,000 were found to be increased in amount in the S2 fraction by both 1D-PAGE and 2D-PAGE analyses. This enrichment was also found in analyses of the NHCP of fractions of DNase II-digested nuclei.

Similar enrichments have been reported by other workers. The S2 fraction of rat liver chromatin digested with DNase II was found to be enriched for NHCP components of approximate molecular weight 40,000 by 1D-PAGE (Gottesfeld et al., 1974). This may correspond to the enrichment for NHCP of approximate molecular weight 45,000 observed by 1D-PAGE in the present study of mouse liver chromatin. The S2 fraction of DNase II-digested chromatin from Friend Erythroleukaemia cells also contains prominent protein bands at molecular weight 40,000 as well as at 68,000 and in the 23-30,000 molecular weight range. Similarly by 2D-PAGE analysis of mouse liver chromatin fractions, in the present work NHCP of approximate molecular weight 60,000, 35,000 and 29,000 were shown to be enriched in S2.

A complete analysis of the histones was not carried out in the work described here but fraction S2 could be seen to be lacking in H1 and depleted of the other histones. Using acid-urea polyacrylamide gel electrophoresis, Gottesfeld et al. (1974) found histone H1 absent, and histone H4 depleted, in the S2 fraction.
of DNase II-digested rat liver chromatin. Histone H1 is also absent from the S2 fraction of Friend Erythro-leukaemia cell chromatin (Lau and Ruddon, 1977). These findings are consistent with the results of experiments which implicate histone H1 in the restriction of template activity (Georgiev, Amanieva and Kozlov, 1966) and condensation of chromatin (Bradbury et al., 1975).

The analyses of the protein contents of chromatin fractions derived by DNase II digestion are thus fairly consistent between the workers who have used this method. Similar results (i.e. enrichment for specific NHCP and absence of histone H1) have been found by workers who fractionated sonicated chromatin by ion-exchange chromatography (Simpson and Polacow, 1973; Reeck, Simpson and Sober, 1974). Other methods have provided a variety of results. Shearing and gradient centrifugation have provided an "active" fraction that is enriched both quantitatively and qualitatively in NHCP and shows little differences in histones (Rodriguez and Becker, 1976b) and also an "active" fraction which contains histone H1 and is deficient in all other histones (Doenecke and McCarthy, 1975). Sheared chromatin fractioned by differential centrifugation has yielded "active" fractions with a full complement of histones which show specific NHCP (Comings and Harris, 1975; Comings et al., 1977) or no significant changes in NHCP (Warnecke, Kruse and Harbers, 1973).

These findings may reflect the differing methods used to prepare active and inactive chromatin fractions.
and the varying criteria set to judge the validity of these methods. There is much evidence to suggest that the DNase II method of Gottesfeld et al. yields a valid separation into active and inactive chromatin. In the studies reported here the NHCP of active and inactive chromatin obtained by this method have been shown to differ quantitatively and qualitatively. The following section will further examine the nature of these differences.
SECTION III

Proteins of Nuclear Ribonucleoprotein Complexes (nRNP)

It was pointed out in the Introduction that care must be exercised in the interpretation of results of experiments designed to examine the NHCP content of chromatin in relation to changes in gene transcription. Any differences are often assumed to represent alterations in regulatory molecules (i.e. NHCP effecting changes in gene transcription) but they may represent alterations in proteins binding to nascent RNA (i.e. differences as a result of changes in gene transcription) and co-isolating with chromatin (Pederson, 1974). Active chromatin is, by definition, that fraction whose DNA is being transcribed into RNA. As discussed in Section II there are problems associated with the adoption of enrichment in nascent RNA as a criterion for an active chromatin fraction. If a valid fractionation is attained, enrichment for NHCP in the active fraction might, as above, reflect increased amounts of proteins binding to nascent RNA. Thus observed increases in amounts of specific proteins in the active fraction of chromatin cannot be taken as revealing increases in specific regulatory or structural proteins associated either with the DNA or histones.

