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THE ROLE OF HISTONE PROTEINS AND IONIC ENVIRONMENT
IN THE STRUCTURE AND ORGANIZATION OF AVIAN LIVER
AND ERYTHROCYTE NUCLEI

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A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Carleton University

Ottawa, Ontario.

July, 1971

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ABSTRACT

This study consisted basically of microscopic analysis of chromatin structure and organization in nuclei isolated from avian tissues in relation to histone protein composition and ionic environment. Nuclei were isolated in saline from chicken erythrocytes and liver parenchyma and from immature and mature goose erythrocytes. Histone proteins were fractionally dissociated by sequential extraction in acid. At each step nuclei were fixed both at the pH of extraction and at neutral pH, and prepared for examination by electron microscopy. Intact nuclei in situ and isolated but unextracted nuclei served as controls. Freshly isolated liver nuclei and erythrocyte nuclei at all stages of histone extraction were also examined by electron microscopy following suspension in water and return to saline after water. In addition, individual nuclei were observed under interference-contrast optics during exposure to saline, water and back to saline.

Unextracted nuclei both in situ and isolated in saline, showed chromatin fibrils about 200 Å in diameter. Exposure to water caused a reversible change to fibrils about 75 Å thick. Since no evidence of multiple parallel strands was found, this data is considered to support a proposed model for chromatin fibril structure involving supercoiling of a single nucleoprotein strand, the A fibril, 60-100 Å in diameter, to give a 150-250 Å thick B fibril. Configuration of nucleoprotein within the B fibril is sensitive to ionic strength but apparently does not depend on other soluble components of the nucleus.

Removal of histone fraction I (lysine-rich) from liver and immature erythrocyte nuclei caused irreversible change to tightly intermeshed 100 Å chromatin fibrils. In similarly extracted mature erythrocyte nuclei fibrillar structure was usually not visible, while removal of both fraction I and V (serine-rich) revealed fibrils 100 Å and less thick. Extraction of further histone fractions gave fibrils of variable diameter down to 20 Å. It is concluded that all histone fractions, including the loosely bound, lysine-rich fractions, are essential for structure of the 200 Å native chromatin fibril. Possible conflicting data from X-ray diffraction studies is consistent if it is assumed that A fibrils already have supercoiled structure and B fibrils contain an additional order of coiling.

All nuclei isolated in saline exhibited overall chromatin distribution very similar to that visible in comparable nuclei in situ. In mature erythrocyte nuclei most chromatin was localized in peripheral heterochromatic masses. Most chromatin in liver and immature erythrocyte nuclei was dispersed throughout the nucleus with some heterochromatin near the periphery and the nucleolus. With progressive histone extraction the pattern of distribution of heterochromatin became somewhat distorted in all nuclei, however its general aspect remained remarkably similar to that visible in the respective nuclei in situ. These observations suggest that probably only tightly bound histones are primarily involved in the gross-distribution of chromatin in nuclei. The secondary organization of chromatin seems to be largely determined by DNA in association with non-histone components

of nuclei.

Studies were undertaken designed to cause dispersal and recondensation of heterochromatin in nuclei without extraction of major macromolecular components. When saline isolated nuclei were suspended in water, condensed chromatin was reversibly dispersed. Interference-contrast examination of individual, unfixed nuclei showed that the configurational pattern of condensed chromatin which reformed in saline after water dispersal was structurally identical to that present before dispersal. It is concluded that the condensation of chromatin into heterochromatic areas is probably not a form of random aggregation but may reflect tissue-specific, ordered arrangement of interphasic chromatin.

Histone proteins may control the availability of DNA as template for RNA synthesis and thus partially determine both structure and genetic activity in chromatin. Studies on RNA transcription, paralleling the present structural work, showed that fraction V is responsible for about half the total template restriction related to histones in chicken erythrocyte chromatin. In chicken liver chromatin, where fraction V is not present, template restriction of similar magnitude could not be linked with extraction of any major histone. This data, in combination with structural studies here reported, may indicate that fraction V imparts excessive template restriction by inducing structural modification in erythrocyte chromatin. However, the precise function of this histone in vivo remains largely undetermined. Template studies accompanying changes in ionic environment,

revealed no major qualitative or quantitative differences in the RNA synthesized in vitro by nuclei in saline, in water and returned to saline. These observations suggest that ion-mediated condensation and dispersal of chromatin does not appear to directly regulate the template activity of chromatin. The regulation of both condensation and template capacity probably involves as yet undetermined changes in DNA-protein interactions in the chromatin complex.

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LIST OF ABBREVIATIONS

Å	Ångstrom units
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNH	Deoxyribonucleohistone (nucleohistone)
DNP	Deoxyribonucleoprotein (nucleoprotein)
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscope
μ	Micron units
NAP	Non-histone protein
RNA	Ribonucleic acid
RNase	Ribonuclease

INTRODUCTION

Much is known about the chemistry and structure of genetic systems in prokaryotic organisms (e.g. viruses and bacteria), where all hereditary information is usually contained in a single molecule of nucleic acid. It is known, for example, that the "chromosome" of Escherichia coli is a circular DNA molecule of determined length. In several bacteria the exact loci of many individual genes have been specified and particular genes can be transferred experimentally from one bacterial cell to another. DNA in bacteria is localized in a region of the cell termed the nucleoid, but a specific site on the plasma membrane probably regulates the onset and rate of DNA synthesis and may segregate the two daughter molecules once replication is completed (Ryter et al., 1968; Sueoka and Quinn, 1968).

While the fundamental genetic properties, such as linear organization of genes, the triplet code and mechanisms of transcription and translation, are alike in both prokaryotic and eukaryotic organisms (animals and plants), the structural and chemical make-up of genetic systems in higher organisms is far more complex than in bacteria. The genome of eukaryotic cells is segregated into at least as many separate units as there are chromosomes in a given organism. Beyond this, however, it is not known whether each chromosome contains

only one DNA molecule or several molecules lying in parallel or joined in tandem. Nor is it fully understood how and to what extent nucleoproteins and other macromolecular components interact chemically with DNA in the chromosome to determine its structure and genetic activity. In several organisms chromosomes have been categorized with respect to size and morphology, and in some organisms, specific genes have been associated with individual chromosomes. Chromosomes, however, are generally visible as distinct entities only during cell division and it is unclear how their individual structural and genetic characteristics are combined for integral function during interphase when chromosomes uncoil and become intimately associated in the nucleus. In this regard, Comings (1968) has written: "Few structures in nature appear to be arranged with as much abandon as the chromatin of the interphase nucleus. And yet, this random appearance may be deceiving, since any degree of order would be well hidden by the very mass of the chromatin itself. When somatic cells enter mitosis and germ cells enter meiosis, the chromatin arranges itself into distinct chromosomes which separate to the opposite poles of the cell with pleasing precision. This impressive display of nature's ability to create order from apparent disorder may be aided by a non-random arrangement of the nucleus". In the present investigation, an attempt was made to study the structural organization of interphase nuclei by means

of light and electronmicroscopy and, hopefully, find ordered arrangement in chromatin which could be related to specific nuclear constituents and to the state of genetic activity of the particular nucleus under examination.

Histone proteins are structural constituents which may be involved in regulating both the genetic activity and the structural arrangement of chromatin. These proteins are closely associated with DNA, forming the so-called nucleohistone complex. This complex, in combination with non-histone proteins and some RNA, forms the chemical basis of chromatin. Histones can be fractionated into several distinct components, which are remarkably similar in overall composition in most organisms and tissues (Hnilica, 1967). This similarity in overall composition suggests that histones probably play a similar role in all organisms in which they are found. Evidence is available suggesting that histones are involved in the control of gene expression by regulating the availability of DNA as a template for the transcription of RNA (Bonner et al., 1968). Other evidence indicates that histones may determine both the primary and the secondary structural organization of chromatin in the nucleus and the chromosome (DuPraw, 1968). These two proposed roles for histones, i.e. regulatory and structural, may go hand in hand. All proposals for histone function, however, are largely hypothetical and await further analysis.

In the present study the structural role of histone function was investigated. Specifically, this included an electron microscopic examination of nuclei from several avian tissues, before and after the selective extraction of histone fractions. Changes in both the primary and secondary organization of chromatin could then be related to the removal of specific fractions from the nuclei. An effort was also made to correlate this structural work with parallel studies on RNA transcription (Seligy and Neelin, 1970), with the aim of relating structural organization to genetic regulation.

There is some evidence (Comings, 1968), that the apparent chaotic aspect of chromatin revealed by the microscope in interphase nuclei may be deceiving, and that the jumbled mass of chromatin actually represents complex ordered structure. In this way, during the cell's development, different portions of its genetic complement may be brought into appropriate configuration for integral function. One way in which order might be reflected in nuclei is in the pattern of distribution of chromatin into compact areas and extended areas. The state of compactness (or condensation) of chromatin, in turn, may be a reflection of its relative capacity to synthesize RNA (Harris, 1967).

In the present work, the arrangement of chromatin and its state of condensation in isolated nuclei were varied through manipulation of ionic strength of the suspension medium. In this way, it was possible to

provide evidence that chromatin in individual nuclei is not distributed randomly but arranged in orderly fashion. Concurrently, it was possible to relate changes in the state of chromatin condensation to parallel assays on its ability to support RNA synthesis in vitro (Brasch et al., 1971).

For these studies, nuclei were used from tissues which had differentiated along widely separate lines, i.e. avian erythrocyte and liver parenchyma. In mature avian erythrocytes the nuclei show almost complete condensation of chromatin (Davies, 1961) and severely restricted capacity for RNA synthesis (Cameron and Prescott, 1963; Seligy and Miyagi, 1969). In addition to histones commonly found in other tissues, these nuclei also contain an apparently unique component not found in other tissues (Neelin and Butler, 1961). Liver parenchyma nuclei, on the other hand, are less specialized than mature erythrocyte nuclei. Much of the chromatin in liver is present in extended form, and is actively engaged in RNA synthesis (Seligy and Miyagi, 1969). Moreover, chicken liver nuclei do not contain anomalous histone components, in addition to the normal complement (Neelin and Butler, 1961). In addition to mature erythrocytes and liver, the nuclei of immature erythrocytes were studied. This was done in an effort to examine nuclei with structural and synthetic characteristics somewhat between those of mature erythrocytes and liver. It was hoped that by examining this spectrum of nuclei,

differences in chromatin structure and organization could be correlated with variations in composition and metabolic activity.

LITERATURE REVIEW

This review is primarily concerned with the structure and organization of chromatin and the relation of structure to gene expression. In this regard, the fine structure of chromatin and the role of cations in the maintenance of chromatin condensation are covered in detail. Functional and biochemical properties of histone proteins are described and the poorly understood, non-histone nuclear proteins are briefly discussed. Specialized aspects of chromatin organization such as the polytene condition and the synaptonemal complex have been largely omitted, since they have little relevance to the study.

I. Basic Structure of Chromatin

The genetic material of eukaryotic organisms consists of a stable complex of nucleic acids and proteins known as chromatin. During most of the life cycle of a cell i.e. interphase, the chromatin exists in a partially dispersed state and is confined within a membrane, forming the organelle called the nucleus. During cell division, however, chromatin becomes tightly condensed and separate units known as chromosomes are revealed. Chromatin exhibits structural organization at two distinct though not necessarily independent levels. The first level involves the

basic molecular interaction and structural relation between DNA and proteins to form the deoxyribonucleo-protein or DNP complex. The second structural level concerns the large scale spatial configuration of chromatin within the interphase nucleus and condensed chromosomes during cell division.

Electron microscopic (E.M.) observations have shown that chromatin is universally organized into fine fibrils of indeterminate length and having a diameter between 100 and 250 Å (Hyde, 1965; DuPraw, 1968; Ris and Kubai, 1970). These morphological units of chromatin will be referred to as "basic fibrils" in this review.

The nature of basic fibrils has been the subject of much study. Ris (1961; 1962; 1966a; 1966b) and his co-workers (Ris and Chandler, 1963; Ris and Kubai, 1970) made extensive E.M. studies of chromatin from many organisms using both sectioned material and isolated preparations spread on air-water interfaces. These workers concluded that a unit about 100 Å in diameter occupies a central position in the make-up of chromatin. Noting that in many organisms a fibril of this diameter predominates, Ris termed this unit the "elementary fiber". Thicker fibrils (200-250 Å), observed in the chromatin of newt erythrocytes (Ris, 1966a) and many other organisms, were thought to be composed of intertwined 100 Å fibrils or a single such fibril repeatedly folded back over itself. It was further suggested

(Ris and Kubai, 1970), that the 100 Å fibrils represent active chromatin such as that found in calf thymus nuclei, while the 200-250 Å fibrils represent inactive chromatin as found in metaphase chromosomes. Active in this sense, refers to the relative participation in RNA transcription in vivo.

Variations in diameter at the basic fibril must be viewed with caution, however. The diameters of basic fibrils as seen in the E.M. are affected by such variables as ionic environment, method of fixation and dehydration, and the means of specimen preparation, i.e. sectioning or spreading on an air-water interface (Dales, 1960; Davies and Spencer, 1962; Gall, 1966; Wolfe, 1968). Consequently it remains difficult to determine with certainty whether reported differences in the diameter of basic fibrils are biologically meaningful or simply due to preparatory artifacts. Moreover, it seems highly unlikely that 100 Å fibrils represent active chromatin while 200-250 Å fibrils are inactive as suggested by Ris and Kubai (1970). Fibrils 200-250 Å in diameter are found in many cells whose chromatin is not inactive; for example, bee embryonic cells (DuPraw, 1968) and human leucocytes (Bastia and Swaminathan, 1967). Furthermore, nuclei in tissue such as calf thymus should contain two classes of basic fibrils since presumably only part of their genome is active at any given time. Instead,

in the calf thymus preparations of Ris and Kubai (1970), fibrils 100 Å in diameter predominated. The simplest interpretation therefore is that basic fibrils are of essentially similar make-up in all types of chromatin, and that they are not multiples of some common elementary unit. Variations in diameter in different preparations probably then represent differential response of the fibrils to preparative procedures (Wolfe, 1968).

Detailed E.M. investigations into the structural relationship between DNA and protein in basic fibrils were made by Ris and his co-workers (Ris, 1959; 1961; 1962; Ris and Chandler, 1963). When preparations of calf thymus chromatin were subjected to treatment with mild acid or proteolytic enzymes, the basic fibrils gave way to 30 to 60 Å thick subunits. Since double-stranded DNA in pure form appears about 25 Å in diameter (Kellenberger, 1962), Ris proposed that in chromatin, DNA and histone proteins were intimately coupled to form a DNP unit 30 to 60 Å in diameter. It was originally thought (Ris 1961; 1966b) that each basic fibril was composed of two DNP subunits and hence contained two parallel DNA molecules, but this proposal was not supported by the E.M. observations of other workers (DuPraw, 1965; Wolfe, 1965; Gall; 1966), and has since been abandoned by Ris (Ris and Kubai, 1970; Bram and Ris, 1971).

The precise manner in which DNP is compacted in basic fibrils is still unclear. Evidence is ac-

cumulating, however, which indicates that some form of super coiling is involved. E.M. evidence to this effect has been obtained primarily by treating chromatin with dilute acids or proteolytic enzymes at various concentrations and for increasing periods of time (DuPraw, 1965; Bastia and Swaminathan, 1967; Abuelo and Moore, 1969; Itzaki and Rowe, 1969). Typically, it is found that when isolated chromosomes or interphase chromatin are treated in this manner, progressive and irregular reduction in the diameter of basic fibrils takes place. Ultimately, only a series of amorphous globules remain, which are joined like beads on a string by filaments, 20-30 Å in diameter. The filaments appear to consist solely of DNA since they are resistant to proteolytic enzymes but are destroyed by treatment with DNase. DuPraw (1968) noted that in partially digested preparations of chromatin, the basic fibrils retain apparent undigested portions 200 to 300 Å thick and digested portions 40 to 60 Å in diameter. The presence of these two portions, which were termed B and A fibrils respectively, suggests that partial removal of proteins causes some regions of a solid basic fibril to unwind.

The above observations have been incorporated into a working model of basic fibril structure (DuPraw, 1968). According to this proposal, the A fibril consists of a single DNA molecule which is sheathed by tightly bound histone proteins and forms a DNP unit

40 to 60 Å thick. More loosely bound histones then direct the A fibril into a supercoiled configuration, a B fibril, which is 200 to 300 Å in diameter and which constitutes the basic chromatin fibril commonly present in interphase nuclei and in condensed chromosomes. The evidence for this model rests primarily on observations of chromatin prepared by spreading techniques and so is subject to the difficulties facing those techniques; notably artificial stretching. Moreover, the contention that basic fibrils unwind when proteins are extracted rests entirely on a series of static E.M. pictures. Evidence of a more direct nature in support of supercoiling was provided by an analysis of the distribution of total mass in human chromosome fibrils (Bahr and DuPraw, 1969; Bahr, 1970). Calculations showed that the ratio of the length of double stranded DNA per unit length of chromatin fibril were of the order of 100:1 to 150:1. The authors argue that the only way in which this much DNA could be accommodated in a single basic fibrils is through extensive supercoiling. Other direct evidence of supercoiling is provided by another type of E.M. observation. Davies (1967; 1968) noted that in many cell types, condensed chromatin adjacent to the nuclear membrane consists of ordered layers of basic fibrils. By tilting sections of such material in the E.M., it was shown that the tightly packed fibrils exhibit hollow

cores and resemble microtubes when viewed parallel to the optic axis. This suggests that the microtubes, which are 130 to 170 Å in diameter, are formed by a supercoiled nucleoprotein unit of lesser diameter.

An independent approach to the study of chromatin structure is provided by X-ray diffraction analysis of isolated nucleohistone (DNE). Unfortunately, the usefulness of this approach is restricted in that it is subject to a high degree of artificiality. Results depended heavily on the state of hydration and the degree of denaturation of the sample under examination (Richards, 1964; Zubay and Wilkins, 1964). Nevertheless, some useful information on chromatin structure has emerged from X-ray data.

In older studies, Zubay and Doty (1959) showed that purified DNE in water exists in gel-form in the absence of cations and in sol-form in the presence of cations. This gel-sol transition is reversible and it was suggested that this results from changes in the orientation of DNA in the DNE complex. X-ray studies on DNE gels revealed low angle reflections around 35 to 38 Å which were not found in DNA or histone alone (Wilkins et al., 1959; Zubay and Wilkins, 1964). It was suggested (Zubay, 1964), that the 35 Å reflection represented a spacing between adjacent DNA helices which were being stabilized laterally by DNA-protein cross-linkages. Luzzati and Nicolaieff (1963) drew different conclusions from their X-ray work on DNE gels under

various conditions of hydration. Their results were best explained by postulating that DNA molecules were bundled into micelles in the DNH complex, and that up to four DNA molecules could be included in each micelle.

It remained difficult to evaluate these interpretations of X-ray patterns, since it was often difficult to maintain a sample of DNH in a steady-state of hydration and orientation for sufficiently long periods of time (Zubay, 1964). Recently, however, Pardon et al., (1967) and Richards and Pardon (1970) succeeded in obtaining detailed X-ray patterns of calf thymus DNH under rigidly controlled conditions. At high relative humidity (92-98%) stabilized fibres of DNH exhibited characteristic ring patterns at 22, 35, 55 and 105 Å and poorly oriented DNA reflections. After the fibre was stretched and allowed to form a stiff gel in water, the ring patterns disappeared and DNA reflections of greatly improved orientation became visible. When the DNH fibre was allowed to relax to its former state, all features of the original pattern reappeared. These findings are explainable in terms of a model where DNA, in association with proteins, forms a supercoil with a pitch of 120 Å and a diameter of 100 to 120 Å (Richards and Pardon, 1970). The DNA is inclined to the axis of the supercoil, which in its relaxed state generates ring patterns at 22, 35, 55 and 105 Å. When the supercoil is stretched, the

DNA becomes re-aligned along the axis of the fibril, the ring patterns are reduced in intensity and strong reflections due to DNA orientation appear.

This model has several important features. In addition to providing independent support for the concept of basic fibrils with superhelical substructure, it explains why reflections characteristic of DNA are not usually seen clearly in X-ray diffraction patterns of DNH. Moreover, the model implies that basic fibrils may have elastic properties and like a coiled spring, probably do not require links between adjacent loops to maintain their helicity. Before this or any other model of basic fibril structure can be scrutinized in detail, however, a fuller understanding of interactions between DNA and specific histones is required.

II. Secondary Structure of Chromatin

During cell division all chromatin fibrils in the nucleus become tightly interwoven and form condensed chromosomes. In interphase nuclei on the other hand, chromatin is usually present in a mixture of condensed and diffuse forms. The structure of basic fibrils does not appear to be altered during condensation and decondensation of chromatin, only the degree to which they are physically compacted seems to change. The term heterochromatin was introduced by Heitz (1929) on morphological grounds to denote chromosomal regions which remain condensed during interphase. Chromatin

which becomes dispersed at this stage was called euchromatin. Examples of classical heterochromatin include the Barr body or condensed X chromosome in mammals, the chromocenter in the nuclei of Drosophila salivary glands (Swanson et al., 1967) and B chromosomes in maize (Himes, 1967).

Heterochromatin may be sub-classified into at least three types. One form may be termed permanent heterochromatin. This is exemplified by the Y chromosome in Drosophila which always remains condensed and with which relatively few genes have been associated (Cooper, 1959). A second type of heterochromatin, often called facultative, is chromatin which is condensed only under certain circumstances. An example of this type of heterochromatin is found in many mammalian females, where only one of the two X chromosomes disperses during interphase and the second chromosome remains condensed (Brown, 1966).

A third type of heterochromatin can be termed differential heterochromatin. This type of heterochromatin appears, for example, during the differentiation of red blood cells, where all the interphase chromatin becomes progressively condensed as the cells approach maturity (Cameron and Prescott, 1963; Grasso and Woodard, 1966). In addition, several intermediate or transitional forms of heterochromatin probably exist but are not readily categorized.

It has become customary to use the terms heterochromatin and euchromatin broadly, and to apply them universally to condensed and diffuse chromatin respectively (Hearst and Botchan, 1970). This generalization has been made because a number of distinctive properties may be common to most forms of heterochromatin. For example, the B chromosome in maize (Himes, 1967) and heterochromatin in most other organisms does not incorporate RNA precursors readily, as shown by autoradiography (Hearst and Botchan, 1970). The heterochromatic X chromosome in many organisms replicates late in the S period of cell division (Stubblefield and Gay, 1970) and contains DNA of anomalous density and base composition (Yunish and Yasmineh, 1970; Arrighi et al., 1970). However, it has also been shown (Ris and Kubai, 1970) that not all forms of heterochromatin have all these properties and therefore cannot be equated indiscriminately. Thus unlike some forms of classical heterochromatin, all chromatin which condenses during interphase is not necessarily genetically impoverished and does not contain anomalous DNA, even though it may have restricted template capacity for RNA synthesis.

