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I am submitting herewith a dissertation written by Kathleen Hall Larsen entitled "Fractionation of Chromatin by Nuclease Digestion and Sucrose Gradient Centrifugation: Implications for Ultrastructure of the Nucleus." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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FRACTIONATION OF CHROMATIN BY NUCLEASE DIGESTION AND SUCROSE GRADIENT CENTRIFUGATION: IMPLICATIONS FOR ULTRASTRUCTURE OF THE NUCLEUS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Kathleen Hall Larsen

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DEDICATION

To

the memory of Gordon Hunt,

a good friend,

for helping to get me into this,

and then for helping to get me out

and To

my husband, John,

and my daughters, Elaine and Kirsten,

for their pride and their patience,

for putting up with and pitching in,

for keeping me going, for keeping me sane.

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ABSTRACT

Recent experimental work underlying the rapid increase in knowledge of chromatin ultrastructure is reviewed. The validity of the evidence for a non-chromatin structural framework within nuclei is discussed.

Preparations of nuclei from mouse spleen were shown to contain contaminating fibers. In the presence of EDTA, nuclei and fibers aggregated into dense clumps. Preparations of liver nuclei did not contain fibers and did not aggregate in the presence of EDTA.

Chromatins prepared by different methods were compared using micrococcal nuclease as a probe for nucleosome integrity. Intranucleosomal DNA was more susceptible to the nuclease in sonicated nuclei than in intact nuclei. Repeated washing of nuclei with chelating agents and buffers of low ionic strength produced a chromatin gel that resisted digestion unless first dispersed by sonication. After sonication, nucleosomes in the washed nuclei were most susceptible to nuclease cleavage of all preparations tested.

After digestion with micrococcal nuclease, mouse liver nuclei separated into slowly and rapidly sedimenting fractions in 5-65% sucrose gradients. Fractionation was obtained after digestion periods ranging from 1 minute (1% of the DNA acid soluble) to 2 hours (50% of the DNA acid soluble). The slowly sedimenting fraction contained chromatin fragments produced by nuclease cleavage. After brief digestion, this fraction was enriched in a specific nonhistone protein.

Residual nuclear structures were observed with the light microscope in unfixed wet mounts of the rapidly sedimenting fraction. After

complete digestion with the nuclease, 15% of the nuclear DNA remained associated with these structures. This DNA was in the form of mononucleosomes and could not be dissociated except by treatments that disrupted the residual nuclei. The presence of these structures in unfixed material prepared under relatively mild conditions is further evidence for the existence of a nuclear matrix in vivo. It is suggested that chromatin is attached to the nuclear structure through individual nucleosomes. Examination of stained preparations indicated that the attachment sites are uniformly distributed at the level of resolution of the light microscope.

The effects of changes in ion concentrations on the fractionation was investigated. Digestion products were not released from nuclei in the continued presence of divalent cations, suggesting participation of these ions in nucleosome-nucleosome or nucleosome-matrix interactions. In the presence of 0.06 M salt, octanucleosome subunits of a higher order of chromatin structure were detected. The data indicate the possibility that stability of these subunits is not dependent on preservation of the linker DNA between core particles and that close association between higher order subunits may not require continuity of the connecting DNA.

The implications of highly ordered structure for the organization and regulation of nuclear activity and experimental approaches for future investigation of these mechanisms are discussed.

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LIST OF ABBREVIATIONS

A	Angström unit
A_{260}, A_{230}	Absorbance at 260 or 230 nm (optical density)
CD	Circular dichroism
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycolbis(β-aminoethyl) tetraacetic acid
hnRNA	Heterogeneous nuclear ribonucleic acid
PMSF	phenylmethylsulfonylfluoride
Poly(A)	poly(adenylic) acid
pTp	Thymidine-3',5'-diphosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
SDS	Sodium dodecylsulfate

CHAPTER 1

REVIEW OF RECENT LITERATURE

The model of the basic chromatin subunit proposed by Kornberg (1974) and now known as the nucleosome has been abundantly confirmed in its major features. The model was originally based on studies of the interactions between histones in solution (Kornberg and Thomas, 1974), on electron micrographs showing strings of spherical particles spilling out of lysed nuclei (Olins and Olins, 1974, 1973; Woodcock, 1973) and on nuclease digestion experiments suggesting a periodic structure in chromatin (Noll, 1974; Hewish and Burgoyne, 1973). The model is now supported by a large body of biophysical, biochemical and ultrastructural evidence described in detail in several recent reviews and symposia (Botchan and Watson, 1978; Callan and Klug, 1978; Felsenfeld, 1978; Kornberg, 1977; Bradbury and Javaherian, 1977). The core of the nucleosome is composed of 140 base pairs of double-stranded DNA coiled around an octamer of histones ($H2A_2H2B_2H3_2H4_2$). Linker DNA, variable in length between species and between different cell types but averaging 45-60 base pairs, connects the nucleosomes in a linear array and is loosely associated with the outside of the cores. Histone H1 binds to linker DNA and stabilizes further coiling and condensation of the chromatin fiber (for references, see the reviews cited above).

X-ray diffraction studies of crystallized core particles show them to be discs 110 A in diameter and 57 A high (Finch et al., 1977). The exact arrangement of the histones and the path followed by the DNA are not yet known, but the electron density map suggests a bipartite

structure with the DNA making almost one complete turn around the periphery of each of the apposed halves of the histone complex. It has been suggested that the core is a dimer of heterotypic histone tetramers $(H2A:H2B:H3:H4)_2$ (Weintraub et al., 1976; D'Anna and Isenberg, 1974). However, studies designed to detect an equilibrium between tetramers and octamers of histones in solution have given contradictory results. Chung and coworkers (1973) report the existence of such an equilibrium, but Eickbush and Moudrianakis (1973) observed a different mode of core dissociation into hexamer and dimer. The concept that the core particle contains a two-fold axis of rotational symmetry is widely accepted and has been incorporated into several recent models of nucleosome structure (Worcel and Benyajati, 1977; Richards et al., 1977; Finch et al., 1977). Hayashi and associates (1978) assert that assymmetry in the binding of histone H1 to the nucleosome precludes the existence of a dyad axis of symmetry in the nucleosome; they do not, however, consider the possibility that the assymmetry of the association may result solely from the lack of symmetry in histone H1. Recently published electron micrographs (Tsanev, 1978; Oudet et al., 1978, 1977) indicate that nucleosomes separate into half nucleosomes at low ionic strength, suggesting that an octamer to tetramer equilibrium is possible in chromatin irrespective of the behavior of histones in solution. Short range crosslinking experiments indicate, however, that half nucleosomes are in very close contact at least part of the time (Wyns et al., 1978).

One assumption about nucleosome structure, that the core histones interact with DNA chiefly through their highly basic N-terminal tails,

has become less tenable. Whitlock and Stein (1978) have shown that core histones deprived of N-terminal tails by trysin digestion re-associate with DNA into particles very similar to nucleosomes. The globular portions of the core histones therefore provide both binding sites and sufficient energy (from DNA-histone or histone-histone interactions) to coil the DNA (see also Kornberg, 1977). Isolated nucleosomes are able to bind additional histones (Voordouw and Eisenberg, (1978) so nucleosome structure is not produced by binding the greatest possible amount of histone, i.e. by maximal charge neutralization. Additional factors must enter into formation of a stable structure. Studies of core particles by nuclear magnetic resonance show that the C-terminal portions of the histones occupy fixed positions, providing a rigid framework for the DNA (Lilley et al., 1977). The fact that core particles can be crystallized provides further evidence for regularity of structure (Finch et al., 1977).

The nature and regularity of associations between nucleosomes to achieve the degree of condensation of interphase chromatin have recently been the focus of much investigation and speculative thought. Nucleosome cores are close together in unsheared chromatin, since extensive crosslinking generates not only an octamer of core histones, but a 16-mer and a 24-mer as well (Thomas and Kornberg, 1975). Neutron scattering patterns provided early evidence for regularity in higher orders of structure (Bram et al., 1975), and differences in circular dichroism spectra and dye-binding properties between chromatin and isolated nucleosomes have been interpreted in terms of internucleosomal associations (Lawrence et al., 1976; Mandel and Fashman, 1976).

Electron micrographs of nuclei and chromatin reveal fibers of several different thicknesses. (The literature on this subject is extensive, extending back fifteen years or more; for references and discussion, see Ris and Korenberg, 1978; Rattner and Hamkalo, 1978; Worcel and Benyajati, 1977; Franke et al., 1977; DuPraw, 1970.) Formation of the 100 A fiber is generally believed to occur by coiling of the linker DNA on and between the cores. There is no consensus, however, on the mode of organization of the next level of structure, the 250 A fiber. Two models have been proposed, continuous supercoiling of the 100 A fiber (Finch and Klug, 1976) and discontinuous clustering of nucleosomes in a beaded fiber (Hozier et al., 1977). These proposals are not necessarily contradictory and may well represent different states of a structure capable of varying degrees of condensation. Several laboratories have recently demonstrated sites of increased susceptibility to micrococcal nuclease spaced approximately eight nucleosomes apart in a large fraction of intranuclear chromatin (Butt et al., 1979, Strätling et al., 1978a,b; Varshavsky et al., 1977a). This evidence for conformational discontinuity in higher order structure favors the beaded model as the major form of the 250 A fiber. Granular structures of similar diameter have also been observed in condensed chromatin within thinly sectioned nuclei (Franke et al., 1977). Histone H1 is required for maintaince of the 250 A fiber (Worcel and Benyajati, 1977; Thoma and Koller, 1977), and changes in the subspecies of H1 present or in post-translational modifications of the H1 molecule are possible mechanisms for regulation of chromatin condensation.

Evidence that chromatin fibers are folded into supercoiled domains has been obtained from yeast (Pinon and Salts, 1977), cultured Drosophila cells (Benyajati and Worcel, 1976), mouse mammary carcinoma cells (Ide et al., 1975) and HeLa cells (Cook and Brazell, 1975). The folded configuration is maintained primarily by nonhistone protein, possibly in association with an RNA component (Pinon and Salts, 1977; Benyajati and Worcel, 1976; Ide et al., 1975). The rotational constraints imposed at many points in the genome indicate that chromatin strands are fixed at many separate sites to each other and/or to a non-chromatin supporting structure. Photomicrographs published by Warren and Cook (1978) show that supercoiled interphase and mitotic chromosomes are retained within a residual nuclear structure in 1 M salt. Points of association between chromatin and the nuclear matrix have been observed in electron micrographs (Comings, 1978; Schatten and Thoman, 1978). Igo-Kemenes and Zachau (1977) report that some relatively small chromatin fragments remain associated with nuclei after prolonged digestion with restriction endonucleases, and propose that these are sites of chromatin attachment to the nuclear structure and determine the subdivision of the fiber into domains. Domains appear to be heterogeneous in size within the cell (Warren and Cook, 1978; Igo-Kemenes and Zachau, 1977) and thus are not manifestations of higher order chromatin packing in which an entire loop is incorporated into a structure of discrete size. A set of permissible sizes, multiples of the size of smaller packing units within each loop, are quite possible.

The locations of the attachment sites in the chromatin may be

a permanent feature of the genome, encoded in the DNA sequence and preserved through the cell cycle. Histone-depleted metaphase chromosomes consist of a scaffold of nonhistone proteins from which the DNA extends in loops of a size comparable to domains in interphase chromatin (Jeppesen, et al., 1978; Paulson and Laemmli, 1977). The DNA remaining attached to the scaffold when the loops have been digested away with a restriction endonuclease is enriched in reiterated base sequences (Razin et al., 1978). Digestion of interphase nuclei with a different restriction endonuclease resulted in solubilization of reiterated sequences (Igo-Kemenes et al., 1977); in this case, however, the nature of the unreleased sequences was not investigated. Dissociated matrix protein does not bind to specific DNA sequences (Comings, 1978), although a preference for thymidine-rich sequences was detected. This finding is not conclusive, however, since the capacity for specific binding may be a property of a minor matrix component and may be destroyed by dissociation of the matrix. Thus, neither of these results directly contradicts the supposition that the attachment sites are encoded by one or more classes of repetitive DNA. Models of chromosome organization incorporating this feature have recently been proposed (Bak et al., 1979; Lerman and Degtyarev, 1978). If it is true that domains are determined by base sequence, they are probably of functional as well as structural significance, and may constitute units of transcription as in the loops of lampbrush chromosomes. This concept is admittedly speculative, and supporting evidence is fragmentary. It suggests, however, that large scale changes in chromatin condensation, as occur during the cell cycle, may

be accomplished by expansion and contraction of the scaffold, thus changing the relative positions of the attachment sites. The major scaffold proteins are reportedly present in interphase nuclei (Campbell et al., 1979; Adolph et al., 1977b).

New evidence has recently been provided for a concept more commonly held in earlier decades: that the interphase chromosomes, although swollen, retain their individuality and that decondensation does not imply extensive intermingling of chromatin fibers from different chromosomes (Murray and Davies, 1979; Stack et al., 1977, and references therein; Brasch and Setterfield, 1974). The results of Sedat and Manuelidas (1977), demonstrating a high degree of order in interphase nuclei, are consistent with this hypothesis. This spatial organization is implicit in the proposal that interphase chromosomes remain attached at specific sites to an extended version of the mitotic chromosome scaffold.

There is now considerable evidence for the existence of a proteinaceous structural framework within the nucleus. The existence of residual proteins in chromatin-depleted nuclei has been known to biochemists for over thirty years (for review, see Comings, 1978). The most prominent feature of the proposed framework seen in electron micrographs is a fibrous layer, the nuclear lamina, underlying the nuclear membrane (Patrizi and Poger, 1967; Fawcett, 1964). Extraction of the lipids from isolated nuclear envelopes reveals a structure in which the nuclear pore complexes are connected by fibers (Agutter et al., 1978; Dwyer and Blobel, 1976; Scheer et al., 1976; Aaronson and Blobel, 1975). In cross-section, amorphous material is associated

with the inner side of this membrane skeleton. Blobel and coworkers (Dwyer and Blobel, 1976; Aaronson and Blobel, 1975) suggest that the pore-connecting fibers are components of the inner nuclear membrane and the amorphous layer is the residue of the fibrous lamina observed by Fawcett (1964) and by Patrizi and Poger (1967). Scheer and co-workers agree on the identification of pore-connecting fibers as membrane components, but find no convincing evidence for the existence of a fibrous lamina in amphibian oocytes. In a later paper, however, they report that further purification of the nuclear envelopes removes many polypeptides, some of whch appear to be derived from intranuclear fibers isolated with the membrane fraction (Krohne et al., 1978). Schatten and Thoman (1978) have published a remarkable series of scanning electron micrographs of Xenopus oocyte nuclei, including views of both surfaces of the inner and outer nuclear membranes. Pore complexes are visible on all membrane surfaces except the inner surface of the inner nuclear membrane. This surface is covered by a fibrous network in which are seen holes of the approximate size of the nuclear pores. Numerous particles 50-60 nm in diameter and occasional strands of chromatin are associated with the inner surface of this fibrous layer. Extraction of the fibers with 0.6 M KI reveals the underlying pore complexes in the inner nuclear membrane. When the inner aspect of the fibrous lamina was examined in oocytes which had been torn open and rapidly plunged into glutaraldehyde, funnel-shaped channels were seen leading into the lamina toward the cytoplasm and chains of particles, identified by autoradiography as RNA, were seen

inserted into some of the channels This structure was, however, unstable. In oocytes prepared by less rapid methods, the lamina appeared as a flattened layer of fibers. This lability of structure may explain the amorphous appearance of the lamina in specimens prepared for transmission electron microscopy.

Extraction of nearly all the nucleic acid, histone and lipid from intact nuclei leaves residual structures which retain the shape of nuclei and contain internal elements as well as the pore complex-lamina layer. Such structures have been found in rat liver (Miller et al., 1978a; Berezney and Coffey, 1974), mouse liver (Comings and Okada, 1976), CHO cells (Hildebrand et al., 1975), Tetrahymena (Herlan and Wunderlich, 1976), HeLa cells (Ghosh et al., 1978; Herman et al., 1978, 1976; Hodge et al., 1976) and PtK1 rat kangaroo cells (Ghosh et al., 1978). The term nuclear protein matrix was proposed for this structure by Berezney and Coffey (1974).

The internal elements of the matrix are not uniformly distributed At low magnification, electron micrographs show clumps of electron-dense material in chromatin-depleted nuclei. These are sometimes in contact with the peripheral lamina or the fibrillar residue of the nucleolus and are frequently connected by fibrous bridges (Miller et al., 1978a; Herman et al., 1978; Hodge et al., 1977; Herlan and Wunderlich, 1976; Berezney and Coffey, 1977, 1974). Collapsed empty shells of the pore complex-lamina layer are sometimes seen (Berezney and Coffey, 1977; Comings and Okada, 1976); these no longer have the roughly spherical shape of the intact matrix, indicating that the internal elements provide structural support for the lamina. The

clumps of matrix material in the nuclear interior often resemble the clusters of intrachromatinic granules seen in intact cells (Berezney and Coffey, 1977) and the entire matrix structure, as seen in electron microscope, bears a striking resemblance to electron micrographs of nuclei stained by the regressive EDTA procedure which selectively bleaches chromatin, thereby enhancing the contrast of ribonucleoprotein structures (Fakan et al., 1976; Monneron and Bernhard, 1969). The clumps and bridges of the matrix define a set of open spaces within the nucleus reminiscent of the arrangement of condensed chromatin in intact nuclei (Miller et al., 1978a; Berezney and Coffey, 1977; Herlan and Wunderlich, 1976; Comings and Okada, 1976). At higher magnification, fine matrix elements are sometimes seen extending into the open spaces (Miller et al., 1978a; Berezney and Coffey, 1977; Comings and Okada, 1976). These are smaller in size and lower in electron density than the elements comprising the compact interchromatin clumps. In negatively stained or metal shadowed preparations, both fibers and granules are apparent in the internal parts of the matrix (Comings and Okada, 1976).

The residual matrix obtained after extraction of nuclei with nonionic detergent, salt, RNase and DNase is composed largely of nonhistone protein, although small amounts of RNA, DNA and phospholipid are reproducibly found. Matrix proteins differ in amino acid composition from other fibrillar proteins, including actin, myosin and tubulin (Comings, 1978; Comings and Okada, 1976). Three to six major protein components are commonly resolved by SDS gel electrophoresis. In particular, three protein species of moderate size have been found

in matrices from a variety of sources; the molecular weights assigned to these bands are listed in Table I. Four proteins are listed for CHO cells, a major band (58,000 daltons) and three minor bands (68,000, 70,500 and 71,500 daltons). Minor bands at 68,000 and 75,000 daltons were also found in the HeLa cell nuclear matrix. While there are some similarities to be noted between the various liver preparations, no two matrix preparations have the same protein composition.

Somewhat different results were reported for the nuclear membranes of Xenopus oocytes by Krohne and coworkers (1978). The highly purified preparation contained only two detectable polypeptides of 73,000 and 150,000 daltons. These are almost certainly components of the nuclear pore complexes; because these membranes contain such a large number of pore complexes, other polypeptides may have been obscured. Less purified preparations, in which the layer underlying the inner membrane is visible in electron micrographs, contain additional protein species of which two have apparent molecular weights of 59,000 and 65,000 daltons (Krohne et al., 1978).

Matrix proteins quite possibly differ between species, and differences in preparative and analytical techniques between laboratories may account for some of the variability observed in matrix protein composition, but there are two reasons why identities that may exist might prove difficult to demonstrate. Pederson and Bhorjee (1979) have shown that some nonhistone nuclear proteins are tightly bound to nucleic acid fragments even in 0.4 M guanidine hydrochloride-6 M urea, and that the nucleic acid fragments comigrate with the proteins in SDS gels, particularly in the region where the major matrix proteins

TABLE I. MOLECULAR WEIGHTS OF SOME MATRIX PROTEINS

Mol. Weight (daltons)	Source	Reference
62,000; 66,000; 69,000	Rat liver (matrix)	Berezney and Coffey, 1974
66,000; 68,000; 69,000	Rat liver (pore complex-lamina)	Aaronson and Blobel, 1975
65,000; 67,000; 68,000	Mouse liver	Comings and Okada, 1976
62,000; 64,000; 67,000	Sheep liver, lung and endometrium	Agutter et al., 1978
54,000; 57,000; 70,000	Tetrahymena macronuclei	Herlan and Wunderlich, 1976
49,000; 53,000; 57,000	HeLa cells	Hodge et al., 1976
58,000;	CHO cells	Hildebrand et al., 1975

are found (Bhorjee and Pederson, 1976). The effect of bound nucleic acids on the mobility of these nonhistone proteins in SDS gels or the existence of nucleic acid fragments in matrix protein bands has not been investigated, but association of both RNA and DNA with the matrix has been reported (see below) and the possibility exists that some apparent differences in matrix protein composition may result from differences in associated nucleic acid fragments. The presence of neutral sugars in the matrix and lamina has also been reported (Herlan and Wunderlich, 1977; Hodge et al., 1976; Scheer et al., 1976; Hildebrand et al., 1975) and matrix proteins can be labeled with glucosamine (Hodge et al., 1976). Glycosylation is known to alter protein mobility in SDS gels (Ohno et al., 1975) and this factor may also contribute to differences between preparations. More information on the chemical composition of these proteins is required before valid comparisons can be made.

A different type of residual structure has been obtained by treatment of detergent-washed nuclei with 0.5 M $MgCl_2$ (Riley and Keller, 1976; Riley et al., 1975). These structures, termed nuclear ghosts, contain more than ten times as much DNA as the DNase-treated matrix and appear in electron micrographs to be composed of rod-like elements connected in polygonal arrays and attached to electron-dense annuli. Similar images can be produced in matrix preparations by incomplete removal of the DNA (Comings, 1978) or artificially in mixtures of DNA with basic proteins (Comings, 1978; Laemmli, 1975; Olins and Olins, 1971). Thus it is likely that the unusual appearance of nuclear ghosts is caused by aggregation of nuclear DNA with nuclear proteins. This

interpretation is supported by the fact that digestion with DNase I or DNase II dissociates the rods and collapses the ghosts (Riley and Keller, 1978). Matrix proteins may be present in nuclear ghosts. The arrangement of ghost components, however, does not resemble anything seen in whole nuclei and probably represents, at best, a significant alteration of nuclear structure.