It is perhaps worthwhile here to reiterate the earlier definition of non-histone chromatin proteins given in the Introduction, i.e. that the NHCP are those proteins associated with chromatin which are not histones. Proteins which bind to chromatin via its RNA (and are not
histones) are NHCP by this definition. This section concerns an attempt to determine whether the enrichment for specific NHCP in active chromatin (derived by DNase II digestion; Section II) was the result of proteins binding to nascent RNA enriched in this fraction. For this a study of the proteins of isolated nuclear ribonucleoprotein complexes (nRNP) was undertaken. These complexes can be isolated essentially free of DNA and have been demonstrated to contain rapidly labelled nuclear RNA of heterogeneous molecular weight (HnRNA; Georgiev and Samarina, 1971; Niessing and Sekeris, 1971; Pederson, 1974; Augenlicht and Lipkin, 1976b). HnRNA must be intimately associated with chromatin during its synthesis and several groups have recently reported that a considerable proportion of HnRNA is found in chromatin (Monahan and Hall, 1975; Tata and Baker, 1975; Augenlicht and Lipkin, 1976b). Evidence has also been presented that some of the chromatin HnRNA is precursor to HnRNA found free of chromatin in the form of nRNP (Augenlicht and Lipkin, 1976b; Monahan and Hall, 1975). An examination of the proteins of isolated nRNP may thus give some insight into the proteins bound to chromatin via HnRNA. A comparison of the proteins of nRNP with the NHCP of the active fraction of chromatin might also provide further information on the true nature of the NHCP found enriched in this fraction of chromatin.

In the work for this thesis attempts by various methods to prepare sufficient nRNP from mouse liver for analysis of their proteins by staining in gels were unsuccessful. Other workers have chosen to stimulate transcriptional
activity by hormone treatment and label RNA in vivo to facilitate nRNP isolation from liver. In the present study, it was decided to attempt isolation of nRNP from mouse cells grown in culture rather than induce elevated levels of transcription in mouse liver cells. Mouse A9 cells were grown in the presence of 0.25 μCi/ml \( ^3H \)-uridine (3.3 Ci/m mole) for 45 min. (see Materials and Methods) to label newly-synthesized RNA. The cells had previously been exposed to \( ^3S \)-methionine (Materials and Methods) in order that proteins obtained could be analyzed by 2D-PAGE and fluorography.

The first methods used in attempt to isolate nRNP involved washing the isolated nuclei in a Tris-saline buffer at pH 8 (see Materials and Methods). This procedure has been reported to release nRNP from rat liver and HeLa cells (Samarina et al., 1968; Albrecht and Van Zyl, 1973; Beyer et al., 1977). The proteins released from mouse A9 cell nuclei by this procedure are highly heterogeneous when examined under 2D-PAGE (figure 33b). When compared to those of nuclei (figure 33a) the pH 8 extract of nuclei (figure 33b) can be seen to be enriched for many non-histone proteins (e.g. at 53/30, 56/100, 62-64/50-60). Several prominent non-histone proteins of nuclei are absent from the pH 8 extract (e.g. around 52-56/50-70). Extensive heterogeneity in similar pH 8 extracts of nuclei has been observed by other workers (Monahan and Hall, 1975). The pH 8 extract is obtained under conditions similar to those by which a nucleoplasmic fraction may be obtained from nuclei (see Materials and Methods); the protein
Mouse A9 cells were grown in the presence of $^{35}$S-methionine (1μCi/ml medium) for 48 hr. Nuclei and a pH 8 extract of nuclei were prepared as described in Materials and Methods. Samples from these were prepared as for 2D-PAGE as described in Materials and Methods.

a) Nuclei, sample load $2 \times 10^5$ cpm, exposure time 32 days.
b) pH 8 extract of nuclei, sample load $1.7 \times 10^5$ cpm, exposure time 32 days.

Fluorography as described in Materials and Methods.

Figure 33

2D-PAGE analysis of non-histone protein of nuclei and non-histone protein of pH 8 extract of nuclei.
pattern obtained from the pH 8 extract is more similar to that of nucleoplasm (figure 17c) than that of total nuclei.