Although much descriptive work has been done on the secondary structure of chromatin in both chromosomes and interphase nuclei, relatively little is known about the biochemical mechanisms or controlling agents which might be responsible for this organization. Ris

and Mirsky (1949) and Anderson and Wilbur (1952) initially showed at the light microscope level that chromatin condensation and the volume of isolated nuclei were sensitive to changes in the ionic environment, the pH and the tonicity of the suspension medium. It was found that exposure of nuclei to hypotonic concentrations of cations resulted in swelling and the disappearance of granular structure in chromatin. These effects could be reversed by returning the nuclei to physiological cation concentrations. Exposure of nuclei to pH 5 or lower, resulted in shrinkage and heavy condensation of chromatin. In contrast, alkaline pH (8.5 - 9.0) caused nuclear swelling and gradual disruption. Most of these changes could be reversed by returning the nuclei to neutral pH.

Observations of the above sort were extended to the E.M. level and included work on both interphase nuclei and mitotic chromosomes. Crawley and Harris (1963) found that when isolated HeLa cell nuclei were swollen and fixed in hypotonic media, the chromatin became totally dispersed and basic fibrils appeared stretched. When swollen nuclei were returned to isotonic saline before fixing, they shrank in volume and the chromatin became coarsely clumped. Monneron and Moule (1968) noted that when rat liver nuclei were isolated and fixed in media containing less than 0.5 mM Mg^{+2} their chromatin was more diffuse than it appeared

in situ. Several workers (Davies and Spencer, 1962; Barnicot and Huxley, 1965; Lafontaine, 1968), reported that for best preservation of chromatin in tissues fixed in unbuffered osmium tetroxide, the presence of 10^{-2} to 10^{-3} M Ca^{+2} or Mg^{+2} was essential. When these ions were absent from the fixative, chromatin always exhibited swelling and low affinity for electron stains. With respect to pH, Barnicot and Huxley (1965) found that best fixation of mitotic chromosomes in situ was obtained near neutrality in isotonic media. Material fixed under pH 6.0 exhibited abnormal chromosome shrinkage and condensation.

Attempts to relate changes in the structure of chromatin to specific ions have concerned largely Na^{+} , K^{+} , Mg^{+2} and Ca^{+2} . Kroeger (1963) noted that differential induction of puffing in polytene chromosomes was initiated by solutions of varying $\text{K}^{+}/\text{Na}^{+}$ ratios. Puffing in polytene chromosomes is a form of chromatin decondensation which is associated with gene activation (Swanson et al., 1967). This work was extended (Lezzi, 1967; Lezzi and Gilbert, 1970) to show that Na^{+} , K^{+} , Mg^{+2} and Ca^{+2} in hypertonic concentrations and in specific combinations, could induce swelling and puffing in selected chromosomal regions. In contrast, in many tissues hypertonic cation solutions induce marked condensation of interphase chromatin in situ (Whitfield and Youdale, 1966; Gaulden and Jones, 1966). Working

with HeLa cells, Robbins et al., (1970) and Pederson and Robbins (1970) found that exposure of interphase cells to 1.6 to 2.8 times the isotonic concentration of either NaCl, KCl or $MgCl_2$ brought about prophase-like condensation of chromatin. These structural changes were accompanied by sharp decreases in the uptake of labelled precursors of DNA, RNA and protein. Both the structural and metabolic changes were reversed to near normal when the cells were returned to isotonic salt solutions. None of the foregoing effects were produced when isolated nuclei instead of whole cells were used (Robbins et al., 1970). Moreover, Pogo et al., (1966) reported that exposure of isolated liver nuclei to hypertonic NaCl concentration led to their gradual rupture and an increase in RNA synthesis.

Certain polyanionic compounds, including heparin, phosphoproteins and polyaspartic acid, caused swelling and dispersal of chromatin in a variety of isolated nuclei (Anderson and Wilbur, 1950; Whitfield and Perris, 1968; Kraemer and Coffey, 1970a). Such treatments also stimulated DNA synthesis in direct proportion to the degree of nuclear swelling and the quantity of polyanion applied. Although these observations indicate that certain polyanions may mediate specific structural and metabolic changes in chromatin, they must be treated with caution. It was found that in most cases significant amounts of DNA were lost

from the nuclei which had been exposed to polyanions (Whitfield and Perris, 1968; Kraemer and Coffey, 1970b).

It remains difficult to assess the significance of the various structural changes induced in chromatin through changes in ionic environment. Clearly, shifts in the electrolyte balance will affect charge interactions in the DNP complex and through either exposure and/or neutralization of specific groups may alter the state of condensation of chromatin. The interactions between chromatin and ions are not simple charge equilibrium reactions, however. Examination of the titration behaviour of purified DNP (Walker, 1965) and isolated metaphase chromosomes (Cantor and Hearst, 1969), showed that in both cases, titration was only partially reversible over an extended pH range. Isolated chromosomes yielded more complex titration curves than DNP and included first and second order OH^- exchange reactions. Titration of chromosomes was not affected by 10^{-3} M Na^+ , but was appreciably altered by the presence of 10^{-3} M Mg^{+2} or Ca^{+2} (Hearst and Botchan, 1970). Thus chromatin is differentially sensitive to several charge interactions, including pH, ionic strength and charge valency. These factors, coupled with osmotic effects, probably account for the different structural alterations which chromatin undergoes depending on whether whole cells, isolated nuclei or purified DNP are examined. The secondary organization of chromatin

in vivo may in part depend on interactions with specific ions, but it is not yet possible to elaborate such mechanisms in detail.

In addition to ions, several other agents or treatments may affect changes in the configuration of chromatin. In many cases such structural alterations take the form of premature chromosome condensation in interphase nuclei. Mild trypsinization of G² Vicia faba nuclei induced interphase chromatin to form prophase-like chromosomes (Wolfe, 1969). In cells of Jerusalem artichoke tuber cycloheximide blocked cell division at various phases of mitosis and induced supercondensation of chromosomes in prophase (Rose, 1970). Working with HeLa cells, Johnson and Rao (1970) showed that premature chromosome condensation could be induced in cells in interphase when they were fused with cells already in mitosis. This phenomenon could also be produced when hetero-karyons were formed between HeLa cells and cells from a variety of other species. Most interestingly, chromosome condensation was achieved in heterokaryons between mitotic HeLa cells and such differentiated cells as fowl erythrocytes and horse lymphocytes, which do not normally enter mitosis (Johnson et al., 1970). Since these cells were in the G₁ phase of the cell cycle, only single chromatids appeared in the premature condensation process. Observations such as these indicate that specific intercellular agents may be responsible for changes in the structural con-

figuration of chromatin in vivo, in particular when cells prepare for division. Although the nature and mode of action of such agents remain unclear, the fact that trypsin and cycloheximide are able to have related effects suggests that protein alterations may be involved.

The nuclear membrane probably plays a major role in the structural organization of chromatin. Studies with bacteria indicate that replication of the prokaryotic chromosome is initiated and terminated at a site situated on the plasma membrane (Ryter et al., 1968; Sueoka and Quinn, 1968). It has been postulated (Ryter et al., 1968) that this site, termed the replicator, controls the onset of DNA synthesis and serves as a segregation point for the separation of daughter chromosomes. Evidence is accumulating, which suggests that a somewhat analogous structural relationship may exist between chromatin and the nuclear membrane in eukaryotic organisms.

Electron micrographs of sectioned cells and isolated nuclei generally reveal masses of condensed chromatin in close association with the nuclear membrane. Davies (1967; 1968) and Everid et al., (1970) showed that in many cell types, chromatin located at the nuclear periphery exhibits complex structure, including ordered layers of basic fibrils which are oriented along the nuclear membrane. By spreading

whole nuclei on air-water interfaces, DuPraw (1965; 1968) demonstrated that many chromatin fibrils are individually attached to the inner portion of the nuclear envelope. There is evidence as well, that portions of the nuclear membrane remain associated with chromatin during cell division. Detailed microscopic studies of mitosis in protozoans (Roth and Daniels, 1962), amphibians (Davies and Tooze, 1966) and plant endosperm (Bajer and Mole-Bajer, 1969) indicate that remnants of the nuclear membrane remained in contact with the chromosomes throughout cell division, and are incorporated into the new nuclear envelope at telophase. Recently, Comings and Okada (1970a) showed that remnants of what appear to be membrane fragments remain attached to mitotic chromosomes isolated from several mammalian tissues. These workers suggested that these fragments represent specific points where chromosomes attach to the nuclear membrane during interphase.

As with bacteria, associations between membranes and chromatin in eukaryotic cells may be involved in the regulation of DNA synthesis. Autoradiographic studies of cultured human cells labelled with ^3H -thymidine, revealed that DNA synthesis began simultaneously at several points on the nuclear membrane and progressed toward the interior of the nucleus (Moses and Coleman, 1964; Comings and Kakefuda, 1968). In

similar work on liver and root tip cells, Alfert and Das (1969) related the amount of labeled thymidine incorporated to the total surface area of the nucleus, and suggested that the nuclear membrane might regulate the rate of DNA synthesis. These observations led to the proposal that an individual chromosome consists of a single DNA molecule or arrays of DNA subunits linked linearly (Comings, 1968; Hearst and Botchan, 1970). During interphase, each chromosome becomes extended and is attached to the nuclear membrane at several points, which function as replicator loci. Although speculative, this model is supported by observations that DNA molecules isolated by gentle methods from human and other mammalian tissues are several millimeters long (Sasaki and Norman, 1966; Lett et al., 1970), and this in turn is in keeping with estimates that the largest human chromosomes contain enough DNA to form a continuous unit up to 20 millimeters in length (Hart, 1968). It appears possible therefore, as suggested by recent E.M. work (Comings and Okada, 1970b) that the nuclear membrane maintains structural order in the nucleus during interphase by providing multiple attachment points for extended chromosomes. In addition, the membrane may coordinate the time of onset and rate of DNA replication.

An important consideration concerning the organization of chromatin is whether changes in secondary

structure are involved in the control of gene expression. In this regard, there is evidence that relative to extended chromatin, condensed chromatin is restricted in its ability to synthesize DNA and to serve as a template for RNA synthesis. In polytene chromosomes (Clever, 1964) and in lampbrush chromosomes (Callan, 1963), RNA synthesis is associated primarily with areas of extended chromatin, namely puffs and loops. In interphase nuclei from various organisms, autoradiographic studies have shown that the uptake of labeled RNA and DNA precursors is confined chiefly to areas of extended chromatin (Hsu, 1962; Hay and Revel, 1963; Littau et al., 1964; Granboulan and Granboulan, 1965). These observations were amplified by studies on interphase chromatin which had been partially fractionated into extended and condensed components (Frenster et al., 1963; Dolbeare and Koenig, 1970). It was shown that while up to 80% of the total DNA resided in the condensed fraction, the extended chromatin fraction was responsible for the bulk of RNA transcription.

Studies of a more kinetic nature also correlate the state of condensation of chromatin with metabolic activity. During the maturation of nucleated erythrocytes of birds and amphibians, a distinct interrelation is found between the progressive condensation of chromatin and the gradual inactivation of the genome, as

judged by the uptake of labeled RNA and protein precursors (Cameron and Prescott, 1963; Grasso and Woodard, 1966; Scherrer, 1966). An apparent reversal of the foregoing sequence was observed when mature erythrocyte nuclei were introduced into the cytoplasm of human or mouse cells (Harris, 1967; Bolund et al., 1969). In heterokaryons, erythrocyte nuclei were reactivated to resume the synthesis of DNA and RNA, a change which was preceded by nuclear swelling and the dispersal of previously condensed chromatin. Similar reactivation and swelling were noted in Xenopus, when nuclei of mature brain cells were transplanted into the cytoplasm of egg cells (Graham et al., 1966). Finally, Tokuyasu et al., (1968) and Milner (1969) provided evidence that in lymphocytes which had been stimulated with phytohaeagglutinin, DNA synthesis did not take place until the chromatin was almost completely decondensed.

It remains to be established, however, whether alterations in the state of condensation of chromatin are a cause of or a reflection of changes in gene expression. In this regard, Harris (1967) found that although swelling and dispersal of chromatin were an integral part of the reactivation of erythrocyte nuclei in heterokaryons, the two processes could be separated. The synthesis of RNA could be arrested through U.V. radiation of the erythrocyte nuclei, however, this did not inhibit the increase in volume and the dispersal

of chromatin. Sieger et al., (1970) showed that in some fibroblastic cells in the vole, part of the heterochromatic X chromosome became euchromatic, but the chromosome did not take up labelled RNA precursors. Finally, DeBellis et al., (1969) found that when fractionated liver chromatin was supplied with an excess of exogenous RNA polymerase, no differences in priming capacity could be detected between the condensed and extended chromatin fractions. Thus it appears that factors other than the state of condensation of chromatin are involved in the direct control of gene expression. The availability of enzymes, histone-DNA interactions and specific chemical agents probably regulate genetic activity at the molecular level, while the state of chromatin condensation is an associated secondary phenomenon.

III. Biochemistry of Chromatin

A. Characteristics of Isolated Chromatin

Chromatin has been isolated in relatively pure form from a variety of organisms and tissues, from both interphase nuclei (Frenster et al., 1963; Dingman and Sporn, 1964; Bonner, 1965) and fractionated chromosomes (Huberman and Attardi, 1966; Maio and Schildkraut, 1967; Cantor and Hearst, 1970). Purified chromatin always contains DNA-associated basic and non-basic proteins and chromosomal RNA. The exact quantitative

make up varies, however, depending both on the organism and the type of cell under examination. This variation is illustrated by a comparison of the gross-chemical composition of chromatin from several tissues (Table I).

Purified chromatin can serve as a template for DNA-dependent synthesis of RNA in vitro (Frenster et al., 1963; Bonner, 1965; Georgieff et al., 1966). The template activity of chromatin may be amplified by the addition of exogenous RNA polymerase. Under comparable conditions, however, chromatin always remains less effective than pure DNA as a template for RNA synthesis (Marushige and Bonner, 1966; Bonner et al., 1968; Sellgy and Miyagi, 1969). Measurements of in vitro template activity provides a means for comparing the relative potential genetic activity of various cells and tissues. Flickinger et al., (1965) demonstrated that chromatin preparations from frog embryos at various stages of development differ in their ability to synthesize RNA. Work of this nature was extended through DNA-RNA hybridization experiments to show that RNA synthesized in vitro by chromatin from a given cell type is similar to the natural RNA from the same source (Bonner et al., 1968; Paul and Gilmour, 1968; Smith et al., 1969; Tan and Miyagi, 1970). At the same time, it was found that RNA synthesized from one source of chromatin differed from the natural and synthetic RNA obtained from other sources

of chromatin. This demonstrates that the RNA transcribed in vitro by the chromatin from one cell type is unique to that genome. Furthermore, this work shows that DNA is repressed in a cell-specific way in vivo and that this repression is retained in isolation (Paul and Gilmour, 1968).

Studies on isolated chromatin and RNA transcription and hybridization must be interpreted cautiously however. Britten and Kohne (1968) demonstrated that in eukaryotic organisms many DNA sequences may be extensively repeated in the genome. Smith et al., (1969), noted that the incubation times commonly employed for DNA-RNA hybridization are much too short to permit the annealing of RNA molecules which arose from unique sites on the DNA. Thus RNA transcribed from repeated sequences may dominate DNA-RNA hybrids (Paul et al., 1970). A further difficulty which faces transcription studies is that the isolation of chromatin might lead to the loss or inactivation of endogenous RNA polymerase or other factors whose presence may be essential for completely efficient RNA synthesis.

Despite these qualifications, data from in vitro transcription provides clear biochemical evidence that chromatin from various sources is differentially restricted as a template for RNA. Studies on the nature of this restriction indicated that such factors as the relative viscosity and solubility of chromatin are not

primarily involved (Seligy and Miyagi, 1969). In fowl, when the solubility and viscosity of restricted erythrocyte chromatin were made equivalent to that of active liver chromatin, no changes in their relative template activities were noted. These observations suggested that specific restriction of template activity in chromatin must involve molecular interactions rather than mere changes in the general physical state of chromatin.

B. Histone Proteins

i. Characterization of Histones

Gross-chemical chemical analysis of chromatin shows that it contains DNA and histones in roughly equal proportions (Table I). Histones are relatively small proteins with molecular weights in the range of 10,000 to 20,000. Their high content of the amino acids lysine and arginine gives them basic properties with iso-electric points in the range of pH 10 to 11. In the DNP complex, the DNA phosphate groups are probably coupled with the cationic groups in histones through salt-linkages (Hnilica, 1967; Butler et al., 1968). Protamines are a class of proteins similar to histones but are found only in the sperm of some birds and fish (Stellwagen and Cole, 1969). Protamines moreover, are smaller proteins (molecular weight: 3,000 - 5,000) with a very high content of arginine

TABLE 1 Chemical composition of several chromatins
expressed relative to DNA

	Chicken liver (1)	Chicken erythro- cyte (1)	Vegetative pea bud(2)	Human HeLa cells(2)
DNA	1.00	1.00	1.00	1.00
Histone protein	0.80	0.80	1.30	1.02
Non-histone protein	1.40	0.50	0.10	0.71
RNA	0.10	0.01	0.11	0.09

(1) Dingman and Sporn, 1964

(2) Bonner et al., 1968

(up to 50% of their total amino acid content) and an isoelectric point around pH 12. Histones and protamines are probably functionally analogous since their physical and chemical properties are similar and there are transitions between the two types of nucleoproteins (Hnilica, 1967).

Histones have been extracted completely from isolated nuclei and chromatin with strong acids like H_2SO_4 and HCl at pH 1.5 to 1.0 (Murray, 1964) or with high salt concentrations such as 2M or 4M NaCl (Zubay and Wilkins, 1962; Phillips, 1964). Whole extracts like this readily separate into major fractions on column chromatography (Murray, 1964; 1966). Histone components may also be extracted in sequential fashion from isolated nuclei or chromatin. This was achieved with the use of weaker acids like 0.1 M citric acid (Littau et al., 1965), by stepwise titration with acid to progressively lower pH (Murray, 1966; 1969; Murray et al., 1968), or by dissociation with increasingly higher salt concentrations (Ohlenbush et al., 1967; Wilhelm and Champagne, 1969). One advantage of selective fractionation is that as various histone components are removed sequentially from chromatin the remaining DNP complex is left relatively intact for subsequent structural and biochemical examination.

The application of several analytical techniques including column chromatography, starch and acrylamide electrophoresis and amino acid analysis, indicates

that histones isolated from various tissues and organisms are remarkably alike in overall composition (Butler et al., 1968; Stellwagen and Cole, 1969). Similar histones have been found in such diverse organisms as plasmodium (Mohberg and Rush, 1969), molluscs (Cozcolluela and Subirana, 1968), pea buds and calf thymus (Fambrough and Bonner, 1968). At the same time, these studies demonstrate a common heterogeneity within the histones. Typically, in calf thymus nuclei at least five chromatographic fractions have been identified (Butler, 1964; Murray, 1966; 1964). Similar or analogous fractions are found in almost all other organisms and tissues, and are considered to represent the spectrum of major histones fractions (Enilica, 1967; Bonner et al., 1968; Butler et al., 1968).

Some ambiguity exists in the number and designation of the major histone fractions. This is due mainly to differences in the analytical techniques used by various investigators. The major fractions are usually distinguished numerically in their approximate order and ease of elution from chromatographic columns. Fractions rich in lysine are extracted first, followed sequentially by moderately lysine-rich fractions and arginine-rich fractions. Table 2 summarizes the most common nomenclature systems and some of the properties of the principal histone fractions.

Electrophoresis in polyacrylamide and starch gels reveals further histone heterogeneity. With these tech-

TABLE 2 Identity of the major histone fractions and some of their characteristics

	Lysine-Rich	Moderately Lysine-Rich	Arginine-Rich
Unilica, 1967			
Butler et al., 1968	F1	F2a2, F2b	F3, F2a1
Murray et al., 1968	Ia, b, c	IIB1, IIB2	III, IV
Fambrough and Bonner, 1969	I	IIB, IIB	III, IV
pH of extraction			
Murray et al., 1968	2.5-2.1	1.8-1.3	1.4-1.0
Molar concentration of NaCl of extraction			
Bonner et al., 1968	0.6	0.8-1.8	0.9-2.0

niques at least a dozen electrophoretic bands are found in isolated histones from many tissues (Neelin and Butler, 1961; Lindsay, 1964; Murray, 1966; Vendrely and Picaud, 1968). The biological validity of some of the minor electrophoretic bands may well be questioned (Butler et al., 1968), since they could represent artifacts introduced through proteolytic degradation, cross-contamination and/or aggregation (Murray, 1964; Stellwagen, et al., 1968). However, parallel studies on histones prepared by different extraction methods and by the combined application of various analytical methods indicate that most heterogenous components are authentic (Neelin et al., 1964; Murray, 1966; Hnilica, 1967).

Detailed studies on the amino acid composition and the primary structure of the various histone fractions remain incomplete. The lysine-rich fraction (F 1) probably contains several (3-6) components, whose exact numbers vary in different organisms and tissues (Reid et al., 1968; Bustin and Cole, 1969a; Kinkade, 1969; Nelson and Yunis, 1969). The four components of rabbit thymus F 1 were shown to be molecularly homogenous with respect to amino acid composition (Kinkade and Cole, 1966). Studies on the primary structure of one of these components, reveal that most lysine residues (45 out of 60) are situated near the carboxyl-terminal of the molecule, suggesting that this strongly cationic region is a binding site for DNA (Bustin et al., 1969;

Bustin and Cole, 1969b). Preliminary work on the primary structure of other components of rabbit thymus F1 indicates that the amino-terminal sequences of these molecules are variable, but that the carboxyl-terminal regions are very similar (Bustin and Cole, 1970).

In contrast to lysine-rich histones, moderately lysine-rich and arginine-rich components are less numerous and relatively homogenous in the various organisms in which they have been studied. Identical peptide maps and very similar amino acid composition were reported in preparations of fraction F2b (moderately lysine-rich) from several different organisms (Hnilica, 1967). The complete amino acid sequence of the main component of F2b revealed that basic amino acids were clustered near both ends of the molecule and were relatively infrequent in the middle portion (Iwai, 1970). Similar work on arginine-rich fractions (F2a1, F3), indicated that there were only two kinds of arginine-rich histones in any one tissue or organism, and that they were virtually identical in amino acid composition (Fambrough and Bonner, 1969; Desai and Foley, 1970). Structural work on F2a1 from pea bud and calf thymus (DeLange et al., 1969; Sautiere et al., 1970) showed that basic amino acid residues were present in both halves of the molecule with some concentration (17 residues vs 10) in the N-terminal half. Although

incomplete, these studies on primary structure indicate that the individual histone fractions consist of distinct types of molecules, each of which must interact with DNA in a different manner.

Although variations in histone composition among different tissues and organisms are rare, a notable exception in this regard is a characteristic histone found in nucleated erythrocytes. First detected electrophoretically in chicken erythrocytes (Neelin and Butler, 1961), this histone was later characterized as a distinct fraction (V, F5) by column chromatography (Neelin et al., 1964; Hnilica, 1964). This histone is of the moderately lysine-rich type, but is distinguished by a relatively high content of serine residues (Neelin et al., 1964). Histone V is found in mature erythrocytes of various avian species (Neelin, 1968), but is apparently present in lesser amounts in embryonic chicken erythrocytes (Mazen and Champagne, 1969), and is definitely lacking in other avian tissues (Purkayastha and Neelin, 1967). Similar, though not identical, histones have been reported in erythrocytes of amphibians, reptiles and fish; each group of animals apparently having its own distinct molecular form of this histone (Vendrelly and Picaud, 1968). Histone F5 may be instrumental in the severe restriction of metabolic activities which is exhibited in mature nucleated erythrocytes and includes the absence of mitotic divisions,

heavily condensed chromatin and the near absence of RNA and protein synthesis.