The larger question of how closely the nuclear matrix is related to the structure of the nucleus in a living cell cannot yet be answered unequivocally, except in the general sense that all components separated from living cells are artifacts, especially when fixed, stained, dessicated and placed in a vacuum for electron microscopy. There is, however, some evidence that the residual structure which survives detergent and salt extraction and nuclease digestions does represent a structure existing in the nucleus. The fact that many nuclear components are recognizable in the isolated matrix has already been mentioned: the pore complexes, inner nuclear membrane, fibrous lamina, nucleoli, clusters of interchromatinic granules and the locations of clumps of condensed chromatin can be identified despite the fact that most of the nucleic acid and phospholipid and much of the nuclear protein were removed before fixation. Many different combinations of fixative and post-fixation treatments have been employed by different laboratories to prepare matrix specimens for electron microscopy, so there is little likelihood that the matrix is an artifact of a particular method. Moreover, the matrix is visible in unfixed preparations by phase contrast microscopy, although few structural details are discernable at such low magnification (Deppert, 1978;

Chin and Maizel, 1977; Hodge et al., 1976). The results of Skaer and Whytock (1977) on the finding of chromatin-like artifacts in nuclear sap are sometimes cited as evidence that the matrix is an artifact produced by precipitation of soluble proteins. In fact, Skaer and Whytock did not use a soluble fraction but examined whole nuclei in which the chromatin was either condensed into polytene chromosomes or packed into one side of the nucleus by centrifugation. It is not at all surprising that they found there fibrous structures resembling the matrix. Nor is it shown that these structures are artifacts, only that they are probably not chromatin. Matrix-like elements can also be seen in whole nuclei after the chromatin has been condensed naturally during prophase (Ghosh et al., 1978) or artificially by inhibition of protein synthesis (Brasch and Sinclair, 1978; Ghosh et al., 1978; Herlan et al., 1978). The resemblance of the isolated matrix to the intact nucleus stained by the regressive EDTA method has already been mentioned. Thus, treatment with detergent, salt and nucleases is not necessary to demonstrate the existence of a matrix-like network. In scanning electron micrographs of nuclei lysed directly onto grids by the method of Olins et al., (1975), smooth fibers of regular diameter are observed along with the knobby chromatin fibers which vary in diameter (Daskal and Busch, 1978; Daskal et al., 1977). The identity of these fibers has not been established, but they do not appear to be chromatin and their diameter is similar to that of matrix fibers. All of these considerations taken together provide some assurance that the isolated nuclear matrix is derived from a structure existing in the living cell. The evidence discussed

below for changes in matrix structure and composition that parallel changes in nuclear structure and function supports this interpretation.

The relationship between the nuclear matrix and the chromosome scaffold is not clear. The mitotic chromosome scaffold contains proteins of about the same electrophoretic mobility as matrix proteins (Adolph et al., 1977a; protein standards were not shown with these gels so precise comparison is not possible). The same authors report that scaffold proteins are present during interphase. Plagens (1978) has shown that the proteins of salt-extracted polytene chromosomes and salt-extracted nuclear envelopes of Chironomus salivary glands are very similar. Thus, it is possible that the scaffold is the intrachromosomal form of the matrix. If so, it is not well preserved in most matrix preparations. The entire matrix cannot become incorporated into the mitotic chromosomal scaffold because chromosomes can be visualized within a matrix structure during mitosis (Warren and Cook, 1978).

Several lines of evidence suggest that the proposed matrix may participate in nuclear metabolism. All matrix preparations seems to contain small amounts of RNA and DNA (e.g., Herman et al., 1978; Berezney and Coffey, 1978, 1976; Herlan and Wunderlich, 1976; Hodge et al., 1976). Associations between matrix fibers and DNA (Comings and Okada, 1976) or chromatin (Schatten and Thoman, 1978) have been observed in electron micrographs. Newly synthesized DNA is bound to the matrix (Berezney and Coffey, 1975).

Significant amounts of heterogeneous nuclear RNA (hnRNA) are also

associated with the matrix (Herman et al., 1976; Faiferman and Pogo, 1975). With no ribonuclease treatment, Herman and coworkers (1978) report that 54% of the hnRNA remains in chromatin depleted nuclei. Miller et al., (1978a) found that 100% of the hnRNA was retained if protease inhibitors were present during the isolation procedures, but only 30% of this sedimented with the nuclei through a sucrose gradient. Thus, much of this RNA appears to be loosely associated with the residual structure and a smaller fraction more tightly bound. After ribonuclease digestion, Miller and coworkers found 12% of the original RNA still associated with the nuclear framework in acid-precipitable form. According to Herman et al., (1978) the double-stranded regions and poly(A) tails of matrix-bound hnRNA are particularly resistant to RNase and are probably protected by protein. Possibly these segments of hnRNA are sites of attachment to the matrix; an alternative explanation, that these protected regions are bound to non-matrix proteins as free RNP, does not explain why they resist extraction from the residual structure.

Newly synthesized RNA becomes rapidly bound to the matrix (Herman et al., 1978, 1976; Miller et al., 1978a). Autoradiographic studies of RNA synthesis in intact liver cells stained by the regressive EDTA technique show newly synthesized RNA initially localized in the perichromatin fibers which surround the chromatin. The label later becomes more evenly distributed throughout the interchromatinic ribonucleoprotein and can be seen passing into the cytoplasm at a few sites (Fakan et al., 1976; Nash et al., 1975). The number of perichromatin fibers increases and decreases in parallel with the

transcriptional activity of the cell (Derenzini et al., 1978; Petrov and Bernhard, 1971; Pretrov and Sekeris, 1971). The arrangement of the perichromatin fibers in normally active liver nuclei is very similar to the network of ribonucleoprotein fibers observed by Miller and coworkers (1978a) after DNase and salt treatment of liver nuclei. The exact function of these fibers is not certain. The autoradiographic experiments indicate that they are newly synthesized RNA bound to or part of the interchromatinic structure. However, a direct identification of perichromatin fibers with the diffuse fibers of the matrix cannot be made on the basis of the information available. Moreover, localization of the enzyme complexes for DNA transcription or replication on the nuclear framework has not been demonstrated. Thus, the suggestion that the matrix is actively involved in DNA and RNA metabolism (Herman et al., 1978; Comings, 1978; Berezney and Coffey, 1977, 1976), while presenting attractive prospects for the organization and control of these processes, must presently be regarded as an interesting possibility.

The fact that pulse-labeled RNA remains associated with the interchromatinic structures during a four hour chase (Fakan et al., 1976) suggests that processing and transport of nuclear RNA may occur in this network. Supporting evidence for this proposal is still fragmentary. The labeled RNA is reduced in size after a four hour chase, compared to newly synthesized RNA and has migrated away from the chromatin masses into the interchromatinic material (Fakan et al., 1976). Several authors have suggested that perichromatin fibers and perichromatin granules, both ribonucleoprotein in nature, contain RNA

in transit to the cytoplasm, the fibers possibly becoming packaged into granules for storage or transport (Cervera, 1979; Risueño et al., 1979; Nash et al., 1975; Monneron and Bernhard, 1969). Near the nuclear periphery, granules have been observed unfolding into fibers before passage through the nuclear pores (Monneron and Bernhard, 1969). This suggests the view of the nuclear periphery shown by Schatten and Thoman (1978) with particles on the inner surface of the fibrous lamina and RNA-containing fibers inserted into channels in the lamina. If a framework of structural fibers does permeate the nucleoplasm, obviously RNA must pass through it en route to the cytoplasm. The relationship between hnRNA, perichromatin fibers and other interchromatinic structures and the exact role of a structural framework, whether active or passive, in RNA processing and transport remains to be determined. On the basis of current evidence, it is probable that some relationship does exist.

In addition to rapidly metabolized RNA, stable nuclear RNA species are found in association with chromatin-depleted nuclear structures. Within the nucleus they are bound to the residual ribonucleoprotein framework (Miller et al., 1978b), but some small RNA species also occur in the cytoplasm, associated with the endoplasmic reticulum, where they may be components of a cytoplasmic skeletal framework (Miller et. al., 1978b; Lenk et al., 1977; Sonenshein and Brawerman, 1977; Brañes and Pogo, 1975; Shiokawa and Pogo, 1974). Three small nuclear RNA species appear to be unique to the nuclear matrix; they do not appear in the cytoplasm and are not involved in the processing of ribosomal RNA or assembly of ribosomes (Miller et al., 1978b).

Goldstein and coworkers (1977) have reported, on the basis of autoradiographic experiments with amobae, that small stable RNAs of the nucleus become concentrated in chromosomes during prophase and anaphase when the degree of chromatin condensation is rapidly changing (these RNAs were found in the cytoplasm during metaphase). In similar experiments, the small nuclear RNAs became localized in chromatin condensed by treatment with actinomycin D (Goldstein et al., 1978). In normal interphase nuclei, these RNA species were evenly distributed over the nucleus. This affinity of small nuclear RNAs for condensing or decondensing chromatin may result from a passive replacement of nonhistone proteins, the interpretation favored by Goldstein and coworkers (1978, 1977), or it may reflect a more active role for small RNA species, possibly as components of a ribonucleoprotein structure, in regulation of chromatin structure. Pederson and Bhorjee (1979) have recently reported that three of the small nuclear RNA species can be isolated from HeLa cells in a form covalently linked to fragments of double-stranded DNA. These molecules are not RNA-DNA hybrids nor intermediates in DNA synthesis, but it has not been shown that they are at any time integrated into chromosomal DNA. One of these RNA species is probably the same as one of the three species unique to the nuclear matrix (SnG' in HeLa cells, 5Sa in rat liver; Miller et al., 1978b). The other two (SnC and SnD in HeLa cells, N3 and N4 in rat liver) are among the most abundant species in nuclei but are also represented in the cytoplasm. Only a fraction of the nuclear complement of these RNA species is found linked to DNA in a random population of HeLa cells, a fraction similar to the proportion of the cell cycle taken up by

mitosis. The evidence presented by Pederson and Bhorjee (1979; reviewed here only in part) is compatible with a model in which these RNA species become integrated into chromosomal DNA at widely distributed sites during mitosis (when the chromosomes are condensed) and are extrachromosomally located during the rest of the cell cycle. This proposal is highly speculative, and none of the evidence thus far produced is unequivocal. Convincing evidence of chromosomal integration will be difficult to obtain and evidence of temporary integration at a specific stage of the cell cycle even more difficult. The combined findings of matrix localization, affinity for condensed chromatin and covalent linkage to DNA do indicate, however, that small nuclear RNAs are probably structural components of nuclei involved in chromatin condensation.

The protein components of the nuclear matrix also appear to be metabolically active. They incorporated labeled amino acids more rapidly than other nuclear protein fractions (Hemminki, 1977), suggesting rapid turnover uncharacteristic of passive structures. Phosphorylation of matrix proteins has also been reported (Allen et al., 1977; Böhm et al., 1977), with elevated levels of matrix phosphorylation occurring shortly before the onset of DNA synthesis (Allen et al., 1977). This also suggests that the matrix is metabolically active, but interpretation of protein phosphorylation is complicated by the possibility that part of the labeled phosphate may be in associated nucleic acid fragments (Bhorjee and Pederson, 1976). In adenovirus-infected HeLa cells, viral DNA and certain virus-specific polypeptides become associated with the matrix (Deppert, 1978; Chin and

Maizel, 1977; Hodge et al., 1976). The matrix of Tetrahymena macronuclei is reportedly reversibly contractile in response to changes in divalent cation concentration (Wunderlich and Herlan, 1977); this contraction is not dependent on ATP nor inhibited by salyrgan and thus does not appear to be caused by an actin-myosin reaction. In macronuclei with intact membranes contraction is accompanied by changes in lipid fluidity and in the distribution of lipid molecules within the membrane (Wunderlich et al., 1978). The magnitude of the membrane lipid thermal phase transition and the temperature at which it occurs decrease on contraction of the macronucleus. The authors propose that the intermolecular organization of the membrane lipids is modified by interaction with the underlying structural framework of the nucleus. The relevance of the reversible alterations in matrix dimensions observed in vitro to changes in nuclear size occurring in vivo is not clear, nor have these observations been extended to nuclei from other cells.

In summary, there is sufficient evidence to support a reasonable assumption that a structural framework, the matrix, exists in nuclei, but there is not conclusive proof. Additional information on the composition of different matrix preparations is necessary to determine if some components are common to different cell types or different species. The evidence for imvolvement of the matrix in nucleic acid metabolism is largely based on the association of nucleic acid species with the residual structure. The nature of these associations must be demonstrated and the presence of enzymes and/or regulatory factors on the matrix demonstrated if this proposal is to be confirmed. The

methods currently in use to isolate the matrix, employing exposure to high concentrations of salt and extensive digestion with nucleases, will probably not be suitable for such experiments since they are likely to remove the structures and activities in question. The fact that chromatin can be released from nuclei under fairly mild conditions following digestion with micrococcal nuclease (Noll et al., 1975) suggests that this method might be adapted for preparation of chromatin-depleted nuclei. This approach has been utilized in the experiments described in this dissertation. Mouse liver nuclei digested with micrococcal nuclease were separated by density gradient centrifugation into a slowly sedimenting fraction containing chromatin fragments released by digestion and a rapidly sedimenting fraction containing residual nuclei with associated chromatin fragments. The nature of the chromatin associated with the residual nuclei and the stability of the association was investigated. Ionic strength and divalent cations were found to affect the fractionation. Two of the experiments done in the preliminary stages of this research have been included because they contain some previously unreported observations and because they influenced the design of later experiments.

The results are discussed in the context of current models of nuclear structure and chromatin packing. The proposal of a dynamic structural framework within the nucleus raises the possibility of complex supramolecular mechanisms for regulation of nuclear activity. The method described in this dissertation for preparation of nuclear skeletons under relatively mild conditions may be suitable, with some modifications, for analysis of such mechanisms.

CHAPTER 2

CONTAMINATING FIBERS IN PREPARATIONS OF SPLEEN NUCLEI

A. INTRODUCTION

During the course of experiments on the sedimentation properties of mouse cell nuclei digested with micrococcal nuclease, stained nuclei were examined under the light microscope. Fibrous contaminants were observed in preparations of spleen nuclei but were absent from preparations of liver nuclei. In the presence of EDTA, these fibers aggregated with the spleen nuclei to form an intractable, gelatinous mass.

B. METHODS AND MATERIALS

The procedures for preparation of liver and spleen nuclei and for photomicroscopy are described in the appendix.

C. RESULTS

Nuclei of liver or spleen cells formed a turbid suspension when dispersed at 750 μg DNA/ml in 0.25 M sucrose, 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0. On the addition of neutralized EDTA to a final concentration of 2.5 mM, the suspension of spleen nuclei became translucent, with visible aggregates which coalesced upon mixing or standing until the entire sample was incorporated into a clear gelatinous mass. This gel could not be dispersed by vigorous agitation and was incompletely solubilized by sonication for 45 seconds. The appearance of liver nuclei suspensions did not change appreciably on addition of EDTA. Turbidity decreased slightly, no visible aggregates appeared and the

nuclei could be uniformly suspended by gentle shaking after they had completely settled.

Figure 1 shows spleen nuclei before addition of EDTA. Fibers similar to that shown (arrow) were present throughout the preparation. These fibers were not detectable when unstained preparations were examined under phase contrast. Figure 2 shows the changes caused by addition of EDTA. The aggregates shown are relatively small since larger aggregates, though more numerous, were too dense to permit resolution of individual nuclei. Fibers are seen in and around the aggregates, adhering to each other and to the nuclei (arrows). No fibers were observed in any preparation of liver nuclei.

Several attempts were made to separate the fibrous elements from the spleen nuclei by increasing the concentration of Triton X-100 in the wash buffer to 1%, by increasing the number of washes and by sedimenting the nuclei through a layer of 2.3 M sucrose. None of these procedures reduced the number of fibers observed or prevented aggregation of spleen nuclei in the presence of EDTA.

D. DISCUSSION

The fibrous contaminants in preparations of spleen nuclei probably derive from the reticular network of the spleen, a scaffold of fibrous material supporting the reticular cells of the white pulp. Elements similar in appearance are seen in histological specimens of spleen tissue, particularly in silver-stained sections (Weiss, 1966; von Herrath, 1966; Rhodin, 1963). No references to the presence of such fibers in preparations of spleen nuclei could be found in the literature.

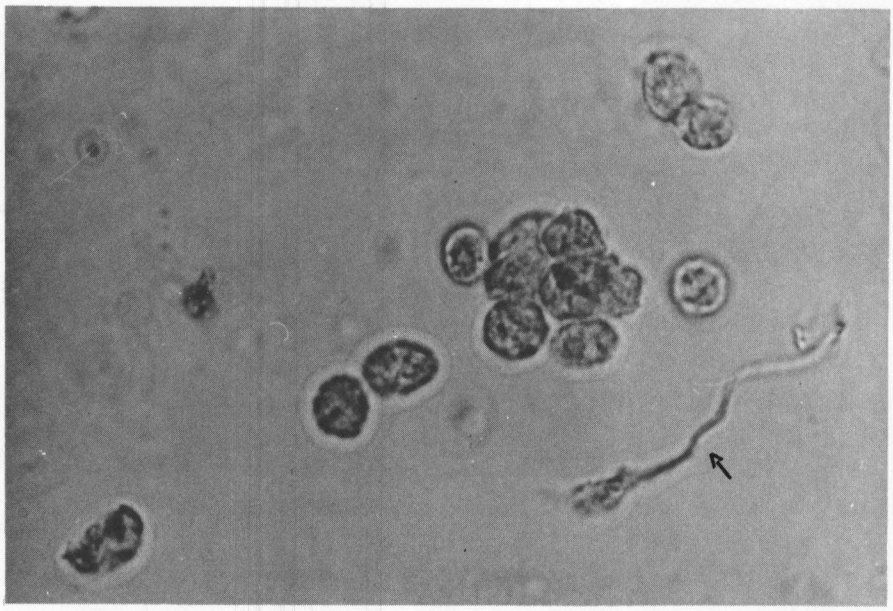

Figure 1. Spleen nuclei in the presence of Ca^{++}. Nuclei suspended in 0.25 M sucrose, 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0, were stained with crystal violet as described in the appendix. The arrow indicates a contaminating fiber (x 938).

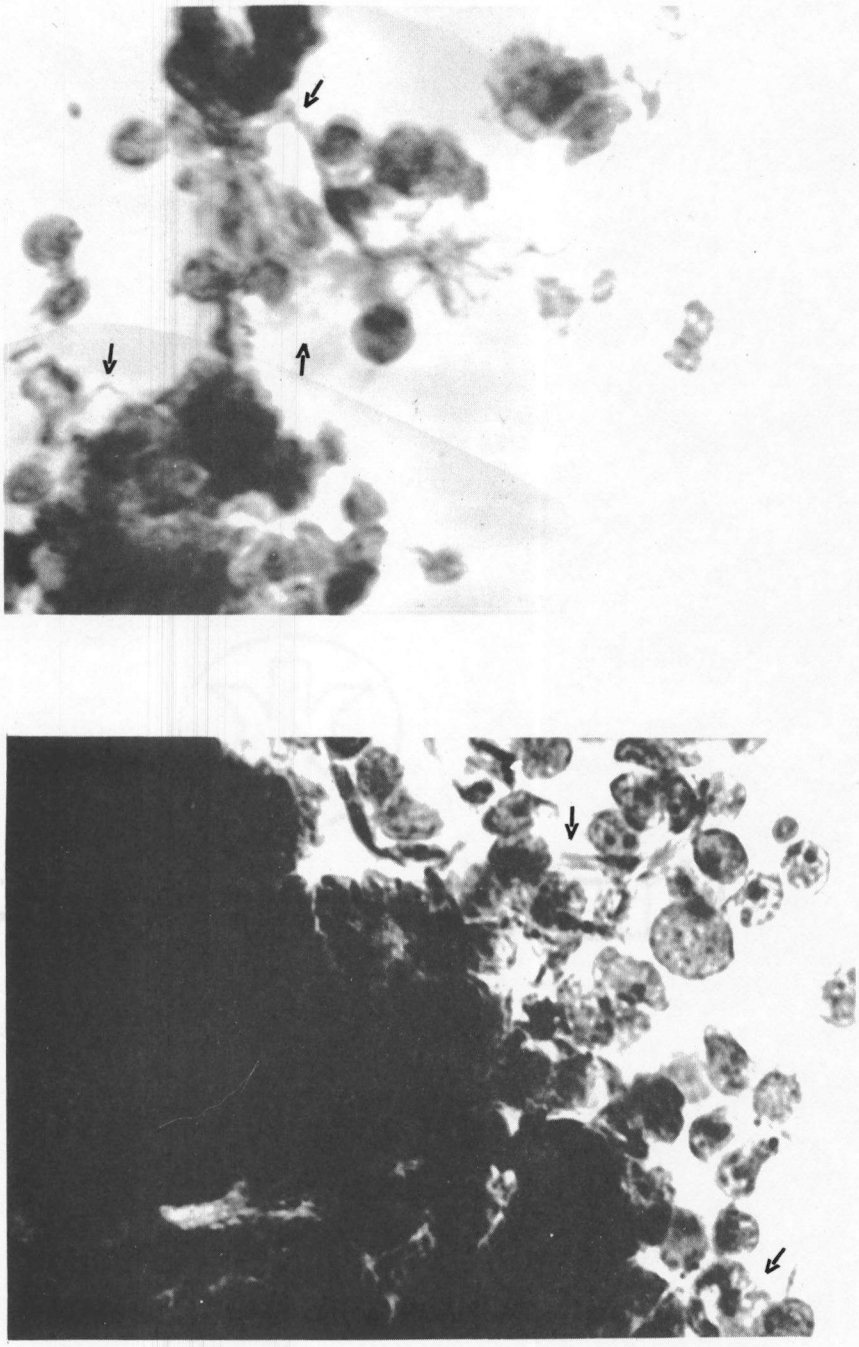

Figure 2. Spleen nuclei in the presence of EDTA. Two photographs of the same preparation of nuclei as shown in Figure 1. EDTA has been added to the nuclear suspension to a final concentration of 2.5 mM. Arrows indicate fibers in the clumps of nuclei (x 938).

The fibers contribute to formation of nuclear aggregates in the presence of EDTA. However, differences in the surfaces of Triton-washed nuclei from liver and spleen could also be important in aggregate formation. Differences in the methods for preparing nuclei from the two tissues cannot be responsible for the observed difference in behavior since spleen nuclei prepared by the methods used for liver nuclei still aggregated on addition of EDTA.

The presence of contaminating fibers does not affect digestion of nuclei by micrococcal nuclease (of necessity occurring in the presence of calcium ions), nor does it affect the position to which nuclei ultimately sediment in sucrose gradients (procedures described in Chapter 4). Information on the rate of sedimentation was not obtained. The existence of large aggregates indicates, however, that all measurements obtained with spleen nuclei or chromatin in the presence of EDTA, particularly measurements of hydrodynamic parameters, should be interpreted with caution.

