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Title: PHYSICAL STRUCTURE AND RECONSTITUTION OF CHROMATIN CORE PARTICLES

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The nucleosome appears to be the basic structural unit of the eukaryotic chromosome. The usual nucleosome preparation, isolated from a micrococcal nuclease digest of nuclei, is heterogenous in DNA length and protein composition with some particles containing spacer DNA and associated protein. This heterogeneity complicated physical and reconstitution studies. As a first step in studying the physical structure of the nucleosome, I have isolated and studied the core particle. It was separated from those nucleosomes containing spacer DNA and histones H1 and H5 by differential precipitation in 0.1 M NaCl. Large quantities of a very homogenous core particle preparation were later prepared by depleting chromatin of H1 and H5 and then digesting it with micrococcal nuclease.

The DNA in the core particle, while being more resistant than free DNA to thermal denaturation, denatures in two transitions. Forty base pairs of DNA in the core particle reversibly denature at 60°C in

1 mM cacodylate. The rest of the DNA, approximately 100 base pairs, denature irreversibly in the second transition at 74°C. Using circular dichroism spectroscopy to monitor the independent changes of the protein and DNA components, I found that the protein denatures only in the second transition. Nucleosomes containing spacer DNA were isolated and thermally denatured. The presence of H1 and H5 increased both the temperature and the amount of DNA melting in the second transition back to a value of 100 base pairs.

Core histones and DNA were isolated and reconstituted by salt gradient dialysis to reform the core particle. The reconstituted particles, which could be prepared in 80-90% yield, were found to be identical to the native particles by the various physical and biochemical tests available.

DNA molecules with 50 to 125 base pairs were isolated by preparative gel electrophoresis and reconstituted with core histones. Particles with less than 120 base pairs of DNA undergo intermolecular association reactions that depend on the size of the DNA and ionic strength. Particles containing 100 base pairs of DNA completely precipitate from solution at 0.15 M ionic strength while 120 base pair particles are completely soluble at the same ionic strength. Particles containing 110 base pair DNA undergo an intermediate degree of association.

Core particles were reconstituted with two pieces of DNA each

65 base pairs long. While nearly identical to the native particle under most conditions, the 65 base pair particle appears to dissociate at very low ionic strength. This conformational change may be a more drastic example of a low ionic strength change observed in nucleosomes using electron microscopy or hydrodynamic methods.

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ABBREVIATIONS

EDTA	(ethylenedinitrilo)-tetraacetic acid
PMSF	phenylmethyl sulfonyl fluoride
Cac.	cacodylic acid (hydroxydimethylarisine oxide)
HAP	hydroxyl apatite
bis	N, N'-methylene-bis-acrylamide
TEMED	N, N, N', N'-tetramethyethylenediamine
BMCE	2-mercapto-ethanol
TCA	trichloroacetic acid
TLCK	N-p-tosyl-L-Lysine chloromethyl ketone
CD	circular dichroism
bp	base pair of DNA
b	base of DNA
Tris	tris(hydroxymethyl) amino methane

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PHYSICAL STRUCTURE AND RECONSTITUTION OF CHROMATIN CORE PARTICLES

I. INTRODUCTION AND BACKGROUND

Our perception of the structure of eukaryotic chromosomes has changed dramatically in the last five years. Prior to 1972, chromatin was perceived as a continuous supercoil of duplex DNA, constrained by very basic lysine and arginine rich proteins called histones. These proteins were thought to interact with the DNA and with each other in some manner to supercoil DNA into a 100 Å diameter coil. The exact conformation of the histones was not well defined and nothing was known about the location of other proteins, lumped into the category of non-histone proteins. Higher order chromosomal folding was considered to be just further coiling of the 100 Å fiber.

Primary evidence for this model came from interpretations of the chromatin X-ray diffraction data of Luzzati and Nicolaieff (1959, 1963), Wilkins, Zubay and Wilson (1959) and Pardon, Wilkins and Richards (1967). While models such as four stranded helices of DNA (Luzzati and Nicolaeieff (1963)) were proposed, the most highly regarded model was the 100 Å diameter supercoil proposed by Pardon and co-workers (Pardon, Wilkins and Richards (1967), Richards and Pardon (1970), Pardon and Wilkins (1972)).

Of course, the supercoil model was not accepted without some dissent. Braum and Ris (1971) and Bram (1972) interpreted their

X-ray diffraction data in terms of a nonuniform supercoil. The supercoil was about 100 Å in diameter but had a 45 Å periodicity along the supercoil. Knobby 100 Å wide chromatin fibers were observed in electron micrographs by Bram and Ris (1971) and by Slayter et al. (1972). However, the chromatin model most generally accepted was the uniform supercoil.

In the early 1970's independent lines of research finally led scientists to accept a particulate model of chromatin structure. Clark and Felsenfeld (1971) discovered that microccal nuclease would digest only 50% of the DNA in chromatin to acid soluble fragments. Free DNA exhibited no such protection from nuclease. Furthermore, the protected fragments were low molecular weight, approximately 100-200 base pairs. This evidence plus data from polylysine binding studies indicated that "exposed" and "protected" areas of DNA in chromatin alternated in short repeats.

Hewish and Burgoyne (1973) observed that DNA fragments, produced by digestion of nuclei with endogenous nucleases, were multiples of a unit size, suggesting the sites of nuclease digestion were regularly spaced along the chromatin fiber. Williamson (1970) had earlier observed the same DNA pattern by gel electrophoresis but interpreted the fragments incorrectly as cytoplasmic DNA. Electron micrographs also supported the idea of a particulate structure of chromatin. Olins and Olins (1973, 1974) and Woodcock (1973)

observed beaded structures on chromatin strands from lysed nuclei. The 70 Å diameter beads were spaced regularly along the fiber, spaced with short connecting strands of DNA.

Rill and Van Holde (1973) first isolated and characterized a soluble chromatin fraction produced from micrococcal nuclease hydrolysis. The protein-DNA complexes appeared as globular particles that unfold with light trypsin digestion (Sahasrabuddhe and Van Holde, 1974). Electron micrographs of the particles (Van Holde et al., 1974a) were similar to those of the beads observed in chromatin. Noll (1974a) demonstrated that similar particles could be isolated from nuclei digested in situ with micrococcal nuclease.

At the same time, Isenberg and coworkers were characterizing the interactions between purified histones. They observed that the large aggregates formed from solutions of individual histones (Li et al., 1972; Smerdon and Isenberg , 1974; D'Anna and Isenberg, 1974b) could be inhibited by mixing specific combinations of histones (D'Anna and Isenberg, 1973, 1974a, c). Specific histone complexes formed, made up of two or more different histones. A thorough study of the different histone complexes led to a model of histone interaction involving all four core histones (H2A, H2B, H3, H4) (D'Anna and Isenberg, 1974c).

Kornberg and Thomas (1974) obtained a histone octomer, made up of the four core histones, by protein crosslinking in chromatin. Their study and the work of Isenberg and coworkers indicated that

histones formed very specific interactions with each other and were probably arranged as an octomer complex in chromatin.

Models for chromatin structure that developed (Olins and Olins, 1974; Van Holde et al., 1974b; Kornberg, 1974) all had chromatin arranged as "beads on a string" with histones organizing the DNA of each bead into a compact globular structure. Further micrococcal nuclease digestion studies indicated that while the repeating structure contained about 200 bp of DNA (Noll, 1974a), about 140 bp of the DNA was more highly resistant to nuclease (Sollner-Webb and Felsenfeld, 1975; Shaw et al., 1976). This more resistant structure (the core particle) did not contain histone H1 (or histone H5 in avian erythrocytes) indicating that these histones were associated with spacer or linker DNA between core particles.

At least 85-90% of the genome was judged to be organized in the subunit structure (Noll, 1974a; Axel, 1975) and all sequences, including specific active gene sequences were represented in the subunit structure (Axel et al., 1975). A repeating structure in yeast (Lohr and Van Holde, 1975) and pea (McGhee and Engel, 1975) provided further evidence for the ubiquity of the repeating subunit structure in eukaryotes.

The most recent models for the core particle structure, based on data from neutron diffraction (Pardon et al., 1977) and X-ray crystalography (Finch et al., 1977) call for 140 bp of DNA making 1.75 turns around an octomer of histones (two each of the four core histones H2A, H2B, H3, and H4). The independently derived models

both call for flat disc shaped structures, about 50 Å high and 110 Å in diameter. Additional DNA is thought to connect adjacent core particles. The conformation of this spacer DNA and the position of H1 is unfortunately not well understood but recent evidence supports either a superhelix of nucleosomes (Carpenter et al., 1976; Finch and Klug, 1976; Campbell et al., 1978) or superbeads of nucleosomes made up of 8 to 10 nucleosomes (Renz et al., 1977).

Detailed reviews of the recent advances in chromatin structure (Van Holde and Isenberg, 1975; Kornberg, 1977; Felsenfeld, 1978) give a more detailed account of the recent progress.

The importance of nuclease digestion studies on the development of our understanding of chromatin structure cannot be overemphasized. On the one hand, the nucleases have been powerful probes of chromatin structure. On the other hand nucleases provided scientists with a tool to make soluble chromatin particles, representative of whole chromatin in DNA and histone content. This has been extremely important, for now many of the sophisticated techniques used to study proteins could be used to study chromatin structure. Hydrodynamic, spectroscopic, and crystallographic studies that generally require relatively low molecular weight homogenous material have now become possible and informative.

This thesis is a study of the chromatin fragments produced by digestion with micrococcal nuclease. I have attempted to isolate,

characterize, and reconstitute homogenous chromatin subunits with the hope that knowledge of core particles, their conformational changes and associations may tell something about the structure and function of whole chromosomes.

II. THE PREPARATION AND FRACTIONATION OF NUCLEOSOMES FROM CHICKEN ERYTHROCYTE NUCLEI

Introduction

Nucleosomes prepared by the standard procedure (digestion of chromatin or nuclei by micrococcal nuclease) contain DNA fragments ranging from 120 bp to 200 bp. The size of the DNA fragments depend upon the extent of digestion: more extensive digestion producing nucleosomes with smaller DNA molecules (Axel, 1975; Sollner-Webb and Felsenfeld, 1975; Shaw et al., 1976). Along with heterogeneity in DNA molecular weight, small amounts of histones H1 and H5 are found in such nucleosome preparations, specifically associated with particles containing DNA 160 bp in length or larger (Varshavsky et al., 1976; Noll and Kornberg, 1977; Todd and Garrard, 1977).

The physical properties of nucleosomes could be profoundly influenced by histone content and DNA molecular weight. Indeed, preliminary evidence for this was found in nucleosome solubility and thermal denaturation experiments. I therefore investigated methods for fractionating the nucleosome into more homogeneous components. The two most common techniques for separating nucleosomes from larger and smaller chromatin fragments, gel filtration (Shaw et al., 1974, 1976) and sedimentation through sucrose gradients (Noll, 1974a), require size and shape differences for separation. But small

differences in DNA length or protein content probably do not significantly change the size and shape of nucleosomes. Thus, gel filtration or sucrose gradient sedimentation will not fractionate the nucleosome into components. It is not surprising therefore, that the protein or DNA profile across the nucleosome peak of a gel filtration chromatogram is constant (Shaw et al., 1976).

Techniques based on properties other than size and shape must therefore be found to fractionate or isolate more homogenous nucleosome preparations. I have investigated three such techniques: 1) hydroxylapatite chromatography, 2) solubility of nucleosomes in 0.1 M NaCl, and 3) micrococcal nuclease digestion of chromatin depleted of histones H1 and H5.

Hydroxylapatite chromatography holds some promise for the fractionation of nucleosomes but high ionic strengths were necessary to completely elute all the chromatin from the hydroxylapatite. The salt fractionation procedure separates a nucleosome preparation into two components by differences in solubility in 0.1 M NaCl. The soluble component contains mostly 144 bp DNA and only the core histones. The insoluble fraction contains nucleosomes with larger DNA fragments (165-185 bp) and histone H1 and H5 in addition to the core histones. A very homogeneous nucleosomal particle (core particle) was obtained in large quantities by nuclease digestion of chromatin depleted in H1 and H5. These preparations and fractionation procedures

are described in this chapter. The products of these procedures will be studied in the following chapters.

Results

I originally used hydroxylapatite chromatography in an attempt to fractionate nucleosome oligomers. Unfortunately, the individual nucleosome oligomers were not resolved and I terminated the experiments. Partial fractionation of the nucleosome monomer was obtained, however, therefore the results of the preliminary hydroxylapatite experiments are presented here.

Chromatin fragments, obtained from a micrococcal nuclease digest of nuclei, were absorbed to a hydroxylapatite column at low ionic strength (0.01 M phosphate buffer, pH 6.8) and then eluted off the column with a salt gradient. The column elution profile is presented in Figure 1. A major disadvantage with this technique as a method of fractionating chromatin is that high ionic strength buffer is necessary to elute all the chromatin, with protein stripping as a possible result. DNA was isolated from column fractions and electrophoresed on polyacrylamide gels. The results are shown in the insert in Figure 1 and from a separate chromatography run in Figure 2. The DNA from fractions a and b of Figure 1 illustrate the partial fractionation of the nucleosome achieved with hydroxylapatite. Fraction a (Figure 2a) contains 140-170 bp DNA while fraction b (Figure 2b) contains

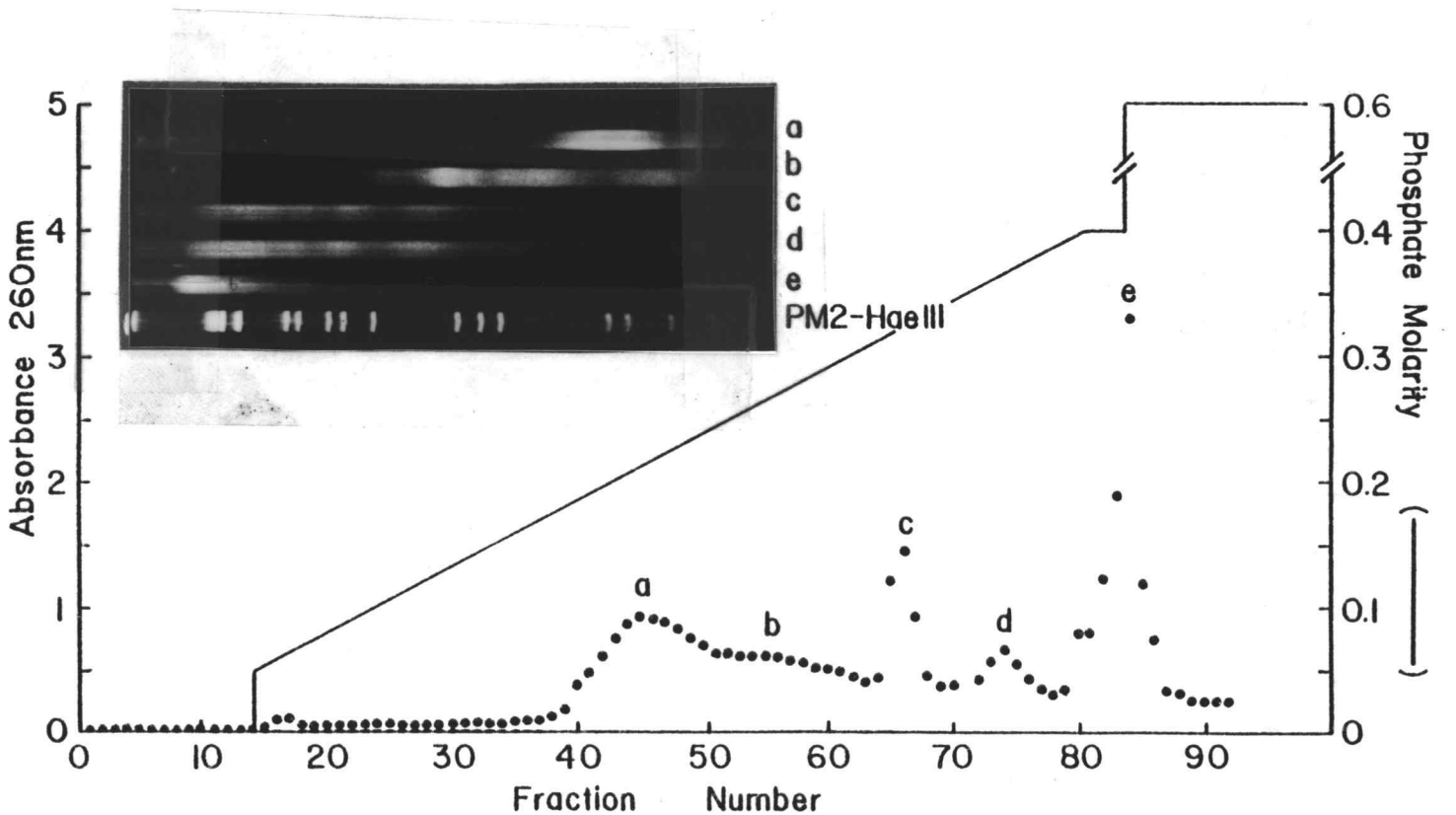

Figure 1. Elution profile of chromatin from a hydroxylapatite column. Chromatin, previously digested with micrococcal nuclease, was absorbed to a hydroxylapatite column and then eluted from the column with a salt gradient. DNA was isolated from the peak fractions and electrophoresced on 3.5% polyacrylamide tube gels. A photograph of the ethidium bromide stained gels is shown in the insert. Electrophoresis is from left to right.