A few additional tissue-specific variations in histone composition have been reported. Sheridan and Stern (1967) noted that an apparently unique histone was present during the meiotic cycle in lily and tulip anthers. Gorovsky (1970) reported electrophoretic differences between the histones of isolate micro and macro-nuclei in Tetrahymena p. Several workers have reported that typical histone fractions are either completely absent, or differentially reduced during the early stages of embryonic development in the newt (Bloch, 1966; Asao, 1969; 1970) and the sea urchin (Johnson and Enilica, 1970; Thaler, 1970). These variations in histone composition may prove highly significant in relation to mechanisms of differentiation, but most of the systems have not been fully investigated and the reports remain to be confirmed and extended. In general, major variations in histone composition appear not to have arisen frequently in the course of evolution, suggesting that a fairly rigid conservation in structure and composition may be functionally significant.

ii. Histone Function: Regulatory

Stedman and Stedman (1950) originally suggested that histones might be involved in the regulation of gene expression, and this proposal has subsequently

received some experimental support. Several workers found that removal of histones from isolated nuclei or chromatin, through acid, salt or enzymatic extraction, leads to a marked increase in transcription of RNA in vitro (Huang and Bonner, 1962; Alfrey et al., 1963; Bonner, 1965). These observations suggest that histones function as gene repressors by interacting with portions of DNA, thereby rendering it unavailable as template for RNA synthesis. To determine whether the various histone fractions play specific repressor roles, the effects of selective histone extraction on chromatin transcription have been examined. It was found that the removal of the lysine-rich fraction (I) had very little effect on template activity, as compared to the marked effects of total histone removal (Butler et al., 1968; Spelsberg and Hnilica, 1971). Koslov and Georgiev (1970) however, noted that RNA chains transcribed after the selective removal of fraction I were four to five times longer than those transcribed from unextracted chromatin, and suggested that lysine-rich histones specifically impeded the movement of RNA polymerase along the DNP complex. The work of Seligy and Neelin (1970) indicated that in chromatin of fowl erythrocytes, the characteristic histone rich in serine (V) was responsible for about half of the total template restriction which was attributable to histones. Clearly, however, this type of work requires further refinement before definite roles can be assigned to histones in

gene regulation. In this regard, Paul and Gilmour (1968) showed that when purified DNA was recombined with histone, the reconstituted chromatin exhibited the same template activity as native chromatin only when non-histone chromosomal protein was also present. Similarly, Bekhor et al., (1969) showed that a distinct type of chromosomal RNA was important in reconstituting chromatin with template capabilities similar to that of native chromatin.

While histone proteins may serve as gene repressors in vivo, available evidence indicates that they are not highly specific regulators of gene expression. Comparable studies of histones have been carried out using cells and tissues which differ markedly in their relative content of active and inactive chromatin and in their RNA synthesis ability. In general, great similarity was found in the qualitative and quantitative composition of histones as determined by various analytical techniques in embryonic and adult tissues of fowl (Dingman and Sporn, 1964; Kirsher and Hnilica, 1967), in bands and puffs of polytene chromosomes (Gorovsky and Woodard, 1967) and in tissues differing markedly in their content of heterochromatin and euchromatin (Comings, 1967). Histones may, however, function as non-specific gene repressors while retaining uniform composition in various tissues. There is evidence for example, that modification of histones through acetylation or phosphorylation may influence DNA-histone

interactions and thereby affect gene expression (Allfrey et al., 1968; Benjamin and Goodman, 1969). Such modifications in histones might be mediated through hormones. Insulin was found to stimulate histone phosphorylation in mammary tissue (Turkington and Riddle, 1969) and liver tissue (Langan, 1969). Insulin also induced histone acetylation in mammary tissue (Marzluff and McCarthy, 1970), while erythropoetin had similar effects on mouse spleen histones (Takaku et al., 1969). In all of the above cases, chemical modification of histones was accompanied by an elevation in RNA synthesis suggesting that this is one way in which gene derepression might be achieved in vivo.

iii. Histone Function: Structural

Histone proteins probably play a major role in the structural organization of chromatin. They contribute to the physical integrity of basic fibrils and probably also govern, at least in part, the secondary configuration of chromatin. The precise nature of the structural role of histones is at present largely unknown, however, since the molecular interactions between DNA and histones is incompletely understood. Certainly this interaction is complex, since as outlined earlier, different histone sub-fractions are found with differential tenacity to DNA.

The physico-chemical properties of chromatin in vitro have been investigated by several techniques, before and after the selective extraction of various histone fractions. In both chromatin and DNP, DNA was shown to be stabilized by histones against thermal denaturation (Bonner, 1965). The selective extraction of weakly bound histones (mostly lysine-rich) resulted in only a slight drop in the melting temperature of thymus DNP (81 to 78°C), while removal of the remaining histones brought about a marked reduction to 56°C (Fredericq and Houssier, 1967). Spectrophotometric and optical rotatory dispersion studies indicated that the conformation of DNA in nucleohistone was largely unaffected by extraction of lysine-rich histones (Tuan and Bonner, 1969; Simpson and Sober, 1970). In contrast, removal of moderately lysine-rich and arginine-rich components, greatly altered the conformation of DNA in DNP (Wilhelm et al., 1970). These observations suggest that lysine-rich histones have only a minor influence on the physical integrity of DNP. The more tightly bound histones in contrast probably confer considerable stability on the DNA helix and may direct its orientation in the DNP complex. Additional evidence to this effect was provided by Murray et al., (1970). It was shown that X-ray diffraction patterns which were attributed to a supercoiled conformation of DNA in DNP, were not abolished after most of histone fractions

I and V were extracted from avian erythrocyte chromatin. Fractions I (lysine-rich) and V (serine-rich) are the most readily extractable histones in nucleated erythrocytes.

Studies on purified histones in solution show that lysine-rich fractions assume an extended chain configuration, while other histones tend to assume an α -helical form (Bradbury et al., 1967; Wilhelm et al., 1970). These observations, combined with information on asymmetrical distribution of basic groups on histone I and on its interaction with DNA (Olins, 1969; Sluyer and Sneller-Jurgens, 1970) could mean that lysine-rich histones are spaced at intervals along the DNA helix. Other histones containing α -helical components may be tightly complexed with DNA and direct its orientation in the DNE complex. An example of the latter is histone IV, whose N-terminal in α -helical form has been shown to fit neatly into the major groove of the DNA double helix (Sung and Dixon, 1970).

Before a more definitive picture can be obtained of the structural role of various histones in the DNE complex and hence the basic fibril, a number of points must be considered. The main difficulty which besets studies on DNA-histone interactions in vitro is that the conditions required to separate these macromolecules from chromatin are severe. The high ionic strength or low pH necessary are far removed from normal physiological

conditions and may well bring about irreversible alterations in the interaction of histones and DNA. In addition, any model of the structure of the DNH complex must make allowances for the fact that many sites on DNA are apparently available for titration with agents like polylysine (Clark and Felsenfeld, 1971), and that DNH can undergo reversible stretching under condition of low ionic strength (Pardon et al., 1967). These respective observations indicate that portions of DNA may not be complexed with protein at all times and that the configuration of DNA can be appreciably altered without loss of histones. Thus histones are probably not rigidly complexed with DNA, and may in fact exist in a state of partial dissociation in vivo.

Investigations into the role of histones in the secondary organization of chromatin have been confined mainly to microscopic studies of isolated chromosomes and nuclei following treatment with specific enzymes and other chemical agents. When unfixed chromosomes isolated from mice (Chorazy et al., 1963) and Chironomus salivary glands (Lezzi, 1967) were treated with either DNase or trypsin, they gradually disintegrated. At the same time, treatment with pepsin or RNase had only minor effects on chromosome morphology. Similar observations on lampbrush chromosomes (Callan, 1963), indicated that DNase alone caused chromosome fragmentation;

trypsin induced chromosome swelling only. In isolated liver nuclei, DNase caused an overall compaction in chromatin (Narayan et al., 1967), while in calf thymus nuclei the dense masses of chromatin were loosened into a fibrous network following trypsin digestion (Allfrey et al., 1963). Since trypsin acted preferentially on histones (Allfrey et al., 1963), the above observations indicated that both DNA and histones were involved in the secondary organization of chromatin. In an attempt to determine the relative importance of histones and DNA in the overall morphology of chromatin, Trosko and Wolff (1965) treated formalin fixed chromosomes and interphase nuclei of Vicia faba with various enzymes. It was found that trypsin affected the morphology of both chromosomes and nuclei to the greatest extent causing initial swelling and eventually complete disruption of structural integrity. DNase caused chromosome swelling, but left them generally intact. Unfortunately, these observations are somewhat ambiguous. They show that histones play a major role in the structural integrity of chromatin and that DNA was not essential to maintain intact chromosomes after stabilization of proteins by fixation. However, since DNase caused chromosome swelling, these observations fail to differentiate whether histones or DNA are primarily responsible for the secondary organization of chromatin.

In this regard two sets of observations are of special interest. Sorsa et al., (1970) showed that when unfixed Drosophila salivary chromosomes were treated with urea-alkali solutions, the entire chromosome banding pattern gradually uncoiled, until the polytene chromosomes looked remarkably like lampbrush chromosomes. This structural transition, attributed mainly to a loss of chromosomal proteins, showed that while proteins may be responsible in large measure for the secondary organization in chromatin, the linear continuity of polytene chromosomes was maintained in the absence of proteins. The results of Littau et al., (1965) and Mirsky et al., (1968) can be similarly interpreted. These workers showed that when lysine-rich histones were specifically extracted from isolated, unfixed thymus nuclei and HeLa chromosomes, the chromatin became markedly loosened. At the same time, however, the overall distribution of peripheral chromatin masses was retained in thymus nuclei, and the linear integrity of HeLa chromosomes was retained even after total histone extraction. These observations showed that while histone proteins are involved in the secondary organization of chromatin, DNA, possibly in conjunction with other nuclear proteins, play an equally important role in the structural integrity of chromatin in general.

C. Non-Histone Proteins

In addition to histone, variable quantities of other proteins are associated with chromatin. Various referred to as acid-insoluble, acidic, non-basic or, most commonly, non-histone nuclear proteins (NHP), these macromolecules are poorly characterized (Hnilica, 1967; Stellwagen and Cole, 1969). The principal hindrances in their study have been the difficulty in dissociating them from DNA and histones and their tendency to form aggregates when purified. NHP are usually recovered from residual DNP after complete histone extraction and have been found to vary both qualitatively and quantitatively, in the various tissues analyzed (Dingman and Sporn, 1964; Wang and Johns, 1968; Bonner et al., 1968). Analysis of NHP by column chromatography, gel-electrophoresis and determination of amino acid composition have revealed that these proteins consist of several heterogeneous components with molecular weights from 5,000 to 100,000 (Elgin and Bonner, 1970). Glutamic and aspartic acid contribute between 15 and 25% of their total amino acid content (Benjamin and Gellhorn, 1968; Shaw and Huang, 1970).

Several preliminary observations indicate that part of the NHP complement may function in the regulation of gene expression. Acidic nuclear proteins were found to both inhibit and stimulate the transcription of RNA

in different tissues (Kruh et al., 1969; Wang, 1970). This led to the suggestion (Paul and Gilmour, 1968; Spelsberg et al., 1971) that NHP may both derepress and repress specific DNA sequences by respectively associating and disassociating with histones. This might function, as suggested by Bonner et al., (1968), through an NHP-RNA complex which temporarily uncouples histone from DNA to open up specific sequences on the genome.

Non-histone proteins were also associated with chromatin structure. Maio and Schildkraut (1967) showed that after most nucleic acid and histone components were apparently extracted from isolated mammalian chromosomes, fibrous chromosome masses could still be recognized microscopically. This observation indicates that NHP might contribute substantially to the structural integrity of chromosomes. Dounce and Hilgartner (1964) and Hilgartner (1968) showed that DNP gels which contained non-histone proteins could be solubilized by agents which dissociated disulphide bridges, and suggested that NHP might act as essential linkers between nucleohistone fibres. Clearly, however, much better understanding of these proteins is necessary before specific functions can be attributed to them.

In this regard, the following observations are of special interest. Non-histone proteins are present in greatest amounts in chromatin which is derived

from tissues which are active in RNA synthesis (Dingman and Sporn, 1964; Shaw and Huang, 1970). For example, chromatin isolated from chicken liver and immature erythrocytes contained relatively more NHP than mature chicken erythrocytes (Seligy and Miyagi, 1969; Sadgopal and Kabat, 1969). A comparative study of NHP from such diverse sources as avian erythrocytes, rat liver and pea buds, revealed that in addition to tissue specific components several homologous fractions were present in all tissues (Elgin and Bonner, 1970). These observations suggest that some NHP components may represent enzymes and/or structural proteins which, like histones, are common to most eukaryotic organisms, while other NHP components may have unique functions which are specific to only some cells.

MATERIALS AND METHODS

Except where explicitly stated, studies were carried out using tissues from mature (3-12 month old) chickens of the Dekalb strain (McCallum Poultry, Carp, Ontario). Two experiments were done using blood from mature ($\frac{1}{2}$ to 1 year old) geese (Central Experimental Farm, Ottawa). All handling of tissues, blood cells and isolated nuclei including fractionation, fixation and dehydration were carried out at $0-4^{\circ}\text{C}$. Except where otherwise indicated, low speed sedimentations were performed on an International clinical centrifuge and high speed ones on a Spinco Model L ultracentrifuge using rotor #30.

I. Preparation of Chicken Erythrocyte Nuclei

Erythrocytes and erythrocyte nuclei were isolated and purified after the method of Purkayastha and Neelin (1966). Birds were bled from the jugular vein and 40 to 80 ml of blood was collected into 8% potassium oxalate solution (1 ml/10 ml blood) to prevent clotting. Blood cells were sedimented by centrifugation at 300 g for 10 min. and then washed twice by dispersal and re-sedimentation in 0.15 M NaCl (saline) solution. The washed pellet was then dispersed in a volume of saline equal to the original blood volume and 1/20 volume of a saline solution containing 3%

(w/v) saponin was added to effect lysis of the cells. The saponin suspension was stirred for 20 to 30 min. and then centrifuged at 300 g for 10 min. to sediment unlysed cells and debris. The resulting supernatant was decanted and centrifuged at 10,000 g for 15 min. to sediment the nuclei. Nuclei were further purified by dispersal in 40 to 80 ml of saline by means of a loose fitting glass homogenizer of the Dounce-type (Kontes Glass, Vineland, N.J.), followed by centrifugation at 10,000 g for 15 min. This procedure was repeated four to five times until the supernatant was free of red colour due to haemoglobin and the nuclear pellet showed a yellow-amber tint. The nuclear preparation was checked by phase-contrast microscopy at this point to ensure that the nuclei were intact and free of gross cytoplasmic contamination. These nuclei constituted the starting material for further fractionation and manipulation.

II. Preparation of Liver Nuclei

Liver nuclei were prepared using a modification of the method of Seligy and Miyagi (1969). Between 40 and 60 gm of liver tissue was excized from freshly exsanguinated birds and immediately placed in 100 ml of sucrose-saline solution (0.35 M sucrose, 0.15 M NaCl). After as much connective tissue as possible was removed, the liver tissue was quickly minced with scissors in sucrose-saline solution and rinsed twice by

decantation. The minced tissue was then homogenized in 100-150 ml of sucrose-saline with 10 to 15 strokes of a loose fitting Dounce homogenizer, filtered through four layers of cheese cloth and centrifuged at 600 g for 15 min. After centrifugation, the supernatant was discarded, the pellet resuspended with the homogenizer in fresh sucrose-saline and the resulting suspension centrifuged again. After this centrifugation, to reduce erythrocyte contamination to a minimum, only the top 4/5 of the pellet was dispersed and decanted. The bottom 1/5, distinguished by its red colour, contained the major proportion of erythrocytes and was discarded. This step was undertaken only once. Dispersal and sedimentation in sucrose-saline was repeated three to four times and was followed by two dispersals and sedimentations in pure saline solution. The final nuclei were checked by phase-contrast microscopy for purity and structural integrity.

III. Preparation of Immature and Mature Erythrocytes from Geese

To obtain blood enriched in immature red cells, anemia was induced in geese by injections of phenylhydrazine (Neelin, 1968). Phenylhydrazine (British Drug House Reagent) was administered in doses of 20 mg/kg of body weight, injected as 1% solution (w/v) in saline. Birds were injected three times, three days apart.

During this time haematocrits (% cell volumes) dropped from 38% to 28%.

Birds were sacrificed by total exsanguination from the jugular vein, the 200 to 250 ml blood being collected into 8% potassium oxalate solution (1 ml/10 ml blood). Blood cells were sedimented at 300 g for 15 min (International B20 centrifuge, rotor #872) and then washed twice by dispersal and sedimentation in Seligman's Balanced Salt Solution (SBSS - Cutts, 1970), containing 0.5% EDTA. Following washing, the cells were resuspended in 400 ml SBSS - EDTA. The cell suspension was then divided into four 100 ml lots and each lot was layered over 100 ml of 30% Ficoll (Pharmacia) in a 250 ml centrifuge tube. These preparations were centrifuged in a swinging-bucket rotor at 5,000 rpm for 30 min (International B20 centrifuge, rotor #947). After centrifugation, mature erythrocytes had settled as a stiff pellet at the bottom of the tube. Immature cells formed a layer about 1 cm thick at the top of the Ficoll layer. The entire supernatant, containing SBSS, Ficoll and immature cells was decanted from each tube and diluted to 200 ml with saline. This mixture was centrifuged at 3,000 rpm for 15 min. The resulting cell pellet was washed twice more by dispersal and centrifugation in 200 ml of saline. Nuclei were isolated from these cell preparations as previously described for chicken erythrocytes.

In a control experiment, mature erythrocytes

were obtained from a normal, healthy goose. In this case, 60 ml cells were washed in SBSS- + EDTA as above and dispersed but not centrifuged in 30% Ficoll for 30 min. This was done to duplicate as closely as possible the conditions to which the immature cells had been exposed. The cell suspensions were then washed free of SBSS and Ficoll as described above, by dilution with 200 ml of saline and centrifugation at 3,000 rpm for 15 min. Nuclei were purified as previously described for chicken erythrocytes.

IV. Extraction and Analysis of Histone Proteins

A. Extraction

The procedure for the fractional extraction of histone proteins from isolated nuclei consisted of an initial extraction with 0.1 M citric acid in 0.125M NaCl (Littau et al., 1965), followed by stepwise extraction with HCl in saline (Murray, 1966; Vidali and Neelin, 1968). Nuclei were routinely suspended in a volume of solution treble the volume of the packed nuclear pellet by dispersal with eight strokes in a Dounce homogenizer. All sedimentations were by centrifugation at 10,000 g for 15 min.

Prior to extraction, purified nuclei in saline were washed by dispersal and immediate centrifugation in 0.01 M citric acid in saline (pH 2.6). This was done to lower the pH gradually and to remove soluble

protein contaminants, but not histones (Murray et al., 1968). After washing, the nuclear pellet was resuspended in a solution of 0.1 M citric acid in 0.125 M NaCl (pH 2.0), and stirred for 30 min. to 1 hr. to extract the first histone fraction. The mixture was then centrifuged and the supernatant decanted and stored on ice for later analysis. The nuclear pellet was resuspended in the same extraction medium, stirred for another 15 to 30 min, sedimented, and the resulting supernatant, which constituted a "washing" was also retained. The residual nuclear pellet was then suspended in saline and the pH was lowered to 1.8 by the dropwise addition of N HCl. Extraction followed by washing, as above, were carried out for 1-3 hr. and 15 - 30 min., respectively. Subsequently the nuclei were extracted and washed in a similar manner in saline adjusted to pH 1.0. In this way, three primary histone extracts, pH 2.0, 1.8 and 1.0, a washing after each extraction and a final residual nuclear pellet were obtained. The entire procedure is summarized in Table 3.

In one experiment with liver nuclei, fractionation of histones was carried out in a parallel rather than the usual sequential manner. This was done to check whether marked degradation of histone components took place during the relatively prolonged sequential extraction. In this experiment the nuclei were divided into three equal aliquots. The first aliquot was extracted with 0.1 M citric acid - 0.125 M NaCl (pH 2.0)

TABLE 3 Flow sheet of procedures for the selective extraction of histone fractions.

for 15 mins. and then centrifuged. The second aliquot was similarly treated twice in succession. The third aliquot was exposed to a single 30 min. extraction in saline-HCl at pH 1.0, followed by centrifugation.

B. Starch-Gel Electrophoresis and Histone Recovery

Starch-gel electrophoresis of the fractionated histones was performed according to Neelin (1968). Electrophoresis was carried out in the vertical manner (Smithies, 1959), with the anode at the top. Each gel was prepared by dissolving 16 gm. of starch (Connaught Medical Research Laboratories, Toronto) and 36 gm. of solid urea in 100 ml. of 0.02 M sodium acetate buffer, pH 4.8, I 0.020. The pH of each histone extract was raised by the addition of 1/10 volume of 0.2 M sodium acetate. From this, a 0.12 ml sample containing 36% (w/v) urea was prepared and added directly to a slot in the starch gel. The external electrolyte solution was 0.1 M NaCl, and a constant current of 36-40 mA was supplied by a Beckman Duostat DC power supply. Electrophoresis was carried out for 6 hr., during which time, the average voltage gradient along the gels declined from 4-5 to 2.5-3 V/cm.

After electrophoresis, each gel was bisected longitudinally with a 17 cm Blair-Brown knife blade (Ingram and Bell, Toronto). One face was stained with saturated nigrosin (Harleco) in methanol: acetic acid:

water (5:1:5) and washed in the same solvent. The other half of the gel was stained with amido black 10B (Harleco) in the same solvent as above and washed with absolute methanol..

In some experiments the protein fractions were recovered quantitatively in order to estimate the relative amounts of histone removed at each stage of extraction. The protein was precipitated from each extract by the addition of 9 volumes of cold (5°C) acetone. After standing in a cold room overnight, the precipitates were collected on fritted glass filters under suction, washed with acetone and ether, dried for several hours over CaCl_2 and scraped off the filters for weighing. In some cases, the acetone precipitates were also examined electrophoretically; usually when the original extracts proved too dilute. For this, the protein samples were redissolved in 0.02 sodium acetate buffer (pH 4.8), containing 36% urea, and applied to starch-gels as described above.

V. Dispersal and Recondensation of Chromatin

Changes in the structural configuration and the state of condensation of chromatin in isolated nuclei were induced by the following treatments. Samples of purified nuclei were diluted with from one to ten volumes of saline and sedimented at 1,000 g for 2 min. The supernatant was decanted and replaced by an equal

volume of distilled water. After a 5 to 10 min. suspension in water, the nuclei were sedimented and resuspended in an equal volume of fresh saline solution. In this manner, the isolated nuclei were alternately exposed to isotonic hypotonic and isotonic salt solutions. The combined effects of progressive histone extraction and sequential exposure to hypotonic and isotonic saline were also investigated. In these experiments nuclei, which had been exposed to acid conditions during histone extraction, were first suspended in saline buffered at pH 6.8 with 0.02 M Na cacodylate-HCl. Subsequently, the nuclei were sedimented at 1,000 g for 2 min. and alternately suspended in distilled water followed by saline as described above. It was found that chromatin was not dispersed by a 5 to 10 min exposure to distilled water in nuclei which had been extracted with 0.1 M citric acid - 0.125 M NaCl (pH 2.0). Samples of these nuclei were therefore subjected to more severe treatment. This included fifty fold dilution and overnight stirring in distilled water and in a solution containing 0.05 M EDTA (pH 7.0).