CHAPTER 3

THE EFFECTS OF PREPARATIVE METHODS ON THE SENSITIVITY OF CHROMATIN TO MICROCOCCAL NUCLEASE

A. INTRODUCTION

Procedures commonly used to prepare chromatin have been shown to alter chromatin structure. Noll, Thomas and Kornberg (1975) first reported that shearing of chromatin in a Virtis homogenizer increased the sensitivity of nucleosomes to micrococcal nuclease, broadening the characteristic pattern of DNA bands and increasing the background between the bands in comparison with digests of nuclei. This is probably the result of changes in the arrangement of chromosomal proteins. Sollner-Webb and Felsenfeld (1975) obtained the same results with chromatin sheared by sonication. Shearing of chromatin increases its ability to bind ethidium bromide (Nicolini et al., 1976), but decreases the cooperativity of the binding (Paoletti et al., 1977), converts some DNA sites previously accessible to micrococcal nuclease into nuclease-resistant form (Block and Cedar, 1976), destroys the "coil of beads" superstructure (de Murcia et al., 1978) and blurs the x-ray diffraction pattern (Sperling and Klug, 1977). Rearrangement of chromatin proteins by shearing has been observed (Maciewicz and Li, 1978; Doenecke and McCarthy, 1976), but the rate of digestion of histones in chromatin is reportedly not affected (Marks and Keller, 1977).

There are conflicting reports on the effects of shearing on the thermal stability and CD spectra of chromatins. Maciewicz and Li

(1978) found only minor effects, except at very high settings. Other investigators report decreases in T_m and increases in molar ellipticity at 276-280 nm (de Murcia et al., 1978; Nicolini et al., 1976; Miller et al., 1976). While the ellipticities reported by Nicolini and coworkers for sheared chromatin are abnormally high, some effect of shearing on the CD spectra and melting properties of chromatin is indicated. On the whole, the evidence demonstrates significant effects of mechanical shearing forces on chromatin structure at the level of the nucleosome.

Buffers of low ionic strength and buffers containing EDTA are frequently used in preparation of chromatin. They cause formation of chromatin gels which expand dramatically as the ionic strength is lowered. This process is frequently called "lysis" of the nuclei, but the term is misused, especially in the case of detergent-washed nuclei. This phenomenon is more accurately described as swelling of the condensed chromatin and nuclear matrix as counterions are removed. The chromatin fibers may be stretched during this expansion, with effects similar to those produced by mechanical shearing forces. Sollner-Webb and Felsenfeld (1975) and Lishanskaya and Mosevitsky (1975) report that the electrophoretic pattern of DNA bands is less distinct after micrococcal nuclease digestion at low ionic strength. Block and Cedar (1976) found that 12% of the sites previously accessible to micrococcal nuclease became resistant after reduction of ionic strength, indicating rearrangement of proteins. Renz and coworkers (1977) demonstrated a change in the mode of binding of histone H1 from cooperative to non-cooperative with decreasing ionic strength, and concurrent loss of associations between nucleosomes in electron micrographs.

Exposure to EDTA causes unfolding of the coiled superstructure of chromatin according to some reports (Vengerov and Popenko, 1977; Finch and Klug, 1976). Coiled superstructure in the presence of EDTA was observed by de Murcia and coworkers (1978), provided the chromatin had not been sheared. Appearance of low melting transitions in thermal melting curves of chromatin exposed to EDTA has also been reported (Vengerov and Popenko, 1977).

In light of these reports, the nuclease sensitivity of chromatins prepared by different procedures was compared by electrophoretic analysis of DNA on polyacrylamide gels.

B. METHODS AND MATERIALS

Nuclei were prepared from mouse spleen as described in the appendix. The nuclei were resuspended at 750 μg DNA/ml in three different media. Type A nuclei were resuspended in 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0, containing 0.25 M sucrose. Type B nuclei were resuspended in the same buffer without sucrose. Type C nuclei were washed twice in each of the following buffers: 75 mM NaCl, 25 mM EDTA, pH 8.0 (saline-EDTA); 50 mM Tris-HCl, pH 8.0; 10 mM Tris-HCl, pH 8.0; 1 mM Tris-HCl, pH 8.0. This is a modification of the procedure of Shaw and Huang (1970). The swollen gel was brought to 750 μg DNA/ml by addition of 1 mM Tris-HCl, pH 8.0, and $CaCl_2$ was added to a final concentration of 1 mM.

Each type of nuclear suspension was prepared in triplicate and three methods were used to solubilize the chromatin. In the first case, digestion with micrococcal nuclease for 5 minutes was carried

out as described in the appendix. Secondly, nuclei were sonicated for 45 seconds at low power as described in the appendix. The third sample was first sonicated and then digested.

The procedures for DNA extraction and analysis on 5% polyacrylamide gels are described in the appendix.

C. RESULTS

Nine chromatin samples were prepared as described in Methods and Materials. Nuclei of Type A or Type B formed a turbid suspension; digestion or sonication increased the amount of time required for the suspensions to settle, but did not much alter their appearance. When nuclei were resuspended in saline-EDTA and collected by centrifugation, a clear gel layer was found coating the inside of the centrifuge tube. On further resuspension in either saline-EDTA or 50 mM Tris-HCl, a large aggregate formed which could not be dispersed by vigorous mixing or homogenization with a hand-held homogenizer. Stepwise reduction of the buffer concentration to 1 mM caused marked swelling of the gel, but it still could not be disaggregated. The gel became opaque and somewhat condensed on addition of $CaCl_2$. Sonication and micrococcal nuclease digestion under the conditions described did not completely solubilize Type C nuclei. Digestion without prior sonication had very little effect and the extracted DNA had a very high viscosity.

The results of electrophoretic analysis of the DNA extracted from the nine different preparations of spleen nuclei are shown in Figure 3. Digestion of unwashed nuclei produced a set of DNA bands corresponding to nucleosome monomers, dimers and trimers (A1, B1),

Figure 3. Polyacrylamide gel electrophoresis of DNA extracted from chromatin samples prepared by different methods. (A) nuclei in sucrose buffer; (B) nuclei in buffer without sucrose; (C) washed nuclei. (1) digested; (2) sonicated; (3) sonicated and digested. The position of mononucleosome DNA in the gels is marked by a vertical arrow in frames A1 and A3. The direction of migration of DNA in the gels is indicated by horizontal arrows under frames C1-3.

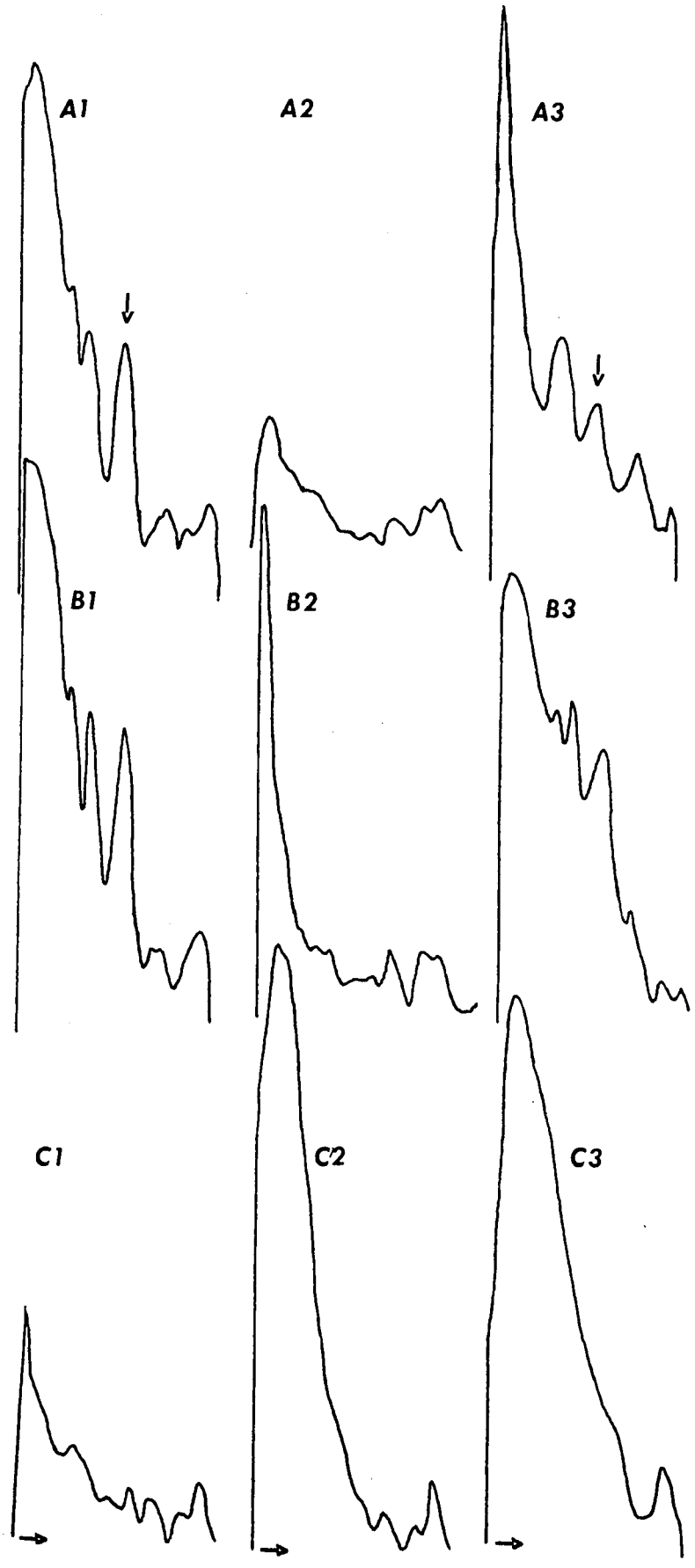

Figure 3.

regardless of the presence of sucrose. No similar bands were produced in washed nuclei (C1). In fact, only a small fraction of the DNA from this sample even entered the gel; accumulated DNA on the top of the gel was frequently observed during staining but became detached during destaining.

Sonication did not produce discrete sizes of DNA in any preparation. Fragmentation of DNA by sonication was more effective in the absence of sucrose (B2) than in its presence (A2), and most effective in the washed chromatin sample (C2). Digestion after sonication produced monomer, dimer and trimer bands in both unwashed preparations (A3, B3), but the background between the bands is high compared to the unsonicated nuclei (A1, B1). Digestion after sonication of the washed chromatin further reduced the size of the DNA fragments, but did not produce discrete bands (C3).

D. DISCUSSION

The results demonstrate that both sonication and washing procedures alter chromatin structure as monitored by sensitivity to micrococcal nuclease. These effects are cumulative, since chromatin subjected to both treatments is more severely damaged than sonicated chromatin.

The washing procedure removes divalent cations, yielding a viscous gel which resists digestion with micrococcal nuclease after readdition of calcium ions. If the added calcium ions were largely bound to sites on the divalent-cation-depleted chromatin so that an insufficient amount remained free for use by the enzyme, digestion

would be largely inhibited. In one experiment, additional $CaCl_2$ was added to the washed nuclei to a final concentration of 5 mM without altering the results. Therefore it seems likely that the comparative resistance of the washed chromatin is due to its highly aggregated state. If the gel is not permeable to the enzyme, digestion will be confined to the chromatin exposed at the surface. Burgoyne and co-workers (1978) have shown that in isolated chromatin 87% of the penetrable space is not accessible to molecules of the size of micrococcal nuclease, in contrast to nuclei where only 58% of the penetrable space is not accessible to the enzyme. This indicates that gross features of nuclear structure are destroyed by the extraction procedure, resulting in tighter packing of the chromatin fibers. Sonication fragments the gel, increasing the area available to the enzyme. But the structure is sufficiently altered by the combination of treatments that nucleosomal core DNA is also accessible.

This experiment demonstrates modest effects of brief sonication and marked effects of extensive washing on the structure of chromatin. The greater effectiveness of sonication in disrupting nuclei in the absence of sucrose is probably a consequence of the increased viscosity of the sucrose solution. Dissolution of the nuclear membrane by Triton X-100 during preparation eliminates the possibility of osmotic effects. The washing procedure has two components, the saline-EDTA extraction and the stepwise reduction in ionic strength. Since all the preparations were suspended at the same low ionic strength prior to digestion, the difference between Type A and Type C nuclei cannot be attributed solely to reduction of ionic strength. The most obvious

change in physical state occurs on exposure to saline-EDTA, but stretching of the chromatin fibers during subsequent swelling may produce significant changes in structure at the molecular level. Further experiments on the effects of ionic strength and divalent cations are reported in Chapter 5.

CHAPTER 4

SEDIMENTATION OF NUCLEI DIGESTED WITH MICROCOCCAL NUCLEASE THROUGH STEEP SUCROSE GRADIENTS

A. INTRODUCTION

Frenster and coworkers (1963) first described fractionation of sheared chromatin by differential sedimentation. Association of rapidly labeled RNA with the slowly sedimenting fraction and its euchromatic, fibrillar character, in comparison with the condensed, heterochromatic masses seen in the rapidly sedimenting fraction, led them to conclude that transcriptionally active chromatin segments were contained in the more slowly sedimenting fraction.

Sedimentation in sucrose density gradients was first used for chromatin fractionation by Chalkley and Jensen (1968). They did not achieve separation into distinct peaks, probably because of extensive shearing of the nuclei with a Virtis homogenizer, but they did find that fractions from the upper part of the gradient had higher template activity with bacterial RNA polymerase than fractions from the lower part of the gradient. Milder shearing of chromatin, using a variety of methods, has increased the resolution of density gradient fractionation, and many investigators have reported separation of chromatin into two distinct peaks (Paul and Duerksen, 1976; Webster et al., 1976; Rodriguez and Becker, 1976; Anderson et al., 1975; Berkowitz and Doty, 1975; Charles et al., 1975; Magee et al., 1975; Murphy et al., 1973; Duerksen and McCarthy, 1971; Dolbeare and Koenig, 1970). Minor differences in the degree of resolution and distribution of chromatin

between the peaks are attributable to variations in procedures for preparation and sedimentation of the chromatin.

Common to all these reports is the conclusion that active chromatin is preferentially released into the slowly sedimenting fraction. In all but two cases, the criteria for determining chromatin activity were (1) association with labeled nascent RNA and/or (2) template activity with bacterial or mammalian polymerases. Duerksen and McCarthy (1971) and Dolbeare and Koenig (1970) used deficiency of satellite DNA sequences as their criterion and are consequently less definite regarding the localization of active sequences. Serious doubt has been cast on the validity of the first two criteria. Artifactual association of labeled exogenous RNA with slowly sedimenting chromatin has been demonstrated by Seidman and Cole (1977), and the capacity of bacterial RNA polymerase to transcribe endogenous RNA obscures the template activity of the chromatin DNA (Zasloff and Felsenfeld, 1977a,b; Giesecke et al., 1977). Moreover, hybridization experiments using probes for expressed sequences have failed to detect enrichment of active genes in slowly sedimenting chromatin fractions (Krieg and Wells, 1976; Howk et al., 1975).

Thus, identification of slowly sedimenting chromatin with active chromatin is doubtful. The basis for fractionation of chromatin in sucrose gradients is an open question. This chapter describes a clean fractionation of chromatin from micrococcal nuclease digested liver nuclei into two distinct peaks by sedimentation through steep sucrose gradients, and provides evidence that the rapidly sedimenting chromatin cosediments with dense nuclear structures to which it is attached.

B. METHODS AND MATERIALS

The following procedures are described in the appendix:

1. Isolation of nuclei from mouse liver.

2. Digestion of nuclei with micrococcal nuclease and measurement of the acid-soluble products of digestion.

3. Sucrose density gradient centrifugation and gradient fraction.

4. Sonication of nuclei.

5. Determination of the DNA and protein concentrations in chromatin fractions.

6. Extraction of DNA from chromatin fractions and estimation of DNA size on 5% polyacrylamide gels.

7. Photomicroscopy.

8. Polyacrylamide slab gel electrophoresis of chromatin proteins.

C. RESULTS

The conversion of DNA to acid-soluble products by micrococcal nuclease is shown in Figure 4. The reaction is complete after two hours, when half of the DNA has become acid soluble. The shape of the curve is similar to those published for reactions at much higher ratios of enzyme to DNA (Noll and Kornberg, 1976; Axel, 1975).

Nuclei digested for 1, 5, 10, 30, 60, or 120 minutes were centrifuged through 5-65% linear sucrose gradients and the results are shown in Figure 5. Two distinct peaks of chromatin were resolved at all stages of digestion, in addition to the small digestion products

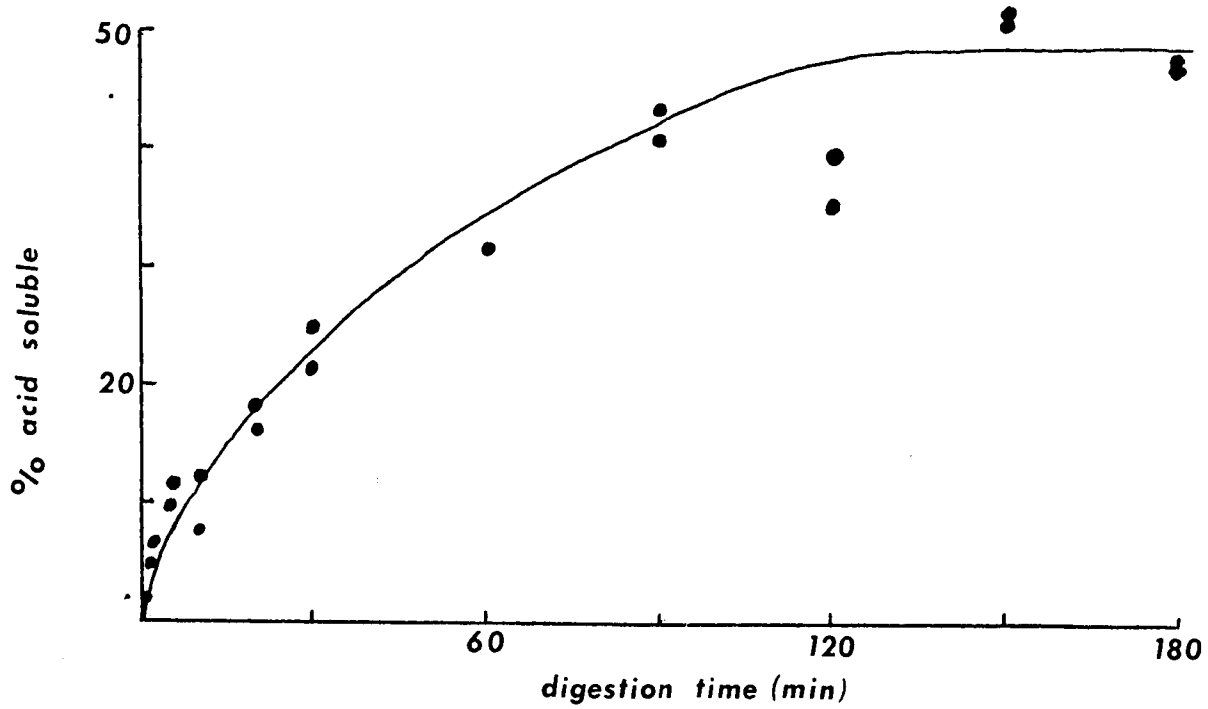

Figure 4. Kinetics of digestion of mouse liver nuclei by micrococcal nuclease. Digestions were performed as described in the appendix for periods of 1-180 minutes. Each point represents the average of two digestions in the same experiment; the total volume of each digestion mixture was 1.0 ml. The method for determination of acid soluble DNA is described in the appendix.

Figure 5. Absorbance profiles of 5-65% sucrose gradients containing nuclei digested for different intervals. Digestion and centrifugation was carried out as described in the appendix. Digestion times are indicated for each gradient. The position of the slowly sedimenting peak (1) and the rapidly sedimenting peak (2) are indicated at the top of the figure.

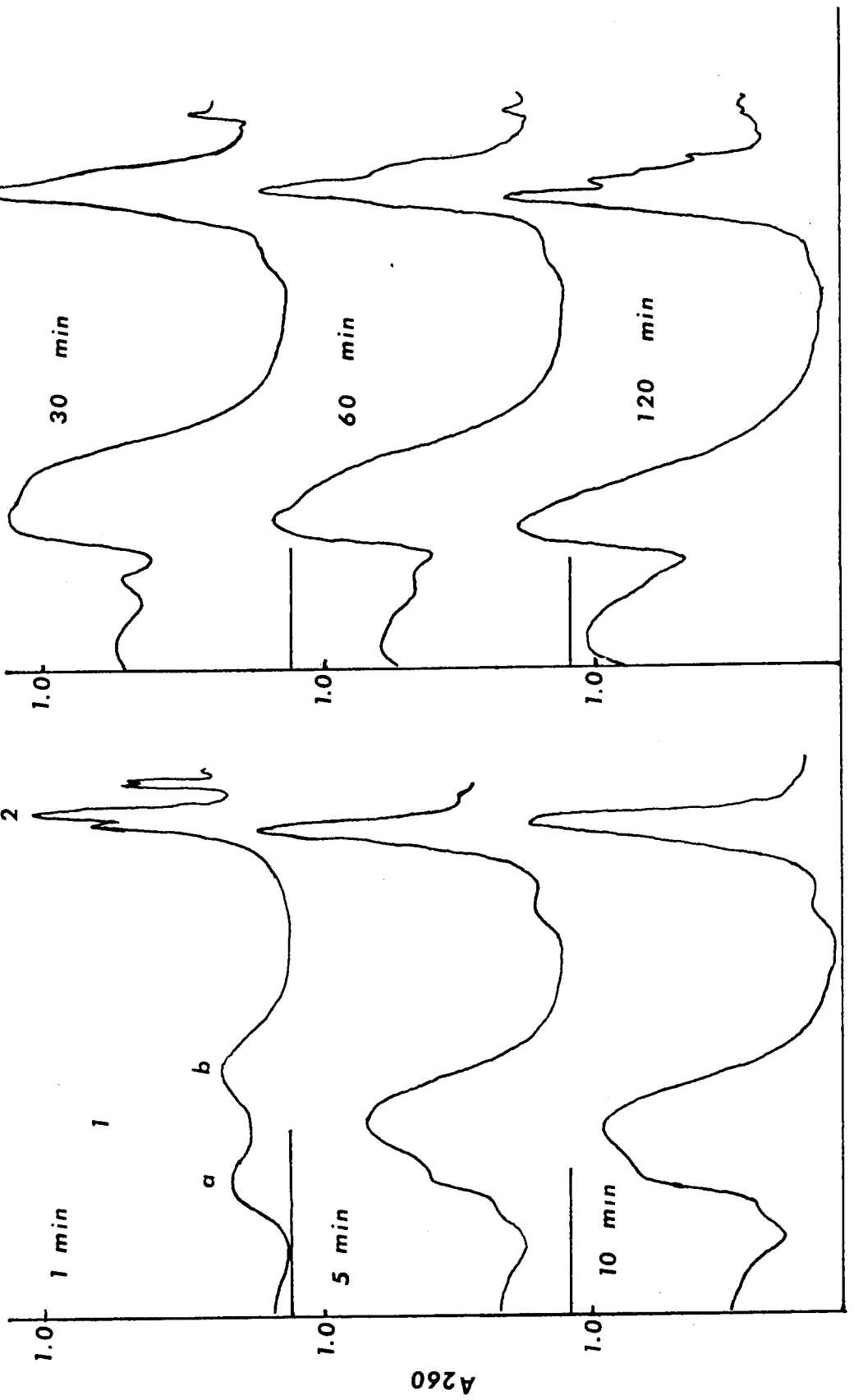
1 min
5 min
10 min
30 min
60 min
120 min
1.0
1
2
a
b
A260
sedimentation →

Figure 5.

remaining at the top of the gradient. The slowly sedimenting peak (Peak 1) was clearly a product of digestion. It was not observed in gradients containing untreated or sonicated nuclei, as seen in Figure 6. After one minute of reaction, Peak 1 contained two components, 1a and 1b. With continuing digestion, component 1b increased more rapidly in amount but decreased gradually in sedimentation rate until, when digestion was complete, all of Peak 1 was found at the position of component 1a. This peak contained nucleosome monomers (vide infra). The position of the rapidly sedimenting Peak 2 did not change throughout the course of the reaction.