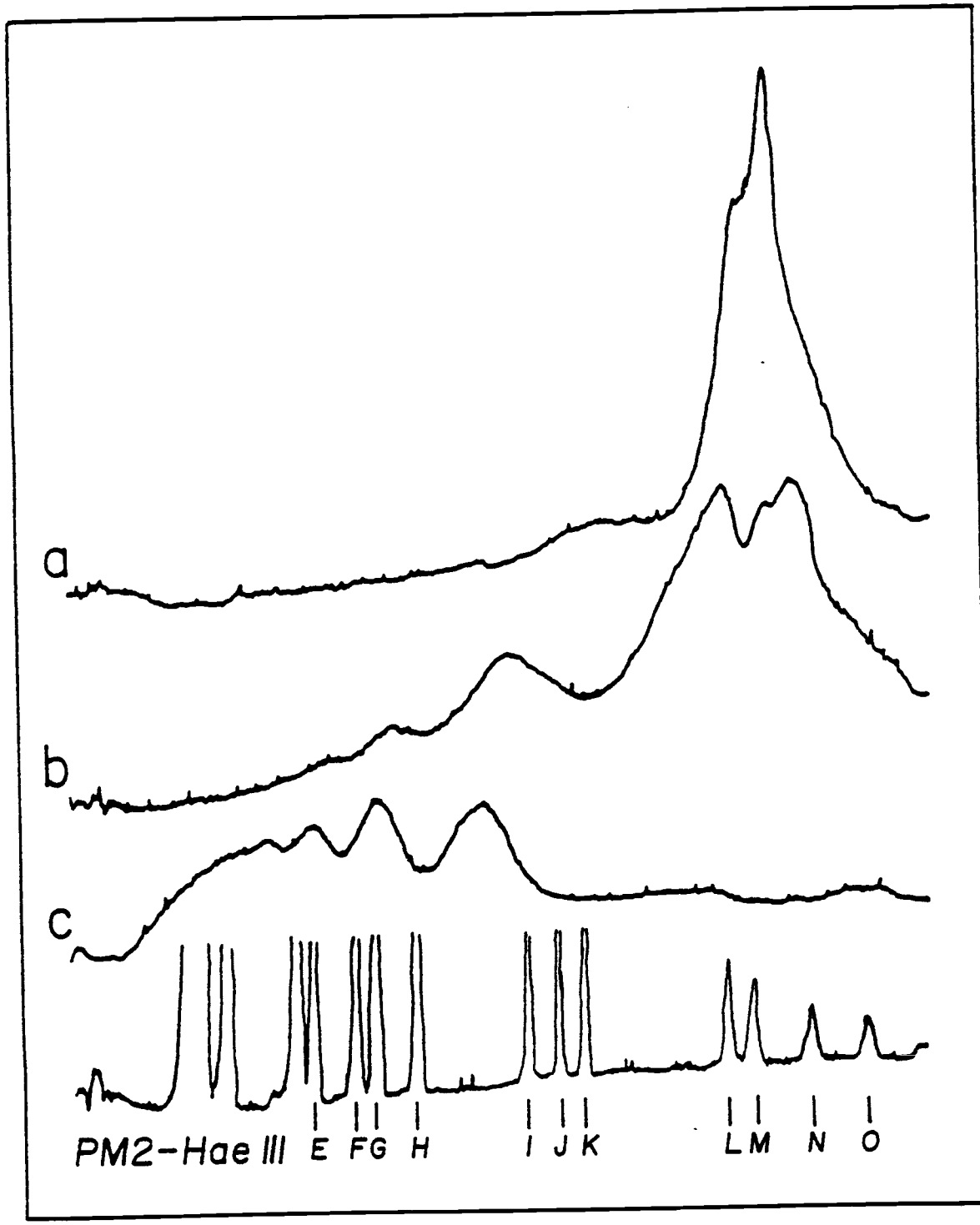

Figure 2. DNA content of chromatin fractions eluted from a hydroxylapatite column. DNA was isolated from peaks a, b, and c of a hydroxyl apatite chromatography run (see Figure 1) and electrophoresced on 3.5% polyacrylamide tube gels. Densitometer scans of the stained gels are presented here along with the scan of PM2-Hae III restriction fragments, used as DNA size markers. The sizes of the restriction fragments are found in Table 1.

DNA larger and smaller than that of fraction a. The proteins present in each fraction were not characterized, but peak a did cosediment with nucleosomes in the ultracentrifuge, indicating most of histones were still associated with the DNA.

Knowledge of the exact size of DNA in a nucleosome preparation is necessary for comparing different preparations. The size of DNA in a particular structure will also profoundly influence the models that can be proposed. DNA standards of known molecular weight are therefore a prerequisite for studies on nucleosome structure. HaeIII restriction fragments of bacteriaphage PM2 DNA have been used as size markers for all electrophoresis experiments. The sizes of these fragments have been determined using sequenced restriction fragments of SV-40 DNA as primary standards. The most recent calibration data was provided by Dr. R. T. Kovacic (private communication). The results, presented in Table 1, are in good agreement with sizes calculated by Noll (1976b) but are slightly larger than the sizes previously reported by Kovacic and Van Holde (1977). The availability, in the last year, of a set of sequenced DNA fragments has allowed a more accurate calibration.

Selective precipitation of nucleosomes in 0.1 M NaCl is a useful technique for fractionating the particles. Jeff Corden, a fellow graduate student, reported to me that a nucleosome preparation was maximally but only partially insoluble in 0.1 M NaCl. I have followed up

Table 1. PM2-Hae III restriction fragments

PM2-Hae III fragment	Fragment molecular weight (bp)[a]
A	1970
B	1824
C	1445
D	900
E	842
F	685
G	632
H	520
I	337
J	299
K	273
L	162
M	148
N	120
O	97
P	50
Q	47

[a]The fragment molecular weights were determined by co-electrophoresis of SV-40 restriction and PM2-Hae III fragments. These results were kindly provided by Dr. R. T. Kovacic.

this observation and the results are presented here.

A nucleosome preparation goes through a solubility minimum at about 0.1 M NaCl with 20-30% of the nucleosomes precipitating out of solution. The actual amount precipitating varies from preparation to preparation (see Figure 3). The soluble and aggregated fractions were separated by low speed centrifugation and analyzed with respect to DNA length and histone content. The precipitate contains most of the 165 bp and larger DNA, and in some cases 120 bp DNA, while the soluble fraction contains more of the 144 bp DNA size class (Figure 4). Histones H1 and H5 are found exclusively in the pellet (Figure 5). Salt precipitation of some preparations led to the isolation of nearly pure 170-185 bp nucleosomes in the pellet; such preparations will be studied by thermal denaturation in Chapter III. The supernatant of the 0.24 M NaCl incubation (Figure 5, slot 4) contained a small quantity of histone H1 and consequently the pellet contained relatively less H1 (Figure 5, slot 5). Olins et al. (1976) have independently developed a similar method of nucleosome fractionation using selective precipitation in 0.1 M KCl.

Exogenous histone H1 causes a similar precipitation of nucleosomes. Purified calf thymus H1 and the nucleosome fraction soluble in 0.12 M NaCl were mixed in a 1:1, mole H1:mole nucleosome ratio at low ionic strength. This mixture of H1 and nucleosomes was completely soluble at low ionic strength (10 mM Tris-HCl, pH 7.2,

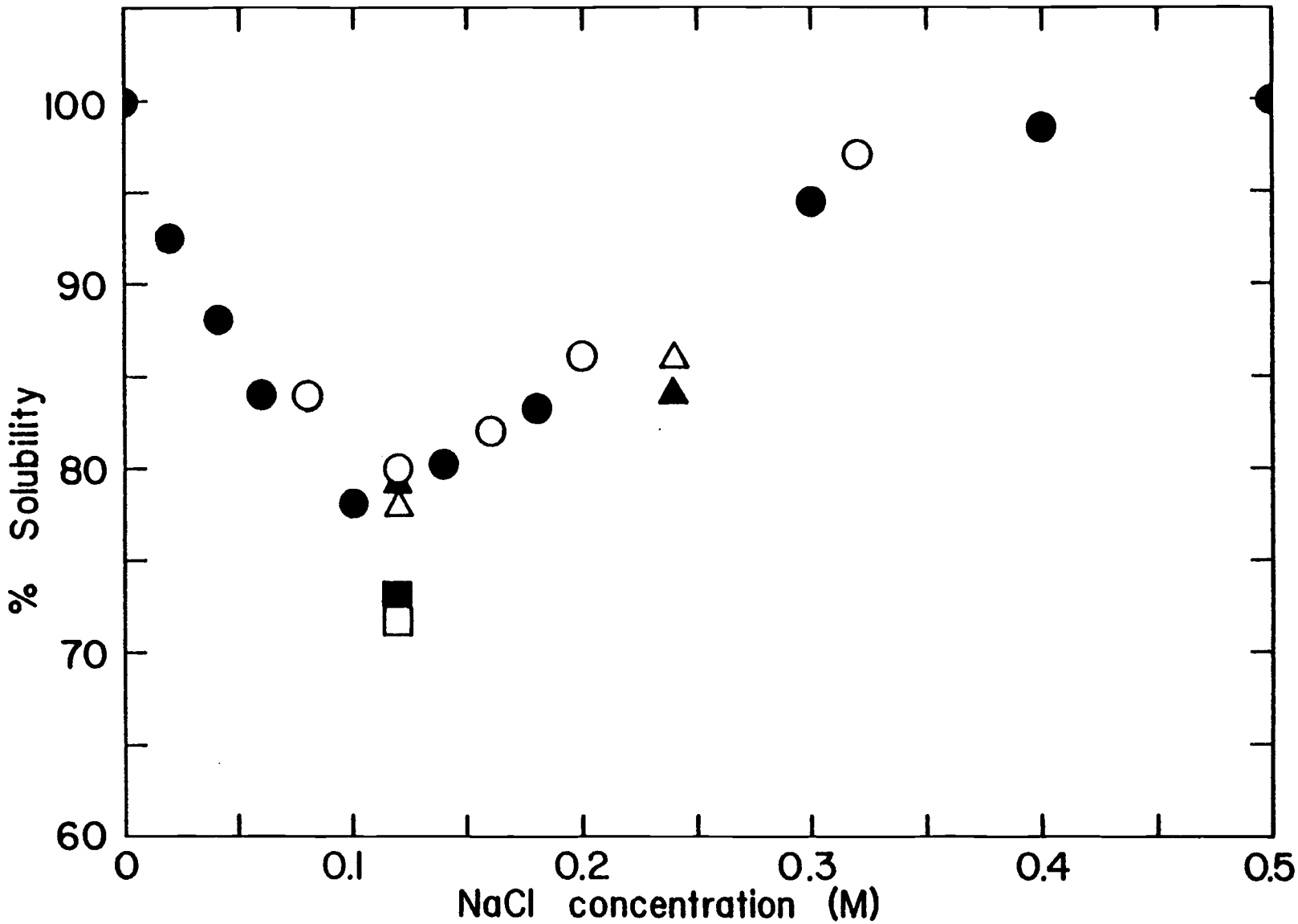

Figure 3. Solubility of nucleosome preparations as a function of salt concentration. The various symbols refer to independent preparations.

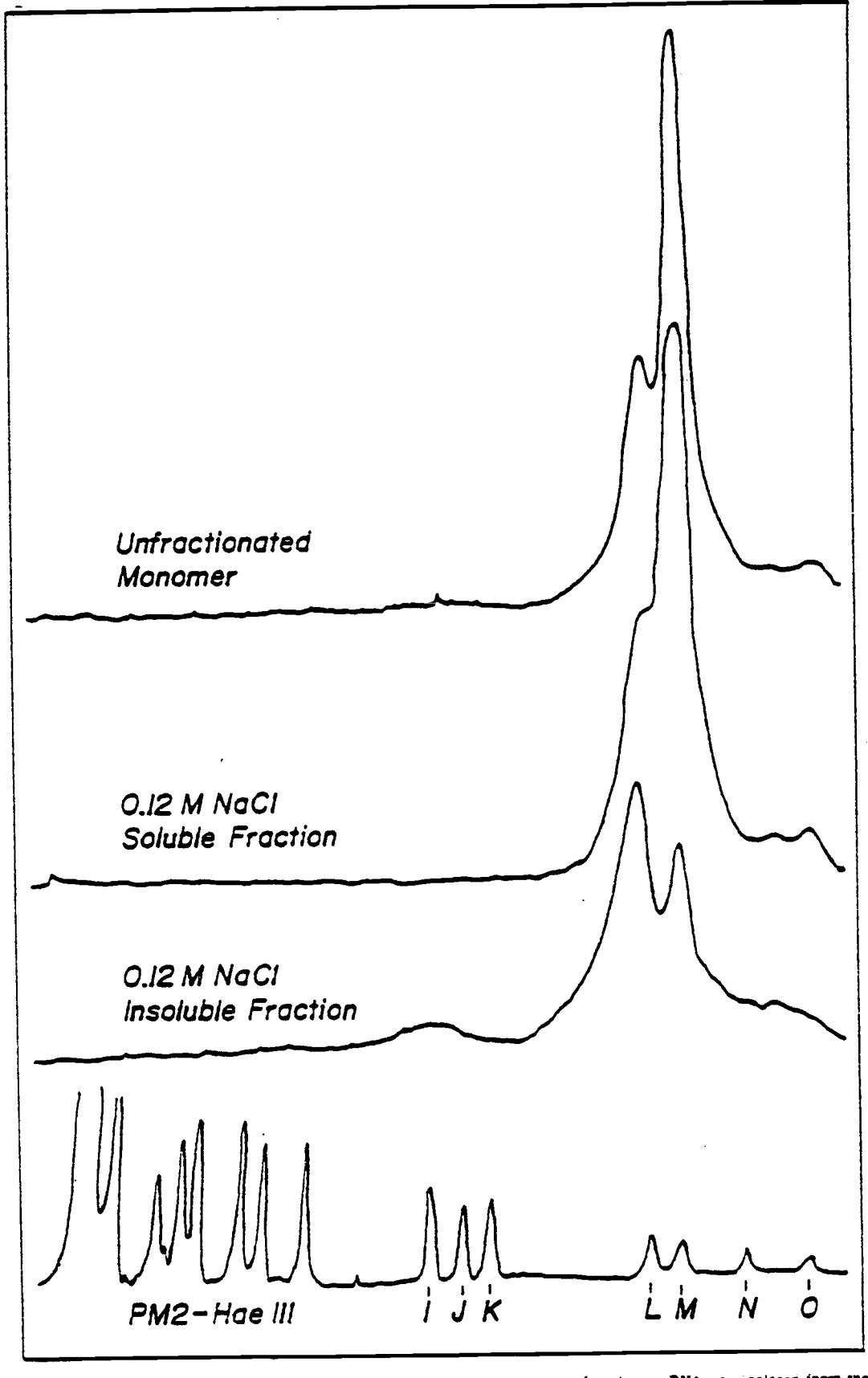

Figure 4. DNA content of 0.12 M NaCl soluble and insoluble nucleosome fractions. DNA was isolated from the various fractions and electrophoresed on 3.5% polyacrylamide tube gels. Scans of ethidium bromide stained gels are shown here.