To ascertain whether the proteins of the pH 8 extract do represent proteins of nRNP or whether it contained particulate nRNP at all, the pH 8 extract was analysed by sucrose gradient centrifugation. Free nucleoplasmic proteins should not enter the sucrose gradient; proteins complexed with RNA in a particulate (nRNP) structure should sediment through the sucrose gradient as a zone with characteristic S value. No such sedimentation was observed indicating that the pH 8 extract contained no intact nRNP. Using rat hepatoma nuclei Albrecht and Van Zyl (1973) were also unable to obtain a definite particle peak of nRNP in sucrose gradients containing a pH 8 extract of nuclei. They suggest that ribonucleoprotein particles in tumour nuclei are more firmly associated with chromatin or less able to pass through the nuclear membrane during extraction. The mouse A9 cell nuclei used for extraction at pH 8 in the present study had been treated with Triton X-100 to remove the nuclear membrane. The lack of nRNP in the pH 8 extract of nuclei in this case may be caused by their firm association with chromatin.

An alternative method, used by Albrecht and Van Zyl (1973) for rat hepatoma cell nuclei, was adopted for the preparation of nRNP from mouse A9 cell nuclei. This consisted of sonicating nuclei in Tris-saline buffer at pH 8.0 until all nuclei were judged to be ruptured by phase
contrast microscopy and subsequently analysing the
sonicate in sucrose gradients (see Materials and Methods).
The resulting distributions of $^{3}$H-RNA and $^{35}$S-protein in
the sucrose gradient are shown in figure 34. A definite
peak of $^{3}$H-cpm can be seen sedimenting in the sucrose
gradient at about 40S (fractions 8-13); at the same
position there is an apparent increase in $^{35}$S-cpm. To
test for association of $^{3}$H- and $^{35}$S-cpm, fractions 8-13
were pooled and dialysed overnight against Tris-saline
buffer (pH 8). The fractions were then concentrated
against polyethylene glycol and re-centrifuged in an
identical sucrose gradient. The $^{3}$H-RNA and $^{35}$S-protein
profiles of this second gradient are shown in figure 35.
The peak of $^{3}$H-cpm is not as defined in this gradient -
this is probably the result of the action of ribonucleases
during the dialysis and concentration steps. However a
definite peak of $^{35}$S-cpm can be seen associated with the
$^{3}$H-cpm peak and little "free" $^{35}$S-cpm remain at the top of
the gradient. This confirms the finding of the first
sucrose gradient that $^{35}$S-labelled protein is in close
association with the $^{3}$H-pulse labelled RNA.

Three clear zones of $^{3}$H- and $^{35}$S-radioactivity can
be seen from the first sucrose gradient (figure 34).
Considerable quantities of both $^{35}$S- and $^{3}$H-radioactivity
can be seen at the top of the sucrose gradient. The
$^{35}$S-protein in this region will be shown later (figure 39)
to correspond closely to that extracted from nuclei by
Tris-saline buffer at pH 8 (figure 33b). The $^{3}$H-
radioactivity may represent breakdown of nRNP particles or
Mouse A9 cells were grown in the presence of \(^{35}\text{S}\)-methionine (1 \(\mu\)Ci/ml medium) for 48 hr. and \(^3\text{H}\)-uridine (0.5 \(\mu\)Ci/ml medium) for 45 min. Nuclei were prepared, sonicated and the sonicate centrifuged in 15-30% sucrose gradients as described in Materials and Methods. 0.9 ml fractions were collected from the bottom of the tubes, 25\(\mu\)l samples removed from these and radioactivity estimated (Materials and Methods) by double-label scintillation counting with correction for spillover.

\[\begin{align*}
\text{3H-radioactivity in RNA} \\
\text{35S-radioactivity in protein}
\end{align*}\]

The peak of \(^3\text{H}\)- and \(^{35}\text{S}\)-cpm (fraction 11) was calculated as having a sedimentation value of 40S from the position of 5, 18 and 28S ribosomal RNAs in a parallel sucrose gradient.
Sucrose gradient re-centrifugation of nRNP complexes.