The material in Peak 2 was not in solution. It was visible in the gradient tube as a sharp, opaque band at the interface between the gradient and the underlying cushion of 70% sucrose. Consequently, the absorbance tracings do not accurately reflect the relative amounts of DNA in the two peaks. To quantitate the fractionation, therefore, fractions under each peak were pooled, and the DNA and protein concentrations were determined spectrophotometrically. The results are shown in Table II. The total amount of material in Peak 2 after one or five minutes of digestion was underestimated because Peak 2 from brief digestions adhered tenaciously to the sides of siliconized centrifuge tubes. Recovery of one minute digests from the gradients was 61-65% of A_{260} units applied, compared to 77-80% for ten minute digests and 85-96% for longer digestion times. The Peak 1 fraction from the one minute digestion contained a higher proportion of protein than the same fraction after longer digestion. When digestion was complete, 15% of the nuclear DNA and 40% of the nuclear protein remained in the rapidly sedimenting peak.

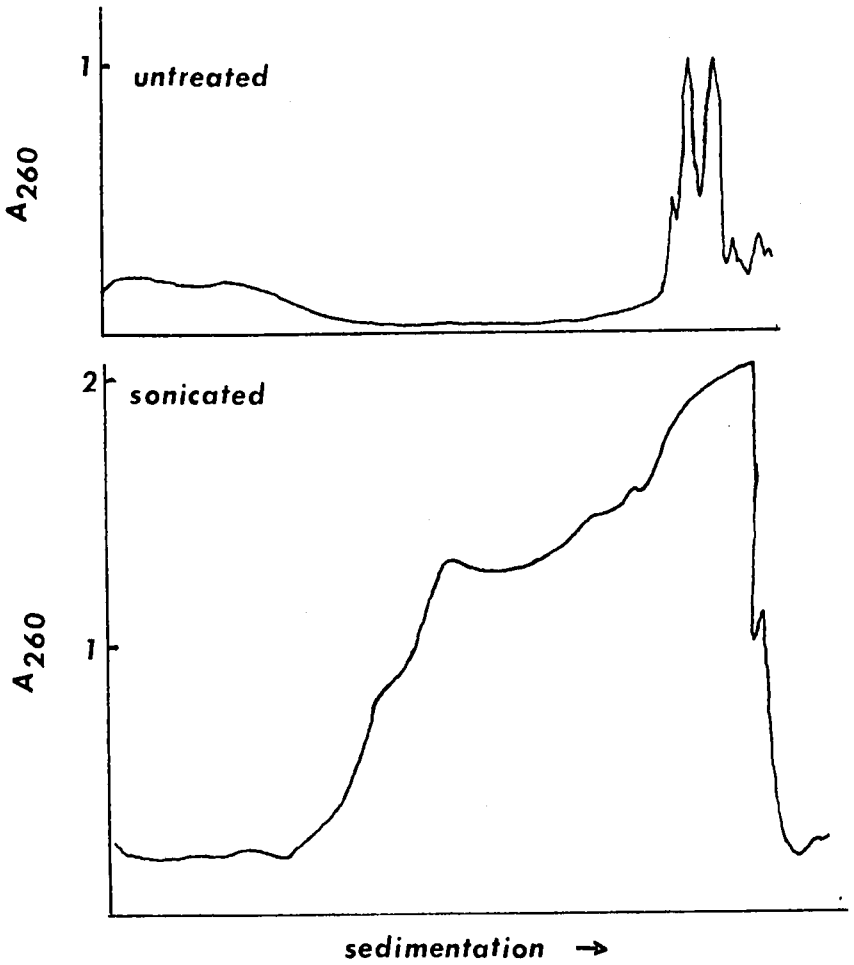

Figure 6. Sucrose gradient profiles of untreated control nuclei and sonicated nuclei. Untreated nuclei were incubated at 37° for 10 minutes without micrococcal nuclease. The sonication procedure is described in the appendix. The 5-65% sucrose gradients used were identical to those used with digested nuclei.

TABLE II. DISTRIBUTION OF DNA AND PROTEIN BETWEEN PEAK 1 AND PEAK 2 FRACTIONS FROM SUCROSE DENSITY GRADIENTS

Peak	Digestion time (min)	DNA (μg)[a]	% Total DNA[b]	Protein (μg)[a]	% Total Protein[b]	Protein / DNA
1	1	7.2	14.4	29.7	30.6	4.1
	5	14.0	28.0	25.6	26.4	1.8
	10	20.8	41.6	34.3	35.1	1.6
	30	33.0	66.0	38.9	40.1	1.2
	60	34.3	68.6	45.6	47.0	1.3
	120	32.4	64.8	39.4	40.6	1.2
2	1	22.6[c]	45.2[c]	46.4[c]	47.8[c]	2.1
	5	14.1[c]	28.2[c]	34.5[c]	35.5[c]	2.4
	10	15.6	31.2	44.5	45.9	2.9
	30	12.9	25.8	43.4	44.7	3.4
	60	11.3	22.6	44.3	45.6	3.9
	120	7.9	15.8	33.8	34.8	4.3

[a]Each value represents the mean of at least 3 determinations.

[b]Based on the content of the gradient.

[c]Underestimated due to losses during fractionation.

The fractions under each peak were pooled, dialyzed to remove sucrose and resedimented through 5-65% sucrose gradients to verify the difference in sedimentation rate between the two peaks. As shown in Figure 7, Peak 1 chromatin resedimented to its original position. Most of the Peak 2 chromatin remained rapidly sedimenting after dialysis, but a minor fraction was found at the position of Peak 1.

The sizes of the DNA fragments extracted from Peaks 1 and 2 were estimated by electrophoresis in 5% polyacrylamide gels. Scans of stained gels are shown in Figure 8 for Peak 1 fractions and in Figure 9 for Peak 2 fractions. A significant fraction of the DNA in the Peak 2 fractions from 1 minute and 5 minute digestions was too large to enter the gels. Mononucleosome- and oligonucleosome-size DNA appeared more rapidly in Peak 1 than in Peak 2. Few fragments of the size of dimers and trimers were seen in Peak 2 at any stage. At the end of digestion, nearly all the DNA in both fractions was monomer size or smaller. Thus, the difference in sedimentation rate did not result from difference in the size of the DNA fragments in the two fractions.

Photomicrographs of unfixed wet mounts of Peak 2 fractions stained with crystal violet are shown in Figure 10. Undigested nuclei (shown in Figure 11) have sharply defined perimeter. After one minute of digestion they have begun to ravel at the edges (Figure 10A, arrows) and after five minutes, fibrous material was seen extending outward from the surface in a profuse tangle (Figure 10B). This extruded material disappeared as digestion continued (Figure 10C-D), but the nuclei remained intact and, except that they stained less intensely,

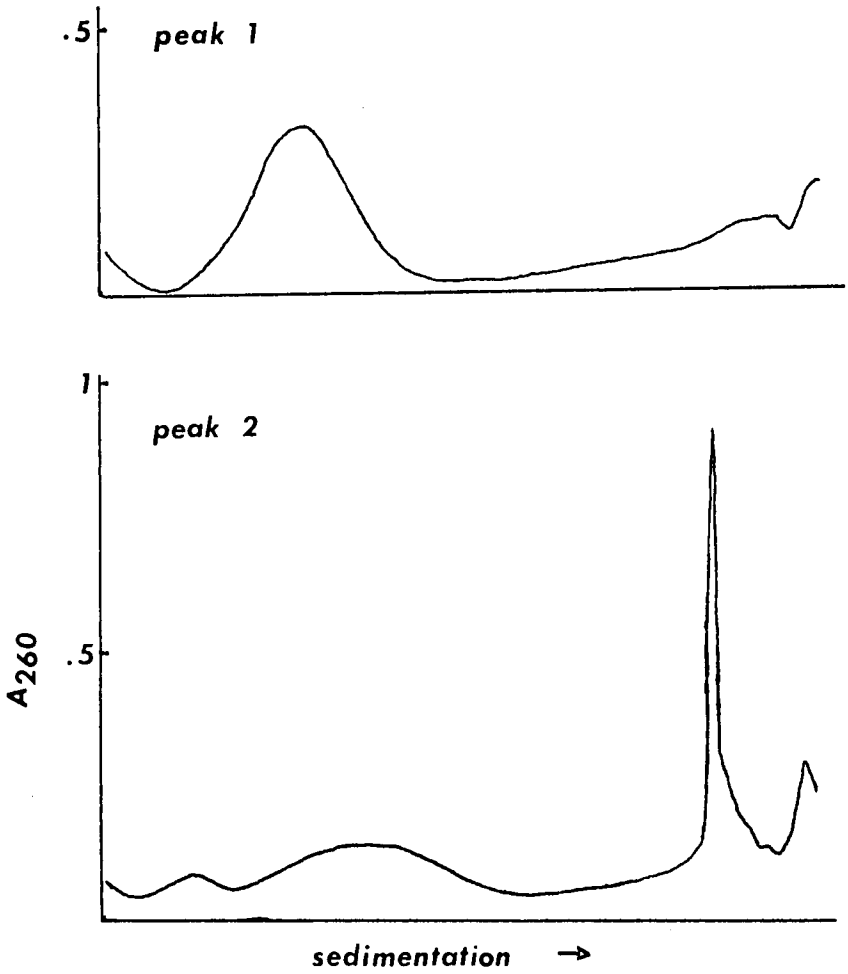

Figure 7. Resedimentation of Peak 1 and Peak 2 fractions. The Peak 1 and Peak 2 gradient fractions of nuclei digested for 5 minutes with micrococcal nuclease were dialyzed for 6 hours against 1 mM EDTA, 1 mM Tris-HCl, pH 8.0, and recentrifuged through 5-65% sucrose gradients.

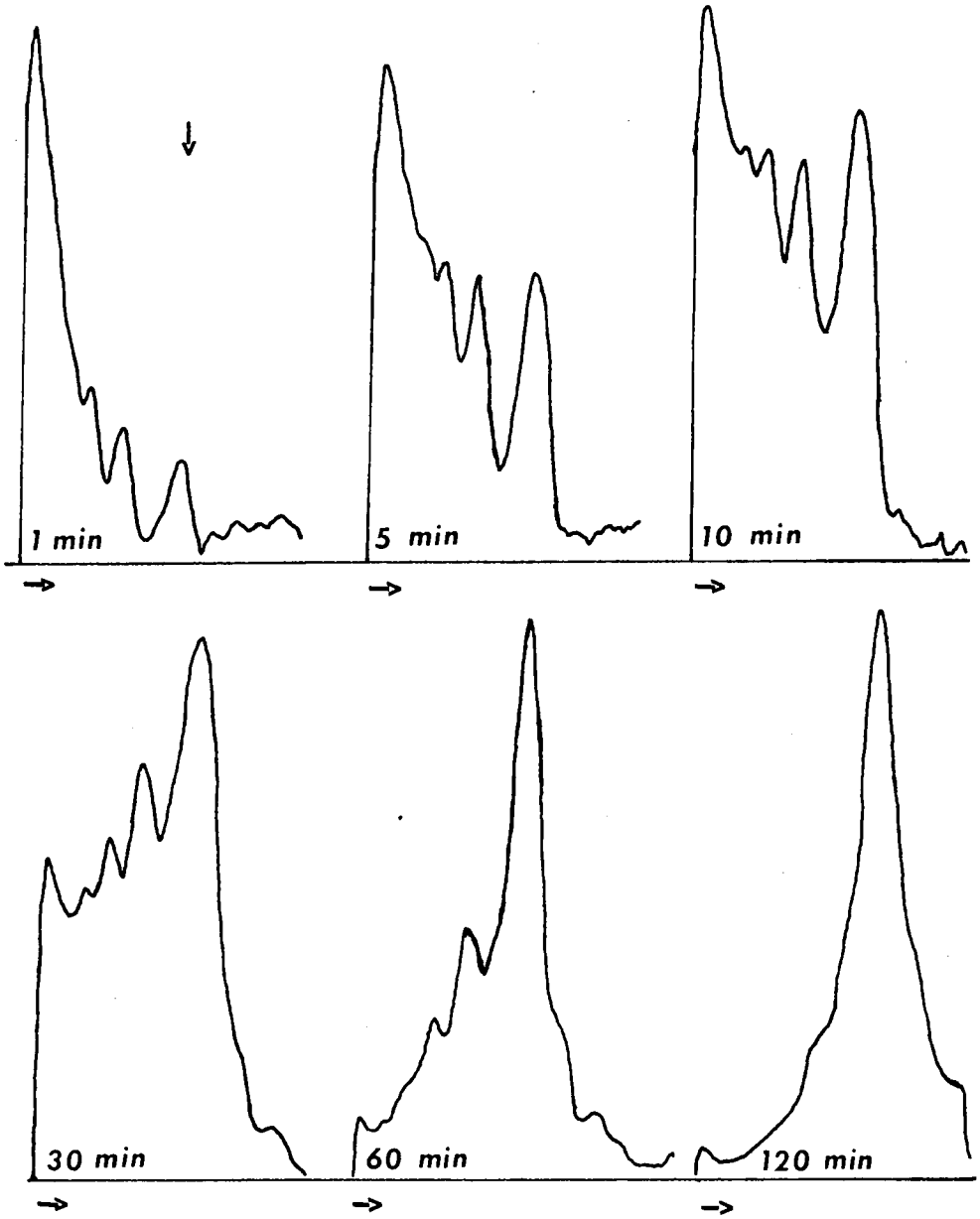

Figure 8. Polyacrylamide gel electrophoresis of DNA from Peak 1 fractions. Digestion time is indicated for each gel tracing. The position of mononucleosome DNA in the 1 minute digest is shown by a vertical arrow. The size of the mononucleosome DNA decreases during the course of digestion. The direction of migration of DNA in the gels is indicated by the horizontal arrows.

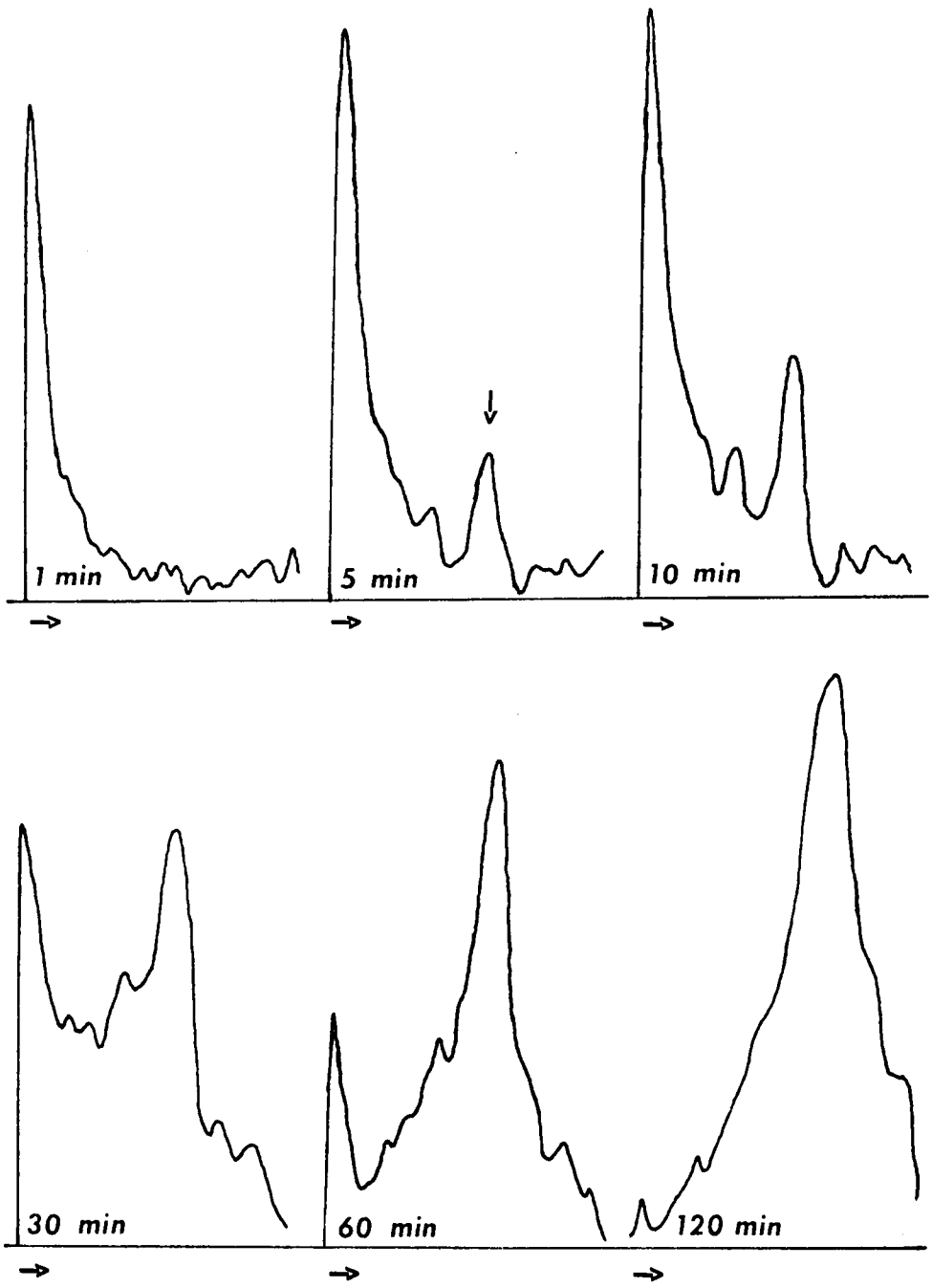

Figure 9. Polyacrylamide gel electrophoresis of DNA from Peak 2 fractions. Digestion time is indicated for each gel tracing. The position of mononucleosome DNA in the 5 minute digest is indicated by a vertical arrow. The size of the mononucleosome DNA decreases during the course of digestion. The direction of migration of DNA in the gels is shown by the horizontal arrows.

Figure 10. Photomicrographs of unfixed Peak 2 fractions. Wet mounts of Peak 2 fractions were prepared and stained with crystal violet as described in the appendix. (A) 1 minute digest. Arrows indicate points where the nuclear periphery is no longer clearly resolved (x 1027); (B) 5 minute digest (x 1094); (C) 10 minute digest (x 1094); (D) 30 minute digest (x 1146); (E) 60 minute digest (x 1094); (F) 120 minute digest (x 1000).

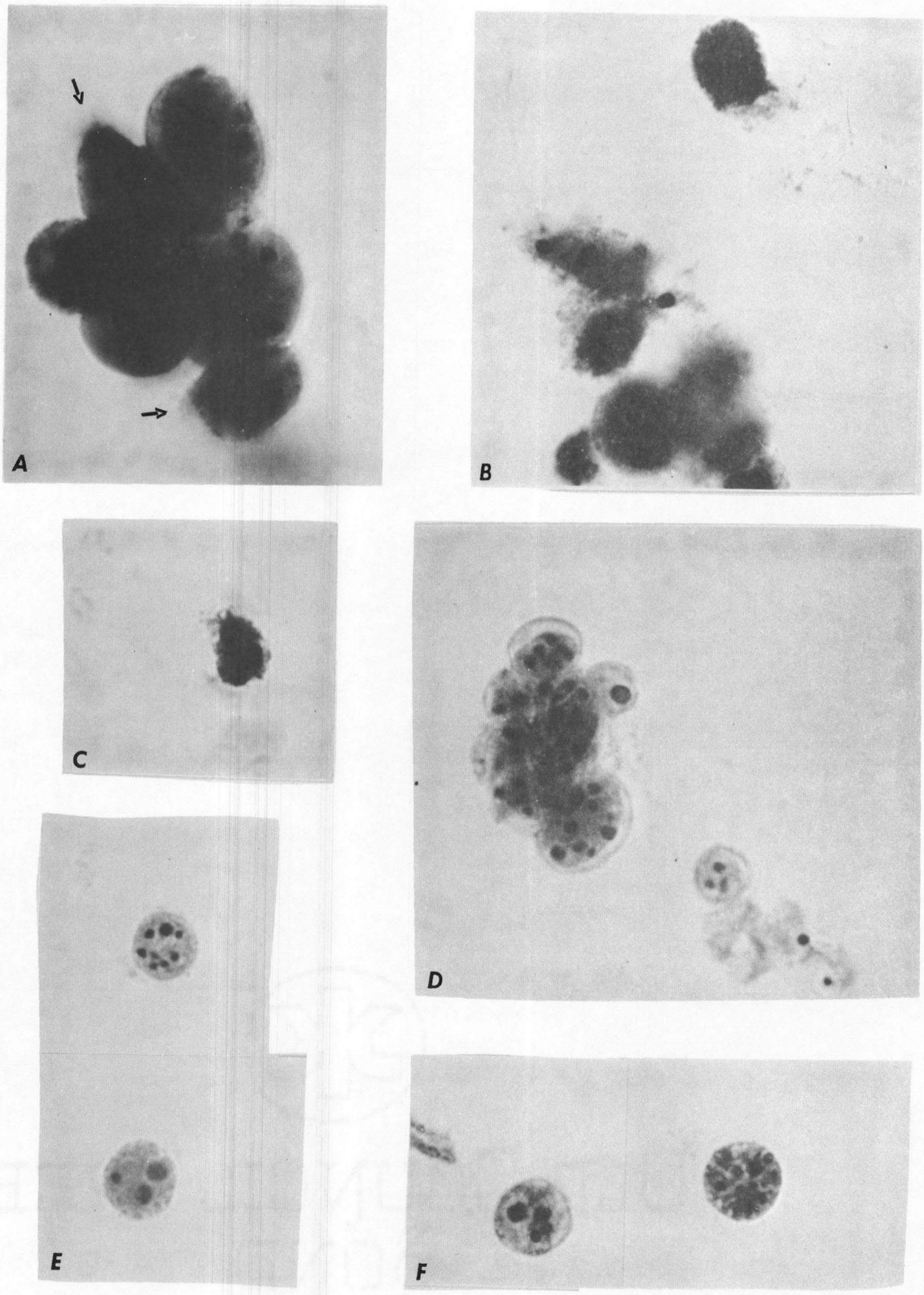

Figure 10.