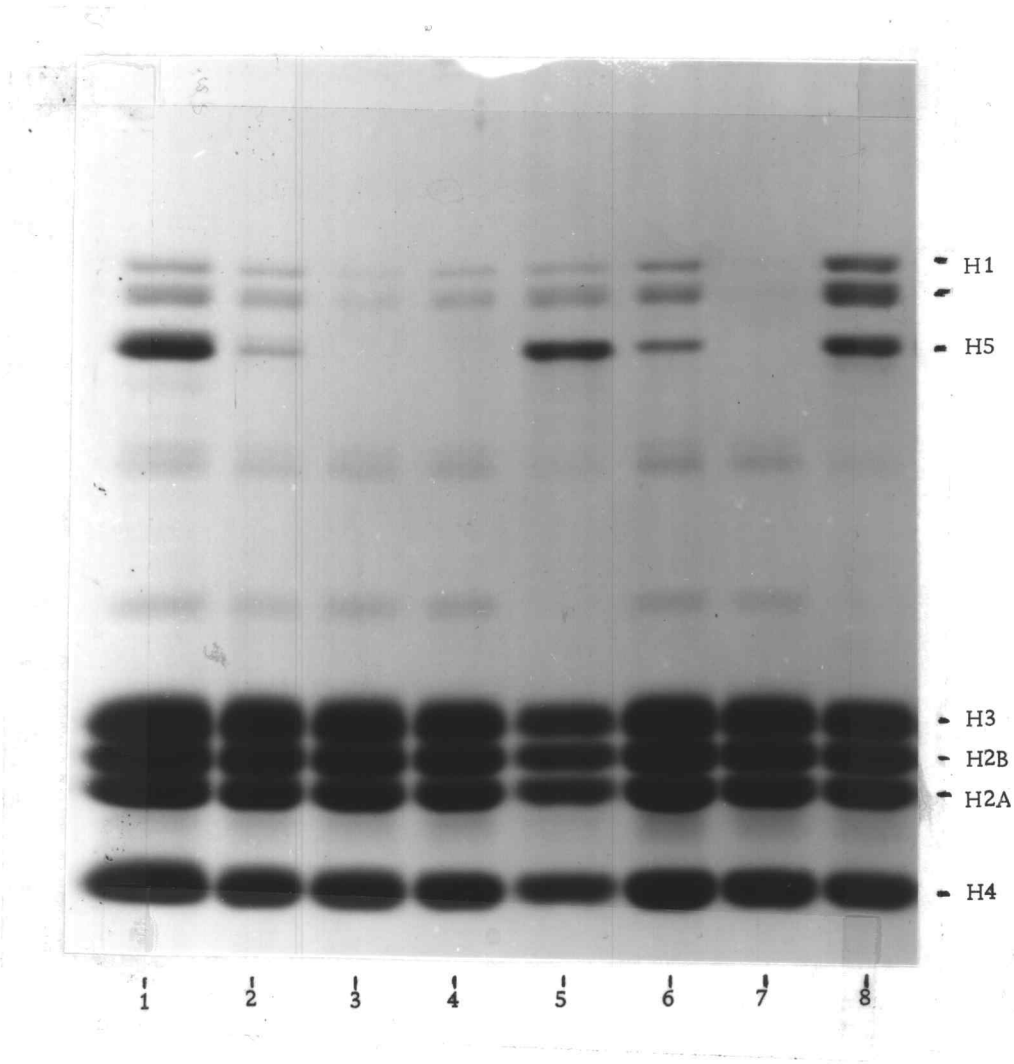

Figure 5. Histone content of NaCl soluble and insoluble nucleosome fractions. Nucleosome fractions were electrophoresed on a 15% polyacrylamide-SDS slab gel, stained with comassie blue, destained and photographed. The channels from left to right are: 1, chicken erythrocyte histone standards; 2, total nucleosome; 3, 0.12 M NaCl soluble; 4, 0.24 M NaCl soluble; 5, 0.24 M NaCl insoluble; 6, total nucleosome; 7, 0.12 M NaCl soluble; 8, 0.12 M NaCl insoluble.

0.7 mM Na_2EDTA) but precipitated maximally at 0.12 M NaCl. In fact, the curve of solubility vs. salt concentration (Figure 6) closely resembled the precipitation curve for native nucleosomes (Figure 3). Almost all the nucleosomes precipitated in 0.12 M NaCl by the addition of only two molecules of H1 per particle. This result is illustrated in Figure 7 as the fraction of nucleosomes soluble in 0.12 M NaCl vs. the concentration of added H1 per nucleosome. The sigmoidal shape of this titration curve is evidence that the precipitation process may be cooperative.

Although the nucleosome fraction soluble in 0.1 M NaCl contains mostly 144 bp DNA and is devoid of histones H1 and H5, there is always some larger DNA present. I obtained more homogeneous preparations of core particles following the observation of Noll (1976a) that nuclease digestion of chromatin depleted of H1 led to a more homogeneous nucleosome preparation. I depleted chromatin of H1 and H5 and most non-histone chromosomal proteins by 0.65 M NaCl washes prior to digestion with micrococcal nuclease. The digestion of H1, H5 depleted chromatin for various lengths of time by nuclease produces the DNA profiles presented in Figure 8. Two important observations from this time course are: 1) The 144 bp DNA size class becomes the predominant product of the chromatin digest. 2) A DNA peak at 265 bp is also a stable digestion product. Fractionation of a micrococcal nuclease digest of H1, H5 depleted chromatin by sedimentation

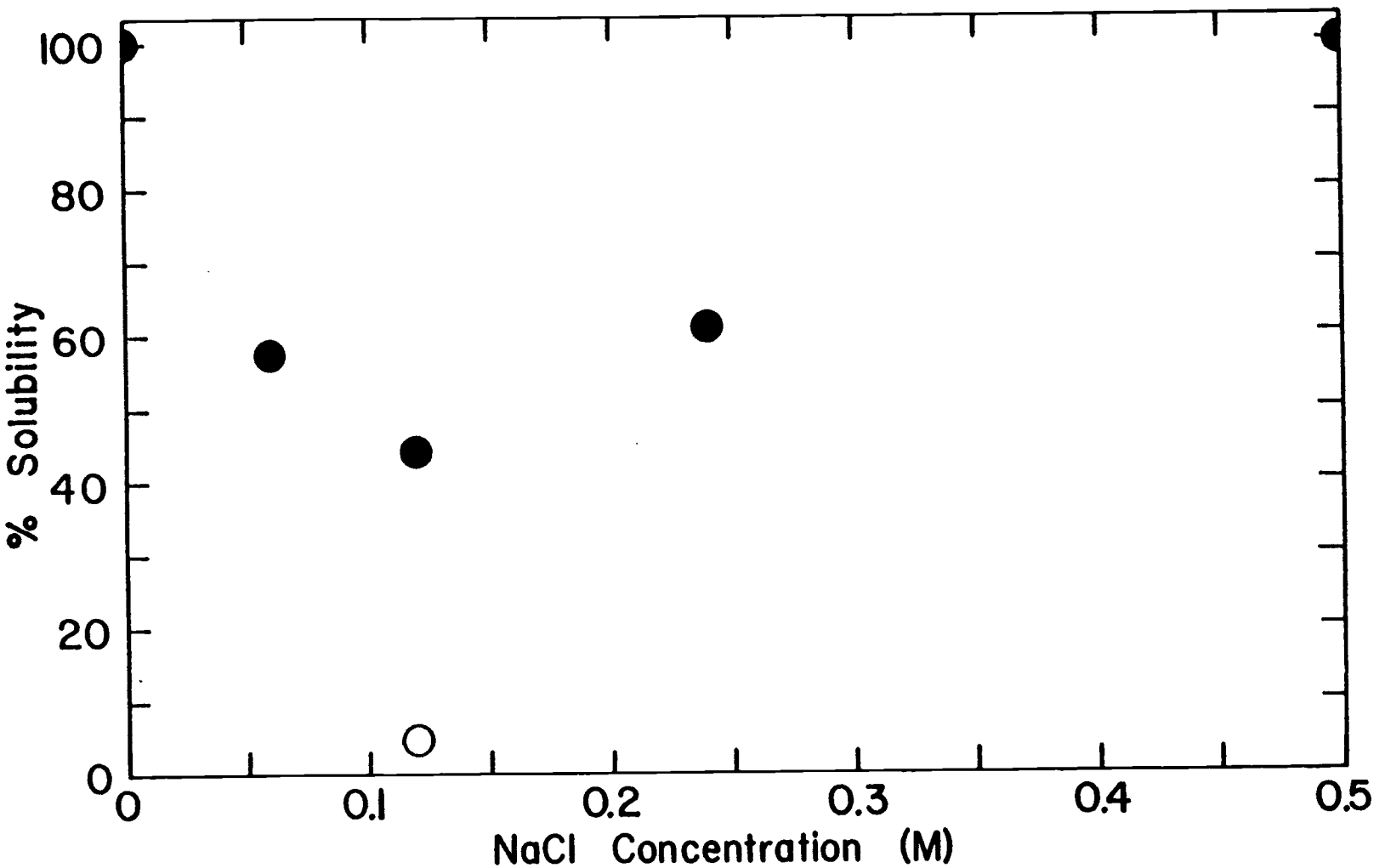

Figure 6. Solubility of histone H1-nucleosome mixtures as a function of salt concentration. (●) 1 Mole H1/Mole nucleosome, (O) 2 Moles H1/Mole nucleosome.

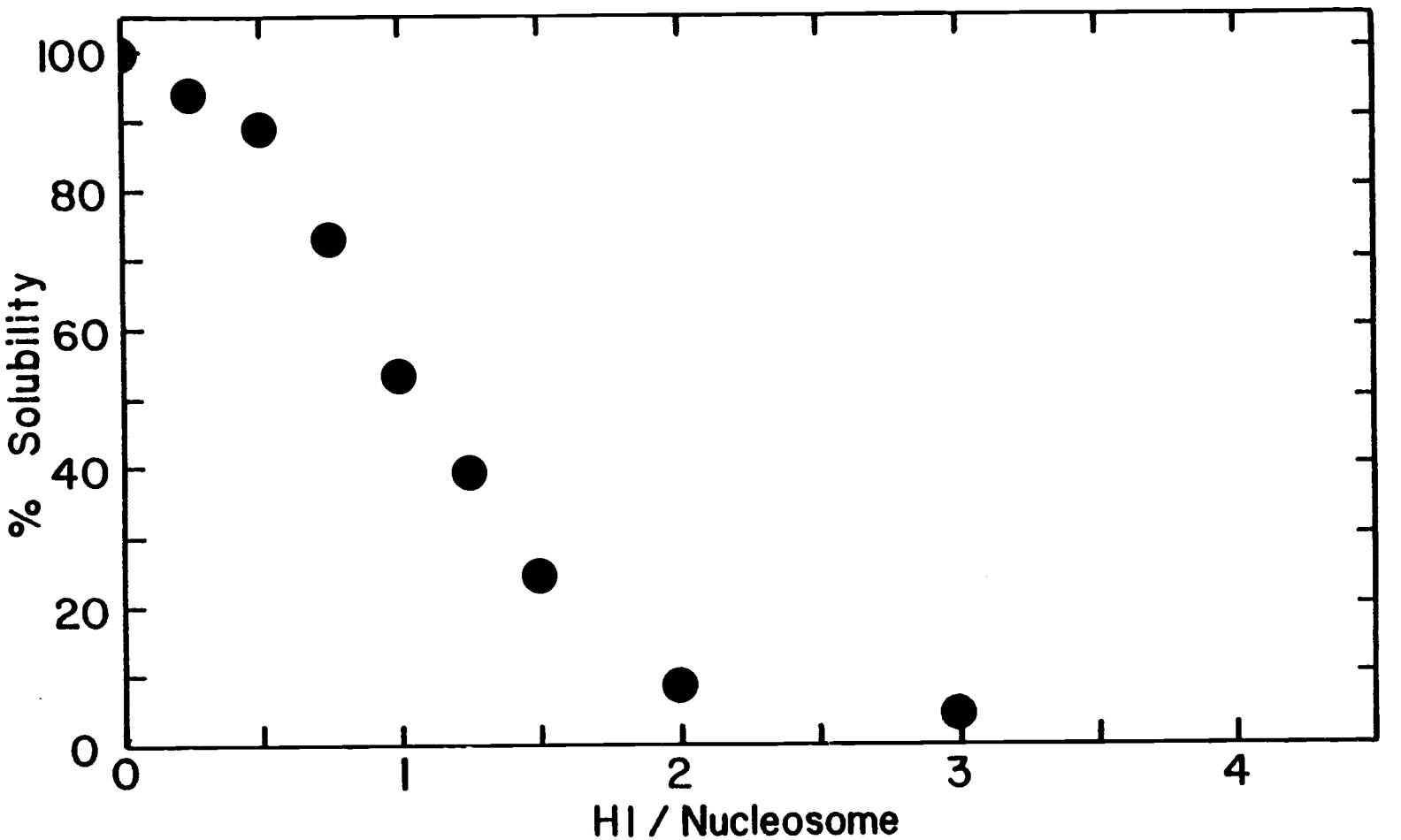

Figure 7. Solubility of H1-nucleosome solutions in 0.12 M NaCl at various H1/nucleosome molar ratios.

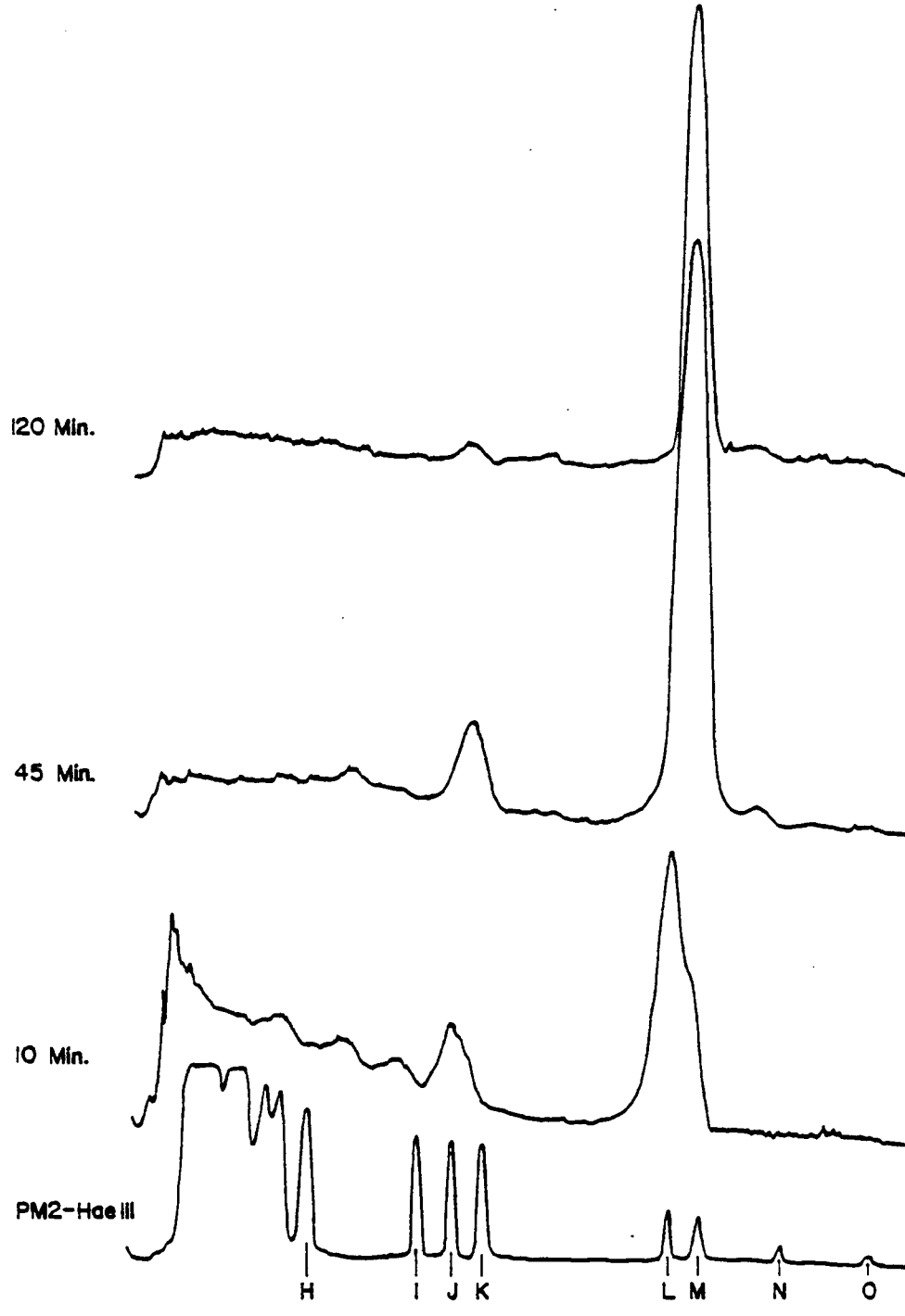

Figure 8. Micrococcal nuclease digestion of H1, H5 depleted chromatin. H1, H5 depleted chromatin was digested with micrococcal nuclease. Portions of the chromatin were removed from the reaction mixture at the designated times and the nuclease digestion was terminated by the addition of EDTA. The DNA was isolated from the various time points and electrophoresed on a 6% polyacrylamide slab gel. Densitometer tracings of the stained gels are shown here.

through sucrose gradients yields a very homogenous core particle in high yield. The half width of this 144 bp core particle DNA is only 5 bp. A small quantity of nucleosome oligomers are also isolated from the sucrose gradient. The dinucleosome contains only 265 bp of DNA, considerably less than the usual dinucleosome isolated from a nuclease digest of whole nuclei. These oliomers, called compact oligomers, will be studied in Chapter V.

Discussion

A number of different procedures for nucleosome fraction have been used to obtain more homogeneous particles. Besides the methods mentioned here, Todd and Garrard (1977) have used gel electrophoresis to fractionate nucleosomes into components. They found a number of components but only in analytical quantities.

The digestion of H1, H5 depleted chromatin gave the best results here in terms of quantity and quality. A 20-40 mg. preparation of core particles could be obtained routinely. This was enough material for calorimetry, lasar raman spectroscopy, dry weight analysis, and crystallization experiments; techniques that require large quantities of material.