Fractions (8-13) containing nRNP of the previous sucrose gradient (figure 34) were dialysed overnight against 0.01M Tris (pH 8.0), 0.14M NaCl, 0.001M MgCl₂, 0.1mM PMSF, concentrated to 2 ml. against polyethylene glycol and re-centrifuged in 15-30% sucrose gradients (Materials and Methods). 0.9 ml. fractions were collected from the bottom of the gradient and radioactivity estimated (Materials and Methods) by double-label scintillation counting with correction for spillover.

- ³H-radioactivity in RNA
- ³⁵S-radioactivity in protein
chromatin-associated RNA as a result of sonication or ribonuclease action. The middle zone of the sucrose gradient (fractions 8-13) shows peaks of both $^{3}H$- and $^{35}S$-radioactivity with sedimentation value $S$ of around 40S. This value is similar to those previously reported by other workers for nRNP (Beyer et al., 1977; Pederson, 1974). Some material sedimented through the sucrose gradient and was found as a pellet at the bottom of the tube. This material was found to contain fairly large amounts of both $^{3}H$- and $^{35}S$-cpm (47% and 55% of the total activity in the sonicate, respectively). Thus some $^{3}H$-RNA was not released from chromatin by sonication and must therefore represent newly-synthesised RNA in strong association with chromatin. Kimmel, Sessions and MacLeod (1976) have also reported strong association of newly-synthesised RNA with chromatin and found this RNA to remain associated with chromatin after shearing by sonication, as determined by sedimentation and equilibrium density gradient analyses. In one experiment of the present study, the chromatin pellet of the sucrose gradient was suspended in 0.01M Tris (pH 7.5), 5mM MgCl$_2$, 0.1mM PMSF and digested for 5 min. at 37°C with RNase A at 100 µg/ml. The undigested chromatin was then pelleted by centrifugation at 1,500xg for 15 min. By this procedure virtually all of the $^{3}H$-RNA of the chromatin pellet was released into the supernatant along with some protein. Five fractions were obtained by the above procedures: a top fraction (of the sucrose gradient), a 40S nRNP fraction, a chromatin pellet, a RNase-resistant chromatin pellet
and a RNase-released pellet fraction.

The protein compositions of the top fraction, 40S nRNP fraction and chromatin pellet were analysed by 1D-PAGE. The fluorogram of the resulting gel is presented in figure 36. The protein of 40S nRNP is enriched both over the chromatin pellet and top fraction for one major band and several minor bands (closed arrows). Thus proteins in the 30,000-40,000 molecular weight range are enriched in this fraction. Pederson (1974) found three major proteins associated with the nRNP of rat liver within the molecular weight range 32,000-40,000. Similarly the proteins of core 40S HnRNP particles of HeLa cells, hamster fibroblasts and mouse ascites cells have molecular weight values in the range 32,000-44,000 (Beyer et al., 1977). The 40S nRNP in the present study can be seen to contain major bands also seen in both the chromatin pellet and top fraction of the sucrose gradient (open arrows).

The above samples, and the RNase-resistant chromatin pellet and RNase-released pellet fractions, were analysed by 2D-PAGE. Figure 37 presents a 2D-PAGE analysis of the chromatin pellet (a) and RNase-resistant chromatin pellet (b). The NHCP analysis of the chromatin pellet of the sucrose gradient (figure 37a) is similar to that previously seen for mouse A9 cell chromatin (figure 17d earlier); this conclusion can also be drawn from 1D-PAGE analyses (compare figure 36a with figure 29c). Several spots present in the chromatin pellet (figure 37a) are absent or much reduced after treatment of that pellet with RNase. These proteins constitute those proteins which
Figure 36

1D-PAGE analysis of proteins of sucrose gradient fractions.

Sucrose gradient chromatin pellet, 40S nRNP peak (fractions 8-13) and top fractions (18-24, see figure 34) were prepared for 1D-PAGE as described in Materials and Methods.

Sample slots:

a) Sucrose gradient pellet; $4 \times 10^4$ cpm, exposure time 7 days.

b) Sucrose gradient nRNP; $1.8 \times 10^4$ cpm, exposure time 14 days.

c) Sucrose gradient top; $2 \times 10^4$ cpm, exposure time 14 days.