Figure 11. Photomicrographs of Peak 2 fractions stained with May Grunwald-Giemsa stain. Slides were prepared and stained as described in the appendix. (A) undigested; (B) 1 minute digest; (C) 5 minute digest; (D) 10 minute digest; (E) and (F) 120 minute digest. All x 1000.

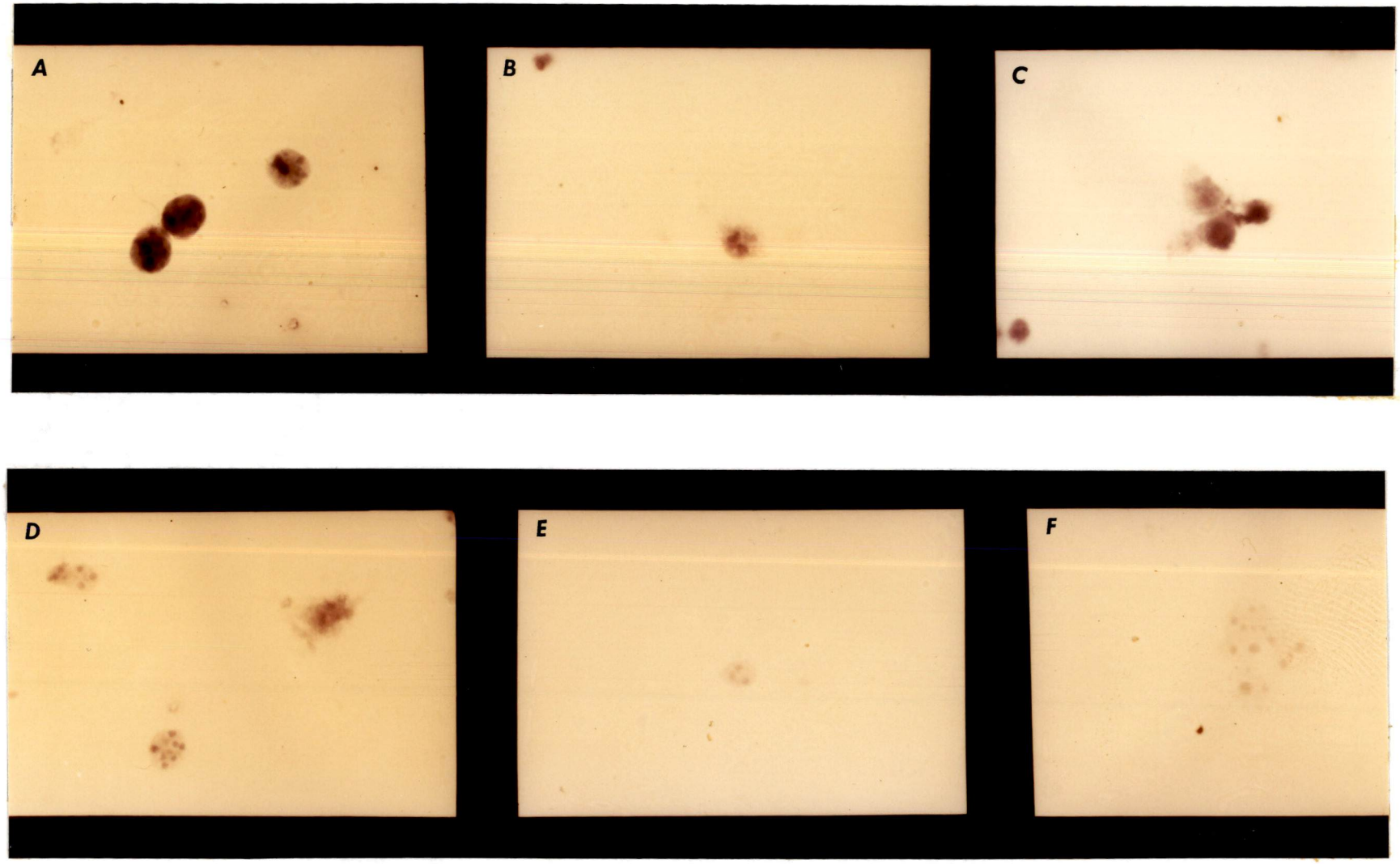

Figure 11.

unchanged in appearance (Figure 10E-F). It should be emphasized that these preparations were neither dried nor fixed, so that the possibility of artifacts was reduced. Peak 1 contained no structures visible in the light microscope at any stage of digestion.

Color photomicrographs of dried and fixed Peak 2 fractions stained with May Grunwald-Giemsa stain are shown in Figure 11. This procedure stains ribonucleoproteins blue, deoxyribonucleoproteins pink. The halo of fibrous material produced after brief digestion is clearly seen to be largely deoxyribonucleoprotein. After complete digestion, the nucleoplasm stains faintly, but the stain is clearly pink and uniformly distributed. Although these are whole mounts, not sections, localization of the residual chromatin at the periphery or around the nucleoli would be noticeable if present. No differences in stain intensity were detected.

The nature of the association of DNA with the residual nuclei in Peak 2 after complete digestion was investigated by attempting to dissociate the DNA from the nuclei. Digested nuclei were dialyzed for 48 hours against 1 mM EDTA, 1 mM Tris-HCl, pH 8.0 (EDTA-Tris) to allow free nucleosomes within the nuclear matrix to diffuse out. The dialyzed nuclei were then sedimented through 5-65% sucrose gradients. The resulting gradient profile is very similar to that of the undialyzed digest (Figure 12). Peak 2 contains 12.5% of the total DNA in the dialyzed digest, 15% of the DNA in the undialyzed digest.

Physical dissociation of the residual DNA by NaCl or urea was investigated in the same manner. Nuclei digested for 120 minutes were dialyzed 48 hours against 2 M NaCl in EDTA-Tris, against 5 M urea in

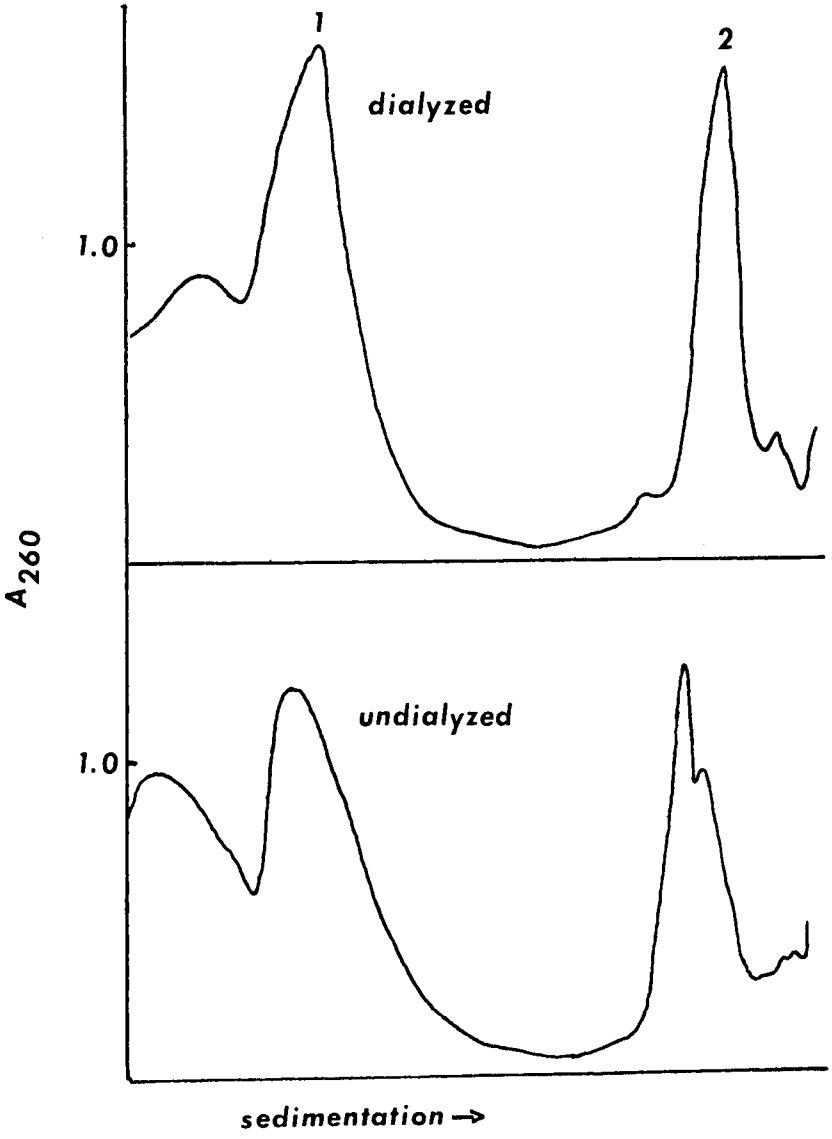

Figure 12. Sucrose gradient profiles of dialyzed and undialyzed samples of digested nuclei. The nuclei were digested for 120 minutes as described in the appendix and then dialyzed for 48 hours against 1000 volumes of 1 mM Tris-HCl, 1 mM EDTA, pH 8.0. The dialyzed digestion mixture was centrifuged through a 5-65% sucrose gradient as previously described. The gradient profile of the undialyzed 120 minute digest from Figure 5 (p 43) is shown for comparison.

EDTA-Tris or against 2 M NaCl, 5 M urea in EDTA-Tris, before sedimentation through gradients containing the same dissociating agents. The gradient profiles are shown in Figure 13. Either 2 M salt or 5 M urea caused Peak 1 to disappear, but both salt and urea were necessary for dissociation of Peak 2. Peak 2 contained 10.4% of the total DNA in the salt gradient and 12.4% of the total DNA in the urea gradient. Complete dissociation of DNA from Peak 2 was also obtained with SDS. A suspension of nuclei digested for 120 minutes was made 0.5% SDS and sedimented through a gradient containing 0.5% SDS. The gradient profile is shown in Figure 14.

The protein composition of Peaks 1 and 2 at different stages of digestion was investigated by electrophoresis in 15% polyacrylamide microslab gels. Nucleosome core histones were the major protein constituents of Peak 1 fractions except at the shortest time sampled (Figure 15). A trace of histone H1 was present in the early samples but disappeared as digestion continued. A nonhistone protein band of 70,000 daltons was the most prominent protein component of the one minute digest, but was not detectable at later times.

Core histones were also the predominant proteins in the Peak 2 fractions (Figure 16). The histone content of this fraction, however, decreased during digestion. Concurrently, a series of faint, non-histone bands became evident, two migrating between 43,000 and 50,000 daltons, another two or three between 60,000 and 70,000 daltons and a third set of at least three bands of greater than 155,000 daltons. As was found for DNA, some core histones remained in the nuclei when digestion was complete.

Figure 13. Absorbance profiles of digested nuclei sedimented through dissociating gradients. Nuclei digested for 120 minutes were dialyzed for 48 hours against 1000 volumes of EDTA-Tris containing 2 M NaCl or 5 M urea or the combination of 2 M NaCl and 5 M urea. The dialyzed digestion mixtures were then centrifuged as described through sucrose gradients containing the same dissociating agents. The small peak appearing just above Peak 2 in the 5 M urea gradient contained broken nuclei.

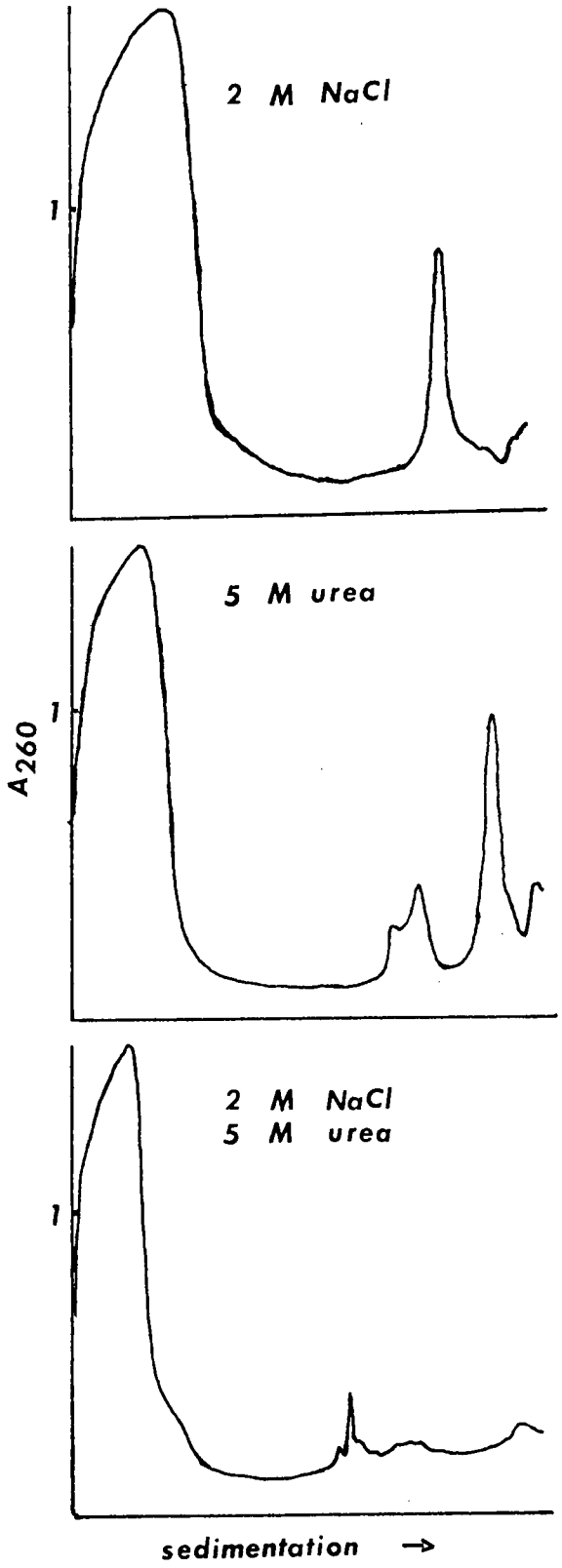

Figure 13.

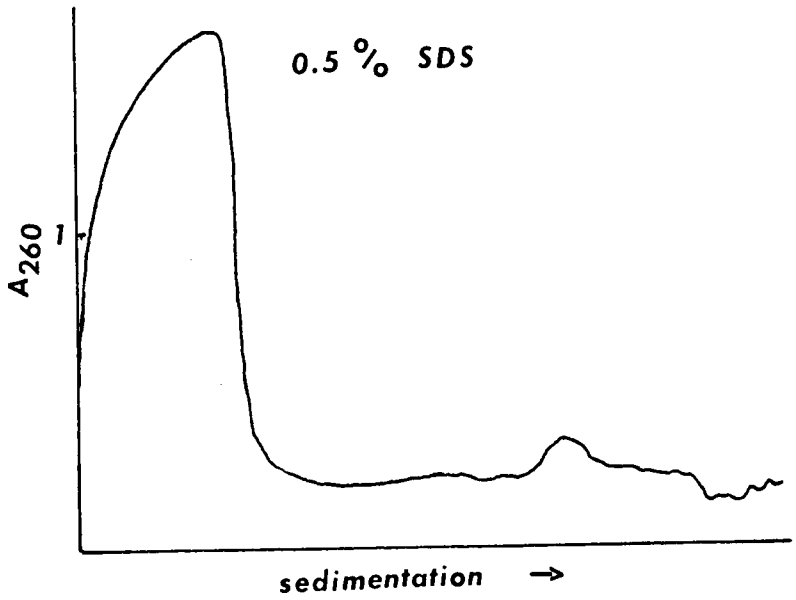

Figure 14. Sucrose gradient profile of digested nuclei treated with 0.5% SDS. Nuclei were digested as described in the appendix. The digestion mixture was then made 0.5% with respect to SDS and incubated on ice for 30 minutes before centrifugation through a 5-65% sucrose gradient containing 0.5% SDS.

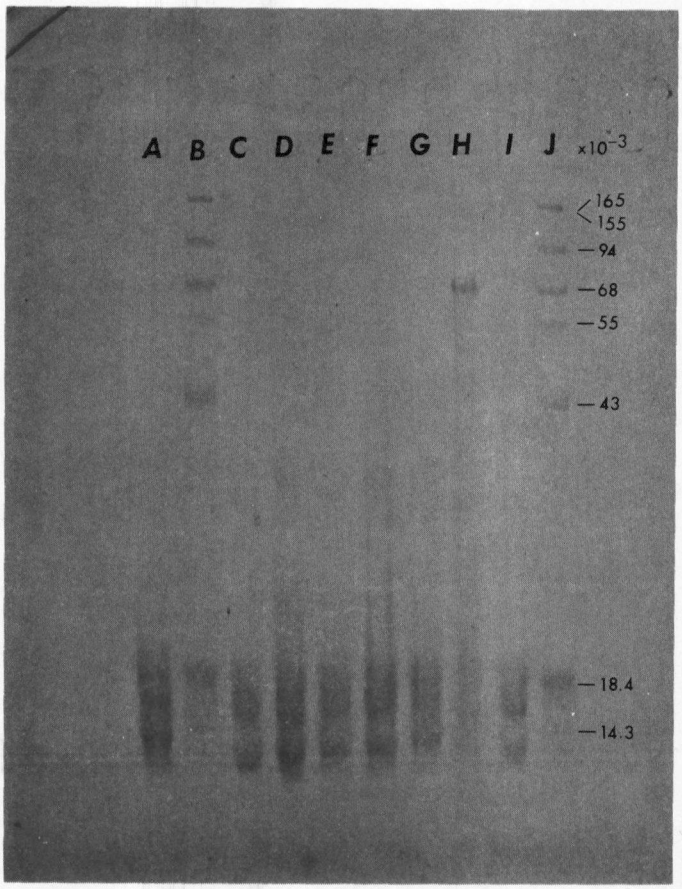

Figure 15. Electrophoresis of Peak 1 fractions on a 15% polyacrylamide SDS microslab gel. (A) 120 minute digest; (B) protein standards; (C) calf thymus histones; (D) 60 minute digest; (E) 30 minute digest; (F) 10 minute digest; (G) 5 minute digest; (H) 1 minute digest; (I) calf thymus histones; (J) protein standards. The molecular weights of the standards are listed at the side of the gel; from top to bottom, E. coli RNA polymerase β and β' subunits, phosphorylase A, bovine serum albumin, E. coli RNA polymerase α subunit, ovalbumin, β-lactoglobulin, and lysozyme.

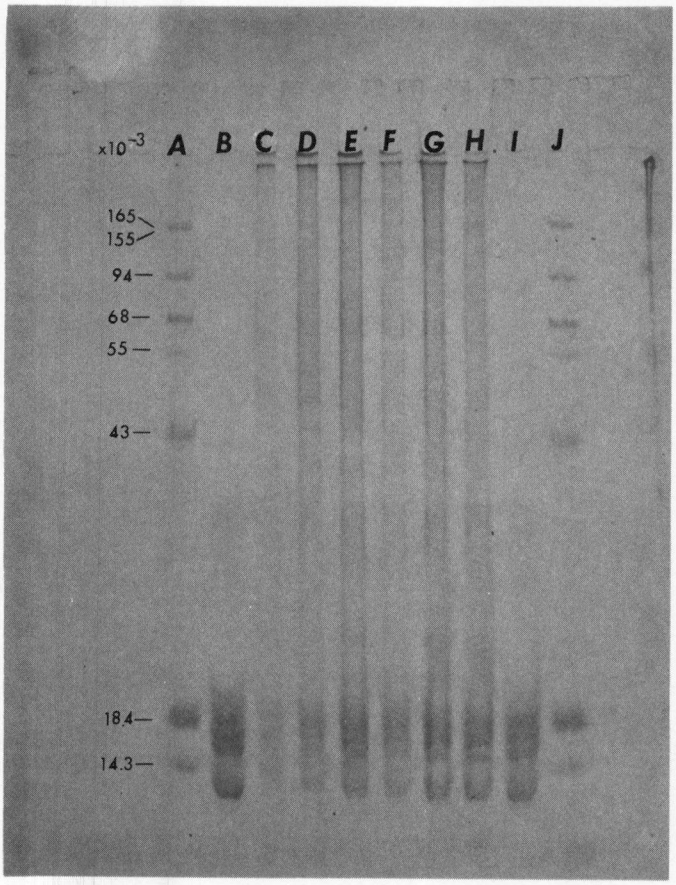

Figure 16. Electrophoresis of Peak 2 fractions on a 15% polyacrylamide SDS microslab gel. (A) protein standards; (B) calf thymus histones; (C) 120 minute digest; (D) 60 minute digest; (E) 30 minute digest; (F) 10 minute digest; (G) 5 minute digest; (H) 1 minute digest; (I) calf thymus histones; (J) protein standards. Molecular weights of the standards are listed at the side of the gel; from top to bottom, E. coli RNA polymerase β and β' subunits, phosphorylase A, bovine serum albumin, E. coli RNA polymerase α subunit, ovalbumin, β-lactoglobulin and lysozyme.