The differential salt precipitation procedure has the advantage of separating a nucleosome preparation into one fraction containing

the lysine rich histones and another containing only the core histones. The observation that the 0.24 M NaCl precipitate contained relatively more H5 than H1 (Figure 5) indicates that this procedure might separate particles containing only H5 from bulk nucleosomes if the optimum conditions could be found. The hydroxylapatite method might also be exploited in fractionating nucleosomes if the optimum conditions could be found.

The mechanism for the differential salt precipitation is unknown at the present time but it seems to involve the action of the lysine rich histones. One possibility could be a simple aggregation of less negatively charged particles containing extra basic proteins. It might also involve ionic strength dependent cross-linking by H1. Evett and Isenberg (1969) found that a solution of polylysine and DNA was most turbid at the ionic strength at which the complex of polylysine and DNA started to disassociate. One model for this phenomena is an intermolecular crosslinking between the polylysine and DNA at the ionic strength where polylysine-DNA disassociation starts. The soluble complexes become "sticky" just prior to complete dissociation, causing large intermolecular aggregates to form. A similar mechanism could be proposed for H1 dependent precipitation of nucleosomes since H1 completely disassociates from chromatin before 0.5 M NaCl.

Renz and co-workers have studied the binding of H1 to

nucleosome oligomers (Renz et al., 1977). They find two modes of H1 binding: a low ionic strength mode (below 20 mM NaCl) and a high ionic strength mode (80 mM NaCl). The binding of H1 to nucleosome oligomers at low ionic strength is independent of the molecular weight of the chromatin whereas H1 binding in 80 mM NaCl increases to a maximum with chromatin containing seven to eight nucleosomes. Renz and Day (1976) had earlier studied the binding of H1 to purified DNA and again found two modes of binding; binding at low ionic strength was non-cooperative but binding at 80 mM NaCl was cooperative.

Interestingly, Smerdon and Isenberg (1976) have discovered that purified H1 folds cooperatively at the same ionic strength that Renz and co-workers observe the high ionic strength binding mode. At very low ionic strength H1 is a random coil or non-globular polypeptide. Hardison et al. (1977) and Bonner (1978) find that H1 can be chemically crosslinked to other H1 molecules and possibly core histones. While these crosslinking studies only indicate the close proximity of proteins and not necessarily protein binding they still indicate the possibility for such interactions. These findings suggest that the condensation or precipitation of chromatin by H1 may be a very complex phenomena. Various interactions of H1 with both DNA and other proteins may be involved in the process.

It is important to point out that this aggregation phenomena does not necessarily require nucleosomes with spacer DNA even though

the lysine rich histones (H1 and H5) are thought to bind to the spacer region *in vivo* (Shaw *et al.*, 1976; Noll and Kornberg, 1977; Varshavsky *et al.* (1976) since most of the nucleosomes in the experiment with exogenous H1 contained only 144 by DNA.

Although there is too little data available to choose a plausible model for the nucleosome precipitation induced by H1, the fact that this aggregation phenomena occurs at the same ionic strength where chromatin precipitates suggests that a mechanism could be related to the precipitation or condensation of chromatin (Bellard *et al.*, 1976; Thoma and Koller, 1977; Christiansen and Griffith, 1977; Muller *et al.*, 1978). Researchers have not been very successful in their effort to elucidate the structure of H1 in chromatin and its interactions with DNA and other proteins. One reason for this lack of success may be due to the plastic nature of H1. The structure of H1 depends on the ionic strength and may vary with the different H1 subfractions or the way in which H1 is enzymatically modified. Experiments with purified H1 and core particles may be one method of studying this complex interaction.

III. THERMAL DENATURATION OF NUCLEOSOMES

Introduction

The nucleosome, containing 140-200 bp of DNA and histones is now recognized as the fundamental building block of chromatin. Since these structures involve a large fraction of the genome, including at least some of the transcriptionally active regions (Lacy and Axel, 1975; Foe et al., 1976; Laird et al., 1976; Gottesfeld et al., 1976; Kuo et al., 1976), analysis of their structure and condition for their stability is of importance. Thermal denaturation has been used to study what forces hold the nucleosome together and what portions of the DNA interact most strongly with the histones. A number of such studies have appeared, both with whole chromatin (Ohlenbusch et al., 1967; Bekhor et al., 1969; Huang and Huang, 1969; Murray, 1969; Henson and Walker, 1970; Spelsberg et al., 1971; Li, 1972; Subirana, 1973; Reeck, 1976; Miller et al., 1976) and nucleosomes (Woodcock and Frado, 1975; Mandel and Fasman, 1976; Lawrence et al., 1976). However, melting studies of chromatin are complicated because different regions of the DNA interact with different proteins, some regions with core histones, some with lysine rich histones, and some with non-histone proteins. Protein-protein interactions could also influence the thermal denaturation studies. Most thermal denaturation studies on nucleosomes are also complicated by heterogeneity in

DNA length and protein content.

I have studied the thermal denaturation of chromatin by starting with the most homogeneous nucleosomal particles presently available, the core particle, and then moving up to the next level of complexity, a nucleosome containing 170-185 bp of DNA and the lysine rich histones. The thermal denaturation was studied using hyperchromicity and in some cases circular dichroism (CD) spectroscopy. This work was part of a collaborative effort with Drs. W. Weischet, K. E. Van Holde and H. Klump (Weischet et al., 1978).

Results

The chromatin core particle, isolated from a micrococcal nuclease digestion of H1 and H5 depleted chicken erythrocyte chromatin, contains 144±5 bp of DNA and only the four core histones H2A, H2B, H3, and H4. The protein and DNA components are shown in Figures 9 and 10 respectively. Such particles sediment as a homogeneous boundary in the ultracentrifuge with an $S_{20,w} = 10.9 \pm 0.1$.

The thermal denaturation of core particles, as followed by hyperchromicity measurements at 260 nm is a reproduceable biphasic process. Figure 11 illustrates a typical melting experiment with the data represented by percent hyperchromicity in the top panel and the first derivative of hyperchromicity in the lower panel. The biphasic profile is reproduceable from preparation to preparation and is even

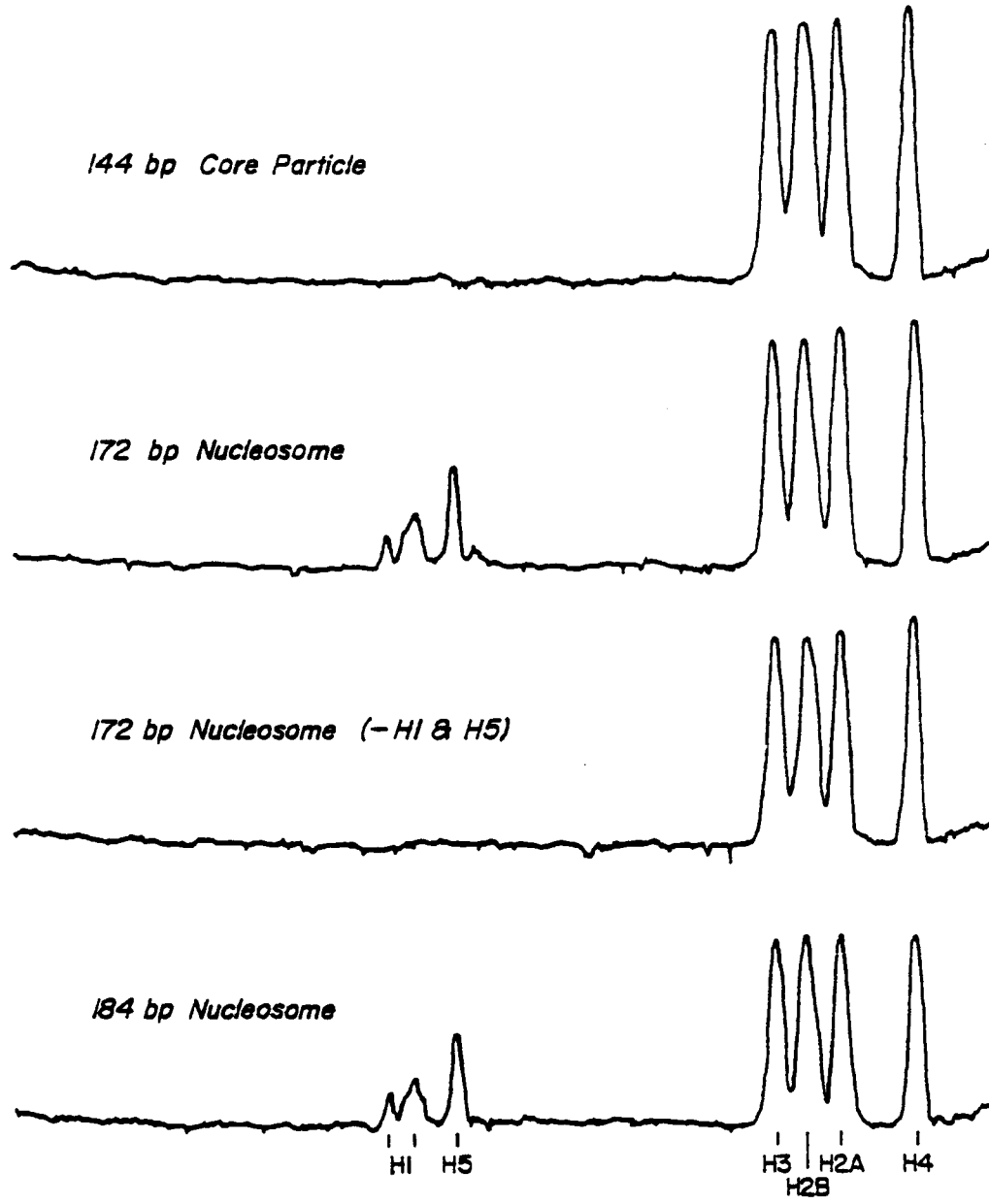

Figure 9. Histone content of different nucleosome preparations. The indicated chromatin samples were lyophilized and then dissolved directly in gel electrophoresis sample buffer. Samples were electrophoresed on 15% polyacrylamide-SDS gels, stained with coomassie blue, and scanned with a densitometer.

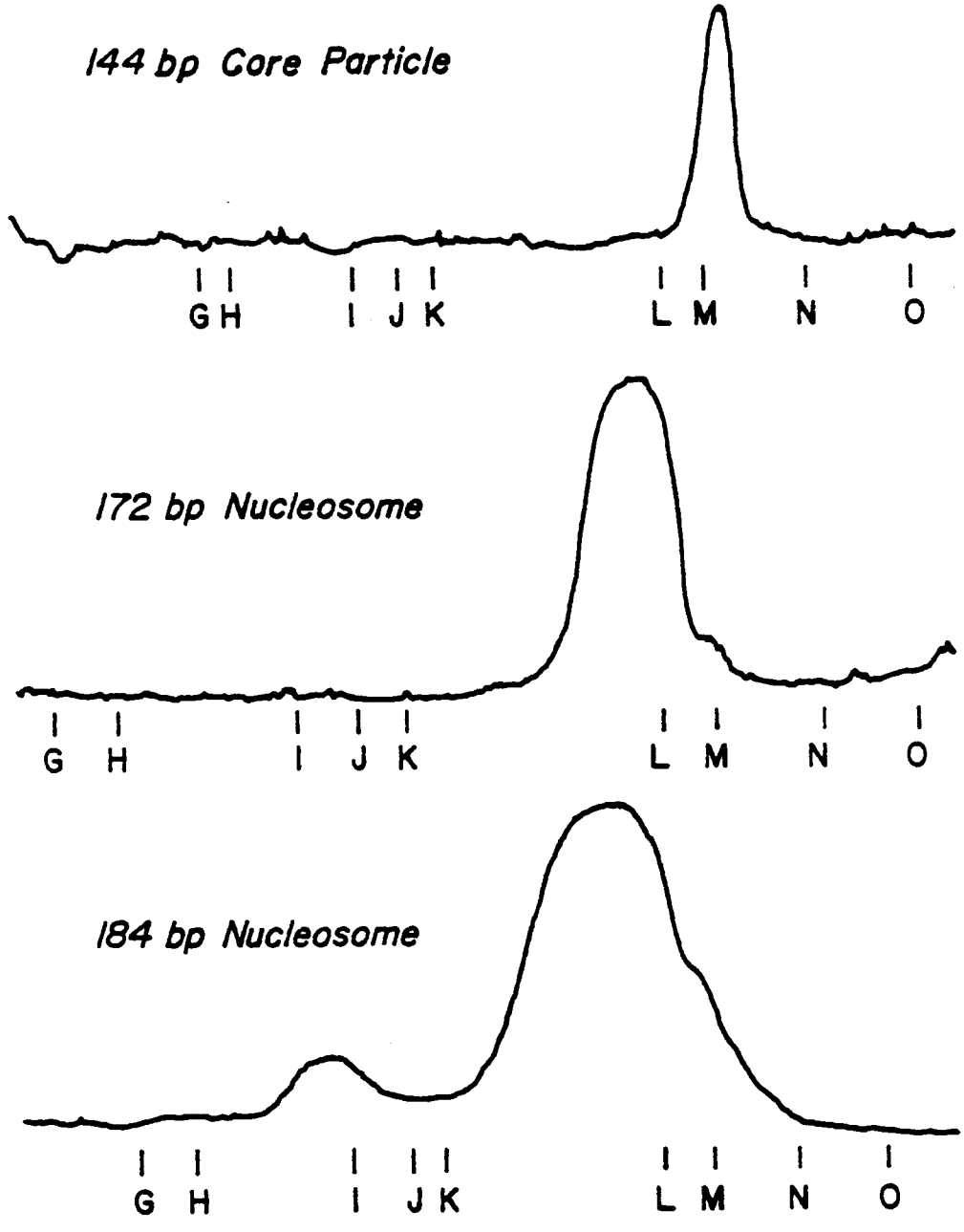

Figure 10. DNA content of different nucleosome preparations. DNA was isolated from the indicated preparations, electrophoresced under nondenaturing conditions on polyacrylamide slab gels, stained with ethidium bromide, photographed and the appropriate wells scanned with a densitometer. PM2-Hae III fragments were electrophoresced on adjacent wells and the positions of migration are marked below each scan. Each sample was electrophoresced on a separate gel so the PM2-Hae III positions are not coincident.

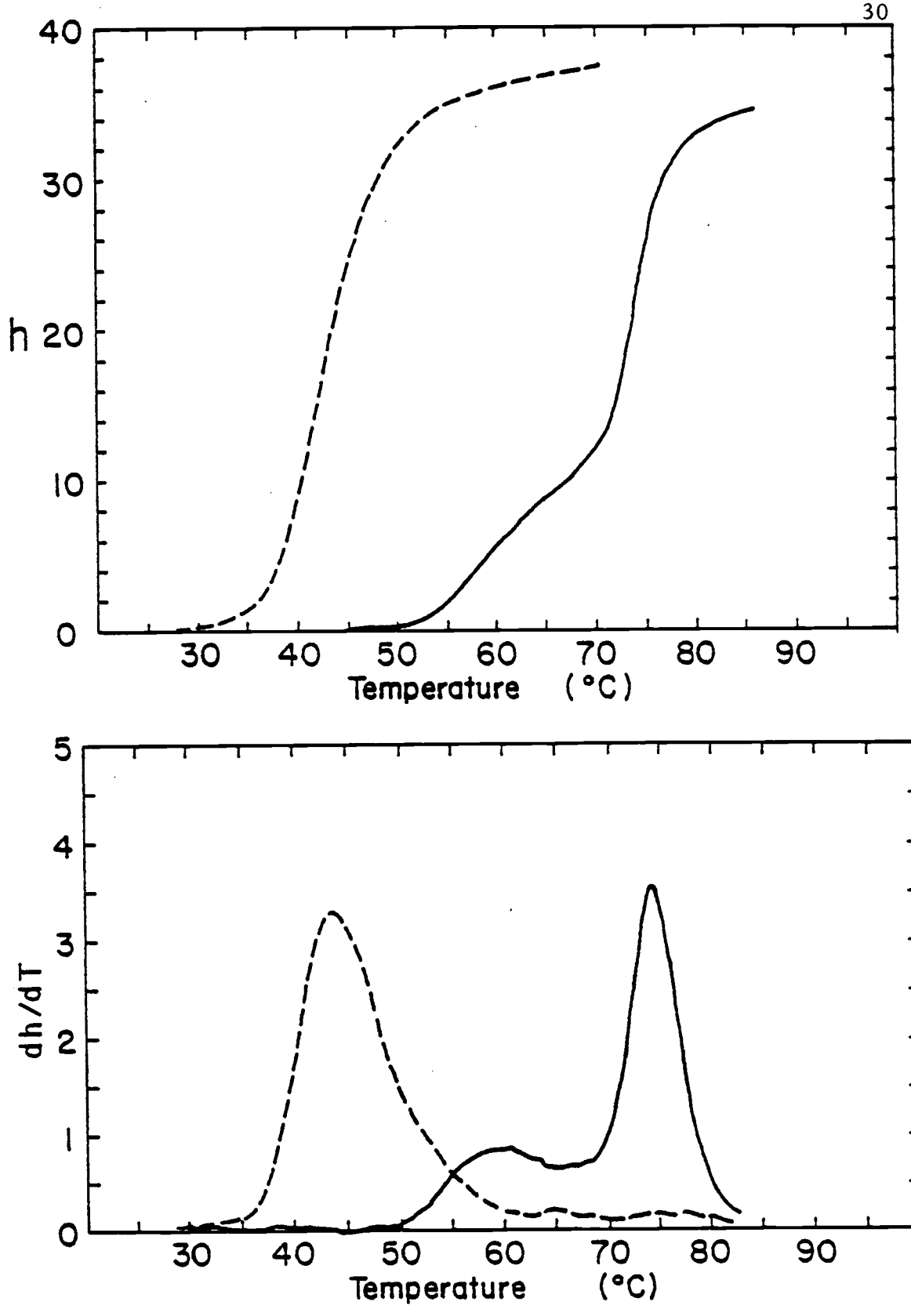

Figure 11. Hyperchromicity (h) and the first derivative of hyperchromicity (dh/dT) at 260 nm. for core particles (———) and core particle DNA (- - - - - -).

identical to the thermal denaturation curve of reconstituted core particles (see Chapter IV). Nevertheless, this profile is strongly influenced by the presence of lysine rich histones and the length of the DNA (see below).