VI. Microscopic Techniques

(A) Electron Microscopy

i. Fixation

Samples of liver tissue, blood cells and isolated nuclei were prepared for electron microscopy at the following stages during histone fractionation:

- (1) Intact immature and mature erythrocytes washed in saline.
- (2) Freshly excised liver tissue.
- (3) Purified erythrocyte nuclei in saline.
- (4) Purified liver nuclei after fractionation in sucrose-saline and washing in saline.
- (5) Nuclei after washing in 0.01 M citric acid-saline.
- (6) Nuclei after extraction in 0.1 M citric acid - 0.125 M NaCl.
- (7) Nuclei after washing in 0.1 M citric acid - 0.125 M NaCl.
- (8) Nuclei after extraction and washing at pH 1.8 in saline.
- (9) Nuclei after extraction and washing at pH 1.0 in saline.

The general fixation regime adopted for routine preparations was the following: all operations were carried out in centrifuge tubes containing about 10 ml of solutions at 0-4°C:

- (1) 1.5% glutaraldehyde.....30 min
- (2) Centrifuged at 1,000 g..... 2 min
- (3) Superantant removed by pipetting.....
- (4) 6% glutaraldehyde..... 2 hr
- (5) Fixative removed, with centrifugation when necessary.....
- (6) Washed three times.....overnight
- (7) 2% OsO₄..... 2 hr
- (8) Washed three times.....1-2 hr

For fixation near neutrality, all fixative and wash solutions contained saline with either 0.025 M sodium phosphate or 0.02 M sodium cacodylate buffer, pH 6.8 (saline-buffer). Since samples fixed in sodium phosphate tended to exhibit fine electron-dense precipitate, sodium cacodylate buffer was preferred. In addition to fixation at neutral pH samples of nuclei were also fixed and washed in the same acid solutions, they were suspended in for histone fractionation, i.e. 0.01 M citric acid-saline (pH 2.6); 0.1 M citric acid -0.125 M NaCl (pH 2.0); saline-HCl (pH 1.8) and saline-HCl (pH 1.0). For comparative purposes, in some cases, samples of chicken erythrocytes and erythrocyte nuclei were fixed for 2 hr. solely in 2% OsO₄ in veronal acetate buffer, pH 7.2, (Palade, 1952). This was followed by centrifugation at 1,000 g for 2 min. and three washes over 1 hr in veronal acetate buffer.

Liver tissue was cut into pieces approximately 2 mm³ and immediately placed in 10 ml of buffered fixative. In all other cases, approximately 0.25 ml samples of blood cells or nuclei were removed from pellets with a fine spatula and dispersed directly into centrifuge tubes containing about 10 ml of fixative or buffer. Nuclei which had been washed or extracted in solutions of low pH were divided; half the sample was dispersed directly in fixative made up in the same acid solution as the nuclei were previously suspended in. The other half sample was dispersed

in 10 ml of saline-buffer (pH 6.8) for 5 to 10 min., and then centrifuged at 1,000 g for 2 min. The supernatant was decanted and replaced with 10 ml of buffered fixative into which the pellet was dispersed.

Samples of intact chicken erythrocyte and liver nuclei which were exposed to low ionic strength were fixed for electron microscopy at the following stages:

- (1) Purified nuclei in saline.
- (2) Nuclei after 5 to 10 min. suspension in distilled water.
- (3) Nuclei after 5 to 10 min. suspension in distilled water, followed by resuspension in saline.

Fixation and washing was carried out in the medium in which the nuclei were initially suspended, i.e., saline or water. The pH of all solutions was adjusted to 6.8 with 0.01 N NaOH.

In experiments designed to study the combined effects of histone extraction and exposure to hypotonic and isotonic solutions, samples were fixed at the following stages:

- (1) Nuclei after extraction and washing with 0.1 M citric acid-saline (pH 2.0).
- (2) Nuclei treated as in (1) and suspended for 5-10 min. in saline-buffer (pH 6.8).
- (3) Nuclei treated as in (2) and suspended for 5-10 min. in distilled water.
- (4) Nuclei treated as in (3) and suspended for 5-10 min. in saline.

Parallel samples of nuclei extracted at both pH 1.8 and pH 1.0 were prepared in identical fashion. Following the final stage of preparation the nuclei were sedimented at 1,000 g for 2 min. and fixed in the

general manner previously described. Fixation and washing were carried out in the medium to which the nuclei had been exposed last, i.e., 0.1 M citric acid-saline (pH 7.0), saline at pH 1.8 or pH 1.0, saline-buffer (pH 6.8), distilled water (pH 6.8) or saline (pH 6.8). The pH of the last two solutions was adjusted with 0.01 N NaOH.

Samples of chicken erythrocyte nuclei which were extracted with 0.1 M citric acid-saline (pH 2.0) and exposed to distilled water and 0.05 M EDTA (pH 7.0) overnight, were fixed in the routine manner in distilled water (pH 6.8) and 0.05 M EDTA (pH 7.0) respectively.

ii. Dehydration - Embedding and Electron Staining

Following fixation, the samples were dehydrated by one of two methods. During some initial experiments, a graded acetone-saline mixture was used:

(1) 1% acetone in saline - 30 min.

(2) 10% " " " "

(3) 25% " " " "

(4) 50% " " " "

(5) 75% " " " "

(6) 90% " " " "

(7) Absolute acetone, two changes; stored at 0°C.

In the majority of experiments, the following simpler dehydration sequence was used with indistinguishable results:

- (1) Methoxyethanol - 2 changes of 1 hr. each.
- (2) Ethanol - " " " " " "

All tissues were embedded in a mixture of Epon-Araldite as described by Fowke (1968):

- (1) Samples in absolute acetone or ethanol at 0-4°C brought back to room temperature.
- (2) Propylene oxide - about 1 ml added over 15 min.
- (3) Propylene oxide - 2 changes, 15 and 30 min. respectively.
- (4) Propylene oxide/resin mixture - 3/1 (v/v) - 1 hr.
- (5) " " " - 1/1 " - 1 hr.
- (6) " " " - 1/3 " - overnight
- (7) Pure resin mixture - 8 to 24 hr.
- (8) Samples about 1 mm³ transferred to fresh resin mixture in #00 gelatin capsules.
- (9) Capsules heated in oven at 75°C - 48 hr.

The resin mixture consisted of 20 parts of Araldite 502, 25 parts of Epon 812, 60 parts of dodecenyl succinic anhydride (DDSA-plasticizer) and 1 drop per ml of tridimethyl-aminomethyphenol (DMP-30 acceleration catalyst) which was added from a syringe with a 22 gauge needle. The mixture without DMP was heated at 80°C for about 15 min. mixed evenly, and stored at room temperature or at -40°C for several weeks. Before use, this mixture was reheated for 10 min. at 80°C, the DMP added and the complete resin mixture was stirred thoroughly.

The embedded tissue was sectioned with diamond

knife. Sections showing silver and light gold interference colours were collected on carbon-formvar coated copper grids and stained by the following method (Stempak and Ward, 1964):

- (1) 25% uranyl acetate in methanol - 20-30 min.
- (2) Rinses - 30 sec. each in methanol and distilled water.

B. Light Microscopy

Using light microscopy the processes of chromatin dispersion and recondensation were followed in individual nuclei from the following sources: purified chicken erythrocyte and liver nuclei; chicken erythrocyte nuclei after extraction and washing with 0.1 M citric acid-0.125 M NaCl, and after extraction and washing at pH 6.8 and pH 1.0 in saline. Erythrocyte nuclei which had been exposed to low pH were suspended in saline buffered at pH 6.8 before all subsequent manipulation.

Observations were carried out using a 100X oil immersion objective and Nomarski interference-contrast optics on a Zeiss Photomicroscope. Suspensions of unfixed nuclei in saline were mounted beneath a coverslip on a slide coated with a thin film of Tissue-Tac adhesive. In this way, many nuclei were prevented from moving during subsequent manipulation. A drop of distilled water was then placed at one edge of the coverslip and drawn under by absorbent paper

placed at the opposite end. When dispersion of chromatin was observed to be complete, saline was again drawn under the coverslip in the same manner. The same nucleus was continually observed and periodically photographed during these procedures.

RESULTS

Ten separate nuclear isolations and associated extractions were carried out during this investigation, including five experiments on chicken erythrocytes, three on chicken liver and one each on mature and immature erythrocytes of the goose. In addition, several separate experiments were carried out with chicken erythrocyte and liver nuclei to study the effects of ionic strength on chromatin organization. Except where explicitly stated, the results of repeat experiments were in general accord and have been integrated and described together.

For electron microscopy, at least three replicate samples were prepared and examined during each stage in any given experiment. The micrographs are examples of cells and nuclei which were considered typical of each preparation. In general, the preparations were quite homogeneous and only when variations in the appearance of cells and/or nuclei in a given preparation were considered significant were they reported (e.g., Figs. 30, 31, 45, 46).

I. Analysis of Histone Fractions

Figs. 1 to 7 are examples of starch-gel patterns obtained on electrophoresis of the fractional histone extracts from chicken erythrocytes, chicken liver and

mature as well as immature erythrocytes of geese. The electrophoretic zones are labelled according to the scheme used by Vidali and Neelin (1968). The nature and the composition of histone components in starch-gel electrophoretograms have been reliably determined (Neelin et al., 1964; Purkayastha and Neelin, 1966; Murray et al., 1970), and their chief characteristics are summarized in Table 4. Fraction V is the "serine-rich" histone specific to avian erythrocytes, while other fractions are common to most or all somatic cells. Relative amounts, in terms of acetone precipitates, of the major histones extracted at different stages of fractionation are summarized in Table 5.

In starch-gels, variations in amounts of a particular protein result in differences in both relative mobility and staining intensity of the electrophoretic zone of that fraction (Neelin, 1968). Examples of this are provided by fraction I and V in Figs. 1 and 2. Fraction I moved farthest from the origin and was most intensely stained in the patterns obtained from the citric acid extracts. This fraction was considerably less obvious and moved less distance from the origin in the citric acid washes and in the pH 1.8 extracts, where it was more dilute. In Fig. 2, fraction V moved about the same distance and stained with equal intensity in the citric acid wash and the pH 1.8 extract, indicating that it was present in similar quantities in both cases.

TABLE 4 Characteristics of the major zones in starch-gel electrophoretic patterns of histone extracts

Chromatographic Fraction	Electrophoretic Zone	Amino Acid Content	Arginine-Lysine Ratio
I	11	lysine-rich	0.1
IIb1	2	moderately	0.5
IIb2	4	lysine-rich	
III	13	arginine-rich	1.0
IV	3	arginine-rich	1.0
V	15	serine-rich	0.5
	d	degradation products	

TABLE 5 Summary of histone fractionations in chicken erythrocytes and liver and in goose erythrocytes.

Source of Histone	0.1M Citric Acid (pH 2.0)	Saline-HCl (pH 1.8)	Saline-HCl (pH 1.0)	Remarks
(a) <u>Typical extractions</u>				
% of total histone extracted:	22.5 ± 2.8	12.5 ± 1.8	65.0 ± 4.8	(see Fig. 1) Values are means of four separate experiments (± one standard deviation).
Main fractions present: I, V		I, V (IIb1, IIb2, IV)	IIb1, IIb2, III, IV	()-trace amounts only.
(b) <u>Atypical extractions</u>				
% of total histone extracted:	17.0	35.0	48.0	(see Fig. 2) Estimates from one experiment.
Main fractions present: I, V		I, V IIb1, IIb2, IV	IIb1, IIb2, III, IV	

TABLE 5 Summary of histone fractionations in chicken erythrocytes and liver and in goose erythrocytes. (cont'd)

Source of Histone	0.1M Citric Acid (pH 2.0)	Saline-HCl (pH 1.8)	Saline-HCl (pH 1.0)	Remarks
(c) <u>Chicken Liver</u>				
(see Figs. 4 & 5)				
% of total histone extracted:	63.0	21.0	16.0	Values are means of two separate experiments.
Main fractions present: I		I, (V)	IIb1, IIb2, III, IV, (V)	()-trace amounts only.
(d) <u>Goose Erythrocytes</u> (mature cells)				
(see Fig. 6)				
% of total histone extracted:	22.5	18.5	59.0	Estimates from one experiment.
Main fractions present: I, V		I, V	IIb1, IIb2, IV, (V)	()-trace amounts only
(e) <u>Goose Erythrocytes</u> (immature cells)				
(see Fig. 7)				
% of total histone extracted:	35.0	25.0	40.0	Estimates from one experiment.
Main fractions present: I, V		IIb1, IIb2	IIb1, IIb2, IV	

Figs. 1, 2, and 3 are illustrations of starch-gel patterns obtained upon electrophoresis of histones from chicken erythrocyte nuclei. The pattern in Fig. 1 was most frequently observed and is an example of the sharpest separation of major histone fractions which was attained. In this gel, the 0.1 M citric acid extract contained two main components, fractions I and V. Two weakly staining components (d) were also present at the top of the gel. Fractions I and V were also evident in the citric acid wash, but the former was much reduced in amount while the latter was more prominent. Fraction V was the most prominent component in the pH 1.8 extract. This was preceded by minor amounts of fraction I, barely discernible traces of fractions IIb1-III and the two components (d). Minor traces of most of these fractions could also be distinguished in the pH 1.8 wash. The pH 1.0 extract contained the series of fractions IIb1, IV, IIb2 and the very prominent fraction III. Trace amounts of all these components were also visible in the pH 1.0 wash.

Figs. 2 and 3, illustrate the range of variability encountered in fractionating the various histones of erythrocytes. In the citric acid extracts in Fig. 2, fraction I was the only major component present, with only a trace of fraction V evident. At pH 1.8, in addition to fractions I and V, substantial amounts of fractions IIb1, IIb2 and IV were also extracted. In some cases (Fig. 3), fraction V showed very variable

Figures 1 to 3

Starch-gel electrophoretograms of chicken erythrocyte histones. In each gel the origin is at the bottom and the cathode at the top. All gels were stained with amido black.

Figure 1

Gel pattern showing sharpest separation of major histone components. Fraction I and V are fully extracted at pH 1.8.

Figure 2

Atypical gel pattern showing considerable overlap in extraction of fractions I, V, IIb1, IIb2 and IV at pH 1.8.

Figure 3

Gel pattern showing variable extraction of fraction V, which was still present in pH 1.0 extract.

CATHODE

citric
acid

citric
wash

pH1.8

pH1.8
wash

pH1.0

pH1.0
wash

CATHODE

response and was not completely extracted until pH 1.0. Although electrophoretic patterns shown in Figs. 2 and 3, deviated significantly from the more typical patterns in Fig. 1, they were obtained only once each in separate experiments. The variability exhibited in these starch-gels was reflected to some extent in the relative quantitative values of histone recovered at various stages during the fractionation (Table 5). In typical extractions, as shown in Fig. 1, about 35% of the total acid extractable histone was recovered when pH 1.8 was reached and included essentially only fractions I and V. In the atypical extraction shown in Fig. 2, about 52% of the total histone had been removed at pH 1.8 and included all of fractions I and V as well as significant amounts of fractions IIb1, IIb2 and IV. As will be shown later, this variable overlap in extraction proved advantageous in assessing the relative contribution of various histone components to the structure of erythrocyte chromatin.

The data on fractionation of histones from chicken erythrocytes can be summarized as follows. Citric acid at pH 2.0 yielded an extract which comprised about 22% of the total histone, and included primarily the lysine-rich histone, fraction I, and some serine-rich histone, fraction V. Two fast-moving, weakly staining components (d) judged to be degradation products were also generally present. At pH 1.8, the remaining portions of fractions I and V were usually

extracted and comprised roughly 13% of the total histone. In some cases, however, not all of fraction V was removed at this pH and in other cases, quantities of moderately lysine-rich (IIb1, IIb2) and arginine-rich (IV) histones were also extracted. At pH 1.0, the bulk (65%) of histone components was usually extracted; it comprised fractions IIb1, IIb2 and IV and all of the arginine-rich fraction III.

Starch-gel patterns of histones fractionated from chicken liver are shown in Figs. 4 and 5. The gel in Fig. 4, shows the results of a sequential fractionation, identical to the type carried out with chicken erythrocytes. Starch-gel patterns obtained after parallel extraction of three separate aliquots (a, b and c) of liver nuclei are shown in Fig. 5. Both types of extractions exhibited essentially the same major histone components.

The citric acid extract was characterized by the presence of only one main component, fraction I, and this was present in the accompanying citric acid washes as well. The fast, featureless smears (d) which preceded fraction I, probably represent contaminants and degradation products (Neelin and Butler, 1961). The pH 1.8 extract in Fig. 4, contained the (d) components, a presumed faint remnant of fraction I and, in addition, a slow component near the origin. The latter was probably a trace of fraction V from small quantities of erythrocytes in the preparation

Figures 4 and 5

Starch-gel electrophoretograms of chicken liver histones. In each gel the origin is at the bottom and the cathode at the top. All gels were stained with amido black.

Figure 4

Gel pattern obtained with sequential extraction of histones. Fraction I is present in the citric acid extracts and is sharply separated from other components.

Figure 5

Gel pattern obtained with parallel extraction of three separate aliquots (A, B and C) of liver nuclei. Note absence of fraction V and separation of fraction I into four zones in extract C.

CATHODE

CATHODE

4

5

of liver nuclei. At pH 1.0, in Fig. 4, several distinct components were evident in both the extracts and washes and included fractions IIb1, IIb2, III and IV. The gel pattern yielded by a one-step extraction of all histones at pH 1.0 is shown in Fig. 5 (c). All major histone components were clearly resolved in this pattern and fraction I appeared to consist of a complex of four overlapping zones. Some differences were evident between the electrophoretograms of sequentially fractionated histones (Fig. 4) and histones extracted in parallel (Fig. 5). In the citric acid extracts, the component identified as fraction V in Fig. 4 was absent. In addition, fraction I was more sharply resolved in the one-step extraction of all histones at pH 1.0.

A comparison of quantitative values (Table 5) and the corresponding starch-gel patterns (Fig. 4) of the various extracts, strongly supports the contention that the liver histones were contaminated by non-histone and degradation products. After extraction and washing with citric acid, about 63% of the total acetone precipitable material had been removed. Clearly, this was not solely histone, since only fraction I had been removed at this point as judged by electrophoresis. The contamination in these extracts probably originated with cytoplasmic material which was present in substantial amounts in the preparation of liver nuclei. Consequently, the quantitative

estimates of histone extracted in chicken liver are of limited significance.

Taken overall, however, the qualitative profile of the sequential fractionation of major histone components of liver, were consistent with the results from chicken erythrocytes. Extraction with citric acid yielded essentially only the lysine-rich histone (fraction I), while extraction at pH 1.8 yielded little recognizable protein, indicating the absence of fraction V. As with erythrocytes, the moderately lysine-rich fractions (IIb1 and IIb2) and the arginine-rich fractions (III and IV) were largely removed only at pH 1.0.

The histones of populations of mature and immature goose erythrocytes were examined with the aim of studying cells whose nuclear characteristics might be intermediate between those of liver and erythrocytes. The electrophoretic profiles of all the major histone components in mature goose erythrocytes (Fig. 6) closely matched the profiles found in mature chicken erythrocytes (Figs. 1, 2, and 3). Fraction I was largely extracted by citric acid, with a small amount remaining until treatment at pH 1.8. Most of fraction V was extracted by citric acid and treatment at pH 1.8, but traces remained until pH 1.0 had been reached. As with chicken erythrocytes (Fig. 3), the prominent fractions IIb1, IIb2 and III were only extracted in goose erythrocytes at pH 1.0. The generally faint

Figures 6 and 7

Starch-gel electrophoretograms of goose erythrocyte histones. In all gels the origin is at the bottom and the cathode is at the top. All gels were stained with amido black.

Figure 6

Gel pattern obtained with histones from mature erythrocytes. Note general similarity between this pattern and that obtained with chicken erythrocyte histones. Compare with Figure 1.

Figure 7

Gel pattern obtained with histones from immature erythrocytes. Fractions I and V are completely removed after extraction and washing with citric acid. Contrast with Figures 1 and 2.

CATHODE

6

7

CATHODE

fraction IV, was probably masked by fraction IIb2 at pH 1.0 in the goose since this extract was slightly more concentrated than usual in the gel shown. A comparison of the relative quantities of histone recovered during the fractionation of chicken and goose erythrocytes (Table 5 (a) and (d)) further reflected their similarities. In both cases, citric acid yielded about 22%, and the bulk (65 and 59% respectively) of the total extractable histone was recovered at pH 1.0.

Fig. 7 illustrates the electrophoretogram obtained with histones extracted from a sample of goose erythrocytes enriched in immature cells. The distinguishing aspect of this gel pattern is that fraction I and V were apparently fully removed by citric acid alone. Unlike the situation in mature goose and chicken erythrocytes, fractions I and V were not present in the pH 1.8 or the pH 1.0 extracts from the immature cells. Fractions IIb1 and IIb2 were present in traces at pH 1.8 and were fully extracted along with fraction III at pH 1.0. Fraction IV was again probably masked by fraction IIb2. When the values in Table 5 are considered in relation to their corresponding starch-gel patterns, the differences in histone fractionation between the mature and the immature cell samples are further demonstrated. In the latter sample, the citric acid extract and wash accounted for about 35% of the total histone extracted. This figure is comparable to the combined quantities of histone recovered after

extraction with citric acid and HCl at pH 1.8 from mature erythrocytes of the goose (41%, Table 5(d)) and chickens (35%, Table 5(a)). In all cases, essentially only fractions I and V had been extracted at the points mentioned.

In summary, the histone fractionation data obtained from mature erythrocytes of the goose was both quantitatively and qualitatively similar to that found in mature chicken erythrocytes. In immature goose erythrocytes, the same kinds and relative proportions of histones were found as those present in mature cells. Fractions I and V, however, were apparently fully extracted with greater ease (i.e., less stringent acidic conditions) from the nuclei of immature cells than of mature erythrocytes.

II. Chromatin Structure in Relation to Histone Composition

Except where specifically indicated, all of the micrographs show cells and nuclei fixed in glutaraldehyde, post-fixed in osmium tetroxide and stained with uranyl acetate. This section includes a detailed comparative study of the effects of the selective extraction of major histone fractions on the structure and the organization of chromatin in chicken erythrocyte and liver nuclei followed by a similar study with nuclei of mature and immature erythrocytes of the goose.