D. DISCUSSION

In contrast to mechanically sheared nuclei, nuclei digested with micrococcal nuclease separated cleanly into two fractions in steep sucrose gradients. Other investigators have reported somewhat better resolution of sonicated chromatin in gradients than reported in this work (Rodriguez and Becker, 1976; Anderson et al., 1975; Murphy et al., 1973), but none have achieved the degree of resolution shown for digested nuclei. Moreover, this fractionation was not dependent on the extent of treatment as with mechanically sheared preparations. Peaks 1 and 2 were easily separable after one minute of digestion (1% of the DNA acid soluble) and after digestion was complete (50% of the DNA acid soluble). The fractionation was not due to aggregation/dissociation phenomena as shown in the resedimentation experiments, nor to separation based on DNA size. The photomicrographs show that Peak 2 chromatin is associated with a rapidly sedimenting structure; It is not intrinsically larger or more dense than slowly sedimenting chromatin. At early stages of digestion, the matrix-bound chromatin was visible, spilling out of the nuclei but still attached. This halo was probably formed by unwinding of supercoiled chromatin fibers following cleavage by the nuclease. Evidence will be presented in Chapter 5 that this unwinding does not occur until divalent cations in the digestion mixture are sequestered by addition of EDTA. After longer digestion, most of the chromatin was trimmed away from the nuclei, but small fragments remained bound at the points of attachment when digestion was complete.

The observation that DNA remains with digested nuclei is not

novel. Preparations of nuclear matrix generally contain a small amount of DNA. Using DNase I and salt to remove most of the DNA, Berezney and Coffey (1974) report 3% of the DNA remaining with the matrix. Comings and Okada found less than 1%, Miller and coworkers (1978a) 1% and Herman and coworkers (1976) 4%. Since nucleosomes are not stable under these digestion conditions (high salt), the amount of residual DNA is expected to be substantially smaller than the 15% found in the present work with micrococcal nuclease. Only one value for micrococcal nuclease has been reported in the literature; Herman and coworkers (1976) found 20% of the DNA remaining with the nucleus after digestion. Hemminki (1977) found 16% remaining after digestion with DNase II under conditions where nucleosomes are not cleaved. These two values agree well with that reported here.

The residual DNA observed by Herman and coworkers (1978, 1976) after micrococcal nuclease digestion was localized at the nuclear periphery. Comings (1978) and Comings and Okada (1976) have found DNA attached to both peripheral and internal elements of the nuclear matrix. No localization of the deoxyribonucleoprotein stain was observed in this work (Figure 11, p 54), in contrast to the report of Herman and associates. Crystal violet staining, however, was frequently localized at later stages of digestion (Figure 10E-F, p 51), but not at the periphery of the nuclei. There appear to be channels extending from the nuclear surface to the interior. The nonspecificity of crystal violet staining makes interpretation of this phenomenon difficult. Large open spaces and a spongy structure resembling the appearance of crystal violet-stained digested nuclei have been

observed in electron micrographs of nuclei extracted with DNase I and high salt (Miller et al., 1978a; Comings and Okada, 1976), with the interpretation that masses of heterochromatin might have occupied the spaces. Electron microscopic examination of the residual structures in Peak 2, produced by micrococcal nuclease digestion at low salt, might well reveal non-uniform distribution of the associated chromatin at a level of resolution below that of light microscopy.

The DNA remaining in the residual nuclei after complete digestion was primarily in the form of mononucleosomes. This conclusion is based on the size of the residual DNA fragments and on the presence of core histones in the residual structures. Association of chromatin with the nuclear skeleton is therefore largely through mononucleosomes. If adjacent nucleosomes are attached to the matrix, in most cases the linker DNA is not protected against cleavage.

The nucleosomes are quite firmly bound to the residual structure. Resedimentation, even after prolonged dialysis, removed little DNA from the Peak 2 fraction; this loss was probably due to degradation of the material during further handling as well as to release of trapped chromatin. Trapping of large numbers of mononucleosomes within the residual nuclei is unlikely for several reasons. Oligonucleosomes were not found in Peak 2 during the early stages of digestion in amounts that would indicate that they were intermediates in the production of trapped monomers. Inspection of the gel tracings in Figure 8 (p 49) shows that fragments considerably larger than mononucleosomes were readily released from the nuclei. In fact, large masses of chromatin escaped from the nuclei, as seen in the

photomicrographs. Retention of monomers by remnants of the nuclear membrane is also unlikely. There is ample evidence that 1% Triton X-100 removes almost all nuclear phospholipid, leaving only tiny shreds of trilaminar membrane on a few nuclei (Miller et al., 1978b; Berezney and Coffey, 1977; Scheer et al., 1976; Faiferman and Pogo, 1975; Aaronson and Blobel, 1974). Thus it is improbable that large numbers of free nucleosomes were prevented from diffusing out of the residual nuclei.

Complete dissociation of DNA from the residual nuclei was achieved only with SDS or with the combination of 2 M salt and 5 M urea. Urea alone removed only 25% of the residual DNA; salt alone removed 33%. Since either 2 M salt or 5 M urea causes unfolding of the nucleosomes, it is possible that more extensive dissociation occurred but was not detected because much of the extended DNA was trapped. Alternatively, the DNA may be attached through tightly-bound nonhistone proteins which are not dissociated by salt and urea. These two possibilities are not experimentally distinguishable. The combination of salt and urea dissociates the entire residual structure; microscopic examination revealed nothing resembling a nucleus in the lower two-thirds of the salt-urea gradient.

The nonhistone proteins of the Peak 2 fraction after 120 minutes of digestion are similar to those reported for the matrix of mouse liver nuclei (Comings and Okada, 1976), although none of the bands are prominent. The level of background stain in the channels containing Peak 2 fractions probably indicates the presence of additional proteins not resolved in these small gels. A more complex protein

composition would be expected in Peak 2 fractions than in matrix fractions that have been extracted with 2 M salt.

The Peak 1 fraction of the one minute digest contained a prominent nonhistone protein of 68-70,000 daltons. At later times this protein is not observed in Peak 1. The increased histone content of this fraction after longer digestion may dilute the nonhistone component so that it is no longer detectable. Alternatively, association of this protein with the chromatin of Peak 1 may be unstable, resulting in loss of the protein from Peak 1 early in the course of digestion. The Peak 1 fraction from the one minute digest also contained two distinct components in the sucrose gradient profile, the lighter component sedimenting at the position of mononucleosomes. There have been several reports that transcribed sequences are more readily released by micrococcal nuclease than inactive sequences (Levy-Wilson and Dixon, 1979; Levy W et al., 1979; Tata and Baker, 1978; Johnson et al., 1978; Reeves et al., 1978, 1977; Bellard et al., 1977). Recently, Bloom and Anderson (1978) have reported fractionation of micrococcal nuclease-digested nuclei by a sedimentation method similar to that used by Frenster and coworkers (1963). The minor fraction released after very brief digestion and before addition of EDTA was composed of mononucleosomes and enriched in transcribed sequences. The protein composition of this fraction was not reported. It is suggestive, however, that fractions derived by a very similar method and containing mononucleosomes generated by brief digestion should be enriched in active gene sequences on one hand and in a specific nonhistone protein in the other.

The molecular weight estimate for this protein is based on comparison of its mobility in SDS-polyacrylamide gels with that of bovine serum albumin (BSA, molecular weight 68,000). A nonhistone protein of very similar mobility in relation to BSA has been identified in a variety of nuclear fractions. It can be seen in nuclear matrix preparations from rat liver (Berezney and Coffey, 1974), rat lung (Hemminki, 1977) and HeLa cells (Herman et al., 1978; Hodge et al., (1976). A protein of 68,000 daltons has been reported in the nuclear matrix of mouse liver (Comings and Okada, 1976), one of 70,000 daltons in Tetrahymena nuclear matrix (Herlan and Wunderlich, 1976) and one of 70,500 daltons in CHO cells (Hildebrand et al., 1976), but in these three cases a BSA marker was not shown. Proteins of similar mobility relative to BSA are seen in the salt-extracted nuclear envelopes of Chironomus salvary glands (Plagens, 1978) and in the nuclear pore complex-lamina of rat liver (Aaronson and Blobel, 1975); none was found in the envelope of Xenopus oocytes, although it was present in the gelled nuclear contents (Krohne et al., 1978). Salt-extracted polytene chromosomes of Chironomus salivary glands (Plagens, 1978) and detergent-extracted nuclei of Krebs ascites cells (Böhm et al., 1977) also contain a protein which comigrates with BSA. The folded supercoiled DNA complexes from mouse carcinoma cells lysed in SDS also contain a tightly bound nonhistone protein with a mobility very close to that of BSA (reported molecular weight 70,000; Ide et al., 1975). The mitotic chromosome scaffold may contain a protein of this mobility as well (Adolph et al., 1977a), but a molecular weight estimate or protein standard was not given. A very rough estimate of 70,000

daltons for the apparent molecular weight of this protein was made by this writer, based on the relative mobilities of this band and others in the same gel to which molecular weights were assigned (53,000 and 35,000 daltons) and careful comparison with other published gel patterns containing a range of molecular weight standards.

Thus, protein species of similar electrophoretic mobility (apparent molecular weight range from 68,000-70,500) are found in nuclear matrix, polytene chromosomes, folded supercoiled DNA complexes, a mononucleosome fraction released by brief micrococcal nuclease digestion and, possibly, in mitotic chromosome scaffolds. Electrophoretic mobility is a poor criterion for protein identity and, in any case, a protein of 68,000 daltons is not identical to a protein of 70,500 daltons. However, the range covered by the estimates is not large considering the imprecision of molecular weight determinations in this range by electrophoresis in SDS gels containing 10-15% polyacrylamide. Some variation between preparations may also be caused by non-protein groups associated with the proteins, as discussed in Chapter 1 (p 12). It is tempting to speculate that these different preparations may all contain a protein species of about 70,000 daltons involved in the maintainance and packaging of chromatin domains in different functional states: total interphase chromatin, active chromatin, and mitotic chromosomes. Association of such a protein with other components of the matrix, scaffold or transcription apparatus could be modulated by cell cycle phase-specific or base sequence-specific modifications to regulate the conformation of the entire genome or a particular set of domains. Further characterization

of these proteins should, in any case, yield information on the structural role of nonhistone nuclear proteins.

CHAPTER 5

DIGESTION AND SEDIMENTATION OF NUCLEI UNDER DIFFERENT IONIC CONDITIONS

A. INTRODUCTION

The physical properties of chromatin vary appreciably with ionic strength. There are numerous reports correlating changes in salt concentration with changes in solubility, sedimentation coefficient, degree of condensation or conformation observed in electron micrographs. But variations in the methods used to prepare the starting material, the composition of the buffers, and the range of salt concentrations employed complicate detailed comparisons of the results. Only the most general conclusions can be drawn.

At very low ionic strength, between 1 and 10 mM NaCl, chromatin undergoes a conformational transition resulting in increased viscosity and decreased sedimentation coefficient (Li et al., 1977). A similar transition is observed with nucleosomal core particles in the same concentration range (Gordon et al., 1978; Zama et al., 1977), indicating alteration in the basic structure of the nucleosome. Specific points of contact between histones H2B and H4 are broken in this transition (Martinson and True, 1979; Martinson et al., 1979).

Chromatin prepared at physiological ionic strength is soluble at that salt concentration (Renz et al., 1977; Rees et al., 1974). It expands as the ionic strength is reduced but this process is fully reversible, and the chromatin does not precipitate until the salt concentration approaches 0.3 M (Lewis et al., 1976). In contrast,

chromatin prepared at low ionic strength precipitates when the salt concentration is raised into the physiological range, 0.12-0.15 M NaCl (Bradbury et al., 1973; Leake et al., 1972; Smart and Bonner, 1971b; Jensen and Chalkley, 1968; Ohlenbusch et al., 1967; Atchley et al., 1964). Oligonucleosomes also precipitate at this ionic strength (Campbell and Cotter, 1977; Gaubatz and Chalkley, 1977). However, chromatin or oligonucleosomes depleted of histone H1 are soluble between 0.05 and 0.3 M NaCl (Campbell and Cotter, 1977; Bradbury et al., 1975, 1973; Smart and Bonner, 1971a). Similar results have been obtained with the SV40 minichromosome. When isolated at physiological ionic strength, this complex contains H1 and has a compact, rapidly sedimenting conformation (Varshavsky et al., 1976a). Removal of H1 or reduction of the salt concentration converts the spherical minichromosome particle to an extended circle of nucleosomes; on return to physiological ionic strength in the presence of H1, the compact conformation is regained (Müller et al., 1978; Christiansen and Griffith, 1977). The condensation of chromatin with increasing salt concentration, therefore, involves internucleosomal associations mediated by histone H1. This view is supported by the fact that continued increase in salt concentration over the range 0.3-0.6 M results in simultaneous dissociation of histone H1 and reexpansion of the chromatin (Lewis et al., 1976; Bradbury et al., 1975; Smart and Bonner, 1971a).

The nature of histone H1 binding to oligonucleosomes changes from noncooperative to cooperative between 10 and 60 mM NaCl (Renz and Day, 1976), and this transition is accompanied by a dramatic increase

in the sedimentation coefficient of the H1-bound oligonucleosomes Renz et al., 1977). However, this transition occurs below the concentration at which the maximum condensation of chromatin is observed. Moreover, H1-depleted chromatin and H1-depleted SV40 minichromosomes do condense as the salt concentration is raised, although not to the extent observed with intact chromatin (Renz et al., 1977; Christiansen and Griffith, 1977; Lewis et al., 1976). Removal of histone H1 from chromatin does not increase the susceptibility of linker DNA to Serratia marcescens endonuclease, but after digestion of the N-terminal regions of the core histone with trypsin, linker DNA is rapidly degraded by this nuclease (Pospelov et al., 1977, 1979). Thus the cooperative binding of histone H1 probably stabilizes intrinsic internucleosomal and linker-core interactions at physiological ionic strength. Salt-induced precipitation of chromatin isolated at low ionic strength may be due to subtle alterations in chromatin structure during repeated exposure to low salt buffers (see Chapter 2). If such alterations interfere with the cooperative intrastrand binding of histone H1, formation of interstrand linkages could be increased, promoting aggregation and precipitation of the chromatin. Changes in the nucleosome spacing, for instance, might produce this effect.

The effects of divalent cations, Mg^{++} and Ca^{++}, on chromatin conformation parallel those observed with NaCl, but much lower concentrations of the divalent cations are required. The transition in core particle conformation observed between 1 and 10 mM NaCl occurs at 10 μM $MgCl_2$ (Li et al., 1977). Condensation and aggregation of chromatin occur between 1 and 10 mM $MgCl_2$ (Bradbury et al., 1975;

Pooley et al., 1974; Leake et al., 1972; Atchley and Bhagavan, 1964). Similar effects have been observed with SV40 minichromosomes Christiansen and Griffith, 1977) and with oligonucleosomes (Campbell and Cotter, 1977). In all cases, the presence of histone H1 is required for condensation to occur. Removal of divalent cations causes unfolding of thick (200-250 A) chromatin fibers and destabilization of nucleosomes (Vengerov and Popenko, 1977; Kiryanov et al., 1977). The supercoil of polynucleosomes observed by Finch and Klug (1976) was formed on addition of Mg^{++}; however, de Murcia and coworkers (1978) report supercoils in chromatin in the presence of EDTA provided the preparation had not been sheared. The greater effectiveness of divalent compared with monovalent ions is only partly explained by their greater charge density (Pooley et al., 1974), suggesting that divalent ions may have a specific role in maintaining chromatin structure in addition to nonspecific charge neutralization.

Two conformations have been proposed for condensed polynucleosome fibers, the supercoil (Finch and Klug, 1976) and the superbead (Hozier et al., 1977). The distinction between them is somewhat arbitrary; closely packed beads might resemble a coil whereas a kinked coil might appear to be beaded. Both structures appear frequently in electron micrographs, sometimes coexisting in the same photograph (de Murcia et al., 1978; Vengerov and Popenko, 1977; Olins, 1977; Franke et al., 1976; Kiryanov, 1976). Varshavsky and associates (1977a) noted preferential cleavage of chromatin by micrococcal nuclease at intervals of seven to eight nucleosomes. Recently, Strätling and coworkers (1978a,b) reported isolation of particles of

the size expected for superbeads after mild digestion of nuclei with micrococcal nuclease. Monomer, dimer and trimer species were detected, containing multiples of eight nucleosomes. The particles unfold to chains of polynucleosomes at salt concentrations below 0.03 M or above 0.2 M, as does the 250 Å chromatin fiber. Up to 75% of the total chromatin could be obtained in the form of these particles. That the periodicity of the higher order structure is manifested as increased susceptibility to the nuclease suggests that the repeating units are physically distinct, not continuous as in a crystal lattice.

This chapter describes the effects of variation in the concentrations of $CaCl_2$ and NaCl on the digestion of nuclei by micrococcal nuclease and on the sedimentation of digested nuclei through steep sucrose gradients. Results from preliminary experiments confirm the observations of Strätling and associates (1978a,b) on the occurrence of monomers and dimers of particles composed of approximately eight nucleosomes.

B. METHODS AND MATERIALS

The procedures for preparation and micrococcal nuclease digestion of mouse liver nuclei, and for analysis of the digestion products by sucrose density gradient centrifugation and gel electrophoresis are described in the appendix. Digestion times are indicated in the text. Where the experiments required the continuous presence of calcium ions after digestion, pTp was used instead of EDTA to inhibit micrococcal nuclease in the digestion mixtures and sucrose gradients (Skup and Millward, 1977).

C. RESULTS

The effect of continued presence of calcium ions on the release of chromatin fragments from digested nuclei was investigated by sedimenting nuclei digested for two minutes through 5-65% sucrose gradients containing either 1 mM $CaCl_2$ and 1.8 μM pTp, or 1 mM EDTA and 1 mM pTp or 1 mM EDTA. Absorbance scans of the gradients are shown in Figure 17. In the absence of EDTA, very little chromatin is found in Peak 1. Addition of pTp along with EDTA does not prevent, in fact seems to amplify, the formation of Peak 1. Removal of most of the divalent cations is therefore necessary for the release of chromatin fragments from the digested nuclei. This experiment was repeated using EGTA in place of EDTA with identical results; since EGTA sequesters only calcium ions, removal of chromatin-bound Mg^{++} alone does not account for the results observed.

Absorbance scans of gradients containing pTp, Ca^{++} and nuclei digested for different intervals are shown in Figure 18. Electrophoretic analysis of DNA from the peak fractions of these gradients is shown in Figure 19. In the presence of Ca^{++}, Peak 1 does not appear at any stage of the digestion. Even when all the chromatin had been reduced to mononucleosomes (Figure 19F), the bulk of the A_{260} sedimented with the nuclei. A small peak of released chromatin was found near the top of the gradient only after very brief periods of digestion (Figure 17A and 18 top). This peak contained primarily monomer-size DNA (Figure 19A). At later stages of digestion the DNA at the top of the gradient was largely degraded (Figure 19B,C).

In an attempt to assess the effects of ionic strength on the

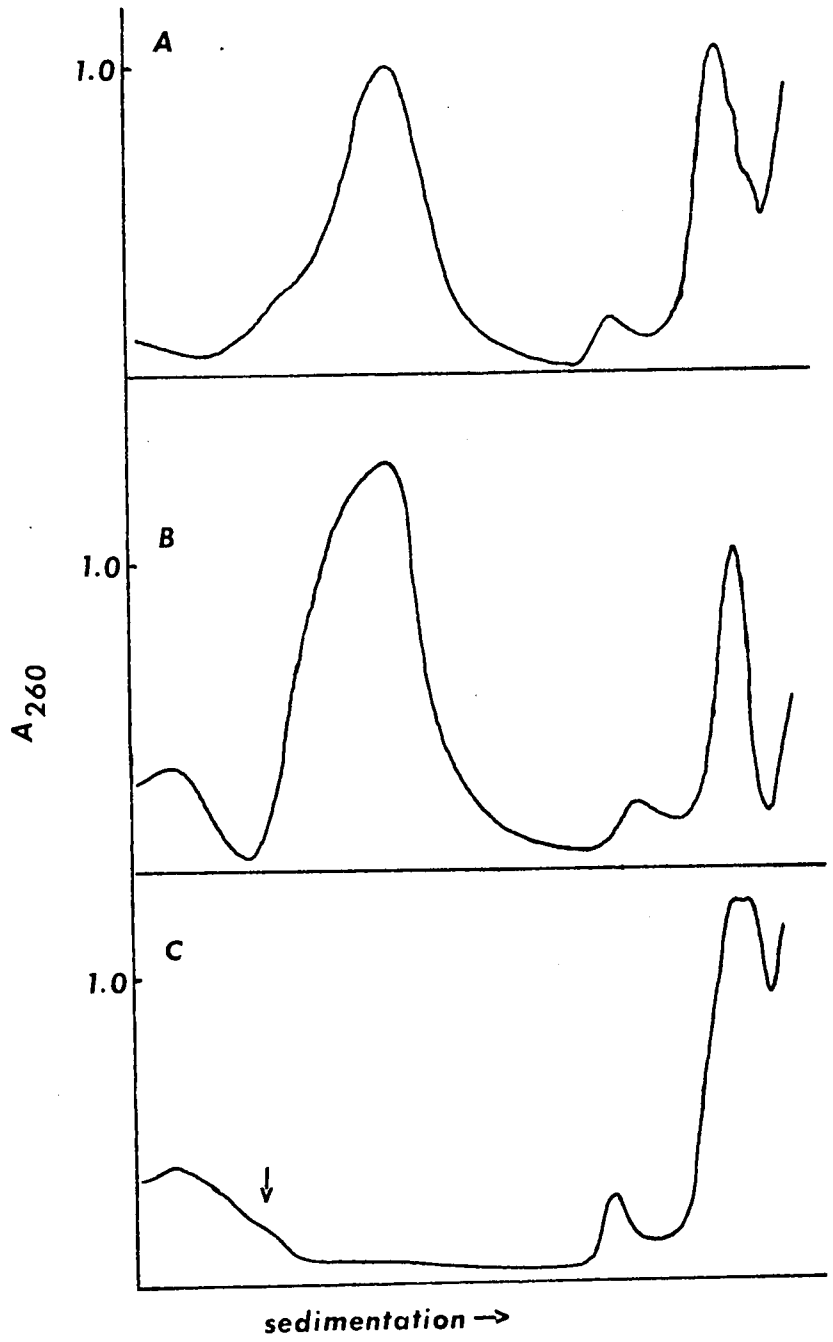

Figure 17. The effects of divalent cations and of pTp on the sedimentation of briefly digested nuclei. The nuclei were digested for 2 minutes as described in the appendix. Sedimentation through 5-65% sucrose gradients containing (A) 1 mM EDTA, (B) 1 mM EDTA and 1.8 μM pTp, (C) 1 mM $CaCl_2$ and 1.8 μM pTp was carried out as previously described. The small peak at the top of gradient C is marked with an arrow.