Fluorography as described in Materials and Methods.
Figure 37

2D-PAGE analysis of proteins of sucrose gradient fractions.

Sucrose gradient chromatin pellet (see figure 34) and RNase-resistant chromatin pellet (after RNase digestion—Materials and Methods) were prepared for 2D-PAGE as described in Materials and Methods.

a) sucrose gradient pellet; $1.2 \times 10^5$ cpm, exposure time 32 days.

b) sucrose gradient pellet after RNase digestion (i.e. RNase-resistant pellet fraction); $1 \times 10^5$ cpm, exposure time 32 days.

Fluorography as described in Materials and Methods.
are released into the supernatant when the remaining RNA of the chromatin pellet is digested with RNase and hence are presumably bound to chromatin via RNA (see figure 38b later).

The proteins of the RNase-released pellet fraction and 40S nRNP fraction are presented in figure 38b and a respectively. As expected, the proteins of the RNase-released pellet fraction (figure 38b) largely correspond to those missing from the RNase-resistant chromatin pellet (closed arrows; figure 37b). The RNase-released pellet fraction (figure 38b) also contains one major (60/75) and several minor (61/75, 60/55 and 52/60) protein spots not seen in the 2D-PAGE analysis of the proteins of the 40S nRNP (figure 38a). The proteins of the 40S nRNP (figure 38a) constitute a major subset of those in the sucrose gradient chromatin pellet (figure 37a) - over 10 spots are common to both analyses, including two of the major spots found in the chromatin pellet (56/60, 60/50). The 2D-PAGE analysis of the proteins of the top fraction of the sucrose gradient, shown in figure 39, shows many similarities with those extracted from nuclei with Tris-saline buffer at pH 8.0 (figure 33b). This sample contains all the protein species found in the 40S nRNP sample.

The most important facet of these studies is the analysis of the proteins of the 40S nRNP. By 1D-PAGE analysis the proteins of this fraction were found to be enriched for specific bands around 30,000 molecular weight. Other proteins were found to be common between the 40S nRNP and chromatin pellet fractions. In the 2D-PAGE
2D-PAGE analysis of proteins of sucrose gradient fractions.

Sucrose gradient 40S nRNP (fractions 8-13; figure 34) and the supernatant of the RNase digestion of the sucrose gradient pellet (see Materials and Methods) were prepared for 2D-PAGE as described in Materials and Methods.

a) sucrose gradient nRNP; \(1.8 \times 10^4\) cpm, exposure time 80 days.

b) supernatant after RNase digestion of sucrose gradient pellet (i.e. RNase-released pellet fraction); 
\(2 \times 10^4\) cpm, exposure time 80 days.

Fluorography as described in Materials and Methods.
2D-PAGE analysis of proteins of sucrose gradient fractions. Sucrose gradient top fractions (18-24, figure 34) were prepared for 2D-PAGE as described in Materials and Methods.

Sample load $1 \times 10^5$ cpm, exposure time 32 days.

Fluorography as described in Materials and Methods.
analysis of 40S nRNP, one protein spot (open arrow, figure 38a) can easily be seen to be enriched in this fraction when compared to the chromatin pellet (figure 37a). This protein (30,000 molecular weight) may correspond to the major protein found enriched in 40S nRNP by 1D-PAGE. It is possible that the other proteins found enriched by 1D-PAGE, but which were not found enriched in the 2D-PAGE analysis, may have isoelectric points above the pH range of the isoelectric focusing gel in the first dimension of the 2D-PAGE system. Beyer et al. (1977) have found that several of the core proteins of 40S nRNP of HeLa cells focused above pH 8 in the isoelectric focusing dimension. The proteins of the 40S nRNP also show considerable homology with those released from the chromatin pellet by RNase treatment of that pellet, although the latter fraction does contain several additional proteins not seen in the 40S nRNP fraction.