The unquestionably biphasic curve suggests that different portions of the DNA in the core particle interact differently with the core histones. A distinguishing characteristic of these transitions is the reversibility of the first transition. If the temperature is raised to 62°, at which point the first transition is largely complete, and then reduced, the original absorbance is regained with the thermal denaturation curve showing only slight hysteresis. A second denaturation of this preparation is indistinguishable from the first. Henson and Walker (1970) and Subirana (1973) have previously observed partial reversibility in the first melting transition of chromatin. On the other hand, if the temperature is now raised to the midpoint of the second transition (74°) the original melting curve is not retraced upon cooling. This is illustrated in Figure 12 as a plot of percent hyperchromicity vs. temperature.

I next wanted to determine what conformational changes were occurring in the protein core during the two melting transitions. To do this the circular dichroism spectra of core particles were examined as a function of temperature. Using this technique, the protein contribution to the spectra can be distinguished from the DNA

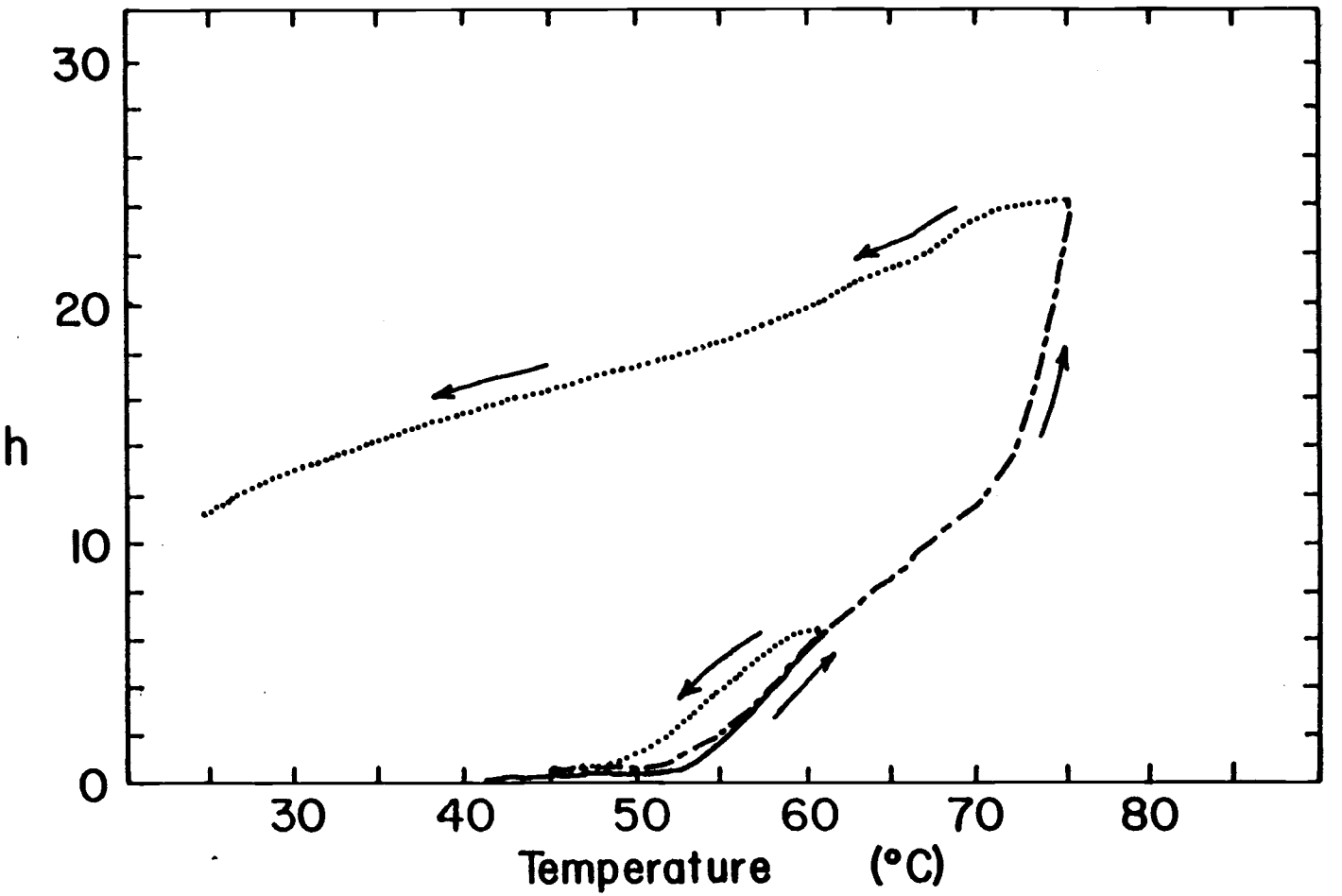

Figure 12. Hyperchromicity as a function of temperature illustrating the partial reversibility of the denaturation process. The solid (———) and dashed (— — —) lines correspond to the first and second temperature increases respectively. The dotted lines (· · · · · ·) correspond to temperature decreases. The rate of heating and cooling was the same (0.25 °C/minute).

contribution. Figure 13 displays circular dichroism spectra of core particles and free DNA in solution as a function of temperature (in two wavelength ranges). The chromatin spectra above 250 nm is almost entirely due to the DNA (Simpson and Sober, 1970; Permogorov et al., 1970; Fasman et al., 1970), and is strongly influenced by conformational changes of the chromatin (Li et al., 1975). At shorter wavelengths both DNA and protein contribute to the spectra (Hjelm and Huang, 1975) but the DNA contribution is minimal (Fasman et al., 1970; Wilhelm et al., 1974). Since the DNA spectra around 220 nm is little effected by thermal denaturation (Figure 13 and Usatyi and Shylahktenko, 1973), the relative contribution of histones and DNA to the overall CD spectra of the core particles can be determined at all temperatures. I am assuming that the DNA contribution to the CD of core particles around 220 nm does not change upon thermal denaturation. Hjelm and Huang (1975)claim that the DNA contribution to the CD of chromatin below 230 nm is the same as that of DNA in solution at low ionic strength. But even this assumption does not preclude the possibility that the DNA contribution may change upon the melting of core particles. Nevertheless, the DNA contribution around 220 nm is probably negligible considering the larger protein contribution.

Inspection of Figure 13 shows that increasing temperature shifts the spectrum above 250 nm towards the spectrum exhibited by free

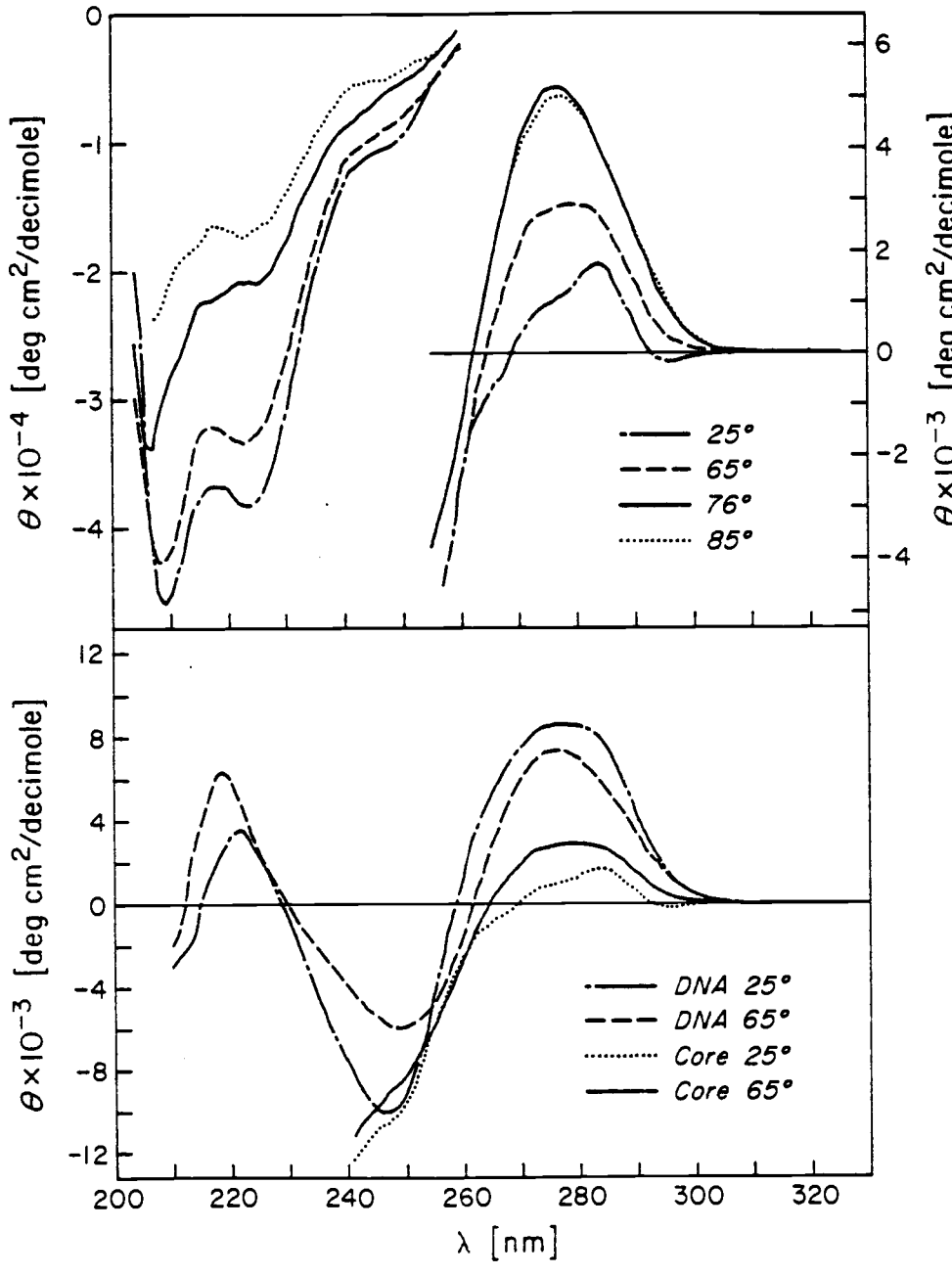

Figure 13. CD spectra of core particles (Top) and core particles and core particle DNA (Bottom) at the temperatures and wavelengths indicated.

B-form DNA. However, neither is a typical B-form spectrum obtained at any temperature, nor are the spectra of denatured core particles ($\geq$ 85°C) or denatured DNA ($\geq$ 65°C) the same. This may indicate the histones still bind to the denatured DNA strands in some fashion.

Below 250 nm the CD spectra of core particles show a decrease in amplitude with increasing temperature. In particular, the minimum at 208 nm and 225 nm disappear or are blue shifted beyond the range of the instrument. The same findings with chromatin have been interpreted as indicating a loss in secondary structure of the histones (Wilhelm et al., 1974; Li et al., 1975).

On examining Figure 13, large CD changes are noted at 223 nm and 273 nm as a function of temperature. The former will measure primarily protein conformational changes because the contribution due to DNA is probably small and nearly invarient with temperature at that wavelength. The latter wavelength will monitor exclusively DNA conformational changes. Therefore the ellipticity was monitored at 223 nm and 273 nm continuously through the melting range of the particle. These results are shown in Figure 14. The most striking result of this study is the monophasic curve at 223 nm; the lower melting transition as seen by hyperchromicity is completely absent indicating no significant protein conformational changes occur until the second transition. In contrast, the curve monitored at

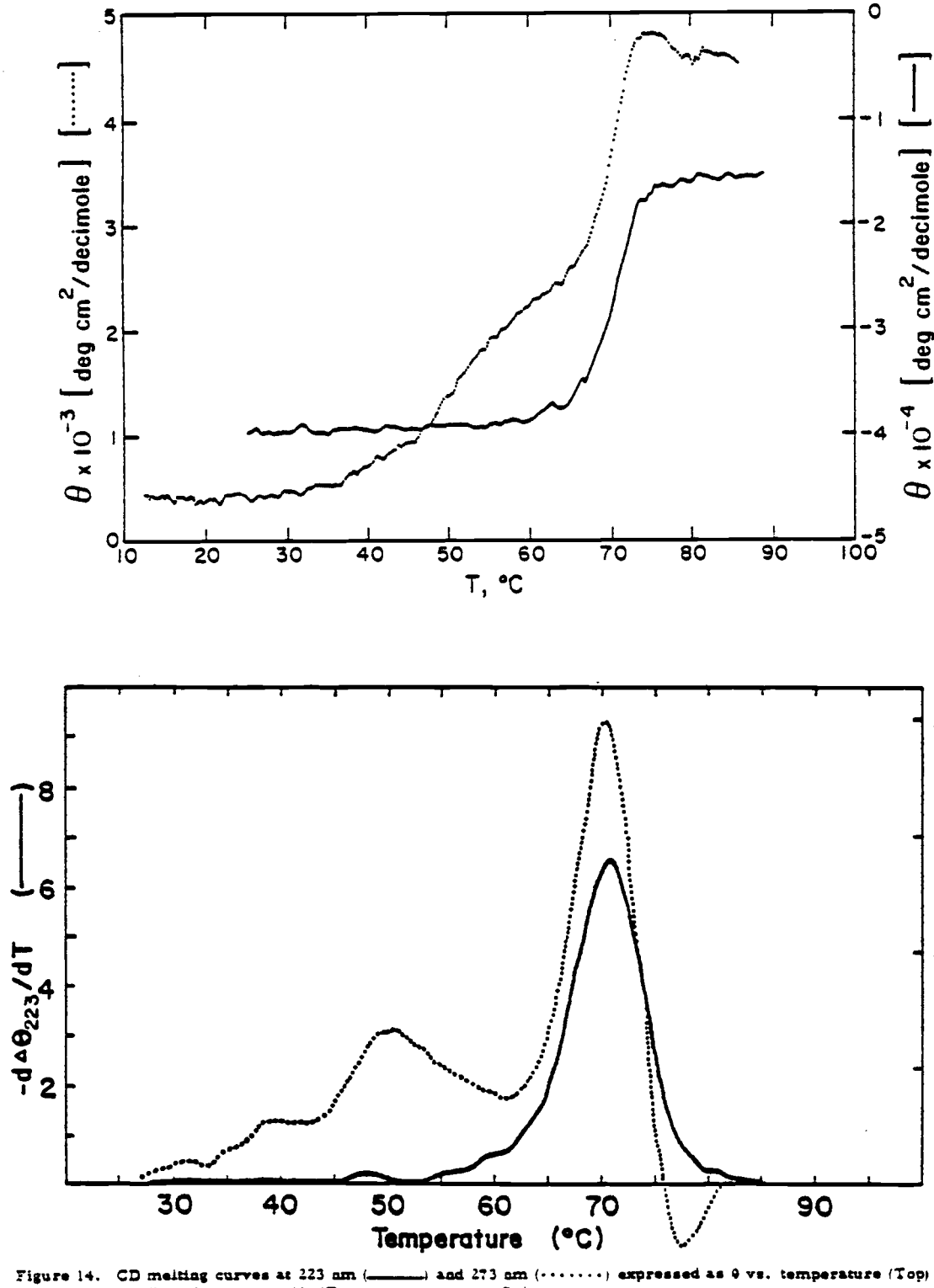

Figure 14. CD melting curves at 223 nm (———) and 273 nm (·····) expressed as θ vs. temperature (Top) or as the first derivative, dθ/dT, vs. temperature (Below).