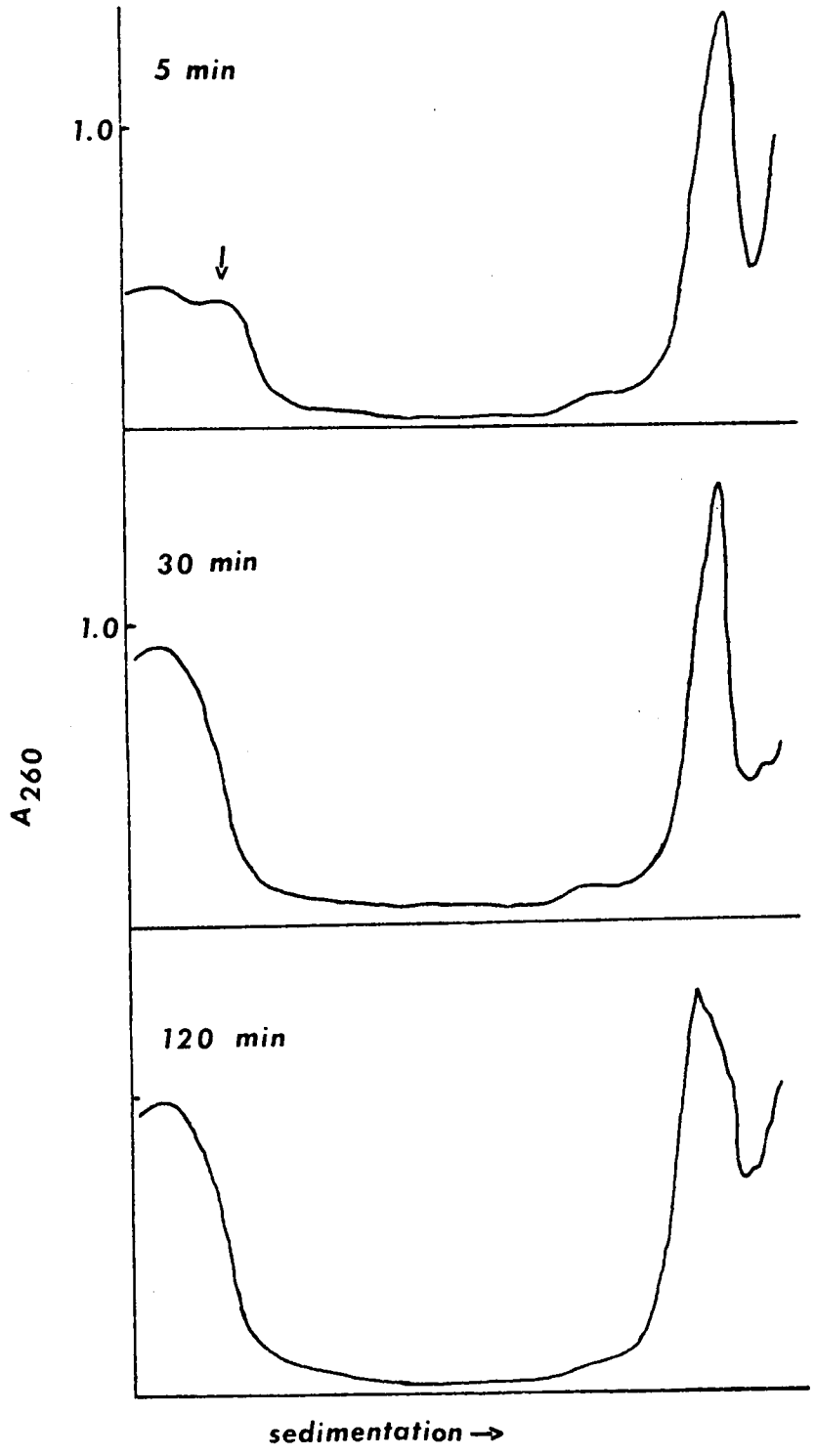

Figure 18. Sedimentation in the presence of $CaCl_2$ of nuclei digested for different intervals. Digestion was carried out as described in the appendix using pTp to stop the nuclease reaction. 5-65% sucrose gradients contained pTp and 1 m$\underline{M}$ $CaCl_2$. The small peak near the top of the gradient in the 5 minute digest is marked with an arrow.

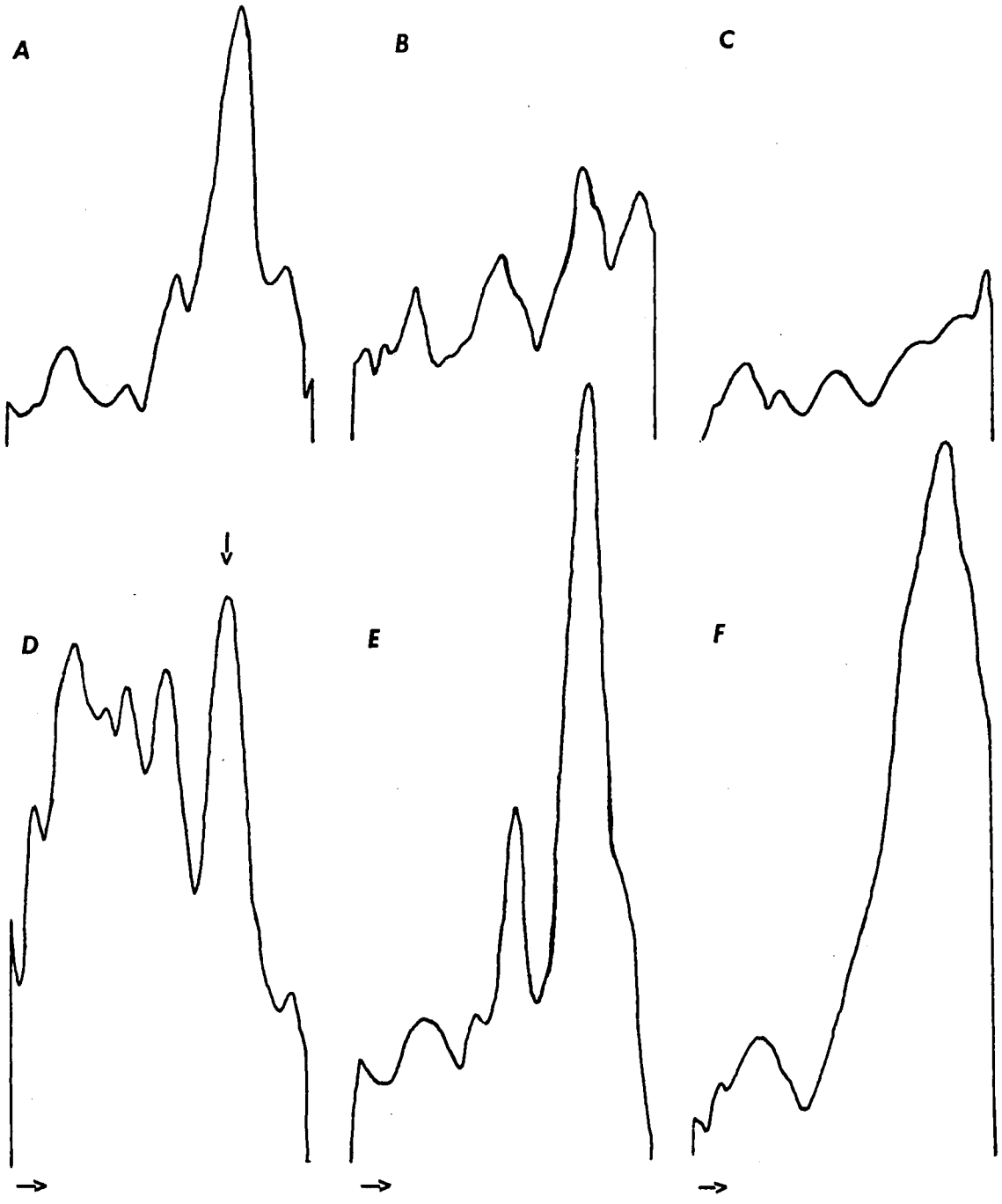

Figure 19. Electrophoresis of DNA from top fractions and Peak 2 fractions of gradients containing $CaCl_2$. (A) 5 minute digest, top fractions; (B) 30 minute digest, top fractions; (C) 120 minute digest, top fractions; (D) 5 minute digest, Peak 2; (E) 30 minute digest, Peak 2; (F) 120 minute digest, Peak 2. The position of mononucleosome DNA is indicated by an arrow in frame D. The size of the mononucleosome DNA decreased with digestion. The direction of migration of DNA migration in the gels is indicated by arrows below the figure.

fractionation procedure, nuclei prepared and digested in buffers containing 0.06 M NaCl were compared with nuclei prepared and digested in the absence of salt. Each of the nuclear preparations was sedimented through 5-65% sucrose gradients containing no salt, 0.06 M NaCl or 0.6 M NaCl. Results are shown in Figure 20. When all steps were carried out in 0.06 M NaCl, Peak 1 was shifted toward the heavier regions of the gradient and two additional peaks appeared between Peaks 1 and 2 (2A in Figure 20D, compare with Figure 20A). These new peaks were much reduced when the 0.06 M NaCl digest was centrifuged in the absence of salt (Figure 20B) and disappeared entirely in gradients containing 0.6 M NaCl (Figure 20F).

Nuclei prepared in the absence of salt aggregated in the 0.06 M NaCl gradient, beginning as soon as the sample was layered onto the gradient, and very little material was found in the Peak 1 region after centrifugation (Figure 20C). In the high salt gradient, the amount of chromatin released from nuclei prepared without salt was markedly increased (Figure 20E).

DNA from the peak fractions of these gradients was analyzed on 5% polyacrylamide gels. Fractions containing the two additional peaks shown in Figure 20D were combined and designated fraction 2A. Recovery of DNA from the Peak 1 region of Figure 20C was insufficient for analysis. Scans of the stained gels are shown in Figure 21 for nuclei prepared in 0.06 M NaCl and in Figure 22 for nuclei prepared in the absence of salt. The principle feature of all these gels is a peak of hexamer- to octamer-size DNA, with octamer the modal size in all cases. A scale showing the locations of the inflections

Figure 20. The effects of ionic strength on digestion and sedimentation of nuclei. Nuclei were digested for 5 minutes as described in the appendix. (A) nuclei prepared without salt, gradient containing no salt; (B) nuclei prepared in 0.06 M NaCl, gradient containing no salt; (C) nuclei prepared without salt, gradient containing 0.06 M NaCl; (D) nuclei prepared in 0.06 M NaCl, gradient containing 0.06 M NaCl; (E) nuclei prepared without salt, gradient containing 0.6 M NaCl; (F) nuclei prepared in 0.06 M NaCl, gradient containing 0.6 M NaCl.

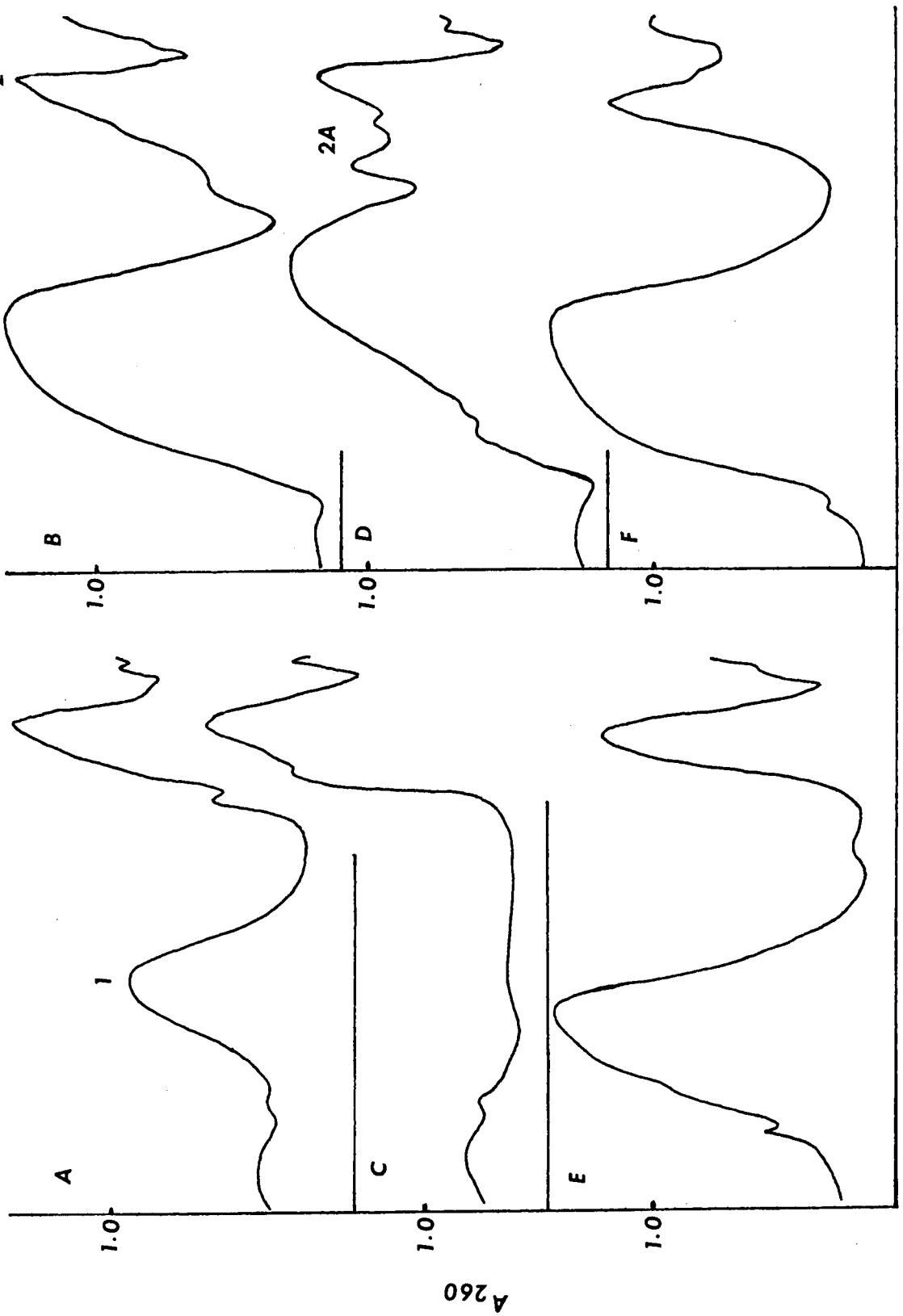

Figure 20.

Figure 21. Polyacrylamide gel electrophoresis of DNA extracted from Peak 1, Peak 2A and Peak 2 fractions of nuclei prepared in 0.06 M NaCl. (A) Peak 1, gradient without salt; (B) Peak 2, gradient without salt; (C) Peak 1, gradient 0.06 M NaCl; (D) Peak 2A, gradient 0.06 M NaCl; (E) Peak 2, gradient 0.06 M NaCl; (F) Peak 1, gradient 0.6 M NaCl; (G) Peak 2, gradient 0.6 M NaCl. A scale showing the locations of the inflections corresponding to monomer through octamer is given in frame D.

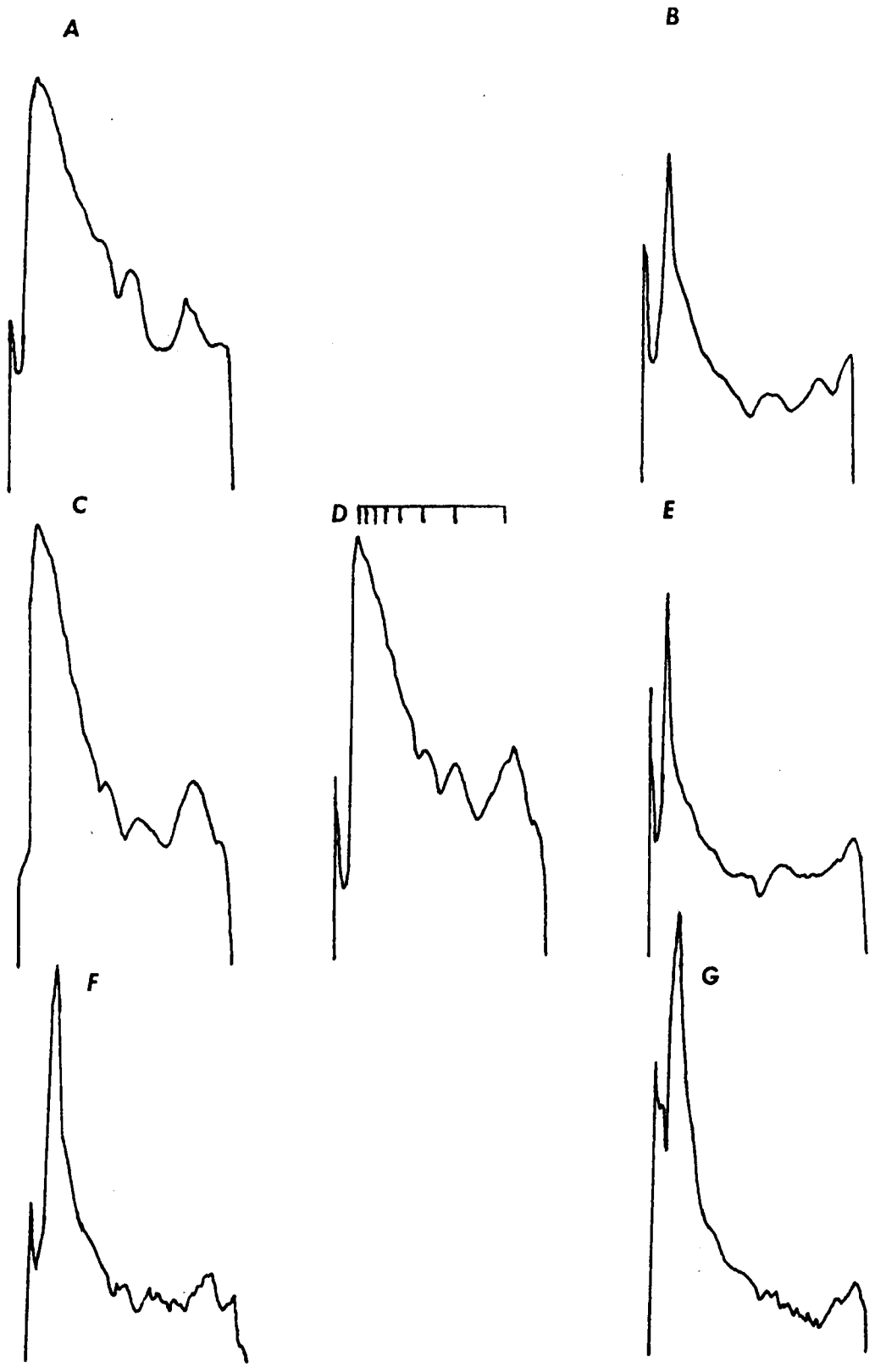

Figure 21.

Figure 22. Polyacrylamide gel electrophoresis of DNA extracted from Peak 1 and Peak 2 fractions of nuclei prepared without salt. (A) Peak 1, gradient without salt; (B) Peak 2 gradient without salt; (C) Peak 2, gradient 0.06 M NaCl (Peak 1 in this gradient contained very little DNA); (D) Peak 1, gradient 0.6 M NaCl; (E) Peak 2, gradient 0.6 M NaCl.

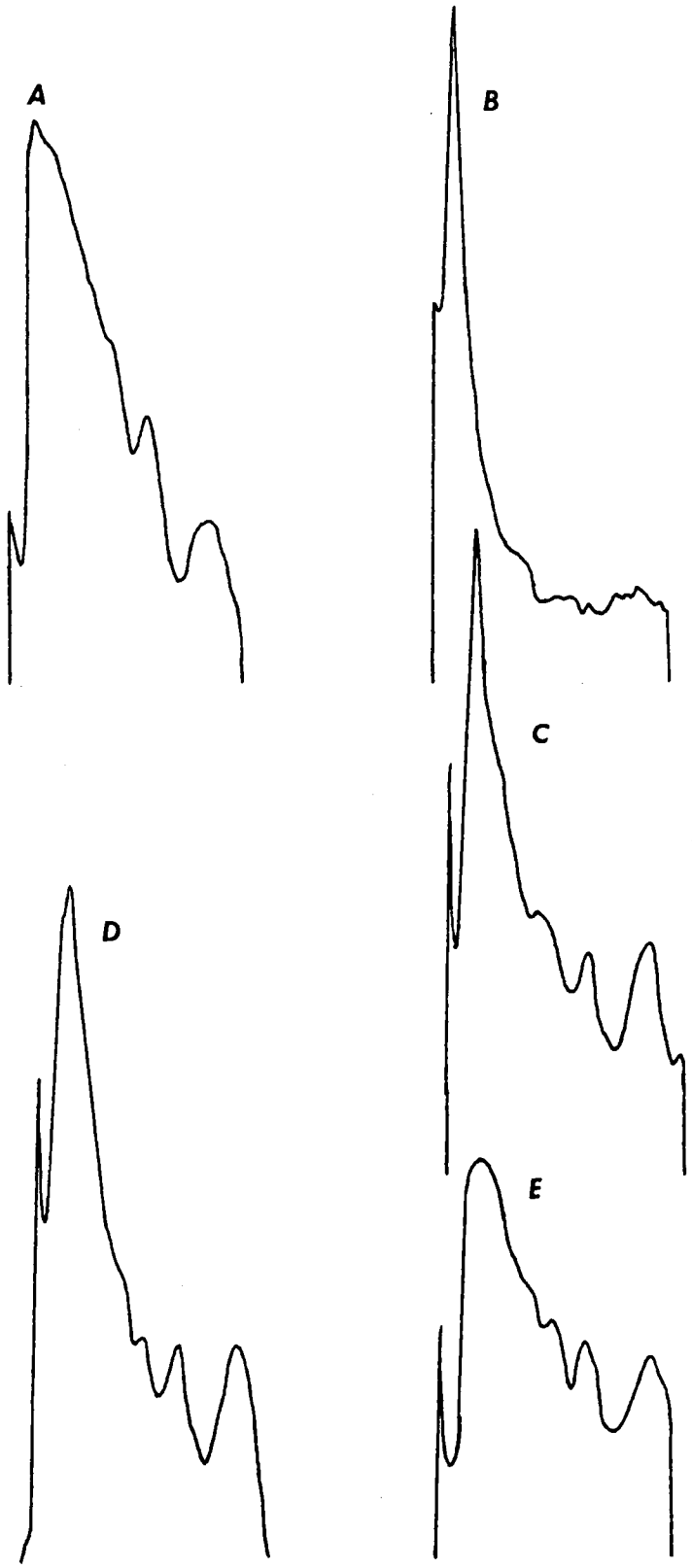

Figure 22.

corresponding to monomer through octamer is included in Figure 21D. At the upper edge of the octamer peak, the amount of DNA dropped sharply. In all the gels except 21C, a narrow band was seen at the very top of the gel. The absence of small chromatin fragments from both peaks of the high salt gradient (Figure 21F,G) is puzzling. This sample of nuclei was digested to the same extent as the others, and small digestion products should be present in the same amount. This gradient did contain a substantial pellet and, although aggregation of chromatin was not expected in 0.6 M salt, the small products of digestion were presumably located there.