A. Histone Extraction and Chromatin Structure in
Chicken Erythrocyte and Liver Nuclei.

(1) Intact Cells

Nuclei and adjacent cytoplasm of an intact mature erythrocyte and a representative hepatic cell are shown in Figs. 8 and 9 respectively. Except for the nucleus, the only organelles found in erythrocytes were occasional mitochondria and vesicle-like structures in the cytoplasm. The cytoplasm typically had a homogeneous texture and was bounded by a single limiting membrane. The structural homogeneity exhibited by the cytoplasm of avian erythrocytes is probably due to the presence of large quantities of hemoglobin (Tooze and Davies, 1963; Schjeide et al., 1964). In contrast to erythrocytes, the cytoplasm of liver cells was structurally varied and rich in organelles (Fig. 9) as previously described (Lentz, 1971). Numerous mitochondria, vesicles and structures tentatively identified as glycogen granules, were scattered throughout the cytoplasm. A complex network of endoplasmic reticulum, lined with ribosomes, occupied much of the cell cytoplasm and usually circumscribed the nucleus.

In erythrocytes, the nucleus was usually separated from the cytoplasm by an electron-transparent gap. This gap, which does not always appear, has been frequently described in nucleated erythrocytes (e.g., Schjeide et al., 1964; Brasch, 1968; MacRae and Meetz,

Figure 8

Intact erythrocyte showing nucleus and adjacent cytoplasm. Presence of hemoglobin causes relatively homogeneous appearance of both nuclear and cytoplasmic contents. Most chromatin is heterochromatic.

Glutaraldehyde-OsO₄ fixation

X 40,000

Figure 9

Intact liver parenchyma cell showing nucleus and adjacent cytoplasm. Nucleus contains mostly euchromatin with some peripheral heterochromatin and prominent nucleolus.

Glutaraldehyde-OsO₄ fixation

X 30,000

Cy = Cytoplasm

Eu = Euchromatin

G = Glycogen

He = Heterochromatin

Ia = Inter-chromatin areas

M = Mitochondria

Nu = Nucleolus

1970) and is probably a preparatory artifact arising from the separation of the two layers of the nuclear membrane (Davies, 1961). The gap was never encountered in liver cells. Nuclear pores, between 800 and 1,000 Å at their widest extent, were visible in both erythrocyte and liver cells. In erythrocytes, the positions of the nuclear pores were emphasized by the gap between the nucleus and the cytoplasm (Fig. 10). Under these conditions, it became evident that the nuclear pores were traversed by electron-dense material which was apparently continuous between the nucleus and cytoplasm.

As with the cytoplasm, the contents of the nuclei of the two cell types were markedly dissimilar. The in situ erythrocyte nucleus was characterized by an overall homogeneity with respect to electron opacity which, as with the cytoplasm, was probably due to the presence of hemoglobin. The bulk of the nuclear volume and nearly the entire periphery were taken up by masses of heterochromatin (Fig. 8). These regions of chromatin were only slightly darker than the inter-chromatin areas, but could be distinguished by their fibro-granular substructure which consisted of densely packed fibrils with cross-sectional diameters between 150 and 250 Å. The inter-chromatin areas, which have been shown to contain hemoglobin (Small and Davies, 1970), were of similar electron-density as the contents of the cytoplasm with which they were apparently joined

Figure 10

Intact erythrocyte showing part of nucleus and cytoplasm.
Note nuclear pores and the ordered layer of chromatin near
the nuclear membrane (arrow).

Glutaraldehyde-OsO₄ fixation

X 100,000

Figure 11

Intact liver cell showing part of nucleus and cytoplasm.
Note nuclear pores and very dense layer of chromatin near
the nuclear membrane (arrow).

Glutaraldehyde-OsO₄ fixation

X 100,000

Eu = Euchromatin

G = Granules

He = Heterochromatin

Np = Nuclear pore

at the sites of nuclear pores. No structure clearly identifiable as a nucleolus could be recognized in these nuclei.

The nuclei of liver cells (Fig. 9) were more heterogeneous in overall appearance than nuclei of erythrocytes. A prominent nucleolus was present and will be fully described later. Regions of heterochromatin, including one or more near the nucleolus, were scattered throughout the nucleus and along most of the nuclear periphery. These regions, which were darker than most other areas of the nucleus, were continuous with an extensive network of euchromatin. The regions of heterochromatin occupied a relatively much smaller area of the nucleus in liver than they did in erythrocytes. The background nucleoplasm (Fig. 11) consisted of a finely textured matrix containing clusters of irregularly shaped granules. As no hemoglobin was present, the contrast between chromatin and background nucleoplasm was much greater in liver.

While the overall appearance of liver and erythrocyte nuclei in situ contrasted, they did have features in common. Both nuclei contained condensed chromatin associated with the nuclear periphery. Chromatin in both nuclei exhibited basic fibrils which were between 150 and 250 Å in diameter. Finally, as shown in detail in Figs. 10 and 11, a layer of an apparently specialized form of chromatin was found in both liver and erythrocyte nuclei in the immediate

proximity of the nuclear membrane. This layer, which looked like an array of basic fibrils in contact with the inner layer of the double nuclear membrane, was between 300 and 400 Å thick and, particularly in liver nuclei, was darker than other chromatin components. In erythrocytes (Fig. 10), indications of a second such layer were often visible immediately within the first.

2. Purified Nuclei

Figs. 12 and 13 are survey views of pellets of purified erythrocyte and liver nuclei respectively. Both types of nuclei looked generally compact and undamaged, and had some contamination by cytoplasmic material. Remnants of membranes were associated with erythrocyte nuclei while some mitochondria and microsomes were present in the preparation of liver nuclei. The isolated nuclei retained the same shapes and outlines which they exhibited in situ. In longitudinal profile, erythrocyte nuclei were ellipsoidal, 2-2.5 μ wide and 3-4 μ long, while liver nuclei always presented circular profiles, 4-5 μ in diameter.

Typical nuclei from both tissues are shown in Figs. 14 and 15. The intrinsic structural differences which existed between erythrocytes and liver nuclei in situ were retained in isolation. The bulk of the chromatin in erythrocyte nuclei was concentrated in large heterochromatic masses, most of which were located near the periphery of the nucleus. In the

Figure 12

Survey view of purified erythrocyte nuclei.

Glutaraldehyde-OsO₄ fixation

X 8,000

Figure 13

Survey view of purified liver nuclei.

Glutaraldehyde-OsO₄ fixation

X 8,000

Figure 14

Erythrocyte nucleus showing heterochromatin with distinct fibrillar substructure in contrast to similar nuclei in situ. Compare with Figure 8.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 15

Liver nucleus containing mainly euchromatin with some heterochromatic areas.

Glutaraldehyde-OsO₄ fixation

X 30,000

Eu = Euchromatin

He = Heterochromatin

Nu = Nucleolus

liver nuclei on the other hand, most of the chromatin was loosely dispersed throughout the nuclear body; although here too, heterochromatin was associated with the nuclear membrane. The structural features which were common to both types of nuclei were also retained in isolation (Figs. 16 and 17). These included basic fibril of 150 to 250 Å diameters and the ordered layer of basic fibrils in the immediate proximity of the nuclear membrane. Nuclear pores and the double nuclear membranes were also retained.

When these nuclei were compared to their respective counterparts in situ (Figs. 8 and 9), several minor differences are evident. During nuclear isolation, hemoglobin was removed from erythrocytes and soluble components from liver. As a result, the nucleoplasms were cleared of electron-dense material and chromatin stood out in sharper contrast than before. In erythrocyte nuclei particularly, the heterochromatin areas were clearly resolved into compact masses of basic fibrils interconnected by a tenuous network of diffuse chromatin fibrils. A similar change had taken place in liver nuclei, although the effect was relatively less pronounced since the chromatin in these nuclei was not obscured by hemoglobin in situ. An important aspect in both erythrocyte and liver nuclei, however, was that despite the loss of soluble nucleoplasmic constituents, the process of nuclear isolation had not brought about any drastic changes in the overall ar-

Figure 16

Portion of erythrocyte nucleus showing intermeshed chromatin fibrils about 200 Å in diameter. Note ordered layer of fibrils near nuclear membrane (arrows).

Glutaraldehyde-OsO₄ fixation

X 100,000

Figure 17

Portion of a purified liver nucleus showing intermeshed chromatin fibrils about 200 Å in diameter. Note ordered layer of fibrils near nuclear membrane (arrows).

Glutaraldehyde-OsO₄ fixation

X 100,000

rangement and distribution of chromatin and other nuclear components. This included the nucleolus in liver, which was virtually unchanged in appearance during purification of the nuclei (Fig. 15).

3. Nuclei after Washing in 0.01M Citric Acid (pH 2.6)

Figs. 18, 19, 20 and 21 show a series of erythrocyte nuclei and liver nuclei after they had been exposed to 0.01 M citric acid. This preliminary low pH wash does not remove histone proteins from the chromatin. The nuclei shown in Figs. 18 and 19 were washed and fixed in 0.01 M citric acid at pH 2.6. A marked change in the appearance of chromatin of both nuclei resulted from this treatment. In erythrocyte nuclei (Fig. 18), the chromatin had a uniformly electron-dense aspect and was virtually devoid of the fibrillar substructure characteristic of the initial purified nuclei (Fig. 14). The chromatin had assumed a gel-like texture, in which all of the fibrils, including the dense layer at the nuclear membrane, appeared to have merged with the rest of the chromatin.

The appearance of liver nuclei (Fig. 19) was also markedly changed from that of the initial nuclei (Fig. 15), following treatment with 0.01 M citric acid (pH 2.6). Portions of the chromatin, particularly the heterochromatic regions, near the nuclear periphery had assumed a smooth texture similar to that seen in the erythrocyte nuclei

Figure 18

Erythrocyte nucleus washed and fixed in 0.01 M citric acid-saline (pH 2.6). Note homogeneous appearance of all chromatin and complete lack of fibrillar substructure.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 19

Liver nucleus washed and fixed in 0.01 M citric acid-saline (pH 2.6). Note homogeneous appearance of peripheral chromatin, with some fibrillar structure in center of nucleus.

Glutaraldehyde-OsO₄ fixation

X 30,000

(Fig. 18). The central portions of the nucleus retained a network of dispersed chromatin fibrils and granular components. This network, however, had become reduced in extent and looked coarser than that found in the initial nuclei. The dark band of chromatin associated with the nuclear membrane could no longer be differentiated from the rest of the chromatin. The nucleolus had become lighter and more skeletonized than it was initially.

Despite the fine structural changes, however, the secondary organization of chromatin was retained in both erythrocyte and liver nuclei. This was especially obvious in erythrocyte nuclei where the masses of heterochromatin were distributed in a pattern very similar to that found in the initial purified nuclei. In addition, nuclear pores and the nuclear membranes were visible in most nuclei.

The nuclei in Figs. 20 and 21, are examples of erythrocyte and liver nuclei which had been resuspended and fixed in saline-buffer (pH 6.8) after washing in 0.01 M citric acid (pH 2.6). When these nuclei were compared to their respective counterparts in Figs. 18 and 19 and in Figs. 14 and 15, it was found that the structural effects brought about in 0.01 M citric acid were largely reversible. The overall appearance of the nuclei shown in Figs. 20 and 21 was similar to that of the initial nuclei in Figs. 14 and 15. Most of the chromatin in both erythrocyte and liver nuclei

Figure 20

Erythrocyte nucleus suspended and fixed in saline-buffer (pH 6.8) after washing in 0.01 M citric acid (pH 2.6). Note partial reappearance of fibrillar substructure in chromatin.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 21

Liver nucleus suspended and fixed in saline-buffer (pH 6.8) after washing in 0.01 M citric acid (pH 2.6). Note partial reappearance of fibrillar substructure in chromatin.

Glutaraldehyde-OsO₄ fixation

X 30,000

again exhibited fibrillar substructure and areas of condensed and dispersed chromatin could be clearly distinguished. The dark bands of chromatin at the nuclear membrane were again evident and, in liver nuclei, the components of the fibro-granular network and a darker nucleolus were visible.

4. Nuclei after Extraction and Washing with 0.1 M citric Acid (pH 2.0).

Erythrocyte and liver nuclei which had been extracted and washed in 0.1 M citric acid are shown in Figs. 22 to 31. At this stage in the fractionation sequence, essentially all of histone fraction I had been removed from liver (see Figs. 4 and 5). In erythrocytes, most of fraction I and about half of the total complement of fraction V, the histone specific to erythrocytes, was extracted (see Figs. 1 and 2).

Erythrocyte and liver nuclei which had been extracted and fixed in 0.1 M citric acid are shown in Figs. 22 and 23. These nuclei looked similar to their respective counterparts in Figs. 18 and 19, which had been washed and fixed in 0.01 M citric acid. The chromatin of the erythrocyte nuclei (Fig. 22) was homogeneously compacted and exhibited no discernible substructure. In the liver nuclei (Fig. 23), the heterochromatic regions had the same compacted appearance as erythrocyte chromatin. The euchromatic areas retained some fibrillar composition, but were coarsely

Figure 22

Erythrocyte nucleus after extraction in 0.1 M citric acid (pH 2.0) and fixation in same medium. Chromatin is congealed as in nucleus shown in Figure 18 and no substructure is visible.

Glutaraldehyde-OsO₄ fixation

X 40,000

Figure 23

Liver nucleus after extraction in 0.1 M citric acid (pH 2.0) and fixation in same medium. Heterochromatin is congealed but euchromatin shows some fibrillar structure.

Glutaraldehyde-OsO₄ fixation

X 100,000

clumped and somewhat reduced in extent.

Most strikingly, when erythrocyte nuclei extracted with 0.1 M citric acid were returned to saline-buffer (pH 6.8) prior to fixation, the chromatin retained the same clumped aspect which it had when fixed at low pH (Fig. 24). Only smooth, featureless masses of chromatin were evident. In contrast, liver nuclei returned to saline-buffer at this point looked as shown in Fig. 25. The chromatin was still relatively dense and compacted but it now had a mottled appearance with fine, granular substructure visible throughout. These liver nuclei differed in appearance from both the unextracted initial nuclei (Fig. 15) and from the nuclei which had been fixed at low pH either in 0.01 M or 0.1 M citric acid (Figs. 19 and 23).

No additional major changes in the appearance of erythrocyte and liver nuclei took place after they had been subjected to a further washing in 0.1 M citric acid. When suspended and fixed in 0.1 M citric acid, the nuclei from both tissues looked as they did in Figs. 22 and 23, showing generally featureless and gel-like chromatin, and when returned to and fixed in saline-buffer the nuclei appeared as shown in Figs. 26, 27, 28 and 29. In all erythrocyte nuclei the chromatin at this point was uniformly electron-dense. Even at high magnification (Fig. 28), no substructure was visible in the gel-like texture of the chromatin. In liver nuclei, which had been treated and prepared

Figure 24

Erythrocyte nuclei after extraction in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-buffer (pH 6.8). Note that chromatin has remained completely congelated as in Figures 18 and 22 and that no fibrillar substructure has returned as with nucleus shown in Figure 20.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 25

Liver nucleus after extraction in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-biffer (pH 6.8). Note that some substructure is visible in chromatin in contrast to similarly treated erythrocyte nuclei (Figure 24, above) and liver nucleus in Figure 23.

Glutaraldehyde-OsO₄ fixation

X 25,000

Figure 26

Erythrocyte nucleus after extraction and washing in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-buffer (pH 6.8). Chromatin remains homogeneously electron-dense.

Glutaraldehyde- OsO_4 fixation

X 45,000

Figure 27

Liver nucleus after extraction and washing in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-buffer (pH 6.8). This nucleus is very similar in appearance to nucleus in Figure 25.

Glutaraldehyde- OsO_4 fixation

X 25,000

Figure 28

Erythrocyte nucleus after extraction and washing in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-buffer (pH 6.8). Note complete absence of substructure in congealed chromatin.

Glutaraldehyde-OsO₄ fixation

X 100,000

Figure 29

Liver nucleus after extraction and washing in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-buffer (pH 6.8). Note fine substructure in chromatin showing units 20 to 100 Å in thickness. Contrast with similarly treated erythrocyte nucleus (Figure 28, above).

Glutaraldehyde-OsO₄ fixation

X 100,000

in identical manner however, a fibrous substructure was visible in all regions of the chromatin (Fig. 27). This detail was poorly defined (Fig. 29) but consisted of interlocked units that were between 20 and 100 Å in diameter.

The erythrocyte nuclei in Figs. 30 and 31 were returned to saline-buffer (pH 6.8) after extraction with 0.1 M citric acid and then fixed directly in osmium tetroxide buffered at pH 7.0. Nuclei prepared in this manner retained their compact appearance, however, the chromatin areas now had a reticulate aspect and fine interlocked fibrils were visible with diameters of 50 to 100 Å. This marked the only instance in the fractionation of erythrocyte nuclei where a significant difference was evident in the fine structure of chromatin that could be related to the use of a specific fixative.

In distinction to the fine-structural changes induced by extraction with 0.1 M citric acid, this treatment caused very little alteration in the gross-organization of chromatin in either liver or erythrocyte nuclei. The overall distribution of chromatin in the extracted nuclei was similar to that in situ, although in liver the pattern of euchromatin was no longer so extensive. The dense band of chromatin adjacent to the nuclear membrane was clearly visible in liver nuclei (Fig. 29) while nuclear membranes and nuclear pores were evident in both types of nuclei

Figure 30

Erythrocyte nucleus after extraction and washing in 0.1 M citric acid (pH 2.0) and resuspension with fixation in saline-buffer (pH 6.8). Note compact chromatin with very fine substructure. Contrast with Figure 26.

Palade's OsO_4 fixation

X 45,000

Figure 31

Same nucleus as above showing detail in chromatin with intermeshed fibrillar units 50 to 100 $\overset{\circ}{\text{A}}$ in diameter.

Palade's OsO_4 fixation

X 100,000

(Figs. 28 and 29). Remnants of a nucleolus remained clearly visible in liver nuclei (Figs. 25 and 27) and continued to show association with heterochromatin.

5. Nuclei after Extraction and Washing at pH 1.8

All erythrocyte and liver nuclei, when extracted and fixed in saline at pH 1.8, exhibited homogeneous chromatin identical to that previously described and shown in Figs. 22 and 23. In addition, when erythrocyte nuclei were returned to saline-buffer (pH 6.8) after extraction at pH 1.8 in the fractionation sequence characterized by the starch-gel in Fig. 3, the chromatin was also dense and compact like that found after exposure to 0.1 M citric acid (Figs. 22, 24 and 26). In this case, extraction at pH 1.8 removed all of histone fraction I but only a portion of fraction V.

The nuclei illustrated in Figs. 32 to 37 had been returned to and fixed in saline-buffer after exposure to pH 1.8. The erythrocyte nuclei shown in Figs. 32 and 33 are typical examples obtained after a pH 1.8 extraction sequence like the one shown in Fig. 1, where both fractions I and V were completely removed from the nuclei while all but traces of other histones remained. The general appearance of these nuclei differed markedly from that of erythrocyte nuclei in the preceeding stages. A distinct loosening of the homogeneous, gel-like texture of the chromatin found after extraction with citric acid had taken place and

Figure 32

Erythrocyte nucleus after extraction at pH 1.8 and resuspension in saline-buffer (pH 6.8). Distinct fibrillar substructure is visible in contrast to nucleus in Figure 26. Overall distribution of chromatin remains similar to that found in initial nuclei (Figure 14).

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 33

Erythrocyte nucleus from same preparation as above, showing detail in chromatin with 20 to 100 Å thick fibrils.

Glutaraldehyde-OsO₄ fixation

X 100,000

a definite substructure was now evident. The main chromatin areas contained a network of apparently interlocked fibrils, whose diameters ranged from 20 to 100 Å (Fig. 33). Scattered amid this network were a number of amorphous centers of condensation. These centers, which were roughly circular in outline, were between 500 and 1,500 Å in diameter and were always interconnected by the fibrillar network. The frequency and the distribution of these centers within different nuclei was highly irregular.

Figs. 34 and 36 illustrate typical erythrocyte nuclei obtained after extraction and washing at pH 1.8 in the histone fractionation shown by the starch-gel in Fig. 2. In this case, in addition to fractions I and V, significant quantities of fractions IIb1, IIb2 and IV had also been extracted. The nuclei shown in Figs. 34 and 36 exhibited chromatin structure that was less compact but basically similar to that found in Figs. 32 and 33. The interlocked network of 20 to 100 Å thick fibrils was more clearly revealed in these nuclei and the centers of condensation appeared more distinct against a less opaque background.

Typical liver nuclei following extraction and washing at pH 1.8 are shown in Figs. 35 and 37. From the corresponding starch-gel shown in Fig. 4, it can be seen that all of histone fraction I was removed from such nuclei and, of course, fraction V was not present initially. The heterochromatin areas in these

Figure 34

Erythrocyte nucleus after extraction at pH 1.8 and resuspension with fixation in saline-buffer (pH 6.8). Chromatin is similar but generally less electron-dense than in nuclei shown in Figures 32 and 33.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 35

Liver nucleus after extraction at pH 1.8 and resuspension with fixation in saline-buffer (pH 6.8). Chromatin is looser than at preceding stage (Figure 27) and generally similar to above erythrocyte chromatin.

Glutaraldehyde-OsO₄ fixation

X 25,000

Figure 36

Erythrocyte nucleus after extraction at pH 1.8 and resuspension with fixation in saline-buffer (pH 6.8). Nucleus from similar preparation as nucleus in Figure 34, showing dense centers and 20 to 100 $\overset{\circ}{\text{\AA}}$ fibrils.

Glutaraldehyde- OsO_4 fixation

X 160,000

Figure 37

Liver nucleus after extraction at pH 1.8 and resuspension with fixation in saline-buffer (pH 6.8). Nucleus from similar preparation as nucleus in Figure 35, showing detail of chromatin structure.

Glutaraldehyde- OsO_4 fixation

X 160,000

nuclei were distinctly looser than at the preceeding stage following extraction with 0.1 M citric acid (Figs. 27 and 29), and were very similar in appearance to the chromatin in corresponding erythrocyte nuclei (compare Figs. 36 and 37). The liver chromatin consisted of a fibrillar network and electron-dense centers of concentration. The fibrils were indistinct and measured between 30 and 100 Å in diameter while the dense centers were relatively smaller in liver than in erythrocyte nuclei (150 to 500 Å vs 500 to 1,500 Å).

Although the fine-structure of chromatin was markedly changed after extraction at pH 1.8, the general distribution and organization of chromatin in both liver and erythrocyte nuclei remained very similar to that in comparable nuclei in situ. Occasionally (Fig. 34), the internal limits of the heterochromatic areas appeared outlined by a relatively dark band, similar in width and in density to the band located adjacent to the nuclear membrane. In both nuclei, the nuclear membrane was present but fuzzy and in liver, remnants of the nucleolus were still visible.

6. Nuclei after Extraction and Washing at pH 1.0

The fine structure of chromatin of both erythrocyte and liver nuclei extracted and fixed at pH 1.0, was again compact and generally featureless, similar to that shown in Figs. 22 and 23. The nuclei

shown in Figs. 38 to 41 had all been extracted and washed at pH 1.0 and then returned to and fixed in saline-buffer (pH 6.8). At this stage, all histone proteins had been extracted. Although both types of nuclei were frequently distorted in shape, they were still generally intact.