All of the proteins of the 40S nRNP fraction can be found both in the chromatin pellet and top fractions of the sucrose gradient. Similarities with the latter could be explained by the existence of pools of these proteins in the nucleoplasm or by some degradation of 40S nRNP taking place during isolation. The presence of 40S nRNP proteins in the chromatin pellet may be due to the proteins binding via nascent RNA to chromatin. However when the nascent RNA of chromatin is almost totally removed from chromatin by treatment with RNase, many of the 40S nRNP proteins are still found in chromatin. This similarity between the 40S nRNP protein composition and a specific
sub-set of the chromatin proteins could reflect contamination of the 40S nRNP fraction with proteins associated with DNA in chromatin. This seems unlikely for the following reasons. The proteins identified in 40S nRNP are a sub-set of chromatin proteins; any major contamination would tend to result in all the proteins characteristic of chromatin being found in the 40S nRNP. The apparent total absence of histones in the 40S nRNP also argues against significant contamination with chromatin. The similarity between the proteins of the 40S nRNP and those released by RNase digestion of the chromatin pellet shows that these proteins are present in a RNase labile component of chromatin, a property expected of RNP, but not of chromatin.

A second possibility is that proteins present in both 40S nRNP and chromatin carry out enzymic functions common to both RNA and DNA metabolism e.g. transcription, modification, etc. Alternatively the proteins could represent structural proteins that bind relatively non-specifically to any polynucleotides or possibly only to single stranded polynucleotides.

At first sight the similarities between the proteins of the 40S nRNP and a subset of those present in the RNase treated chromatin pellet appears to be contrary to the conclusions drawn by other workers on the basis of 1D-PAGE (Augenlicht and Lipkin, 1976,b,c; Pederson, 1974). While these authors stress the differences between proteins in their nRNP and chromatin fractions, careful examination of their published photographs of gels shows extensive similarities existing in the population of proteins present.
in these two fractions. Thus, while a small number of proteins do appear to be specifically enriched in 40S nRNP (both in the present study and those of Augenlicht and Lipkin and Pederson), the over-riding conclusion is that the majority of proteins are common to both nRNP and chromatin.

The proteins of 40S nRNP and active chromatin may now be compared. Figure 38a and b shows respectively the proteins of 40S nRNP and those released from chromatin by treatment with RNase. The non-histone proteins of the S2, transcriptionally-active, fraction obtained by DNase II digestion and those obtained in the control S2 fraction, in the absence of enzyme, are presented in figure 23a and b respectively. The active chromatin fraction is very similar in protein composition to the 40S nRNP. A major spot at 60/80 and a minor spot at 55/80 are absent from the 40S nRNP fraction (figure 38a); however, these spots are seen in the RNase-released pellet fraction (figure 38b). In addition the major protein spots found enriched in the S2 fraction over its control (minus enzyme) S2 fraction at positions 55-52/50-60 are found both in the 40S nRNP fraction and in the RNase-released pellet fraction. The control (minus enzyme) S2 fraction (figure 23b) shows considerable homology with the RNase-released pellet fraction (figure 38b) - it is possible that the protein release in the absence of DNase II is the result of ribonuclease action.

All of the proteins of 40S nRNP are found in active chromatin. These proteins may have structural or enzymatic
functions common to chromatin and 40S nRNP. Alternatively, much of the non-histone protein complement of active chromatin may represent proteins bound to chromatin via nascent RNA. Further work is required to distinguish between these possibilities. The above result, however, does indicate that those proteins found to be enriched in active chromatin do not necessarily represent regulatory molecules. It is possible that, if proteins with such a function are present in the NHCP complement of the active chromatin fraction, they exist at concentrations too low to be detected by the level of resolution attained by this polyacrylamide gel system.