273 nm resembles the curve of absorbance hyperchromicity but with both transitions shifted to lower temperatures. Evidently conformational changes are occurring below the temperature at which the DNA starts to denature. It would be helpful to be able to assign these premelting transitions to the regions of the DNA involved in the first or the second hyperchromicity transitions but, unfortunately, this is not possible. The maxima in the derivative plots at 223 nm and 273 nm are 71°. The corresponding maximum in the hyperchromicity curve is three degrees higher at 74°. A similar difference has been observed by Mandel and Fasman (1976).

Hyperchromicity melting experiments were repeated in tenfold more concentrated and tenfold more dilute buffer than in the above experiments. A solution of core particles (A_{260}=9) in 1 mM cacodylate, pH 7.2 was diluted 1:16 into 0.05 mM cacodylate or 10 mM cacodylate of the same pH. Thus the final concentrations were 0.11 mM and 9.44 mM respectively; I will refer to them as 0.1 and 10 mM. The derivative melting curves at these ionic strengths are shown in Figure 15. The sample in 10 mM cacodylate begins to aggregate at around 78°C as shown by light scattering at 340 nm.

To a first approximation, the main transition seems to be shifted linearly with the logarithm of the buffer concentration. The melting temperatures are 71°, 74°, and 78° for 0.1 mM, 1.0 mM and 10 mM cacodylate respectively. In contrast, the premelt

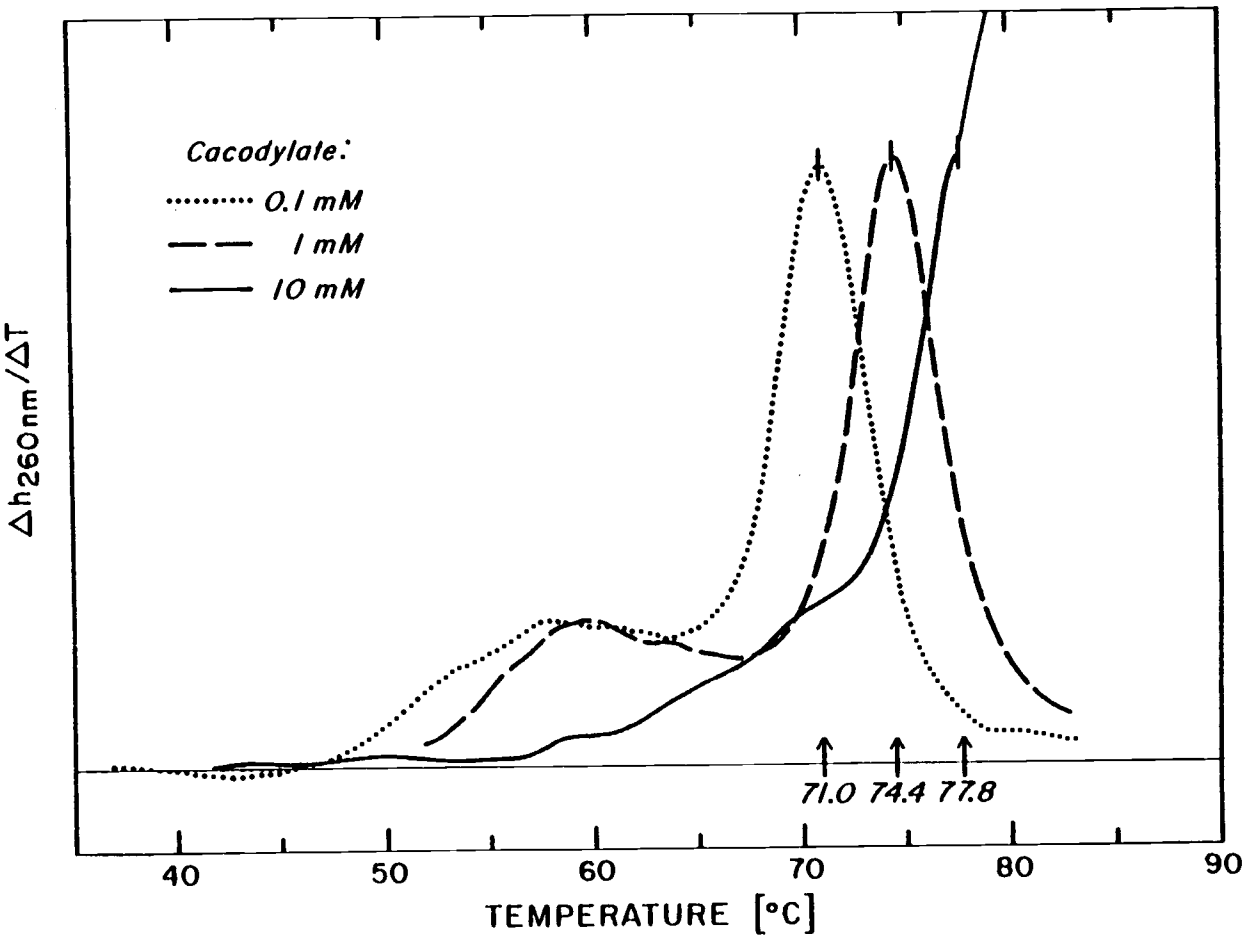

Figure 15. The effect of ionic strength on the melting of core particles. The derivatives of hyperchromicity, followed at 260 nm., are shown for core particles in the buffers indicated. The absorbance at 340 nm. was followed simultaneously in order to observe the onset of any aggregation, in this case at about 78°C in 10 mM cacodylate as noted by the change in line thickness.

undergoes a much larger and non-linear shift. In fact, the premelt, which is well resolved at low ionic strength, is transformed by 10 mM cacodylate to a broad shoulder of the main melting transition.

I next sought to study the thermal denaturation of particles containing the lysine rich histones (H1 and H5). Such particles were obtained by differential precipitation of nucleosomes in 0.12 M NaCl as described in Chapter II. Precipitates of nucleosomes in 0.12 M NaCl were redissolved in low ionic strength buffer and then run on sucrose gradients to remove any remaining nucleosome oligomer contamination. An aliquot of the precipitate was also sedimented through a sucrose gradient containing 0.6 M NaCl to strip the H1 and H5 from the particles. After dialysis to low ionic strength, these particles with and without H1 and H5 were thermally denatured. Two independent preparations were obtained, differing in the length of DNA in the nucleosome preparation, by isolation of nucleosomes from nuclei digested to different extents with micrococcal nuclease. A less extensive digest produced a nucleosome preparation containing on the average 185 bp of DNA while a longer nuclease digest produced a nucleosome containing 172 bp of DNA. Figures 9 and 10 illustrate the protein and DNA composition of these particles respectively while Figure 16 shows the melting curve for each preparation. The thermal denaturation curve for the core particle is included in Figure 16 for comparison. A dinucleosome melting curve, also

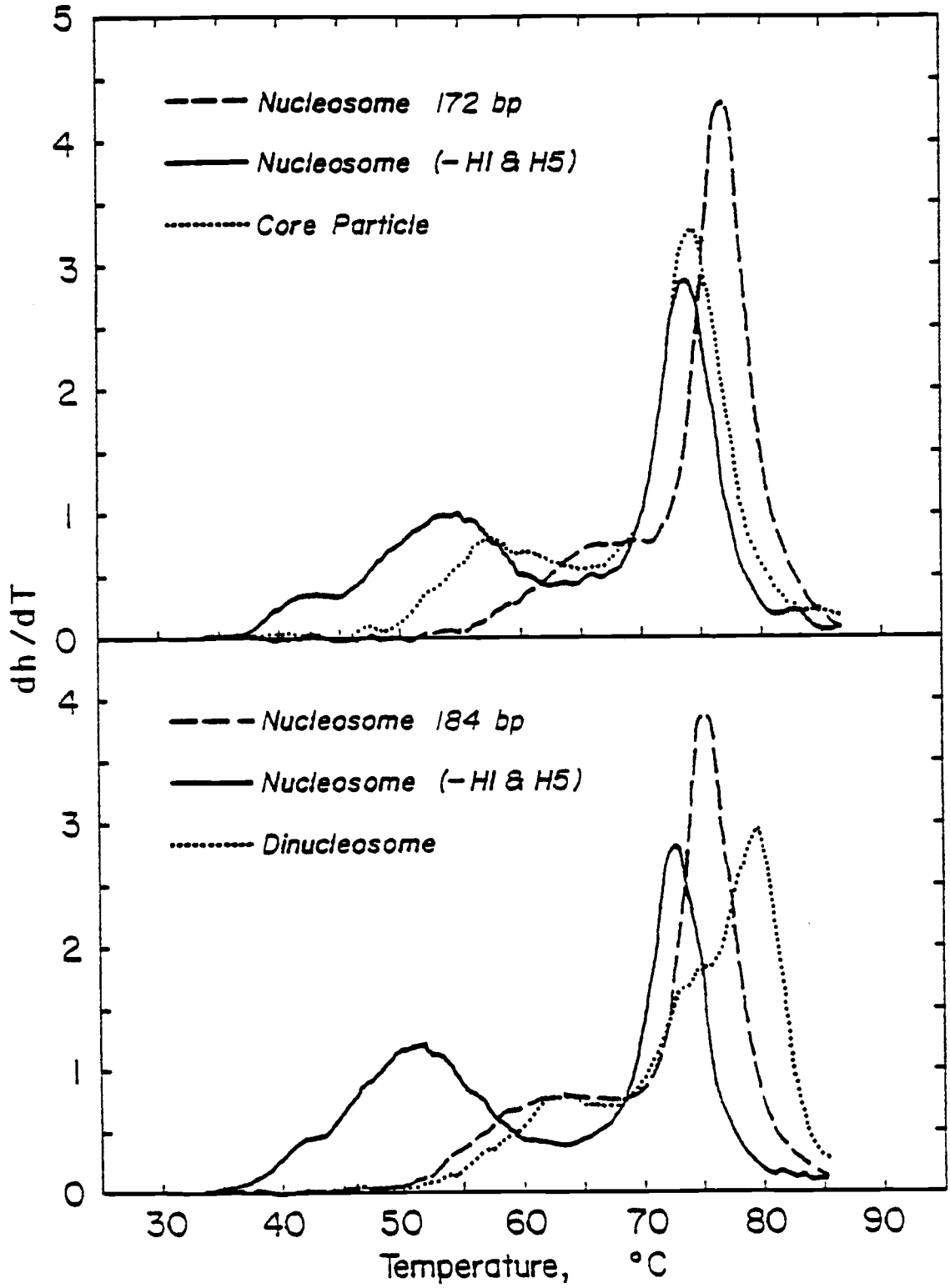

Figure 16. A comparison of nucleosome and core particle melting curves, shown as first derivatives of hyperchromicity vs. temperature.

included in Figure 16, is used only to illustrate the complexity that rapidly develops in thermal denaturation studies.

The lysine-rich histones have two effects on the denaturation curve: the temperature of the main transition is increased and the size of the premelt is decreased. Removal of H1 and H5 by salt stripping lowers the TM to that or below that of the 144 bp core particle and greatly increases the size of the premelt.

Discussion

The thermal denaturation of core particles is a reproducible two step process. Biphasic melting curves have been reported by Mandel and Fasman (1976) but apart from these results, mononucleosomes have been described as having monophasic melting curves with a melting temperature consistently higher than the value observed here (Saharabuddhe and Van Holde, 1974; Woodcock and Frado, 1975; Lawrence et al., 1976; and Whitlock and Simpson, 1976). The discrepancies between these results are probably due to the presence of histone H1 in those particles with monophasic melting curves. Indeed, the particles studied by Sahasrabuddhe and Van Holde (1974) were prepared by precipitating chromatin in 0.15 M NaCl after nuclease digestion. This procedure would select for particles containing H1.

Table 2 summarizes the thermal denaturation data for core

Table 2. Analysis of nucleosome thermal denaturation data

	bp[a]	$S_{20,w}$[b]	TM[c] premelt	TM main trans.	% premelt[d]	bp in premelt	bp in main trans.	presence of H2 & H5
Core particle	144± 5	10.9±1	58	74	29	42	102	-
Nucleosome	172±16	11.3	66	77	22	38	134	+
Nucleosome	184±40	12.0	63	75	29	53	131	+
Nucleosome (-H1 and H5)	172±16	--	54	73	41	71	101	-
Nucleosome (-H1 and H5)	184±40	11.0	51	72	46	85	99	-
Dinucleosome	355±60	16.6	--	79				+
Dinucleosome (-H1 and H5)	355±60	14.6	--					-
Reconstituted 123 bp particle	123± 7	10.4±.3	e	74	21%	26	97	-

a) ± indicates half width of DNA peak at half height in base pairs

b) ± indicates average of more than three runs.

c) TM refers to peak of the transition in the derivative curve.

d) % premelt was calculated by dividing the area under the premelt transition in the derivative curve by the total area of the derivative curve.

e) The premelt in the case of the 123 bp reconstitution was only a shoulder of the main transition. The % premelt was calculated assuming the main transition was symmetric.

particles and larger nucleosomes with and without H1 and H5. The first phase in the thermal denaturation of core particles involves the denaturation of approximately 40 bp of DNA. This transition is completely reversible and most likely involves base pairs at the ends of the DNA molecules. This contention is supported by the melting results of nucleosomes stripped of H1 and H5. I have calculated the number of DNA base pairs involved in each transition, on the basis of the hyperchromicity, and tabulated the results in Table 2. In each case, with particles containing no H1 or H5, the number of base pairs in the main transition is 100 (the presence of H1 and H5 raises this number to 132). The rest of the DNA in the nucleosome, 40-85 bp, corresponds to the first or premelt transition. These results are also consistent with the thermal denaturation results of particles containing only 123 bp of DNA (Figure 44 in Chapter VI). These reconstituted particles have only 26 bp of DNA in the premelt but they still have 97 bp of DNA involved in the main transition. These results are best summarized by Figure 17 in which the amount of DNA melting in the first transition or premelt for each preparation is plotted against the total amount of DNA. For the particles without H1 and H5 the points fall on a line, on which particles containing 100 bp would be predicted to have no premelt. Those particles containing H1 and H5 fall off the line.

The strong correlation between length of nucleosomal DNA and

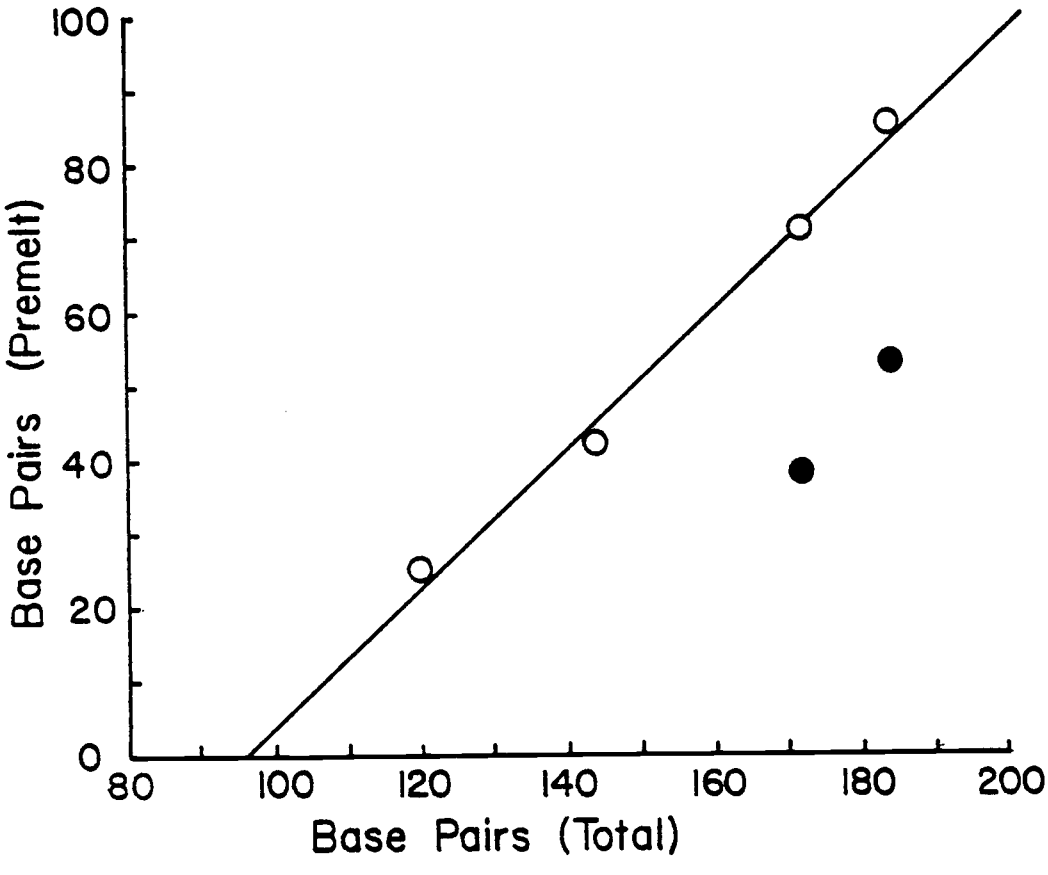

Figure 17. Plot of the amount of DNA melting in the first melting transition vs. the total amount of DNA in each preparation. The open circles (O) are for preparations containing no H1 and H5 while the closed circles (●) are for preparations containing H1 and H5. The line is a least square fit for those preparations containing no H1 or H5.

the size of the premelt suggests an obvious model with the 100 bp of central DNA tightly bound to the histone core and melting in the main transition. Additional DNA, whether 20 or 85 bp, melts prematurely at a temperature that is dependent on the length of the DNA. This model is consistent with the observation that the first regions of DNA in the 144 bp core particle to be tightly bound to the core histones are 20-30 bases from either 5' end (Whitlock and Simpson, 1977; and Mirzabekov et al., 1978).