The detailed appearance of the chromatin in the two types of nuclei was remarkably similar (Figs. 40 and 41). Amorphous dense centers were found in both nuclei, but were relatively larger in erythrocytes. These centers were distributed like islands amid networks of fine fibrils. The fibrils seemed randomly interconnected and while their diameters varied, particularly in erythrocyte nuclei, the thinnest were between 20 and 30 Å thick. The dense centers appeared to have an irregular fibrous substructure and their cores were often relatively darker than the rather diffuse peripheral regions which seemed to merge at some points with the peripheral regions of adjacent dense centers.

The gross-distribution of chromatin was less compact than it had been at earlier stages and the total amount of stainable material present in the nuclei appeared substantially reduced. Nevertheless, the overall pattern of distribution of the major chromatin regions was clearly retained in both types of nuclei. The nuclear membrane was often broken but usually recognizable at the nuclear periphery and

Figure 38

Erythrocyte nucleus after total histone extraction at pH 1.0, followed by resuspension and fixation in saline-buffer (pH 6.8). Note general similarity in chromatin distribution to that in initial nuclei (Figure 14).

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 39

Liver nucleus after total histone extraction at pH 1.0, followed by resuspension and fixation in saline-buffer (pH 6.8). Note general similarity in chromatin distribution to that in initial nuclei (Figure 15).

Glutaraldehyde-OsO₄ fixation

X 25,000

Figure 40

Erythrocyte nucleus after total histone extraction at pH 1.0, followed by resuspension and fixation in saline-buffer (pH 6.8). Detailed view of chromatin with dense centers and 20 to 30 Å thick fibrils.

Glutaraldehyde-OsO₄ fixation

X 160,000

Figure 41

Liver nucleus after total histone extraction at pH 1.0, followed by resuspension and fixation in saline-buffer (pH 6.8). Detailed view of chromatin with dense centers and 20 to 30 Å thick fibrils.

Glutaraldehyde-OsO₄ fixation

X 160,000

occasional nuclear pores were visible. The remnants of the chromatin near the nuclear membrane still formed a dark band, which was frequently in contact with the rest of the chromatin. In liver nuclei (Fig. 39), most of the euchromatic material seemed to have disappeared or become part of the heterochromatin regions. A distinct, though skeletonized nucleolus was retained in liver nuclei.

7. Structural Changes in Nucleoli During Histone Extraction in Liver Nuclei

The general appearance of nucleoli in freshly isolated nuclei did not differ appreciably from the appearance of chicken liver nucleoli in situ (compare Figs. 15 and 76 with Fig. 9). Three structural components were visible in favourably transected nucleoli; one or more clumps of heterochromatin associated with each nucleolus and sometimes extending into the nucleolar body, a distinctly granular zone, and a relatively diffuse fibrillar zone. The granular zone, which occupied about half the nucleolar area, contained granules which were roughly circular and about 250 Å in diameter. The fibrillar zone was relatively more electron-dense than the rest of the nucleolus and was ramified into spaces not occupied by the granular zone. Although no clear substructure was seen in the fibrillar zone, it appeared to be finely textured and not completely

homogeneous. Minor portions of the nucleolus were apparently empty and had an electron-density similar to that of the surrounding nucleoplasm from which soluble components were washed away during nuclear isolation.

Chicken liver nucleoli were affected structurally during the sequential extraction of histone proteins from isolated nuclei. The observations which follow are of limited value only, since they could not be related to extraction of specific macromolecules from the nucleolus.

The major change in the structure of liver nucleoli took place after the nuclei had been simply exposed to 0.01 M citric acid (pH 2.6). A typical nucleolus treated in this manner is shown in Fig. 19. The nucleolus shown was surrounded by clumped chromatin and exhibited only fine granular structure. The fibrillar zone, found in initial nucleoli in saline (Figs. 15 and 76), was absent or at least greatly reduced in relative density. The general appearance of nucleoli which had been exposed to pH 2.6 remained as shown in Fig. 19 in nuclei which were returned to pH 6.8.

During subsequent exposure of nuclei to 0.1 M citric acid (Figs. 23, 25), pH 1.8 (Fig. 35) and pH 1.0 (Fig. 39), the nucleoli became generally tighter and relatively more electron-dense than they appeared in Fig. 19. Apart from this, however, the structure of nucleoli was not appreciably altered by acid treat-

ment. Following exposure to pH 1.0, the remnants of the fibrillar zone were similar in appearance to the chromatin surrounding the nucleolus, and consisted of a tenuous network of interlocked fibrillar components. Distinct granular zones were retained by nucleoli during all stages of acid treatment, and contained granular components with irregular shapes and diameters ranging from 100 to 250 Å.

B. Histone Extraction and Chromatin Structure in Mature and Immature Erythrocytes of the Goose

1. Structural Development of Goose Erythrocytes

Figs. 42 to 46 illustrate the range of cell types found in a sample of goose blood from anemic birds. The relative numbers of various cell types present both before and after the sample had been enriched in immature cells by differential centrifugation, are recorded in Table 6. The cell types were classified as described by Lucas and Jamroz (1961). The data show that before enrichment, the blood sample was composed of about 15% of cells which were less mature than reticulocytes (R). The relative content of these immature cells was roughly doubled by subsequent differential centrifugation. In the enriched sample, over 8% of the population consisted of very young cells of the basophilic erythroblast type (LBE and SBE), 20% was identified as polychromatic erythroblasts (BE)

TABLE 6 Relative numbers of cell types found in anemic goose blood. Counts were performed on smear preparations which had been fixed in neutral formalin and stained with May-Grünwald-Giemsa.*

Cell Types	Relative Numbers of Cells(%) (Pooled from three birds)	
	Circulating Blood (before centrifugation)	Enriched Blood (after centrifugation)
LBE	0.7	1.4
SBE	1.7	7.0
PE	9.0	19.7
OE	2.8	4.8
R+E	81.3	54.9
G	0.3	0.7
L	0.7	0.2
Nuclei	3.5	10.3

Abbreviations:

LBE = large basophilic erythroblast

SBE = small " "

PE = polychromatic "

OE = orthochromatic "

R = reticulocyte

E = erythrocyte (mature cell)

G = granulocyte

L = lymphocyte

* Courtesy of Dr. G. Adams.

and about 5% was orthochromatic erythroblasts (OE). The nearly mature reticulocytes (R) and the fully mature erythrocytes (E) were very similar in appearance and were therefore counted together, forming about 55% of the total population. About 10% of the total count was made up of unidentified nuclei, which probably resulted from cell breakdown due to the abnormal physiological condition of the birds.

The cells shown in Figs. 42 and 43 have been tentatively identified as large and small basophilic erythroblasts respectively. These cells were characterized by the following morphological features, as described previously by Tooze and Davies (1967) and by MacRae and Meetz (1970). Both were rich in cytoplasmic organelles, including mitochondria and assorted membrane-bound vesicles or vacuoles. Centrioles were occasionally visible (Fig. 43) and the cytoplasm showed pseudopodal projections. All areas of the cytoplasm not occupied by other organelles were packed with ribosomes. The nuclei were irregular in outline and occupied a large proportion of the total cell volume, particularly in the LBE. One or two rather ill-defined nucleoli were usually present. The heterochromatin was very electron-dense and was generally confined to relatively small areas near the nuclear periphery and the nucleoli. Euchromatin extended from heterochromatin areas throughout most of the nuclear area, but was always poorly defined against a relatively electron-dense nucleoplasm.

Figure 42

Large basophilic erythroblast (LBE). Note large nucleus, relative paucity of heterochromatin and abundance of cytoplasmic ribosomes.

Glutaraldehyde- OsO_4 fixation

X 17,000

Figure 43

Small basophilic erythroblast (SBE). Note abundance of cytoplasmic organelles, vesicles and ribosomes and presence of prominent nucleolus.

Glutaraldehyde- OsO_4 fixation

X 18,000

Eu = Euchromatin

He = Heterochromatin

Nu = Nucleolus

Polychromatic erythroblasts (PE) (Fig. 44) were present in relatively large numbers and were readily found in E.M. preparations. When these cells are compared to the basophilic erythroblasts described above, several developmental changes are apparent. The cell shape had become ovoid and the number of mitochondria and cytoplasmic vesicles was reduced. Most striking, however, was the change in number and distribution of ribosomes as compared to the cells shown in Figs. 42 and 43. In the PE, ribosomes were grouped into distinct polysomal clusters and showed a sharp reduction in overall number per unit area of cytoplasm. The nuclei in the PE had also changed considerably and tended to show circular rather than irregular profiles. The relative amount of heterochromatin was increased sharply and most was associated with the nuclear periphery. A concomitant reduction in the relative extent of euchromatin was indicated; although this may have been due in part to the increased electron density of the nucleoplasm, which matched that of the cytoplasm.

The cells shown in Figs. 45 and 46 have been tentatively identified as reticulocytes. Cells of this type were most numerous in the samples examined, and therefore probably constitute the bulk of cells marked R + E in Table 6. The cells shown in Figs. 45 and 46 probably represent cross and longitudinal sections respectively of red blood cells approaching maturity. Only a few mitochondria and cytoplasmic

Figure 44

Polychromatic erythroblast (PE). Note relative increase in heterochromatin and reduction in number of cytoplasmic organelles and especially ribosomes. Compare with Figures 42 and 43.

Glutaraldehyde-OsO₄ fixation

X 20,000

Figure 45

Reticulocyte (R). Chromatin is mainly heterochromatic and nucleoli are inconspicuous or absent.

Glutaraldehyde-OsO₄ fixation

X 30,000

Figure 46

Reticulocyte (R). Most chromatin is heterochromatic and only scattered polysomes remain in cytoplasm. This cell is very nearly mature.

Glutaraldehyde- OsO_4 fixation

X 28,000

Figure 47

Survey view of nuclei from blood enriched in immature cells, showing compact heterochromatic nuclei and larger nucleus with mainly dispersed chromatin.

Glutaraldehyde- OsO_4 fixation

X 10,000

vesicles remained in these cells, and ribosomes were confined to a few scattered polysomes. Nucleolar remnants were only occasionally recognizable in the very electron-dense nuclei. Chromatin was highly condensed and occupied a large part of the nuclear volume. Scattered elements of extended chromatin were still visible and the background nucleoplasm had a density very similar to that of the cytoplasm. These cells were very similar in appearance to the mature erythrocyte of the chicken illustrated in Fig. 8.

2. Purified Nuclei

Figs. 47, 48 and 49 illustrate the types of nuclei which were isolated from the sample of immature blood cells. Fig. 47 is a survey view through the pellet of initial purified nuclei; numerous mitochondria, membrane remnants and microsome contaminants were present. The nuclei were generally compact and undamaged and distinctly heterogenous in appearance. The illustrations in Figs. 48 and 49 represent the two extremes of structural differentiation that were found. The majority of the nuclei present were ovoid in shape and rich in heterochromatin (Fig. 48). Apart from the frequent presence of a poorly defined nucleolus, these nuclei looked remarkably like the nuclei purified from mature chicken erythrocytes (Fig. 14). Most of the chromatin was dense and compact and associated with

Figure 48

Nucleus classified as "mature", with heavily condensed chromatin similar to mature erythrocyte nucleus shown in Figure 14.

Glutaraldehyde-OsO₄ fixation

X 40,000

Figure 49

Nucleus classified as "immature", containing mainly dispersed chromatin similar to liver nucleus shown in Figure 15.

Glutaraldehyde-OsO₄ fixation

X 30,000

the nuclear membrane. A prominent dark band was visible around the entire nuclear periphery. Basic chromatin fibrils measured between 150 and 250 Å in cross-section. These are probably nuclei from polychromatic erythroblasts or reticulocytes (see Figs. 44 and 45) and for a lack of a more specific designation, will be referred to as mature nuclei. A number of nuclei of the type shown in Fig. 49 were also present. These nuclei were much less frequent than the mature nuclei but were clearly distinguished from them. Typically, these nuclei were relatively large and generally contained more euchromatin than heterochromatin. One or more prominent nucleoli were usually present, along with various granular components. In many respects, these nuclei looked very similar to the nuclei isolated from chicken liver (Fig. 15). They were designated as immature nuclei since they probably originated with basophilic erythroblasts (see Figs. 42 and 43).

3. Nuclei after Extraction and Washing in 0.1 M Citric Acid (pH 2.0)

All the nuclei illustrated in this section were returned to and fixed in saline-buffer (pH 6.8) after they had been exposed to acid conditions. Parallel samples of nuclei fixed at the pH of the respective extraction media, looked identical to comparable chicken erythrocyte and liver nuclei which were des-

cribed earlier, i.e., the chromatin was homogeneously clumped and lacked visible substructure.

Figs. 50 and 52 show mature nuclei following the initial extraction in 0.1 M citric acid. These nuclei were very compact and electron-dense and appeared essentially identical to chicken erythrocyte nuclei after similar treatment (compare with Fig. 24). No clear substructure was visible in the heterochromatic masses, which had a homogeneous texture that extended to the inner nuclear membrane (Fig. 52). The general distribution of chromatin areas however, remained similar to that of the initial nuclei (Fig. 48).

Figs. 51 and 53 illustrate a presumed immature nucleus after extraction in 0.1 M citric acid. This nucleus was judged immature because nuclei like it were found infrequently. The chromatin in these nuclei was quite dense. However, in contrast to the mature nucleus in Fig. 52, the chromatin was not gel-like, but rather showed fine fibrillar substructure. Moreover, clumped remnants of euchromatin extended into the interchromatin regions at many points. These nuclei were similar in overall appearance to chicken liver nuclei after extraction in 0.1 M citric acid (compare with Fig. 25).

Figs. 54 and 56 show mature nuclei fixed after both extraction and subsequent washing in 0.1 M citric acid. At this stage in the extraction of histone proteins from the sample of immature goose erythrocytes

Figure 50

Mature nucleus after extraction in 0.1 M citric acid. Chromatin is congealed and similar to that in mature nucleus shown in Figure 24.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 51

Immature nucleus after extraction in 0.1 M citric acid. Note its similarity to liver nucleus in Figure 25. The chromatin is relatively dense but not homogeneous like that in mature nucleus above (Figure 50).

Glutaraldehyde-OsO₄ fixation

X 30,000

Figure 52

Mature nucleus after extraction in 0.1 M citric acid, showing complete absence of substructure in homogeneous chromatin. Compare with Figure 28.

Glutaraldehyde-OsO₄ fixation

X 100,000

Figure 53

Immature nucleus after extraction in 0.1 M citric acid showing ill-defined substructure in chromatin, in contrast to nucleus shown above (Figure 52).

Glutaraldehyde-OsO₄ fixation

X 100,000

Figure 54

Mature nucleus after extraction and washing in 0.1 M citric acid. Note diffuse substructure in chromatin in contrast to nucleus at preceeding stage (Figure 50). Compare also with Figure 32.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 55

Immature nucleus after extraction and washing in 0.1 M citric acid. Chromatin is very similar in appearance to that shown above (Figure 54) and in liver nuclei shown in Figure 27.

Glutaraldehyde-OsO₄ fixation

X 30,000

Figure 56

Mature nucleus after extraction and washing in 0.1 M citric acid, showing detail in chromatin with irregular fibrils approximately 100 $\overset{\circ}{\text{A}}$ thick.

Glutaraldehyde- OsO_4 fixation

X 100,000

Figure 57

Immature nucleus after extraction and washing in 0.1 M citric acid, detail in chromatin fine-structure similar to above (Figure 56).

Glutaraldehyde- OsO_4 fixation

X 100,000

(starch-gel in Fig. 7) all of fractions I and V had been extracted. When compared to nuclei which had received only a single citric acid extraction (Figs. 50 and 52), they exhibited significant change. The dense heterochromatic areas had loosened somewhat and diffuse substructure was visible. The latter consisted of an irregular fibrous mass with units less than 100 Å in diameter. A dark band of chromatin was visible near the nuclear membrane and, except for the nucleolar remnants and some extended chromatin, these nuclei looked similar to chicken erythrocyte nuclei after extraction at pH 1.8 and liver nuclei after extraction at pH 2.0 (compare with Figs. 32 and 27).

Figs. 55 and 57 show presumed immature nuclei after extraction and washing in 0.1 M citric acid. The chromatin in these nuclei was somewhat looser than that in its counterpart in Figs. 51 and 53. In general appearance, however, these two nuclei were very similar.

4. Nuclei after Extraction and Washing at pH 1.8

Mature and immature nuclei after extraction and washing at pH 1.8 are shown in Figs. 58 and 59. The starch-gel pattern in Fig. 7 indicates that at this point in the extraction sequence of histones, in addition to fractions I and V substantial amounts of fractions IIb1 and IIb2 had been removed. Once again

Figure 58

Mature nucleus after extraction and washing at pH 1.8, showing fine fibrillar substructure and chromatin distribution similar to nucleus shown in Figure 34.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 59

Immature nucleus after extraction and washing at pH 1.8, showing fine fibrillar substructure and chromatin distribution similar to above nucleus (Figure 58).

Glutaraldehyde-OsO₄ fixation

X 30,000

the nuclei were classified as mature and immature respectively mainly on the basis of their relative content of heterochromatin. The chromatin in both nuclei looked quite similar, showing fine fibrillar substructure and much looser texture than found in similar nuclei after citric acid extraction (Figs. 54 and 55). The nuclei shown in Figs. 58 and 59 were not unlike in overall appearance to comparable chicken erythrocyte nuclei from which the same histone fractions had been extracted (Figs. 34 and 2).

5. Nuclei after Extraction and Washing at pH 1.0

The examples shown in Figs. 60 and 61 are typical nuclei found after total histone extraction at pH 1.0. At this time, it became very difficult to distinguish mature from immature nuclei with any degree of certainty, because in most cases the size of the nuclei and the distribution of chromatin within them were distorted. Fig. 60 is probably a mature nucleus since most of its volume is occupied by chromatin, and the nucleus in Fig. 61 may be immature because a relatively larger proportion of its volume is free of chromatin. Although somewhat ⁵mishapen, these nuclei were generally intact and retained most of their nuclear membranes, nuclear pores and occasionally nucleoli were recognizable. The chromatin regions were relatively empty of total stainable material but were essentially the same as the chromatin in comparable chicken erythro-

Figure 60

Mature nucleus after extraction and washing at pH 1.0.
Compare with Figure 38.

Glutaraldehyde-OsO₄ fixation

X 45,000

Figure 61

Immature nucleus after extraction and washing at pH 1.0.
Compare with Figure 39 and above Figure 60.

Glutaraldehyde-OsO₄ fixation

X 30,000

cyte and liver nuclei (compare with Figs. 38 and 39). As in chicken erythrocytes and liver nuclei, the chromatin at this stage consisted mainly of a network of 20-30 Å thick fibrils which interconnected amorphous centers of condensation. The general peripheral distribution of heterochromatin was retained and the perimeters of chromatin areas were circumscribed by a dark border.

III. Ionic Strength and Nuclear Structure

A. Effects of Variable Salt Concentration on the Structure of Chromatin in Chicken Erythrocyte and Liver Nuclei

1. Chicken Erythrocyte Nuclei

Figs. 62 to 67 illustrate isolated erythrocyte nuclei as they appeared initially in saline, suspended in distilled water and resuspended in saline. The nuclei in Figs. 62 and 63 were typical examples of freshly isolated nuclei as described in detail previously (see Figs. 12, 14 and 16).

Figs. 64 and 65 show intact nuclei the same as those in Figs. 62 and 63, which, however, had been removed from saline and suspended and fixed in water. A marked change in both the gross-organization and the fine structure of chromatin resulted from this treatment. The condensed masses of chromatin became completely

Figures 62 and 63

Erythrocyte nuclei freshly isolated in saline, showing
200 Å thick fibrils and compact heterochromatin areas.

Glutaraldehyde- OsO_4 fixation

X 45,000

X 100,000

Figures 64 and 65

Erythrocyte nuclei isolated in saline and then suspended and fixed in water. Condensed chromatin is dispersed and fibril diameters are reduced to about 75 Å. Note attachment of many fibrils to nuclear membrane.

Glutaraldehyde-OsO₄ fixation

X 35,000

X 100,000

dispersed and basic fibrils were uniformly distributed within the nuclei. The basic fibrils were sharply reduced in average diameter to 50-100 Å (Fig. 65) and showed radial orientation near the periphery of the nucleus where many fibrils were attached to the nuclear membrane. The chromatin as a whole was less electron-dense than it appeared in Figs. 62 and 63. The nuclear membrane was relatively more distinct than before, although the double nature was not so clear, and was often partially ruptured in some places (Fig. 64). Nuclear pores were usually extended to a width of 2,000-3,000 Å from an original 500 to 1,500 Å.

When water-treated nuclei were returned to saline they looked as shown in Figs. 66 and 67. Both the fine structure and the gross-distribution of chromatin returned essentially to the condition found in the nuclei initially suspended in saline (Figs. 62 and 63). Chromatin was again condensed into mainly peripheral, large masses and distinct basic fibrils with cross-sectional diameters of 150-250 Å reappeared. The prominent dark band of chromatin was again evident along portions of the nuclear perimeter, but the nuclear membrane and nuclear pores were generally distorted.

Typical interference-contrast micrographs of unfixed individual erythrocyte nuclei before, during and after water treatment are shown in Fig. 68. In the nuclei which had been freshly prepared in saline the chromatin showed complex internal structure. Sev-

Figures 66 and 67

Erythrocyte nuclei suspended and fixed in saline after suspension in water. Chromatin is recondensed into distinct areas and 200 Å thick fibrils have reappeared. Compare with initial nuclei in Figures 62 and 63.

Glutaraldehyde-OsO₄ fixation

X 45,000

X 100,000

Figure 68

Interference-contrast micrographs showing individual erythrocyte nuclei. Each horizontal series of three micrographs represents the same nucleus : in saline (left), in water (center), and resuspended in saline (right).

X 6,500

eral condensed masses of chromatin were evident in each nucleus, forming an irregular but distinct pattern. The nuclei swelled rapidly when exposed to water, but usually did not rupture. When swelling had reached its maximum extent, all evidence of internal structure was gone and the nuclei appeared smooth and largely featureless. On re-exposure to saline solution, the nuclei shrank rapidly in volume and their chromatin recondensed. Most interestingly, the recondensed chromatin showed a general pattern which was nearly identical to that present before water treatment. Occasionally, the recondensed pattern was not as distinct as the original pattern, although its general outline remained the same (top three illustrations, Fig. 68). In some cases, when the nuclei were ruptured during the swelling process, the original structural configuration could not be recognized when the chromatin was recondensed in saline.

2. Chicken Liver Nuclei

Figs. 69 to 74 are electron micrographs of chicken liver nuclei as they appeared in saline, water and recondensed in saline. The nuclei in Figs. 69 and 70 are typical liver nuclei in saline, which have been described in detail previously (see Figs. 15 and 17).

Liver nuclei after suspension and fixing in water are shown in Figs. 71 and 72. As in similarly

Figures 69 and 70

Liver nuclei freshly isolated in saline, showing 200 Å⁰
thick fibrils and mainly dispersed chromatin.

Glutaraldehyde-OsO₄ fixation

X 30,000

X 100,000

Figures 71 and 72

Liver nuclei isolated in saline and then suspended and fixed in water. All chromatin is dispersed and fibril diameters are reduced to about 75 Å. Note attachment of many fibrils to nuclear membrane.