Addendum
Pederson, T. and Bhorjee, J.S. (Biochemistry, N.Y., 14, 3238-3250, 1975) have examined, by 1D-PAGE, the NHCP of template active and inactive HeLa cell chromatin (derived by DNase II digestion). They find that the NHCP of inactive chromatin correspond closely to those of unfractionated chromatin while the NHCP of active chromatin resemble those complexed with HnRNA in HeLa cells. These results are in agreement with the results of the higher resolution 2D-PAGE analyses described in this thesis.
CONCLUDING REMARKS

The NHCP are a heterogeneous mixture of proteins whose functions in chromatin, despite intensive research, remain unclear. This study has established a two-dimensional polyacrylamide gel electrophoresis system for the analysis of the NHCP. Using this technique the heterogeneity of the NHCP was confirmed, over 100 protein spots being found for mouse liver chromatin. As far as could be detected, little artefactual heterogeneity was induced. The level of resolution attained by 2D-PAGE in the analysis of NHCP can be seen to be a great improvement over one-dimensional techniques and several two-dimensional techniques used previously. The system used, however, has the limitation that only proteins with isoelectric points within the range of the pH gradient of the isoelectric focusing gel can be analysed. Further work to improve the first dimension isoelectric focusing separation could probably overcome this drawback.

Using 2D-PAGE the NHCP were shown to exhibit extensive homology with nucleoplasmic proteins. It was concluded that the NHCP represents a sub-set of nuclear non-histone proteins which are more tightly bound to chromatin.

A DNase II and salt precipitation method was used to fractionate chromatin. That fraction which putatively contained the transcriptionally-active chromatin was enriched for specific NHCP. This result was shown unlikely to be an artefact of the chromatin preparation or digestion procedures used. However the proteins of 40S nRNP contain those proteins enriched in active chromatin. It is suggested
that the NHCP enriched in active chromatin represent proteins binding to chromatin via RNA or structural and enzymic proteins common to active chromatin and 40S nRNP. Further work is required to confirm and extend these findings. The resolution obtained with two-dimensional polyacrylamide gel electrophoresis will facilitate such work.
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BIBLIOGRAPHY


Arnold, E.A. and Young, K.E. (1972), Biochim. biophys. Acta, 257, 482-496


Baserga, R. (1974), Life Sciences, 15, 1057-1061


Coffino, P., personal communication.


Doenecke, D. and McCarthy, B.J. (1975), Biochemistry, N.Y., 14, 1366-1372

Doenecke, D. and McCarthy, B.J. (1976), Eur. J. Biochem., 64, 405-409

Duerksen, J.D. and McCarthy, B.J. (1971), Biochemistry, N.Y., 10, 1471-1478


Elgin, S.C.R. and Bonner, J. (1972), Biochemistry, N.Y., 11, 772-781


Graziano, S.L. and Huang, R.C.C. (1971), Biochemistry, N.Y., 10, 4770-4777

Grouse, L., Chilton, M.D. and McCarthy, B.J. (1972), Biochemistry, N.Y., 11, 798-805

Hemminki, K. (1976a), Mol. and Cell. Biochem., 11, 9-15


Jackson, R.C. (1976), Biochemistry, N.Y., 15, 5652-5667


-118-


Krieg, P. and Wells, J.R.E. (1976), Biochemistry, N.Y., 15, 4549-4558


Miller, O.L. and Beatty, B.B. (1969), Science, N.Y., 164, 955-957
Mullins Jr., D.W., Giri, C.P. and Smulson, M. (1977), Biochemistry, N.Y., 16, 506-513
Oudet, P., Gross-Bellard, M. and Chambon, P. (1975), Cell, 4, 281-300
Paul, I.J. and Duerksen, J.D. (1976b), Archs Biochem. Biophys., 174, 491-505
Paul, J. and Gilmour, R.S. (1968), J. molec. Biol., 34, 305-316
Paul, J. and Malcolm, S. (1976), Biochemistry, N.Y., 15, 3510-3515


Sanders, M.M. and Hsu, J.T. (1977), Biochemistry, N.Y., 16, 1690-1695


Sevaljevic, L. (1973), Biochim. biophys. Acta., 335, 102-108

Sevaljevic, L. and Stamenkovic, M. (1972), Int. J. Biochem., 3, 525-530


Simpson, R.T. and Reeck, G.R. (1973), Biochemistry, N.Y., 12, 3853-3858


Spelsberg, T.C. et al (1973) - see Wilhelm, Spelsberg and Hnilica (1971)


Weintraub, H. and Groudine, M. (1976), Science, N.Y., 193, 848-856