In contrast, the second and main melting transition seems to be initiated by a massive disruption of the secondary structure of the histones, accompanied by an unstacking of the DNA. The narrow width of the main transition indicates that this process is one highly cooperative transition and not, as Wilhelm et al. (1974) have suggested, one conformational change in the proteins followed by another conformational change in the DNA.

It is believed that most of the secondary structure of the histones is located in the more hydrophobic domains (Bradbury and Rattle, 1972; and Li, 1972) which are also the regions that are highly conserved and are thought to involve histone-histone interactions (Spiker and Isenberg, 1978). It is therefore concluded that the CD transition at 223 nm monitors the destruction of the histone core at about 71°C. Coupled with it is a relaxation of conformational constraints of the DNA. One possible explanation for the observed changes is that once the

histone-histone interactions are disrupted, the DNA can extend from the supercoiled state and adopt a conformation more like that found in free DNA. Only in such a relaxed state can base unstacking occur which is manifested as an increase in absorbance. This final DNA denaturation involves all the remaining base pairs (about 100). The conformational changes in both the histone core and the DNA are a necessary prelude to the subsequent base unstacking. Recent electron microscopic evidence of Seligy and Poon (1978) supports this contention. They have followed the thermal denaturation process of core particles by electron microscopy and correlated structural changes with changes in hyperchromicity. Evidence indicates that the basic globular core particle structure is disrupted only in the second melting transition. During the premelt transition the core particle retains its compact globular shape.

As has been pointed out by Mandel and Fasman (1976), the final melting step consists of two processes which have very different effects on the CD spectrum: the relaxation of the DNA conformation which yields an increase in ellipticity and the subsequent melting and base unstacking of the duplex DNA accompanied by a decrease in ellipticity. These opposing two factors may be the explanation for the overshoot in the change of ellipticity at 273 nm at 76° (Figure 14).

Evidence suggests that even after complete denaturation the histones are still bound to the DNA. The CD spectrum for core

particles above 250 nm never reaches that of free denatured DNA, even though the percent hyperchromicity is that of denatured DNA. Seligy and Poon (1978) visualized completely denatured core particles by electron microscopy and find large torus shaped complexes, the circumference of which are 90% that of free 140 bp DNA. The DNA in these complexes is completely denatured, as judged by hypochromicity, yet the two DNA strands are presumably held in close proximity by the histones and constrained into a large torus or circle. I would like to speculate that this phenomena might have some functional significance if polymerases do transcribe through nucleosomes. Complexes of single stranded DNA and histone must exist, if only transiently, during replication or transcription. The DNA-histone complexes found in thermally denatured core particles may be very different from the complexes found during transcription but they illustrate the strong interaction that can exist between single-stranded DNA and histones.

The effect of lysine rich histones on the thermal denaturation of nucleosomes helps explain the contradictory melting results obtained by other investigators. H1 and H5 have the effect of decreasing the size of the first transition and raising the temperature of the second. The highly charged lysine rich histones may exert part of their effect, similarly to increased ionic strength, by charge neutralization of the DNA phosphates. The fact that H1 and H5, thought to bind to the

spacer region outside the 140 bp core regions (Shaw et al., 1976; Noll and Kornberg, 1977), raise the temperature at the main transition is somewhat surprising. Either H1 and H5 interact directly with the core histones as suggested by Bonner (1978), thereby stabilizing the DNA, or their interaction with the spacer somehow indirectly raises the temperature of the main transition.

One problem with these experiments is that the amount of lysine rich histones in the nucleosomes has not been quantified. The three major bands of H1 and H5 also attest that this system is far from homogeneous. Nevertheless, these experiments indicate some possible effects of the lysine rich histone on nucleosome structure.

IV. RECONSTITUTION OF CORE PARTICLES

Introduction

An inherent joy of western science is tearing an object apart and then attempting to put it back together. The inability to do such indicates a priori that the system is not really understood. Conversely, the ability to do so gives assurance that understanding is at hand. In the biochemical sciences this approach is commonplace, with reconstitution of many multienzyme systems as prime examples. Similarly, scientists have been trying to reconstitute chromatin since they were first aware of what it was and how to take it apart.

With one exception, the methods used for chromatin dissociation and reconstitution have changed little in the last 25 years. Fleming and Jordan (1953) studied the dissociation of chromatin by salt treatment. A year later Crampton et al. (1954) reassociated chromatin from high salt, obtaining viscosities and protein extractabilities similar to the native chromatin. Huang and Huang (1969) claimed that chromatin could be reconstituted by dialysis from high salt and urea to give E. coli polymerase transcription products similar to those of native chromatin.

One of the major probes for studying the accuracy of reconstitution has been the use of E. coli DNA dependent RNA polymerase. The RNA products of native and reconstituted chromatin were then

compared by hybridization. Paul and Gilmor (1968), Bekhor et al., (1969), Gilmor and Paul (1969, 1970), Barret et al. (1974), Stein et al. (1975), Ballal et al. (1975), and Gadski and Chae (1978) have all probed reconstituted chromatin with E. coli polymerase and many have concluded that the reconstituted chromatin could give transcription products similar to native chromatin. The significance of these findings is difficult to appraise. In the first place, it is not known exactly what the E. coli RNA polymerase is probing. There is no evidence to suggest eukaryotic promoters are the same as procaryotic promoters. The question of endogenous RNA, RNA polymerases, and the possibility of RNA dependent transcription (Zasloff and Felsenfeld (1977a, b) have also made many of the studies with bacterial RNA polymerases questionable. With all the possible problems taken into account, including the correct polarity of transcription, Konkel and Ingram (1978) found no evidence for specificity of E. coli polymerase-dependent transcription in salt-urea reconstituted reticulocyte chromatin. The best that can be said for these reconstitution studies is that the bacterial polymerases can recognize regions of transcriptionally active chromatin and are therefore probes of chromatin structure and not transcription (Felsenfeld, 1978).

Studies on the physical structure of reconstituted chromatin have been less controversial. Most advances in the last 15 years have not been due to a more native reconstituted product but instead to more sophisticated techniques in analyzing and probing the

reconstituted products. Hence, the development of new probes of chromatin structure have been the basis for major advances. Early probes of chromatin structure (and therefore reconstituted chromatin) consisted of thermal denaturation, electron microscopy, X-ray diffraction, viscosity measurements, optical rotatory dispersion and circular dichroism. More recently, the use of nucleases (micrococcal nuclease, pancreatic DNase I, DNase II) and viral mini-chromosomes (SV-40, polyoma) have been added to list of techniques.

Richards and Pardon (1970) observed that histones could be reconstituted with DNA by dialysis from 2 M salt to give X-ray diffraction patterns very similar to native chromatin patterns. Similar conclusions have more recently been reached by Kornberg and Thomas (1974) and Boseley et al. (1976). Both Richards and Pardon (1970) and Boseley et al. (1976) discovered that reconstitution protocols with urea present gave poorer diffraction patterns than with salt alone. More recently, reconstitutions by Woodcock (1977) and Steinmetz et al. (1978), using electron microscopy and nuclease digestion respectively, have come to the same conclusion about the use of urea. Surprisingly, this is in contrast to some reconstitutions using E. coli polymerase as a probe for reconstitution where urea was found necessary for "correct" reconstitution (Huang and Huang, 1969; Bekhor et al., 1969). This discrepancy could be due to the fact that the two probes, X-ray diffraction and E. coli polymerase, are monitoring different

structures. X-ray diffraction is monitoring the average structure while the polymerase is probing only a small fraction of the total chromatin. Gadski and Chae (1978) recently observed that many non-histone proteins, specifically those that are required for "correct *E. coli* polymerase activity," bind to the DNA before the histones do from a salt gradient in the presence of 5 M urea. This observation suggests that the urea may be necessary for correct nonhistone placement but not necessarily for total chromatin reconstitution.

Paul and More (1972), Olins *et al.* (1975), Oudet *et al.* (1975), Germond *et al.* (1976), Woodcock (1976), and Oudet *et al* (1978a) have all observed reconstituted chromatin with electron microscopy and concluded that reconstituted chromatin was similar to native chromatin. Paul and More (1972) observed 200 Å-300 Å chromatin fibers while the latter workers observed the now characteristic nucleosome bead morphology. The differences between the chromatin fibers observed by Paul and More and the others was probably due to differences in sample preparation. Oudet *et al.* (1975) and Germond *et al.* (1976) compared reconstitutions between eukaryotic and procaryotic DNA and between acid extracted and salt extracted (van der Westhuysen and von Holt, 1971) histones and found no observable differences.

Richards and Pardon (1970) observed that only the arginine rich and slightly lysine rich histones, now more commonly called

the core histones, were necessary for the reconstitution of the "native like" X-ray diffraction pattern; the lysine rich histone (H1) was not necessary. Furthermore, histones H3 and H4 alone gave an X-ray diffraction pattern similar to chromatin (Richards and Pardon, 1970; Boseley et al., 1976). Richards and Pardon's suggestion for a major role for histones H3 and H4 in chromatin structure has recently been substantiated by more recent reconstitution experiments. Camerini-Otero et al. (1976) have carefully shown that H3 and H4 along with DNA give some of the subnucleosomal DNA fragments upon digestion with micrococcal nuclease and DNase I. Sollner-Webb et al. (1976) repeated similar reconstitution experiments and showed that reconstitutions with H3 and H4 alone partially protected the histones from trypsin digestion in a manner similar to whole chromatin (Weintraub and Van Lente, 1974). In both these papers, only reconstitutions containing H3 and H4 gave protection from nucleases and proteases.

Wang (1971) discovered an enzyme in bacteria that unwinds supercoiled DNA. Similar enzyme activity has been found in eukaryotes (Baase and Wang, 1974) as well as prokaryotes and has been named omega, nicking closing enzyme, or unwinding enzyme. Germond et al. (1975) found that the supercoils in SV-40 DNA were not removed by unwinding enzyme if nucleosomes were present on the closed circular DNA. The number of supercoils remaining in

the DNA after unwinding treatment was directly proportional to the number of nucleosomes on the DNA. The core histones therefore supercoil DNA. It has recently been shown that H3 and H4 along have this ability to supercoil DNA; H2A and H2B are not necessary (Bina-Stein and Simpson, 1977; Camerini-Otero and Felsenfeld, 1977; Oudet et al., 1978a). One H3-H4 tetramer was found to supercoil DNA to the same extent as all eight histones of the nucleosome. Furthermore, the "beads on a string" morphology observed in the electron microscope can be reconstituted with H3 and H4 alone (Bina-Stein and Simpson, 1977; Wilhelm et al., 1978; Oudet et al., 1978a). Bina-Stein (1978) has recently shown that H3 and H4 alone can coil 145 bp DNA into a globular structure that sediments and thermally denatures like a nucleosome ($S_{20,w}$=9.80).

Felsenfeld and co-workers (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976) have suggested that the arginine rich histones, H3 and H4, act as a "kernel" for DNA folding in the nucleosome. This suggestion has been substantiated by recent evidence that H3 and H4 bind to DNA prior to H2A and H2B and H1 during reconstitution from 2 M NaCl (Wilhelm et al., 1978; Ruiz-Carrilo and Jorcano, 1978).

Nucleosomes can undeniably be reconstituted by salt dialysis. The data in this chapter will show that the reconstituted core particle is very similar, if not identical to the native core particle.

Nevertheless, core particles are a long way from whole chromatin and nuclei and it is at this point that the success story ends. With one possible exception, no one has been able to reconstitute the native nucleosomal repeat. While nucleosomal core particles can be reconstituted onto high molecular weight DNA, the average spacing between core particles is always less than the usual 180-200 bp (see Chapter V for a discussion of variable repeats). In a detailed study, Steinmetz et al. (1978) were unable to reconstitute the usual 200 bp repeat, with or without the addition of H1. The reconstitution products always had a repeat of 140-150 bp.

The eventual reconstitution of native chromatin may require a different approach than the simple salt or salt-urea dialysis protocols. One such approach, which was alluded to in the above paragraph is a recent discovery by Laskey and co-workers (Laskey et al., 1977) of a factor, isolated from Xenopus laevis oocytes, which will reconstitute nucleosomes from isolated DNA and histones at or near physiological ionic strength. Electrophoresis of DNA fragments produced by micrococcal nuclease digestion of reconstituted chromatin indicates the nucleosome repeat is approximately 200 bp. A multi-subunit protein with a molecular weight of 100,000 daltons is responsible for at least part of the reconstitution activity (Laskey et al., 1978). The results from SDS gel electrophoresis indicate the subunit molecular weight of the protein is 29,000 daltons. The

acidic protein binds four core histones in stoichiometric amounts and reconstitutes nucleosomes onto closed circular DNA. Laskey and co-workers do not yet know if this protein alone can reproduce the correct nucleosome repeat or if it can bind and reconstitute H1 on to chromatin. Nevertheless this approach may revolutionize reconstitution studies in the future.

This chapter describes nucleosome reconstitution experiments starting with isolated 144 bp DNA and core histones. Two questions were of greatest concern: 1) Can quantitative reconstitution be obtained? 2) How do the properties of the reconstituted core particles compare with those of particles obtained by micrococcal nuclease digestion of chromatin? A wide variety of physical and chemical tests were applied to examine the fidelity of reconstitution. Part of this work was reported at the Biophysical Society meeting in February 1976 (Tatchell and Van Holde, 1976), at the Dahlem Conference on Organization and Expression of Chromosomes in May 1976 and published in Biochemistry (Tatchell and Van Holde, 1977).

Results

The reconstitution studies in this chapter were done with particles very homogeneous in DNA size and histone content, identical to the particles studied in Chapter III. There is only insignificant contamination by lower and higher molecular weight DNA size classes.

That there is no significant "nicking" of the DNA can be seen by examining the zero-time columns in Figure 25; the sample remains entirely homogeneous on a denaturing gel. Histone H1 and H5 along with non-histone proteins are present in only low percentages.

A large number of reconstitution experiments have been carried out, using the salt-extracted core histones and core particle DNA. The results are summarized below.

Nearly Complete Reconstitution can be Attained

The typical reconstitution procedure involves mixing core histones (H2A, H2B, H3, H4) with purified 144 bp core particle DNA in 2 M NaCl and then reducing the ionic strength to 10 mM Tris over a period of 24 hours. Step dialysis or gradient dialysis are used to gradually lower the ionic strength.

Figure 18 shows an ultracentrifuge scanner trace from a reconstitution using nearly equivalent amounts of histones and DNA (2 moles of each histone per mole of core DNA) at a DNA concentration of 50 μg/ml. The prominent leading boundary corresponds to reconstituted core particles. In this case, about 80% of the OD_{260} is present in this boundary. The small trailing boundary (< 5%) which has a sedimentation coefficient of about 4.5S (an expected value for 144 bp DNA at this low ionic strength) corresponds to DNA that has not reassociated. The faster boundary (~15%) corresponds to a

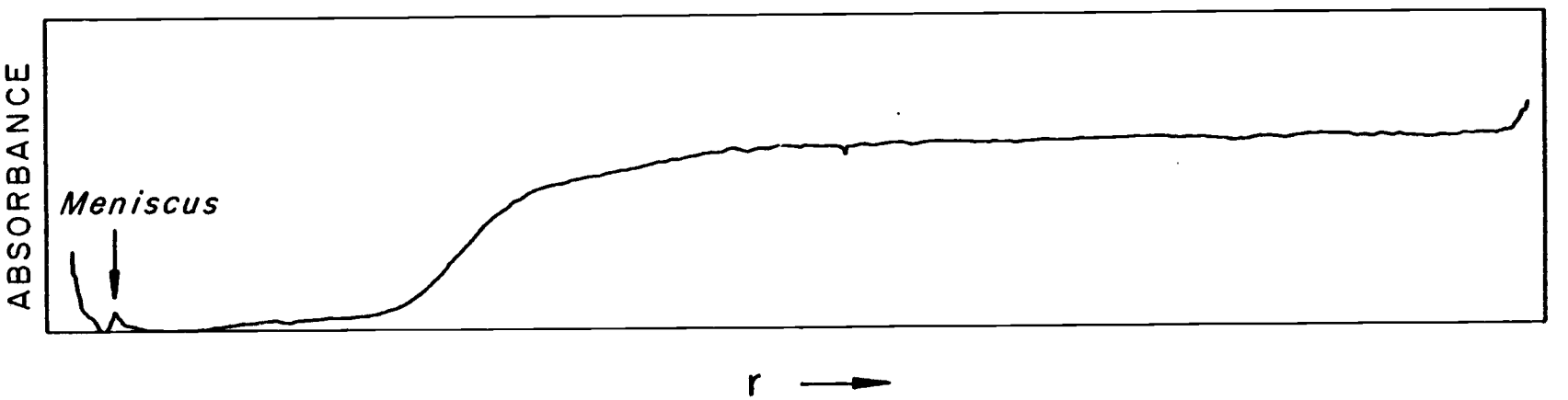

Figure 18. Analytical ultracentrifuge scanner trace of reconstituted core particles. This scanner trace shows the boundary obtained after reconstitution at a 2:1/histone tetramer:DNA ratio. The slower and faster sedimenting components comprise about 5% and 15% of the 11S boundary.

particle with a sedimentation coefficient of ~16S. Since UV absorption of 265 nm will not be very sensitive to histones at the concentration used here, I cannot tell whether there is a small amount of unassociated histone, of if I have not used exactly equivalent quantities. Concentrations were estimated assuming an extinction coefficient of 6600 cm^{-1} $(Mole\ base)^{-1}$ for the DNA at 260 nm and 2.02×10^4 $cm^{-1}\ M^{-1}$ for a histone heterotypic tetramer at 275.5 nm. In particular, I do not have evidence that the latter value is exact for the histone tetramers in 2 M NaCl since the histone extinction coefficient was calculated from individual histones in distilled water (D'Anna and Isenberg, 1974c).