The rate of the nuclease reaction in 0.06 M salt was not measured, but it is evident from comparison of the sizes of Peak 1 on the left side of Figure 20 (digestion without salt) with those on the right side (digestion in 0.06 M NaCl) that more chromatin was released after 5 five minutes of digestion in the presence of salt. This might result from differences in the extent of release of digestion products at the two salt concentrations, but the possibility of a difference in the kinetics of digestion should be kept in mind when comparing the two preparations.

D. DISCUSSION

Retention of digested chromatin fragments within the nucleus in the presence of divalent cations has been noted by other investigators (Sanders, 1978; Bloom and Anderson, 1978; Bernstine, 1978). Bloom and Anderson report release, after brief digestion, of a minor fraction of nucleosomes enriched in transcribed sequences prior to release

with EDTA of the bulk of the digested chromatin. Using isolated chromatin, Varshavsky and coworkers (1976b) found that mononucleosomes released before the addition of EDTA were deficient in histone H1. The small peak of released chromatin observed in the Ca^{++}-containing gradients after two or five minutes of digestion also contained mononucleosomes; this peak disappeared with longer digestion. This peak may be the same as the mononucleosome peak seen when nuclei were centrifuged in an EDTA-containing gradient after one minute of digestion (Peak 1a in Figure 5, p 43). A nonhistone protein, detected in that Peak 1 fraction after one minute of digestion, was not seen after longer digestion. Conceivably, this protein is associated with a subset of rapidly digested mononucleosomes.

Considering the low concentration of divalent cations required to condense chromatin, it is not surprising that chromatin fragments sedimented with nuclei in 1 m$\underline{M}$ $CaCl_2$. It seems probable that divalent cations are involved in the maintainance of chromatin packing. The level of tightly-bound Mg^{++} retained by the nuclei, on the other hand, was not sufficient to maintain the association when Ca^{++} was removed with EGTA. In the continued presence of Ca^{++}, the state of the nuclei in the gradient should not be much altered from that in the digestion mixture, and it is reasonable to assume that the chromatin remaining after brief disgestion is not dramatically altered in structure. Since increasing the ionic strength of the digestion mixture to 0.06 $\underline{M}$ appeared to facilitate the release of chromatin fragments from the nuclei, investigation of the interaction between divalent cation effects and overall ionic strength might identify conditions for

selective release of part of the chromatin. One such experiment has been reported (Sanders, 1978), in which selective extraction of chromatin fractions differing in protein composition and DNA size was achieved. It seems likely that this fractionation method discriminates between regions of different accessibility and degree of condensation within the nucleus, but the functional significance of these differences remain to be demonstrated.

It is evident from the gels shown in Figures 21 and 22 that brief digestion of nuclei produces primarily hepta- and octanucleosomes, irrespective of the salt concentration. Apparently the periodic structure producing this preferential cleavage is stable within the nuclei at low ionic strength, while released oligonucleosomes unfold. The narrow band at the top of most of the gels probably contains DNA from dimers of the higher order subunit. This band is missing from the one fraction where these dimers would not be expected, the Peak 1 fraction from nuclei digested and sedimented in the presence of 0.06 M NaCl. The amount of this putative dimer cannot be estimated since an undetermined fraction has not entered the gel.

When 0.06 M NaCl was present during digestion and centrifugation, two additional peaks were found between Peaks 1 and 2. The presence of a large amount of octanucleosome-size DNA in the Peak 1 fraction from this gradient, and comparison with the gradient profiles shown by Strätling and coworkers (1978a,b) indicates that these new peaks contain dimers and trimers of the higher order subunit and the monomer comprises the leading edge of Peak 1. The presence of DNA corresponding to nucleosome monomers through pentamers in fraction 2A suggests

that the internucleosomal interactions within the superbead may be maintained despite cleavage of the linker DNA between component nucleosomes. The possibility of aggregation of smaller fragments with the higher order subunits has not been ruled out, but aggregation should produce a more uniform distribution of chromatin between Peaks 1 and 2. Thus, it appears that the integrity of the higher order subunit is not dependent on the continuity of the DNA between nucleosomes. The existence of octamer DNA in fraction 2A may be contamination from Peak 1 since resolution in the gradient was incomplete. It is present in sufficient amount, however, to suggest the possibility that superbeads may remain associated when the connecting DNA strand between them has been cleaved. This is consistent with the very compact structure of the dimer observed in electron micrographs (Strätling et al., 1978a). Since no traces of higher order monomers, dimers or trimers were seen when nuclei digested in the absence of salt were sedimented through a gradient containing 0.06 M salt, these considerations do not hold for reassociation of higher order subunits.

In these experiments, EDTA was used to stop the nuclease digestion, and the gradients also contained EDTA. Thus, divalent cations are evidently not required for stability of the superbead subunits. The presence of divalent cations may therefore be necessary for still higher levels of chromatin packing or, conceivably, for association between the bulk of the chromatin and the nuclear framework.

These conclusions are based on a reproducible preliminary experiment. They support the observations of Strätling and coworkers (1978a,b) of a higher order of chromatin structure based on multiples

of eight nucleosomes. The experiment was designed, however, to assess the effects of changes in ionic strength on the sedimentation rate of Peak 1 and Peak 2 fractions, not to detect superbeads. The results are therefore incomplete and inconclusive. The existence of 16-mer and 24-mer particles should be confirmed by analysis of DNA in agarose gels. The use of somewhat higher salt concentrations, between 0.06 and 0.15 M NaCl, and more gradual density gradients might improve resolution of the higher order subunits. The protein composition of monomers and oligomers of the superbeads should also be investigated. The presence of scaffold or matrix proteins in the 250 A fiber is a distinct possibility. The relationship between higher order subunits and the nucleosomes remaining in the residual nucleus after prolonged digestion (described in Chapter 4) would also be of interest. The proportion of DNA remaining Peak 2 after two hours of digestion is strikingly close to 1/8. But the release of significant numbers of higher order subunits, especially their release in dimer and trimer form, is inconsistent with the interpretation that each superbead is attached to the nuclear matrix through a single nucleosome. Some subunits may be attached at several points. If complete digestion were carried out at sufficient ionic strength to maintain the internucleosomal interactions, the entire octanucleosome subunit might remain attached to the nucleus, and might be discernable in electron micrographs. It is also quite possible that nucleosomes attached to the nuclear matrix belong to a chromatin fraction with a different higher order structure. None of the evidence presented here or elsewhere precludes the existence of supercoils or other higher order structures in

addition to superbeads. The current understanding of chromatin architecture and its relation to chromatin function leaves ample scope for speculation and experimentation.

EPILOGUE

The results reported in this dissertation support and extend the hypothesis that the nucleus contains a structural framework of nonhistone protein. Residual nuclear structures were observed in unfixed preparations after 85% of the DNA had been removed. Although these structures were prepared in the absence of salt, they did not dissociate in 2 M NaCl. Mononucleosomes remain associated with the residual nuclei after complete digestion by micrococcal nuclease and appear to be firmly attached. The distribution of attachment sites, if not uniform, is not highly localized.

A survey of the literature, presented in Chapter 1, shows that evidence for the existence of a nuclear matrix is accumulating from a variety of sources. The evidence suggests, but does not prove, that the matrix is a dynamic structure and may participate in several aspects of nuclear metabolism, particularly in synthesis and processing of RNA and replication of DNA. The fact that chromatin seems to be attached to the matrix raises the possibility that matrix proteins may be involved in the maintainance and control of chromatin structure. However, much remains to be done if these possibilities are to be developed into a convincing body of evidence. A method has been described in this dissertation for preparation of residual nuclear structures under mild conditions. The existence of nucleosomes and superbeads in these structures demonstrates that at least some aspects of native chromatin structure are retained and thus it seems possible that other intermolecular associations, perhaps enzymes complexes, might be retained as well.

Some limitations of the method have become apparent during the course of the experiments and modifications will be necessary for full exploitation of this approach. The use of cultured cells as a source of nuclei in future experiments is recommended because they can be conveniently labeled with radioactive precursors, permitting the use of more sensitive analytical techniques. Moreover, the metabolic activity of the matrix fraction could then be analyzed in pulse-chase experiments. Cultured cells can also be manipulated to provide either proliferating or quiescent cell populations and, using synchronized cell populations, specific stages of the cell cycle for investigation of matrix structure and activity in different metabolic states.

Micrococcal nuclease is appropriate for some investigations of chromatin-matrix interactions, but restriction endonucleases are needed for determination of the role of repeated DNA sequences as attachment sites for matrix proteins. These enzymes could also be utilized to obtain matrix preparations containing larger chromatin fragments. The activity of micrococcal nuclease against RNA precludes its use in investigations of matrix-bound RNA species and here, again, restriction endonucleases or DNase II might be used. The ability of DNase I to degrade nucleosomes is a disadvantage for most purposes but might be utilized to detect possible changes in matrix structure or composition when chromatin attachment sites are destroyed.

It is difficult to predict at this stage the extent to which matrix fractions will be useful in the study of RNA and DNA synthesis. The development of isolation procedures which preserve matrix-associated enzyme activities could be time consuming and might lead to the

conclusion that such activities do not exist. It is certainly within the realm of possibility, however, that DNA and RNA polymerases do function in a matrix-bound state in vivo and that factors required for their native activity are left behind when the enzymes are isolated in soluble form. If so, it may eventually be possible, by combining soluble, matrix-bound and chromatin fractions, to reconstitute these activities in a form approaching their in vivo state.

The emerging concept of a highly ordered nuclear structure, in place of the concept of the nucleus as a sack of chromatin awash in the nucleoplasm, has profound implications for understanding nuclear function. Molecules attached to a framework do not take random walks, thus surface chemistry is regulated by mass transport to a far greater extent than solution chemistry. Delivery of reagents, e.g. polymerases, histone acetylases, hormone-receptor complexes, to reactive sites in the nucleus may be regulated by the number and size of channels in the matrix. Processing and transport of transcription products may be similarly regulated. Since these functions are ultimately under genetic control, matrix structure might conceivably be responsive to genetic signals. This raises the possibility that some of the regulatory sequences in the genome could be sites for matrix-chromatin interaction, capable under appropriate conditions, perhaps in conjunction with signal molecules, of initiating changes in the organization of both chromatin and matrix elements. For this reason, nuclei or systems reconstituted from matrix fractions may prove more useful for investigation of genetic regulatory mechanisms in eukaryotes than isolated chromatin.

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APPENDIX

APPENDIX

METHODS

A. GENERAL

Animals. Balb/c (Cum) mice, male, 8-10 weeks of age, were obtained from Cumberland Farms Laboratory, Clinton, Tennessee

Nuclease. Micrococcal nuclease was obtained from Sigma Chemicals, St. Louis, Missouri. The activity of this preparation is reported by the supplier in units of μmoles acid-soluble nucleotides produced per minute. Micrococcal nuclease activity is generally reported in the literature in units of acid-soluble A_{260} produced in 30 minutes. To facilitate comparison with published reports, enzyme concentrations recorded in this work have been converted to units based on absorbance change.

Temperature. Unless otherwise specified, all procedures were carried out at 4° Celsius.

Protease inhibitor. All solutions which came in contact with the nuclei contained 0.2 μM PMSF to inhibit proteases, except for the micrococcal nuclease digestion mixtures. PMSF was omitted from the digestion mixture to avoid possible interference with the enzyme.

ISOLATION OF NUCLEI

Spleen nuclei. Nuclei were isolated from mouse spleen by a modification of the procedure described by Hymer and Kuff (1964). Minced

tissue was disrupted in a hand-held Dounce homogenizer in 9 volumes of homogenization buffer (0.25 M sucrose, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0), filtered through 8 layers of cheesecloth and centrifuged for 10 minutes at 750 x g. The pellet was resuspended in homogenization buffer containing 0.5% Triton X-100, agitated vigorously with a Vortex mixer and recentrifuged as above. The Triton X-100 wash was repeated two more times.

Liver nuclei. Nuclei from mouse liver were isolated by a modification of the procedure of Hymer and Kuff (1964) as described above for spleen nuclei, and further purified by sedimentation through a discontinuous sucrose gradient (Blobel and Potter, 1970). After 5 detergent washes, the pelleted nuclei were resuspended in homogenization buffer, using 1 ml for every 4 livers, and mixed with an equal volume of 2.3 M sucrose, 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0, and layered onto discontinuous gradients composed of 5 ml of 2.3 M sucrose, 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0, over 2 ml of 70% sucrose, 1 mM $CaCl_2$, 1 mM Tris-HCl, ph 8.0, in siliconized 12.5 ml polyallomer tubes. The upper half of the contents of each tube was stirred and the gradients were centrifuged at 30,000 rpm for 30 minutes in a Beckman SW41 rotor. After the overlying portion of the gradient had been removed and the walls of the tube wiped, the layer of nuclei resting on the 70% sucrose cushion was removed carefully with a pipette, dispersed in approximately 5 volumes of 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0, filtered through 2 layers of cheesecloth and collected by centrifugation at 750 x g for 10 minutes.

B. SONICATION OF NUCLEI

The Branson Model W185 Sonifier equipped with a microtip was calibrated by immersing the tip in a beaker of water tared on a Mettler P1200 balance and adjusting the output until the force exerted on the balance produced a reading of 1.09 grams. The setting obtained in this way was always slightly above 2, but varied somewhat from use to use. Small volumes (3-5 ml) of nuclei at 750 µg DNA/ml were immersed in an ice bath and sonicated for three 15 second bursts at this setting. Cooling periods of 15 seconds were allowed between bursts.

C. MICROCOCCAL NUCLEASE DIGESTION

Digestion mixtures contained 0.25% sucrose, 1 mM $CaCl_2$, 1 mM Tris-HCl, pH 8.0, nuclei equivalent to 750 µg DNA/ml and 15 units/ml of micrococcal nuclease. In some experiments reported in Chapter 2, sucrose was omitted from the digestion mixture. Reactions were incubated in a 37° water bath; the digestion was started by addition of enzyme to the prewarmed mixture and stopped by addition of neutralized EDTA to a final concentration of 2.5 mM and chilling the bubes in an ice bath. Where the presence of calcium ions after termination of digestion was required, pTp at a final concentration of 1.8 µM was used to inhibit the nuclease (Skup and Millward, 1977).

D. DETERMINATION OF ACID SOLUBLE DNA

Samples of known volume from the micrococcal nuclease digestion mixtures were precipitated at 4° with 7% perchloric acid and 0.4 M NaCl.

The precipitate was removed by filtration through a glass fiber prefilter and an 0.45 μ membrane filter, and the A_{260} of the filtrate was measured. The acid soluble DNA content was calculated from the A_{260} after corrections for hyperchromicity (Noll et al., 1975) and dilution, using 20 A_{260} units per mg DNA.

E. SUCROSE DENSITY GRADIENTS

Polyallomer tubes for gradients were boiled for 10 minutes in 5 mM EDTA, pH 7.0, submerged in a 1% solution of Siliclad, rinsed with distilled water and allowed to dry. This pretreatment reduced the amount of chromatin adhering to the tubes. Linear gradients of 5-65% sucrose in 1 mM Tris-HCl, pH 8.0, with either 1 mM EDTA or 1 mM $CaCl_2$, were formed on cushions of 70% sucrose using an ISCO Model 570 gradient Former. The contents of a single digestion mixture (1 ml) were layered onto a gradient and centrifuged for 14-18 hours at 25,000 rpm in a Beckman SW27.1 rotor at 4°. Gradients were fractionated with the ISCO Model 183 Gradient Fractionator, using Fluorinert (ISCO) to force the gradients out of tube. Absorbance at 254 nm was monitored with an ISCO Type 4 UV absorbance monitor. Fractions of 12 or 15 drops were collected, and the fractions under the absorbance peaks were pooled for further analysis.

F. DNA AND PROTEIN DETERMINATIONS

Fractions from sucrose density gradients could not be used directly for DNA determination by the diphenylamine reaction because sucrose interferes with color development in this test. Complete

elimination of sucrose by acid precipitation or by extensive dialysis was accompanied by losses of DNA, which greatly increased the variability of the measurements. To circumvent this difficulty, the spectrophotometric method described by Raynaud and Ohlenbusch (1972) was used to estimate the amounts of DNA and protein in fractions obtained from gradients. Using mixtures of known composition of calf thymus DNA and bovine serum albumin, the measured values for DNA and protein were within 5% of the known values and the standard deviation less than 10% of the mean. Chromatin samples were dispersed in 4 M NaCl and the absorbance measured at 400, 260 and 230 nm. The 400 nm reading was used to correct for turbidity; in practice this was required only for Peak 2 samples and the correction was never greater than 20% of the recorded A_{260}. The weight of DNA in the sample was calculated from the corrected A_{260}, using 20 A_{260} units/mg DNA and assuming no contribution by protein to absorbance at this wavelength. Since both DNA and protein absorb 230 nm light, the DNA contribution to the A_{230} was calculated using $A_{260}/A_{230} = 2.07$ (measured for calf thymus DNA), and the protein contribution obtained by substraction. The amount of protein in the sample was calculated using 6 A_{230} units per mg protein (measured for bovine serum albumin).

G. DNA EXTRACTION

Samples containing at least 100 µg DNA were adjusted to 5 mM EDTA, 0.5% SDS, and 50 mM Tris-HCl, pH 8.0. Proteinase K (E. M. Laboratories) was added to a final concentration of 50 µg/ml and the mixtures were incubated 3-4 hours at 37° to digest the chromosomal

proteins. After addition of NaCl to 0.2 M, the digested samples were extracted twice at room temperature with an equal volume of phenol: chloroform:isoamyl alcohol (50:50:1) and once with chloroform:isoamyl alcohol (50:1), and precipitated by addition of two volumes of cold 95% ethanol. After standing overnight at -20°, the precipitated DNA was collected by centrifugation at 41,000 rpm in a Beckman SW41 rotor at -5° for 90 minutes.

H. ELECTROPHORETIC ANALYSIS OF DNA

The procedure described by Maniatis et al. (1975) was followed with modification of the gel size and composition. Cylindrical gels of 5% polyacrylamide (acrylamide:bis-acrylamide 19:1), 0.4 cm in diameter and 7.5 cm long, were pre-electrophoresed on reversed polarity until the voltage reached 75 volts at a current of 1 mA per gel. The buffer used in the gels and reservoirs was 0.09 M Tris-borate, 2.5 mM EDTA, pH 8.3 (TBE). Samples containing 50-75 µg of DNA and 25% glycerol in a 1:10 dilution of TBE were applied and electrophoresed on normal polarity until the bromphenol blue marker on a separate gel had reached the end of the tube. DNA extracted from chicken erythrocyte mononucleosomes was used to calibrate the gels. Gels were stained with toluidine blue (40 mg/ml in distilled water) and destained in distilled water. Transparancies were made by photographing the stained gels with a Polaroid MP4 camera using Type 46L film, and scanned with an E. C. Apparatus Corporation densitometer.

I. PHOTOMICROSCOPY

Wet mounts were prepared by mixing 50 µl of the sample with 5 µl of an 0.1% solution of crystal violet on a clean microscope slide. A coverslip was applied and sealed with clear nail polish.

For dried and stained preparations, a drop of the sample was mixed with 1-2 drops of distilled water on a subbed microscope slide and dried at 30°. The water was added to facilitate spreading of the viscous samples on the slide. Slides were fixed in absolute methanol and stained 10 minutes in May-Grunwald solution and 20 minutes in dilute Giemsa solution. Stained slides were rinsed in distilled water, dehydrated in acetone, cleared in acetone-xylol and then in xylol, then dried and mounted. The Zeiss photomicroscope used was equipped with green filter and automatic exposure control. Black and white photomicrographs were taken with Kodak Panatomic X film and color photomicrographs with Kodak Ektachrome 160 Tungsten Professional film.

J. ELECTROPHORETIC ANALYSIS OF PROTEINS

The microslab gel apparatus used was designed by Matsudaira and Burgess (1978). Separation gels (75 mm high x 10 mm wide x 0.5 mm thick) were 15% acrylamide-0.2% bis-acrylamide and the spacer gels 6% acrylamide-0.08% bis-acrylamide (Joffe et al., 1977). The Tris-glycine buffer formulations of Laemmli (1970) were used. Buffers and gels contained 0.1% SDS. Samples were dialyzed against 500 volumes of 1 mM ammonium acetate to remove sucrose, lyophilized to dryness and redissolved at 0.1-1.0 µg protein/ml in 2% SDS, 10% glycerol, 5% 2-mercaptoethanol in 0.125 M Tris-HCl, pH 6.8. Samples were heated for

1 minute in a boiling water bath and 1-5 µl was layered into the sample well. Bromphenol blue (0.001%) in sample buffer was layered into the wells at either side of the sample wells and electrophoresis was carried out at 200 V in a 6° cold room until the bromphenol blue had run off the bottom of the gel. Gels were stained with 1% Coomassie brilliant blue in methanol:water:acetic acid (5:5:1) for 15 minutes at room temperature, destained in the same solvent for 10 minutes and then in 7.5% methanol, 10% acetic acid for 50 minutes.

VITA

Kathleen Hall Larsen was born July 26, 1941, in Minneapolis, Minnesota. She attended the Minneapolis Public Schools and the University of Minnesota High School. In September of 1958, she entered Jackson College of Tufts University, where she received the Bachelor of Science degree, *magna cum laude*, in June 1962. In September of 1963, she entered Purdue University in the Department of Biological Sciences. She was awarded an NSF biochemistry traineeship during 1965 and 1966, and received the degree of Master of Science in August 1966. She enrolled in the graduate school at The University of Tennessee, Knoxville in September of 1972, and became a candidate for the Ph.D. in the Department of Microbiology in September of 1974. She was awarded an NIH predoctoral traineeship from May 1974, to June 1977, and a University of Tennessee research assistantship from July 1977, through April 1978. In May 1978, she joined the staff of the Environmental Mutagen Information Center at the Oak Ridge National Laboratory.

Kathleen Larsen received the degree of Doctor of Philosophy from the University of Tennessee in August 1979. She is married to the current John W. Larsen, and they have two former children, Elaine Claire Larsen and Kirsten Thora Larsen.