Glutaraldehyde-OsO₄ fixation

X 18,000

X 100,000

treated erythrocyte nuclei (compare with Figs. 64 and 65), exposure to water brought about a marked change in both the gross organization and fine structure of chromatin. The chromatin lost all aspects of compartmentalization and became evenly distributed throughout the nucleus. Basic fibrils were reduced in diameter to 50-100 Å and often radiated from the nuclear membrane toward the nuclear center. The nuclear membrane was relatively more distinct than previously, while the chromatin as a whole was less electron-dense than in nuclei in saline. The nuclear membrane was often ruptured, but its double nature was retained. Nuclear pores were evident, although they were usually extended to 2,000-3,000 Å from their original width of 500-1,000 Å. As in the nuclei suspended in saline, clusters of granular components were scattered throughout the nucleus, though they were more prominent now against a relatively light background of chromatin. In these water treated nuclei it was possible to see that the granular components were clearly continuous with the chromatin fibrils.

When the water-treated nuclei were returned to saline (Figs. 73 and 74), structure very similar to that of the initial nuclei in saline was re-established (Figs. 69 and 70). Basic fibrils exhibited diameters of 150 to 250 Å and the chromatin as a whole appeared dense and compact. Areas of heterochromatin and extended chromatin were present and were distributed in

Figures 73 and 74

Liver nuclei suspended and fixed in saline after suspension in water. Heterochromatin is recondensed and 200 Å thick fibrils have reappeared. Compare with initial nuclei in Figures 69 and 70.

Glutaraldehyde-OsO₄ fixation

X 30,000

X 100,000

apparently normal patterns. A prominent dark band was evident at the nuclear membrane.

Fig. 75 shows light micrographs of individual unfixed liver nuclei as they appeared in saline, water and resuspended in saline again. The internal structure of the initial nuclei in saline consisted of a complex of condensed and extended chromatin and nucleoli. These structural components formed a recognizable heterogeneous pattern, although, as expected from electron microscopy, such patterns were less distinct than in erythrocyte nuclei. When the nuclei were exposed to water, they swelled perceptably and all internal structure disappeared. The smooth featureless nuclei that remained were similar in appearance to the water-suspended erythrocyte nuclei (Fig. 68). When liver nuclei were returned to saline, they quickly shrank in volume and often became more compact than they had been originally. At the same time, their internal components recondensed into a pattern of dispersed and condensed areas that was similar to the pattern seen before nuclear swelling. Liver nuclei did not recondense into their original patterns with quite the same degree of precision as found with erythrocyte nuclei. As with erythrocyte nuclei, if liver nuclei were partially ruptured during exposure to water, the original pattern did not reappear on return to saline.

Figure 75

Interference-contrast micrographs showing individual liver nuclei. Each horizontal series of three micrographs represents the same nucleus : in saline (left), in water (center), and resuspended in saline (right).

X 6,500

B. The Effects of Variable Salt Concentration on the Structure of Nucleoli in Chicken Liver

Nucleoli in isolated chicken liver nuclei suspended in saline, in water and resuspended in saline are shown in Figs. 76, 77 and 78. The appearance of nucleoli in nuclei isolated in saline have been described in detail previously. A marked change in the appearance of the nucleoli took place following the exposure to water (Fig. 77). Although the nucleolus did not appear to have swollen appreciably, it was greatly reduced in overall density. The most striking change, however, was the apparent absence of the entire fibrillar zone. Only granular components with irregular shapes occupied the bulk of the nucleolus at this point. The distribution of granules was also altered in water-treated nucleoli, being grouped homogeneously throughout the nucleolar body. The chromatin that was associated with the nucleolus was greatly reduced in density, though fibrils of 50 to 100 Å diameters were in contact with the entire nucleolar perimeter. These nucleoli looked somewhat similar to the nucleoli in liver nuclei which had been exposed to low pH during histone fractionation (e.g., Figs. 19, 25 and 35).

When liver nuclei were recondensed in saline after their exposure to water, the nucleoli looked indistinguishable in general appearance from the initial nucleoli in saline (compare Figs. 76 and 78). Granular

Figure 76

Liver nucleolus in saline showing two types of zones and associated chromatin.

Glutaraldehyde-OsO₄ fixation

X 55,000

Figure 77

Liver nucleolus in nucleus suspended and fixed in water. Note apparent absence of fibrillar zone but persistence of granular components.

Glutaraldehyde-OsO₄ fixation

X 55,000

Figure 78

Liver nucleolus in nucleus resuspended in saline after exposure to water. The nucleolus is apparently normal in appearance and similar to the nucleolus in Figure 76.

Glutaraldehyde-OsO₄ fixation

X 55,000

fz = fibrillar zone

gz = granular zone

and fibrillar zones were clearly visible in the recondensed nucleoli, and the two zones exhibited the same relative densities as they did before exposure to water. The recondensed chromatin was in contact with the nucleoli at several points.

IV. Ionic Strength and Chromatin Structure During Histone Extraction in Chicken Erythrocyte Nuclei

In an effort to study the relative contribution of the various histone fractions to the structural response of nuclei to solutions of low ionic strength, erythrocyte nuclei were passed through the sequential histone fractionation used above. At each stage of extraction, nuclei were subjected to low ionic strength and subsequent return to saline.

1. Nuclei after Extraction and Washing in 0.1 M Citric Acid (pH 2.0)

In erythrocyte nuclei which had been extracted and washed in 0.1 M citric acid, the chromatin retained the tight gel-like texture described previously in Figs. 24, 26 and 28, even when the nuclei had been returned to pH 6.8 in saline-buffer before fixation. Fig. 79 illustrates such a nucleus which was suspended and stirred overnight in water at pH 6.8, following 0.1 M citric acid and return to pH 6.8. In distinct contrast to intact nuclei, these nuclei retained their

Figure 79

Erythrocyte nucleus after extraction in 0.1 M citric acid, followed by suspension and fixation in water at pH 6.8. The chromatin is dense and congealed (Compare with Figures 24 and 28).

Glutaraldehyde-OsO₄ fixation

X 40,000

Figure 80

Erythrocyte nucleus after extraction in 0.1 M citric acid, followed by suspension and fixation in water with 0.5 M EDTA. Note diffuse substructure and intermeshed fibrils.

Glutaraldehyde-OsO₄ fixation

X 100,000

compact homogeneous appearance in water and no substructure was visible in the gel-like chromatin.

When similar nuclei were dispersed and stirred overnight in a water solution containing 0.5 M EDTA (pH 7.0), (Fig. 80), the chromatin lost its gel-like texture and intermeshed fibrils, 20 to 100 Å thick, were visible in all areas. The gross-distribution of chromatin within the nuclei was also partially disrupted as the major heterochromatin areas seemed to merge with each other at their borders. The nuclear membrane was indistinct and partially ruptured in some nuclei. The chromatin loosening in EDTA is the maximum we were able to obtain with nuclei extracted in 0.1 M citric acid.

2. Nuclei after Extraction and Washing at pH 1.8 and pH 1.0

The nuclei shown in Figs. 81 and 82 were suspended and fixed in saline-buffer (pH 6.8) after having been extracted in saline at pH 1.8 and pH 1.0 respectively. Similar nuclei were described in detail previously (Figs. 34 and 38).

When nuclei from the same preparation as shown in Fig. 81 were suspended and fixed in water at pH 6.8, they appeared as in Figs. 83 and 84. These nuclei were swollen and the chromatin areas were enlarged and dispersed throughout most of the nuclear body. The dense centers of chromatin found in similar nuclei in

Figure 81

Erythrocyte nucleus after extraction at pH 1.8, fixed in saline-buffer (pH 6.8). Compare to similar nucleus in Figure 34.

Glutaraldehyde-OsO₄ fixation

X 40,000

Figure 82

Erythrocyte nucleus after extraction at pH 1.0, fixed in saline-buffer (pH 6.8). Compare to similar nucleus in Figure 38.

Glutaraldehyde-OsO₄ fixation

X 40,000

Figures 83 and 84

Erythrocyte nuclei from the same sample as those shown in Figure 81, but suspended and fixed in water at pH 6.8. Chromatin is broadly dispersed showing fibrils 20 to 100 Å thick.

Glutaraldehyde- OsO_4 fixation

X 30,000

X 100,000

similar nuclei in saline (Fig. 81) had largely disappeared and the chromatin consisted mainly of fine fibrils with diameters from 20 to 100 Å. Although frequently ruptured, the nuclear membrane and its adjacent chromatin were relatively dense. Resuspension and fixation in saline-buffer (Figs. 85 and 86) brought about a partial return to the condition of the nucleus shown in Fig. 81. The nuclei were again relatively compact and their chromatin retained tightly packed fibrils, 20 to 100 Å in diameter. The gross-distribution of chromatin was not as compartementalized as it had been before exposure to water (Fig. 81), but some areas of the recondensed nuclei were free of chromatin.

When nuclei such as shown in Fig. 82, from which all histone proteins had been extracted, were suspended and fixed in water, they appeared as in Figs. 87 and 88. Water-treated nuclei at this stage in histone extraction were swollen and usually partially ruptured. The chromatin was very light, consisting almost exclusively of 20 to 30 Å thick fibrils which were apparently randomly interconnected. Many areas of chromatin were largely free of stainable material and formed empty holes, and the nuclear membrane was folded in many places. The nuclei in Figs. 89 and 90 were returned to and fixed in saline-buffer following exposure to water. Although the recondensed nuclei had irregular shapes and were always partially ruptured, they retained some structural integrity. The chromatin had

Figures 85 and 86

Erythrocyte nuclei like those in Figures 84 and 85, but resuspended and fixed in saline-buffer (pH 6.8). Chromatin is again relatively compact. Compare with Figure 81.

Glutaraldehyde-OsO₄ fixation

X 40,000

X 100,000

Figures 87 and 88

Erythrocyte nuclei from the same sample as those shown in Figure 82, but suspended and fixed in water at pH 6.8. Chromatin is dispersed, showing 20 to 30 $\text{\AA}$ thick fibrils.

Glutaraldehyde- OsO_4 fixation

X 30,000

X 100,000

Figures 89 and 90

Erythrocyte nuclei like those in Figures 87 and 88, but resuspended and fixed in saline-buffer (pH 6.8). Chromatin is very distorted but relatively compact. Compare with Figure 82.

Glutaraldehyde- OsO_4 fixation

X 40,000

X 100,000

an overall density matching that found in similar nuclei before swelling in water (Fig. 82). On the whole, the chromatin had a heterogeneous appearance and contained regions where 20 to 30 Å thick fibrils were loosely interlocked and other areas where the fibrils were packed into dense centers (Fig. 90). Remnants of poorly defined nuclear membrane were evident in some nuclei and no clear pattern of chromatin organization was evident in these nuclei at the E.M. level.

3. Light Microscope Observations of Individual Nuclei

Examinations of individual unfixed nuclei under the light microscope support the E.M. results reported above. The top three illustrations in Fig. 91, show individual, citric acid extracted nuclei as they appeared at pH 6.8 in saline, water and saline again. The nuclei remained compact and their internal structure did not change during exposure to water and subsequent re-exposure to saline.

The three middle illustrations in Fig. 91 show a pair of nuclei, after extraction at pH 1.8 and return to saline-buffer, in saline, water and returned to saline. Nuclei in similar conditions after extraction at pH 1.0, are shown in the bottom three illustrations. In both cases, the internal structure of the initial nuclei in saline formed a recognizable pattern. Compared

Figure 91

Interference-contrast micrographs showing individual erythrocyte nuclei. Each horizontal series of three micrographs represents the same nucleus : in saline (left), in water (center), and resuspended in saline (right).

Top three illustrations - after citric acid extraction.
Middle three illustrations - after extraction at pH 1.8.
Bottom three illustrations - after extraction at pH 1.0.

X 6,500

to similar nuclei before histone extraction (Fig. 75), however, these nuclei looked swollen and their chromatin was less distinct. When exposed to water, the nuclei swell and their internal structure became very indistinct. Nevertheless, when the nuclei were re-exposed to saline, they shrank again, and the original configuration of the internal structure reappeared.

DISCUSSION AND CONCLUSIONS

I. Histone Extraction

An important prerequisite to the present study was selective extraction of major histone components from isolated liver and erythrocyte nuclei. Citric acid has been used to selectively remove lysine-rich components from isolated nuclei (Littau et al., 1965) and histones have been fractionally extracted from nuclei by stepwise titration with HCl (Murray, 1966). The combined use of these two methods gave the following typical results. Citric acid dissociated fraction I almost completely from both liver and erythrocyte nuclei. Additionally, in the latter nuclei, about half fraction V was extracted at the same time; this fraction is, of course, not present in liver nuclei. Subsequent titration to pH 1.8 extracted fraction I fully from liver nuclei, and fraction I plus usually all of fraction V from erythrocyte nuclei. Fraction V was sometimes not fully extracted until pH 1.0. The remaining histone components were completely removed from both nuclei at pH 1.0.

These results are in general agreement with those of other workers. Littau et al., (1965) and Mirsky et al., (1968) found that 0.1 M citric acid extracted lysine-rich histones with relatively high

selectivity from calf thymus nuclei and HeLa cell chromosomes. Similarly, with chicken erythrocyte nuclei, Murray et al., (1968), Vidali and Neelin (1968) and Seligy and Neelin (1970) showed that extraction and washing with 0.1 M citric acid yielded most of fraction I and appreciable quantities of fraction V; titration to pH 1.8 extracted all of fractions I and V and titration to pH 1.0 yielded fractions IIb1, IIb2, III and IV.

The fractional extraction procedure provided the following samples of liver and erythrocyte nuclei for structural analysis: nuclei from both tissues with all histones present; liver nuclei with fraction I absent; erythrocyte nuclei with fraction I absent but with substantial amounts of fraction V remaining; erythrocyte nuclei with both fraction I and V absent; nuclei from both tissues with histones extracted. Due to variation in extraction, erythrocyte nuclei were also available in which fractions I and V and substantial amounts of fractions IIb, III were removed. These samples permitted some rational assessment of the contribution made to chromatin structure by various histone components. Broad comparison was possible between the structural roles of relatively loosely bound components (fractions I and V) and those histones which are tightly complexed with DNA. Additionally, some insight was provided on the possible function of the tissue-specific component

(fraction V) present in erythrocytes, although some difficulty was encountered because this fraction was always partially co-extracted with fraction I. Finally, some analysis of the relative importance of histones and the other DNP constituents in determining organization within the nucleus was possible.

An interesting variation in histone fractionation was encountered with nuclei from goose blood enriched in immature erythrocytes, where both fractions I and V were completely extracted by 0.1 M citric acid. Since fraction V was not fully extracted from mature erythrocytes until pH 1.8 was reached, it appears that this component was less tightly bound to DNP in the nuclei of immature cells. Work by Purkayastha and Neelin (1966) as well as Mazen and Champagne (1969) indicates that during very early stages of erythropoiesis, fraction V may be absent or present in reduced amounts and only later reaches the amount found in mature cells. Conceivably, when histone V is first synthesized in young cells it does not interact with the DNP complex in the same manner as in mature cells. As the cells develop, the DNP complex may be altered in as yet undetermined ways such that fraction V binds to DNP more strongly. The change in the binding of this histone component is probably correlated with the loss of RNA synthetic activity (Adams et al., 1971) and the progressive condensation of chromatin during red cell maturation. In mature cells

tightly bound fraction V is involved in gene repression (Seligy and Neelin, 1970). There is evidence indicating that histone V is extensively phosphorylated only in immature erythrocytes (Seligy and Neelin, 1971) and that as these cells mature, the levels of phosphoprotein kinase fall off several fold (Gershey and Kleinschmidt, 1969). Change in the binding of histone V to DNP may, therefore, come about through dephosphorylation and this process could be an important mechanism in the maturation of the highly specialized erythrocyte nuclei.

II. Primary Structure of Chromatin

The average diameter and general appearance of basic chromatin fibrils in situ was similar in all cells studied, i.e., mature and immature erythrocytes and liver parenchyma. The fibril diameters showed a normal distribution around a mean of 200 Å and there was no evidence for multiple size classes. Isolation and subsequent washing of nuclei in saline removed material from the background nucleoplasm, thereby increasing visibility of the fibrils. Such treatment, however, did not appreciably alter the diameter or general appearance of the fibrils, indicating that soluble components of the nucleoplasm other than salts (e.g., soluble proteins) are probably not essential for maintaining the structure of basic fibrils.

The size and overall aspect of basic fibrils described here are very similar to those reported by some other workers. In nucleated erythrocytes (Davies, 1961; Tooze and Davies, 1967; Zentgraf et al., 1970) and in hepatic cells (Mentre, 1968; Monneron and Bernhard, 1969), the reported thickness of chromatin fibrils in situ and in isolated nuclei ranges between 150 and 250 Å. These values are similar to those commonly reported for chromatin fibrils in various organisms and tissues (Hyde, 1965; DuPraw, 1968; 1970) and indicate that a mean diameter of 200 Å may be a universal figure.

The present investigation provides some insight into the substructure of chromatin fibrils. The observation that the average diameter of the fibrils changed reversibly from about 200 Å to about 75 Å in response to variations in the ionic strength of the suspension medium is consistent with an interpretation that the native chromatin fibril consists of a single linear component which is supercoiled to give a basic fibril of about 200 Å width (Bastia and Swaminathan, 1967; Bahr, 1970; DuPraw, 1970). Careful examination of micrographs of nuclei both dispersed in water and subsequently recondensed in saline gave no evidence for fraying or aggregation of multiple parallel fine strands which might have composed the 200 Å native fibril (Ris, 1961).

The ability to undergo such a marked, reversible change in size while retaining all macromolecular constituents indicates that chromatin fibrils possess a

degree of flexibility which permits them to withstand considerable physical distortion without permanent damage. This characteristic can be explained by comparing basic fibrils to a coiled spring, whose helical structure is maintained by a flexible spiral element (Richards and Pardon, 1970). In most profiles of erythrocyte and liver nuclei in situ and freshly isolated in saline, an ordered array of basic fibrils is located in close proximity to the nuclear membrane. Similar arrays, reported in the nuclei of many cell types, have been shown to consist of tightly packed fibrils with hollow cores which resemble microtubules when viewed at the appropriate angle in thin sections (Davies, 1967; 1968). This may constitute direct evidence, as suggested by DuPraw (1968), that 200 Å basic chromatin fibrils, i.e. B fibrils, are formed of a supercoiled DNP unit, the A fibril, which is less than 100 Å in diameter.

The molecular organization of chromatin fibrils remains obscure. Evidence presented here indicates that the integrity of the 200 Å B fibril, as visualized in sections of fixed material, requires a full complement of organized histones. B fibrils were not visible in nuclei fixed in 0.01 M citric acid (pH 2.6) where no significant amount of histone protein was extracted. The congealed aspect of chromatin in these nuclei probably resulted from neutralization of phosphate groups in the DNP complex. Presumably at pH 2.6 insufficient charge repulsion remains to keep individual

fibrils apart and condensation results. Recognizable B fibrils reappeared when the nuclei washed at pH 2.6 were returned to neutral pH for fixation. The continuing presence of B fibrils could, therefore, be demonstrated as long as no histone proteins were extracted. However, the original appearance of the native basic fibrils was not fully restored even at this point. Apparently exposure to only moderately low pH caused some irreversible molecular rearrangement which prevented complete reformation of discrete B fibrils. This suggests that the molecular organization underlying B fibrils is complex and dependent in part on components which are quite sensitive to pH change.

After only partial extraction of histone fractions I and/or V, chromatin became permanently altered and 200 Å thick fibrils were no longer observable, even with fixation at neutrality. In erythrocyte nuclei it is conceivable but unlikely that B fibrils were present in the congealed chromatin present after extraction by 0.1 M citric acid. The chromatin in these nuclei could not be dispersed by return to neutral pH or exposure to hypotonic solutions. The only way in which substructure was revealed in this congealed erythrocyte chromatin without further acid extraction was by osmium tetroxide fixation or EDTA treatment. The structure revealed by these treatments, however, was not typical of native chromatin with B fibrils. Only fine, tightly interwoven fibrils about 100 Å in diameter were visible.

Presumably in these nuclei partial removal of fraction I leads to some undetermined rearrangement within the fibrils probably involving fraction V (see below), such that the chromatin becomes tightly interlocked. In liver nuclei, where fraction V is absent, extraction of fraction I did not induce irreversible clumping, but even in these nuclei removal of fraction I caused irreversible change in the appearance of the fibrils; the largest units visible were only about 100 Å thick. Moreover, in erythrocytes after complete removal of fractions I and V, basic fibrils were drastically changed in appearance and the fibril diameters ranged from 20 to 100 Å. These results argue strongly that the easily dissociated histones, fractions I and V, probably play an important role in the maintenance of B fibril integrity.

Other workers have presented data from spectroscopic analysis and X-ray diffraction which indicate that neither fraction I, nor fraction V in nucleated erythrocytes, are primarily responsible for the conformational characteristics of DNA in isolated nucleohistone (Tuan and Bonner, 1969; Murray et al., 1970). X-ray studies on thymus nucleohistone revealed that selective dissociation of fraction I did not appreciably alter the diffraction pattern attributed to the supercoiled structure, while progressive removal of other histones did abolish the pattern (Richards and Pardon, 1970). These observations appear difficult to reconcile with the E.M. observations

here reported, since presumably, loss of B fibril integrity and supercoiled structure are related phenomena.

A solution to this discrepancy may be provided by a recent re-evaluation of the configuration of DNA in chromatin fibrils. In the model of the basic fibril originally proposed by DuPraw (1968), DNA was supercoiled only once as shown schematically in Fig. 92a. In this version, the A fibril contains a single DNA helix which is sheathed by tightly bound histones, while loosely bound histones (fractions I and/or V) may serve as bridges between adjacent gyres of the supercoil. Selective removal of loosely bound histones presumably would lead to breakup of the B fibril resulting in loss of supercoiled structure and a change in DNA conformation. This model appears too simple, however. Recent analysis of the total dry mass per unit length of chromatin fibril, led Bahr (1970) and DuPraw (1970) to the conclusion that more than one order of coiling is required to accomodate the known amounts of DNA in a fibril about 200 Å in diameter. These authors, therefore, revised their original model and proposed a coiled supercoil as shown schematically in Fig. 92b. In this version of basic fibril structure DNA assumes a supercoiled conformation in the A fibril, which is then secondarily coiled to form the B fibril. Presumably, the A fibril consists of DNA complexed with tightly bound histones. Loosely bound histones may act as links between adjacent loops

a - first order coil

b - second order (coiled) coil

Figure 92 Proposed models of chromatin fibril structure. Model b is adapted with modifications from DuPraw (1970).

in the B fibril, so that when they are selectively removed, or even disturbed, B fibril structure is lost. DNA would still, however, remain supercoiled in the smaller A fibril. Only when tightly bound histones are extracted is the conformation of the A fibril drastically altered.

The revised model of basic fibril structure can accomodate the E.M. findings reported in this work, and the X-ray diffraction data on the role of histones in fibril structure (Murray et al., 1970; Richards and Pardon, 1970) if it is assumed that the latter data applies primarily to an A fibril containing a supercoil while the E.M. results apply to a B fibril which contains another order of coiling. The nucleohistone fibres used for X-ray diffraction studies were obtained from material prepared as a dilute gel in distilled water (Pardon et al., 1967; Richards and Pardon, 1970). The findings in the present work, and other E.M. (Crawley and Harris, 1963; Tokuyasu et al., 1968), indicate that in water the diameter of basic fibrils is reduced from about 200 Å to 100 Å and less. Presumably in the absence of ions, B fibrils unwind to leave the supercoiled A fibrils core. It is noteworthy that measurements of nucleohistone fibril size obtained from X-ray diffraction indicate that the DNA supercoil is maintained in a fibril about 100 Å in diameter (Pardon et al., 1967; Pardon and Richards, 1970). This is close to the estimated size of the A fibril (DuPraw, 1970), while the basic chromatin

fibril (the B fibril) as visualized in the E.M. is about 200 Å in diameter.

The foregoing interpretation of basic fibril structure is consistent with the sequential decrease in fibril widths found with progressive extraction of histones in liver and erythrocyte nuclei. Presumably as suggested by others (Bastia and Swaminathan, 1967; DuPraw, 1968) the basic fibrils unwind gradually as proteins are removed. After extraction of fractions I and/or V, the largest units visible in both liver and erythrocyte chromatin were between 60 and 100 Å thick. If these units represent more or less intact A fibrils, then this observation suggests strongly that lysine-rich histones are involved in maintaining the secondary coiling of the A fibril which gives the B fibril. These histones seem well suited to function in this capacity, since they adopt extended chain conformation in solution and can probably attach to several adjacent loops of a DNP supercoil. The reversible unwinding of B fibrils in response to ionic strength would require transient changes in the nature of the binding of these proteins in the DNP complex.

As previously shown by specific staining and DNase treatment (Brasch, 1968), the 20-30 Å fibrillar elements remaining in nuclei after complete histone removal consist mainly of individual DNA strands. The 60 to 100 Å thick units (the A fibrils) found after removal of fractions I and V, therefore, are probably composed

of DNA complexed with moderately lysine-rich and arginine-rich histones. By virtue of their relatively high content of α -helical structure, these histone components may achieve maximum bonding with DNA phosphate groups (Bradbury et al., 1967). The tightly bound histones presumably impose restraints on DNA orientation and direct formation of the primary supercoil within the A fibril.

Histone fraction V must be largely responsible for the tight interlocking of chromatin in mature erythrocyte nuclei after partial extraction of fraction I, since only the complete removal of this fraction led to distinct loosening of the gel-like chromatin. Interlocking may be facilitated by charge neutralization at low pH, which tends to bring chromatin fibrils in intimate contact. Recent studies on histone-DNA interactions (Li and Bonner, 1971) imply that all major histone fractions tend to interact with DNA in a biphasic manner, whereby the more basic end of a histone molecule is more tightly complexed with DNA than the less basic end. Possibly, during selective histone extraction, only the weakly-bound terminals of some histone molecules dissociate from DNP and subsequently, by interacting with suitable binding sites on neighbouring DNP strands, effectively cross-link chromatin. In this respect, fraction V may be especially effective since it tends to form extended chains in solution (Wilhelm et al., 1970) and contains the highest number of basic

amino acids found in any major histone in fowl erythrocytes (Murray et al., 1970). To some extent, the homogeneous texture of congealed chromatin was probably exaggerated by fixation with glutaraldehyde, since vague fine structure was visible in similar nuclei fixed in osmium tetroxide. The latter fixative tends to disperse chromatin somewhat (Baker, 1965), probably because it does not cross-link proteins in the same manner as aldehyde fixatives (Sabatini et al., 1963).

III. Secondary Structure of Chromatin

Most eukaryotic nuclei show internal structure dependent on the relative amounts and distribution of euchromatin and heterochromatin. In cytological terms, such chromatin organization may be species-specific, tissue-specific within an organism and/or cell-specific within a tissue. Species specificity of chromatin organization is well illustrated by the so-called prochromosomal nuclei characteristic of certain plant species (Lafontaine, 1968). Tissue and cell specificity of chromatin distribution are illustrated in the present work by the differences between liver and erythrocyte nuclei and mature and immature erythrocytes, respectively. It has not been determined definitely, however, whether organizational differences, like the disparity in the relative content of heterochromatin and euchromatin between liver and erythrocyte nuclei, reflect more or

less random quantitative variations in chromatin condensation or whether they represent differences in highly ordered tertiary structure of the chromatin. Several observations in the present study bear on this important question.

When individual liver and erythrocyte nuclei were exposed to water, their complex internal structure was drastically altered and the chromatin became dispersed throughout the nucleus. These changes were almost completely reversed when the nuclei were returned to saline. Previous reports have shown that exposure of isolated nuclei to hypotonic salt solutions induces swelling and dispersal of chromatin and that these changes are reversible (Ris and Mirsky, 1949; Anderson and Wilbur, 1952). Specifically, however, the observations here presented show that within the limits of light microscopy, the chromatin recondensed into a pattern which was identical to that present before exposure to water. Because of the much more extensive heterochromatin in erythrocyte nuclei the precision of recondensation was more obvious than in liver nuclei, but the same general observation was true for both types. It appears then, that each interphase nucleus has reproducible internal structure and individual masses of chromatin are rigidly positioned with respect to each other. As a corollary, the condensation of chromatin into discrete masses is probably not a form of random aggregation but rather a strictly ordered process.

In addition to the response of individual nuclei to ions, the general tissue-specific appearance and distribution of chromatin in liver and erythrocyte nuclei in situ was retained during isolation and repeated washing in saline. Specific secondary organization of chromatin is therefore probably not determined to any appreciable extent by soluble nucleoplasmic components apart from salts. Furthermore, since in the same ionic environment the intrinsic structural differences between the nuclei remained, specific structural organization in nuclei must be determined primarily by factors inherent in their macromolecular composition. Ion interactions with nuclei may lead to alterations of structure in vivo (Lezzi, 1967; Robbins et al., 1970), but such effects probably bring about only secondary variations on the inherent organization.

Specificity in interphasic structure may be a general phenomenon common to all eukaryotic nuclei. As early as 1888, Boveri noticed that prior to cell division in Ascaris, the chromosomes always reappeared in the same relative position where they disappeared after the preceeding division (DuPraw, 1970). Comings (1968) has summarized other evidence in support of ordered interphase structure and proposes that chromatin is not casually arranged during interphase, but that the apparently jumbled mass of fibrils visible in the nucleus possesses specific tertiary and quaternary structure which probably results from specific packing and folding of chromatin.

The observations here presented provide strong support for this position. It would be of interest to know whether such specificity in chromatin folding and packing is maintained to the point where internal structure is identical in all nuclei of a homogeneous population of cells, as for example mature erythrocytes of a given avian species. To test this possibility would require a means whereby several individual nuclei could be oriented along precisely the same co-ordinates and the structure then analyzed.

In combination with specific folding of chromatin, the nuclear membrane probably plays a major role in the structural organization of the nucleus. This was evident in most profiles of erythrocyte and liver nuclei, where heterochromatin showed preferential association with the nuclear membrane. The association between chromatin and the nuclear membrane was particularly evident in nuclei whose chromatin had been dispersed in water, where chromatin fibrils radiated from the nuclear membrane to the center of the nucleus. Furthermore, an intact nuclear membrane was essential for specific recondensation of the dispersed chromatin on return to saline.

Several authors have recently discussed the intimate association between the nuclear membrane and heterochromatin (Comings, 1968; Davies, 1968; Tokuyasu et al., 1968). The specific attachment of individual chromatin fibrils to portions of the nuclear membrane

has been stressed (DuPraw, 1968; Comings and Okada, 1970b), and recently evidence has been provided indicating that remnants of the nuclear envelope may adhere to specific points on individual chromosomes during cell division (Comings, and Okada, 1970a). Perhaps all chromatin in the nucleus is ultimately anchored to the nuclear membrane through the specialized layer of chromatin visible along the perimeter in most profiles of liver and erythrocyte nuclei. It might be argued, however, that the nuclear membrane is merely responsible for chromatin organization to the extent that it serves as a cytoplasmic barrier against which the chromatin is pressed passively. There is evidence suggesting that the nuclear membrane may control the onset of DNA synthesis (Comings, 1968; Hearst and Botchan, 1970) and act as a focal point of chromosome condensation during prophase (Comings and Okada, 1970a; Stubblefield and Wray, 1971). These observations, make it unlikely that the nuclear envelope has a purely passive role in chromatin organization. They suggest rather, that this membrane may partially co-ordinate both structural and metabolic characteristics of chromatin. The degree of specificity in the interaction of chromatin and the nuclear membrane remains to be established with certainty, however.

The role of nucleoproteins in the secondary organization and distribution of interphase chromatin is largely unknown. Progressive histone extraction

markedly affected the density of packing and fine-structure of chromatin fibrils but the overall arrangement of heterochromatin characteristic of the different nuclei in situ remained remarkably unchanged, even after most histones had been removed. During most stages of the fractional extraction of histones it was relatively easy to differentiate liver nuclei from mature erythrocyte nuclei and immature from mature erythrocyte nuclei by their respective patterns of heterochromatin distribution. Removal of fraction I had almost no visible effect on the heterochromatin distribution in either liver or erythrocyte nuclei. Removal of both fraction I and V from erythrocytes also had very little effect. Some disorganization in pattern was evident after partial extraction of fractions IIb-III, while substantial distortion occurred after all histones were extracted. These observations suggest that tightly bound histone components probably influence the secondary organization more than relatively loosely bound fractions.

Littau et al., (1965) and Mirsky et al., (1968), reported that the selective extraction of lysine-rich histones by 0.1 M citric acid from isolated calf thymus nuclei and HeLa chromosomes, caused dispersal of chromatin and concluded that these proteins are responsible for chromatin condensation. This assertion appears to be an oversimplification. In the present study, liver nuclei are most comparable with calf thymus nuclei, in that both possess the same general classes of histone

proteins. Selective removal of fraction I by citric acid from liver nuclei did not bring about a dispersal of condensed chromatin masses. The fine structure of basic fibrils was affected by removal of this histone but the general distribution of heterochromatic areas remained similar to that visible in situ. Mature erythrocyte nuclei behaved quite differently than calf thymus nuclei after extraction with citric acid, since the chromatin became excessively condensed rather than dispersed. After complete removal of both fractions I and V, erythrocyte chromatin became loosened to the extent that A fibril structure was revealed. A subtle distinction arises here between a general "loosening" of chromatin and the dispersal of condensed masses of chromatin. Certainly extraction of histones, particularly fractions IIb1, IIb2 and III in addition to fractions I and/or V, caused loosening of chromatin in liver and erythrocyte nuclei to the extent that it became less electron dense than before and B fibrils gave way to a network of finer fibrils. In this respect, the chromatin in liver and erythrocyte nuclei after extraction at pH 1.8, appeared quite similar to the chromatin in calf thymus nuclei following extraction with citric acid. However, neither in liver nor erythrocyte nuclei did extraction of lysine-rich or any other histone fraction, cause complete dispersal of heterochromatin similar, for example, to that observed in intact nuclei exposed to water. Moreover, it would appear from pictures of

calf thymus nuclei (Littau et al., 1965), that despite marked fine structural changes, the overall distribution of peripheral heterochromatin was not appreciably altered after extraction with 0.1 M citric acid. Taken overall, the results from histone extraction obtained here suggest that the fundamental patterns of heterochromatin condensation are only partially dependent on DNA-histone interactions. Furthermore, there does not appear to be a simple dependence of chromatin condensation and organization on any single histone fraction.

Euchromatin, particularly in liver nuclei, became noticeably reduced in extent early in the histone extraction. This indicates, not unexpectedly, that because of its relatively diffuse composition, euchromatin is less rigidly ordered in the nucleus than heterochromatin, and its distribution depends to a large extent on the structural integrity of basic fibrils. Presumably disruption of the 200 Å B fibril by extraction in 0.1 M citric acid leads to collapse of the dispersed fibrils and disappearance of the euchromatin regions.

Apart from the nuclear membrane and histone proteins, the other macromolecular element which could be involved in regulating intranuclear organization is non-histone proteins (NEP) associated with chromatin. These proteins constitute some 20% and 40% of the total chromatin material in chicken erythrocyte and liver nuclei, respectively (Dingman and Sporn, 1964). That the NEP may play a significant role in chromatin secondary

structure is indicated by the properties of nuclei which were dehistonized by extraction at pH 1.0. Such nuclei from all tissues still showed considerable residual secondary structure. Further, at all stages of extraction, including complete histone removal, the nuclei continued to show reversible change in gross structure in response to variations in ionic strength.

Analysis of NHP indicates that these proteins constitute a heterogeneous group of molecules, with some components probably common to most tissues and organisms (Banjamin and Gellhorn, 1968; Elgin and Bonner, 1970). Tissues which are relatively active in RNA synthesis usually contain greater amounts of NHP than inactive tissues (Dingman and Sporn, 1964; Shaw and Huang, 1970), suggesting that some of the proteins may be agents in gene derepression and/or enzymes directly necessary for transcription. NHP components, particularly those common to many nuclei, may serve structural roles. This could involve mediating attachment of chromatin fibrils to the nuclear membrane, as discussed above. In addition the NHP might cross-link adjacent fibrils within the body of the nucleus. In this connection, the persistence of a dark band at the periphery of the heterochromatic regions in extracted nuclei might be of considerable interest. The composition, location and function of NHP require much further study.

IV. Chromatin Structure and Gene Regulation

Many of the questions concerning both the primary and secondary structure of chromatin are relevant to mechanisms of gene function and regulation. These relationships are well illustrated by the differentiation of nucleated erythrocytes. As immature avian and amphibian erythrocytes develop, their ability to synthesize RNA and proteins gradually decreases and virtually ceases at maturity (Cameron and Prescott, 1963; Grasso and Woodard, 1966; Attardi et al., 1970). Concomitantly the nuclei become progressively more compact and heterochromatic (Tooze and Davies, 1967; MacRae and Meetz, 1970). Alternatively, mature erythrocytes have been "reactivated" with respect to RNA and protein synthesis through introduction into the cytoplasm of human and mouse cells (Harris, 1967). Such reactivation was preceded by gradual swelling of the nuclei and dispersal of condensed chromatin. These observations indicate that the degree of chromatin condensation in the nucleus may be an index of genome repression, but the precise relations between structure and function are unknown. Some of the structural findings in the present work, taken in conjunction with parallel biochemical studies, bear on this problem.

One important question regarding nuclear structure is whether the process of chromatin condensation is directly responsible for gene repression and vice versa.

In this regard when saline-isolated nuclei were exposed to low ionic strength and the chromatin decondensed, there was no correlated major change in the RNA transcription in vitro. With both chicken erythrocyte (Brasch et al., 1971) and hepatic nuclei (Seligy and Neelin, unpublished data), no appreciable qualitative or quantitative changes were found in the RNA synthesized by the respective nuclei in saline, in water and recondensed in saline. These results demonstrate that mere loss of gross-structural condensation, and B fibril integrity, at least as a result of ion-mediated changes in configuration, are not themselves sufficient to bring about major alterations in the synthetic capabilities of chromatin.

The above assertion is consistent with studies (Seligy and Miyagi, 1969) showing that the template activity of chromatin from chicken liver remained about ten times higher than that from chicken erythrocytes even when such physical properties as solubility and viscosity of the two chromatin were made equivalent. Similarly, Harris (1967) showed that in heterokaryons between HeLa cells and fowl erythrocytes, dispersal of erythrocyte chromatin continued without the usual increase in RNA synthesis if the nuclei were U.V. irradiated. Collectively, these studies suggest that while gene repression and structural condensation of chromatin are associated phenomena, they are not necessarily causally related.

Some treatments or agents which lead to changes in the molecular make-up of chromatin also induce structural decondensation and concomitant gene activation. For example, certain polyanions induce rapid swelling and dispersal of chromatin in nuclei (Whitfield and Perris, 1968; Kraemer and Coffey, 1970b), and often bring about accompanying increases in DNA and RNA synthesis (Kraemer and Coffey, 1970a; Brown and Coffey, 1971). These changes appear to result primarily from dissociation of the DNP complex, since they are generally accompanied by marked solubilization and loss of DNA or histones from the treated nuclei (Whitfield and Perris, 1968; Kraemer and Coffey, 1970b; Miller et al., 1971). It appears, therefore, that regulation of gene expression involves as yet unresolved interactions among the various macromolecular constituents of the DNP complex, which may secondarily lead to microscopically visible structural changes in chromatin.

Although the agents involved in the specific genetic regulation governing the transcription of RNA are as yet unknown, histone proteins are at least partially responsible for differences in gene repression among different tissues (see Literature Review). Further evidence to this effect is provided by a comparison of structural and RNA-transcriptional changes in liver and erythrocyte chromatin, during the selective acid extraction of histones.

The difference in structure found between liver and erythrocyte nuclei after extraction with 0.1 M citric acid, was clearly related to histone fraction V. After extraction of most of fraction I by citric acid, liver chromatin exhibited distinct substructure which probably consisted largely of intermeshed A fibrils. Erythrocyte chromatin, on the other hand, remained tightly congealed until both fractions I and V were removed. Only complete extraction of fraction V clearly revealed the A fibril constituent of erythrocyte chromatin.

Parallel studies on RNA transcription (Seligy and Neelin, 1970) showed that selective removal of fraction I had almost no stimulatory effect on the template activity of erythrocyte chromatin. In contrast, subsequent dissociation of fraction V resulted in an increase in template activity equivalent to about half the total template activity of fully dehistonized chromatin. This increase in template activity took place over the relatively narrow pH range (pH 2.1-1.8), in which fraction V was extracted. All remaining histone-related template restriction was removed after extraction at pH 1.0. With liver chromatin, on the other hand, after extraction of fraction I a gradual increase in template activity was noted over a relatively broad pH range (pH 1.8-1.0) and could not be linked to the extraction of any particular histone fraction (Seligy and Neelin, unpublished observation). These authors concluded, therefore, that fraction V serves as

special repressor of transcription in chicken erythrocytes and may act as a "super-repressor" of a large portion of the genome in these cells.

Results from the present investigation are directly comparable to the above transcriptional studies, and together suggest that fraction V may act as a genome repressor by promoting structural modifications in erythrocyte chromatin. Perhaps by binding to as yet undetermined sites on the genome, this histone can effectively bring the entire chromatin into close physical proximity and restrict its capacity for RNA transcription. It seems likely, however, that chromatin in erythrocyte nuclei becomes structurally modified after extraction with citric acid to an extent quite removed from conditions in vivo. The tightly congealed chromatin may in part represent an artificial rearrangement of DNP constituents, whereby they become structurally interlocked and, secondarily, unavailable for RNA transcription. Thus while fraction V may be involved in both the extreme repression and the structural alteration of chromatin in mature erythrocytes, the exact nature of this involvement remains to be clarified.

Gene regulation in vivo probably involves correlated changes in both primary and secondary structural levels in chromatin and in the interactions of various DNP constituents. It seems probable that part of the regulatory process must involve at least temporary dissociation of histones and DNA. It is unlikely that

such dissociation involves the actual physical removal of histone molecules from nuclei, as is experimentally done in in vitro studies of template capacity, since major variations are not found in situ in qualitative and quantitative histone composition of chromatins with widely differing genetic activities (Gorovsky and Woodard, 1967; Comings, 1967). Perhaps histones bind to DNP with variable tenacity, as histone V possibly does in immature and mature erythrocytes. For example, small differences in molecular weight, amino acid composition, phosphorylation, etc..., might result in alteration of various histone components so that they alternately bind and dissociate from specific regulatory sites on the genome. Such minor variation in the binding and repressive capacity of different histones might well be hard to detect. A recent comparison of histones of polytene and non-polytene nuclei of Drosophila (Cohen and Gotchell, 1971) revealed several minor but distinct differences in fraction I between the two tissues and between Drosophila fraction I and those of several other animals. Conceivably, combined minor variations in several histone components might result in small structural and regulatory modifications in chromatin. The action of histone V is perhaps just an amplification of the function served to varying degrees by all histone components.

V. Nucleolar Structure

When liver nuclei were isolated in saline, the nucleoli remained very similar in appearance to those in situ, indicating that most structural components were not readily solubilized in aqueous media. These nucleoli contained granular and fibrillar zones as well as closely associated chromatin, a structural pattern common to most plant and animal nucleoli (Bernhard and Granboulan, 1968; Lafontaine, 1968). Nucleolar chromatin and the fibrillar zone were markedly affected by exposure to acid during histone extraction. Perhaps proteins of a basic nature were extracted from the fibrillar zone by acid. Such proteins could include histones associated with the chromatin which is thought to be a constituent of the fibrillar zone (Lord and Lafontaine, 1969). In addition, this zone may contain ribosomal proteins, which are known to be basic and acid extractable (Setterfield et al., 1960).

The granular zone was not markedly affected by exposure to acid, although it became somewhat compacted and the granules appeared more irregular in outline than they had been initially. Since the granules probably represent the large ribosomal subunits, acid extraction would be expected to remove some basic proteins. This would account for the changes in granule structure. Presumably the reason that the granular zones holds together is that the granules are embedded in non-basic protein which resists extraction.

The finding that the fibrillar component in liver nucleoli could be dispersed and re-condensed in response to ion concentration indicates that the architecture of the nucleolus is in part dependent on molecular organization similar to that in chromatin. Lord and Lafontaine (1969) and Recher et al., (1970) have shown by autoradiography and enzyme digestion studies, that segments of the nucleolar organizer chromatin are probably extended throughout the nucleolar body. The results here reported lend support to the above observations and suggest that chromatin may be located throughout the fibrillar zone. In this way, nucleolar chromatin may play an important role in determining the structural organization of the nucleolus as suggested by Lord and Lafontaine (1969).

VI. A Model of Chromosome Organization during Interphase

When the present work on nuclear structure is considered with various other current studies, a general model of nuclear organization may be proposed. In a eukaryotic nucleus, individual chromosomes can be likened to the separate polypeptide chains in complex protein molecules. Each chromosome is a discreet genetic entity, which probably consists of one continuous or several tandemly-linked DNA molecules (Sasaki and Norman, 1966; Comings, 1968; DuPraw, 1970; Lett et al., 1970). During the interphase stage, when they become

synthetically active, chromosomes become differentially extended and assume specific tertiary and quaternary structure. Through this specific folding and packing, genes which are widely spaced in the genome could be brought into physical proximity for functional integration. A further possibility in this regard, is that much of the DNA in a chromosome may function in a purely structural manner, like most of the amino acids in a polypeptide chain. This function may be fulfilled by some of the non-transcribed portions of DNA (Flamm et al., 1969) or by some of the redundant DNA sequences found in most eukaryotic organisms (Britten and Kohne, 1968). The spatial arrangement with specific folding of chromatin is probable determined in large measure by NHP and histones with secondary regulation by ionic environment, while the nuclear membrane probably serves as an anchor point for chromatin attachment. In this manner, various regions of a chromosome could fold and unfold as required and yet retain the overall organization of the chromosome complex. The actual transcription of specific genes would require unfolding of chromatin fibrils and in addition correlated molecular changes in the DNP complex, which may or may not be causally related to unfolding (Brasch et al., 1971). In preparation for cell division, the nuclear membrane may regulate DNA replication (Comings and Kakefuda, 1968; Hearst and Botchan, 1970) and during prophase,

chromosomes possibly condense at several points on the nuclear membrane (Comings and Okada, 1970a). The nuclear membrane would then be important to structural order in chromatin during most phases of the cell cycle.

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