In other similar experiments native core particles were dissociated by making the solution 2 M in NaCl and reassociating them in the same manner as the reconstituted cores. In such "reassociation" experiments the stoichiometry should be exact. The scanner trace from the reassociated core particles is very similar to that of the reconstituted cores.

In sedimentation experiments, the scanner profile is somewhat complicated by the fact that some of the boundary spreading is due to diffusion. Thus, a broad boundary may result from diffusion, heterogeneity, or both. Using the boundary analysis method of Van Holde and Weischet (1978b), an integral distribution of sedimentation coefficients can be obtained. This effectively removes the

diffusion spreading and gives a picture of the true heterogeneity of the sample. Figure 19 compares the heterogeneity in reconstituted core particles, reassociated core particles, and native core particles. Notice that the distribution for the native core particle used in this work is nearly a step function with the step at 11S. Both the reconstituted and the reassociated core particles have small amounts of faster and slower sedimenting components. It is of interest that the reassociated core, showing this somewhat heterogeneous distribution, was very homogenous prior to disassociation and reassociation. The similarity between the reconstituted and reassociated cores is evidence that the failure to attain 100% reconstitution is not a result of the wrong stoichiometry of DNA and histone.

The reconstituted and reassociated mixtures were run on sucrose gradients in order to separate the 5S, 11S and 16S components (Figure 20). The fractions corresponding to these 5S, 11S and 16S components were isolated, and the DNA electrophoresed on 6% polyacrylamide gels and the proteins electrophoresed on 15% SDS gels. Figure 21 shows that the reassociated and reconstituted 11S components contained just the 144 ± 5 bp DNA size class. The 16S components in each case contain 90% 144 bp DNA and 10% 265 bp DNA while the 5S component contained sizeable quantities of small DNAs (50 bp and 90 bp). The protein contents of the 11S and 16S fractions are nearly identical. There is almost a total lack of nonhistone

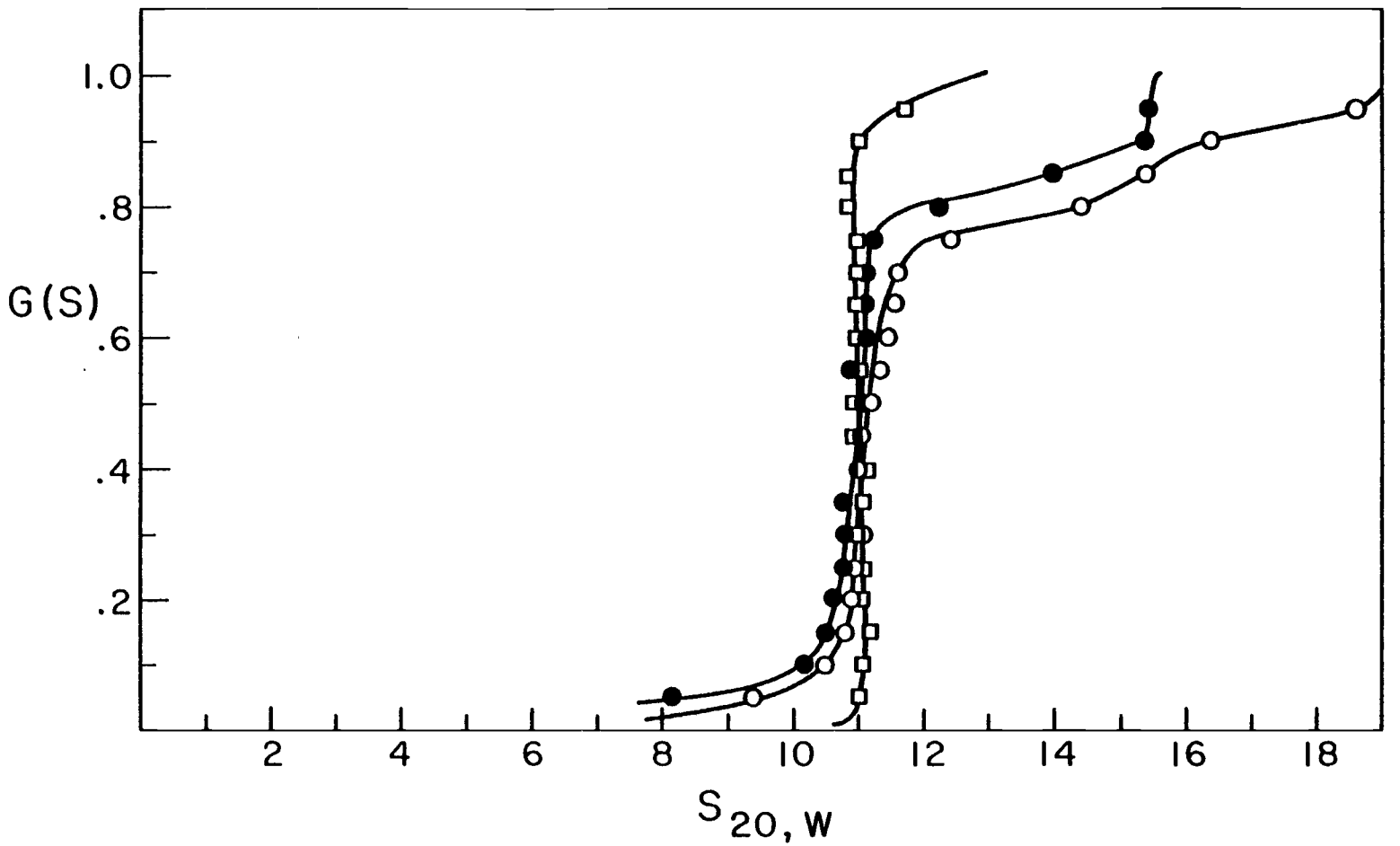

Figure 19. Integral distribution of S. for reconstituted core particle. The integral distribution of S (G(S)) for: (□) native core particle, (●) reconstituted core particle at 2:1/histone tetramer:DNA ratio, and (O) reassociated core particles.

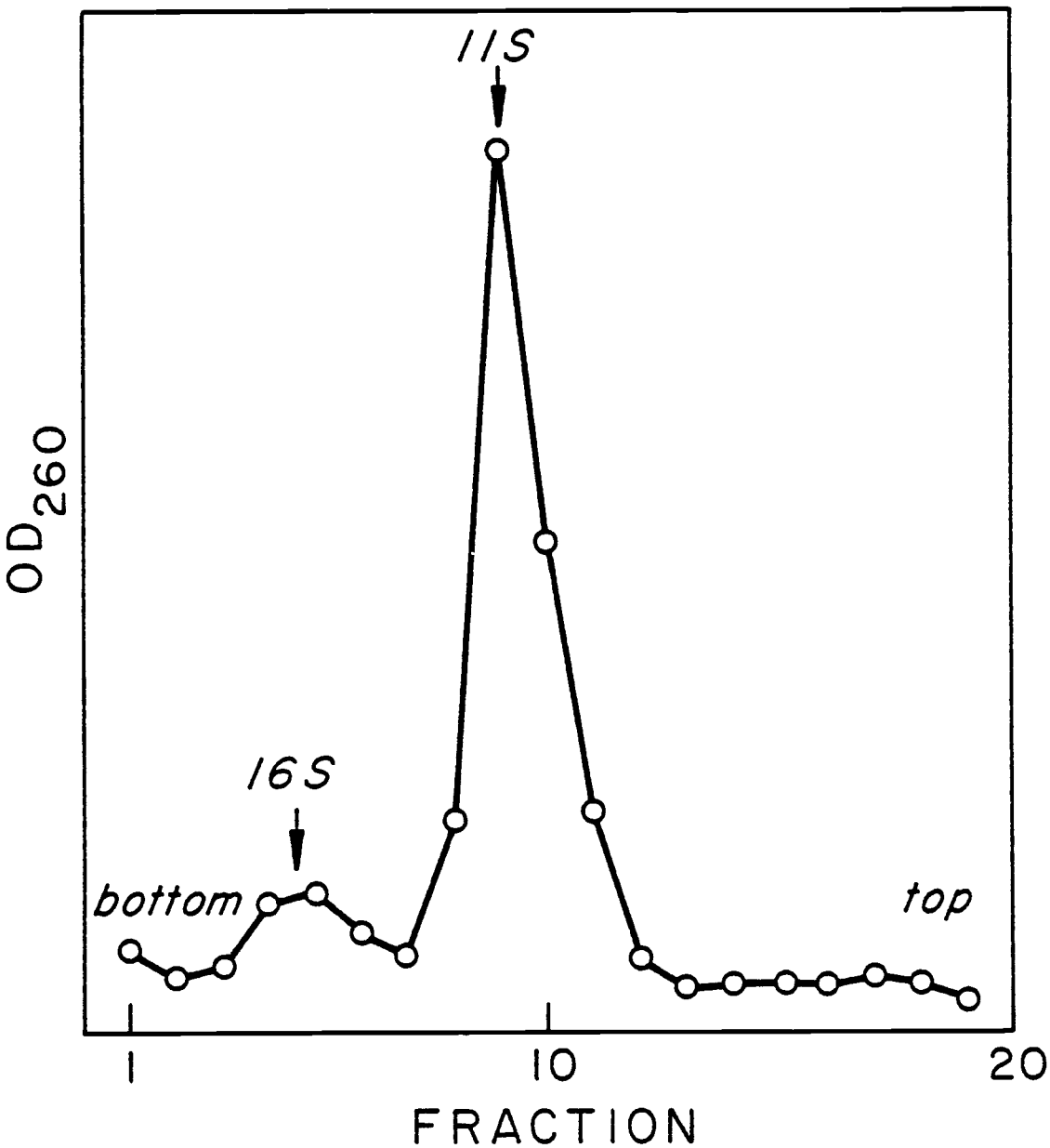

Figure 20. Sucrose gradient fractionation of reconstituted core particles. A reconstituted core particle preparation was run on isokinetic sucrose gradients and gave the fractionation pattern shown. This procedure was used to preparatively separate 5S, 11S, 16S, and larger components after a reconstitution.

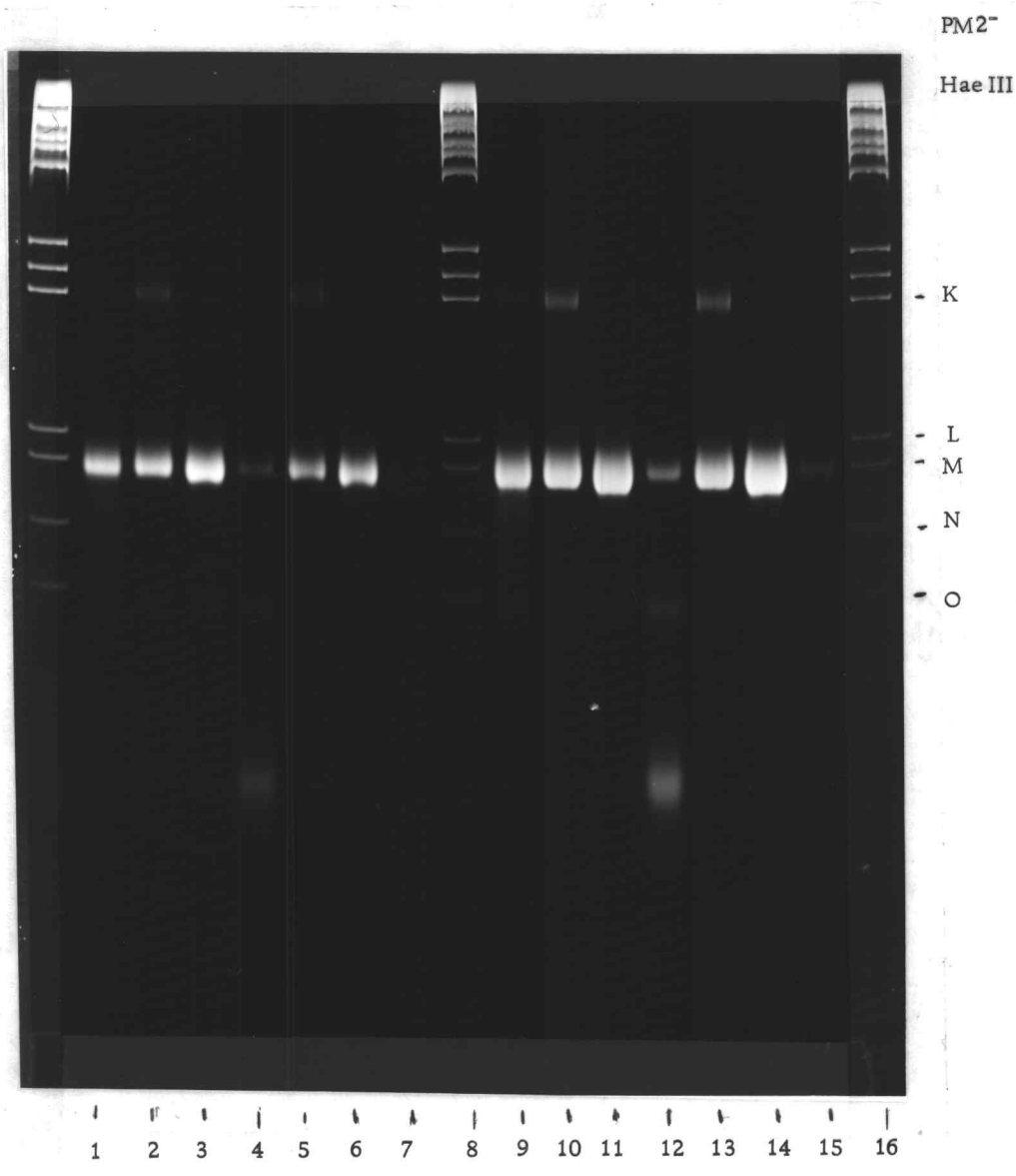

Figure 21. DNA content of reconstituted core particle fractions. DNA was extracted from 5S, 11S, and 16S sucrose gradient fractions of reconstituted and reassociated core particle preparations and run on 6% polyacrylamide slab gel. Channels 8 and 16 are PM2-Hae III fragments. Channels 1 and 9 are free or unreconstituted DNA (5S) from reconstituted preparations. Channels 4 and 12 are free DNA from reassociated preparations. Channels 2, 10 and 5, 13 are DNA from the reconstituted and reassociated 11S fractions respectively. Channels 3, 11, and 6, 14, are DNA from the reconstituted and reassociated 16S fractions. Channels 7 and 15 are the entire DNA before fractionation on sucrose gradients. The higher DNA loading on the right allows better observation of minor DNA size classes, specifically the 265 bp DNA in the 16S components and the low molecular weight DNA in the 5S components.

proteins. Two very faint, slowly migrating bands are occasionally observed in the protein gels. I do not know the nature of these bands. It is of importance that only a very small amount of the histones H1 and H5 are associated with the 16S component (less than 1%). This indicates that H1 and H5 are not responsible for the binding of two 11S cores to form a 16S dimer. This phenomenon will be discussed in more detail below.

Comparative Properties of Native, Reconstituted, and Reassociated Core Particles

I have examined the physical properties of native, reconstituted and reassociated core particles. In each case the purified 11S component, isolated by the sucrose gradient sedimentation was used in the comparative studies.

Histone Composition. Figure 22 compares scans of SDS gels of proteins from native and reconstituted particles. In each case, the only proteins present in significant quantities are the "inner" histones H2A, H2B, H3 and H4, and the proportions of each appear to be the same in each case. Results with reassociated particles and 16S particles appear identical. The histone stoichiometry cannot be determined directly due to the possibility of differential dye binding (McMaster-Kaye and Kaye, 1974), nevertheless, the stoichiometry of the inner histones in native and reconstituted core particles

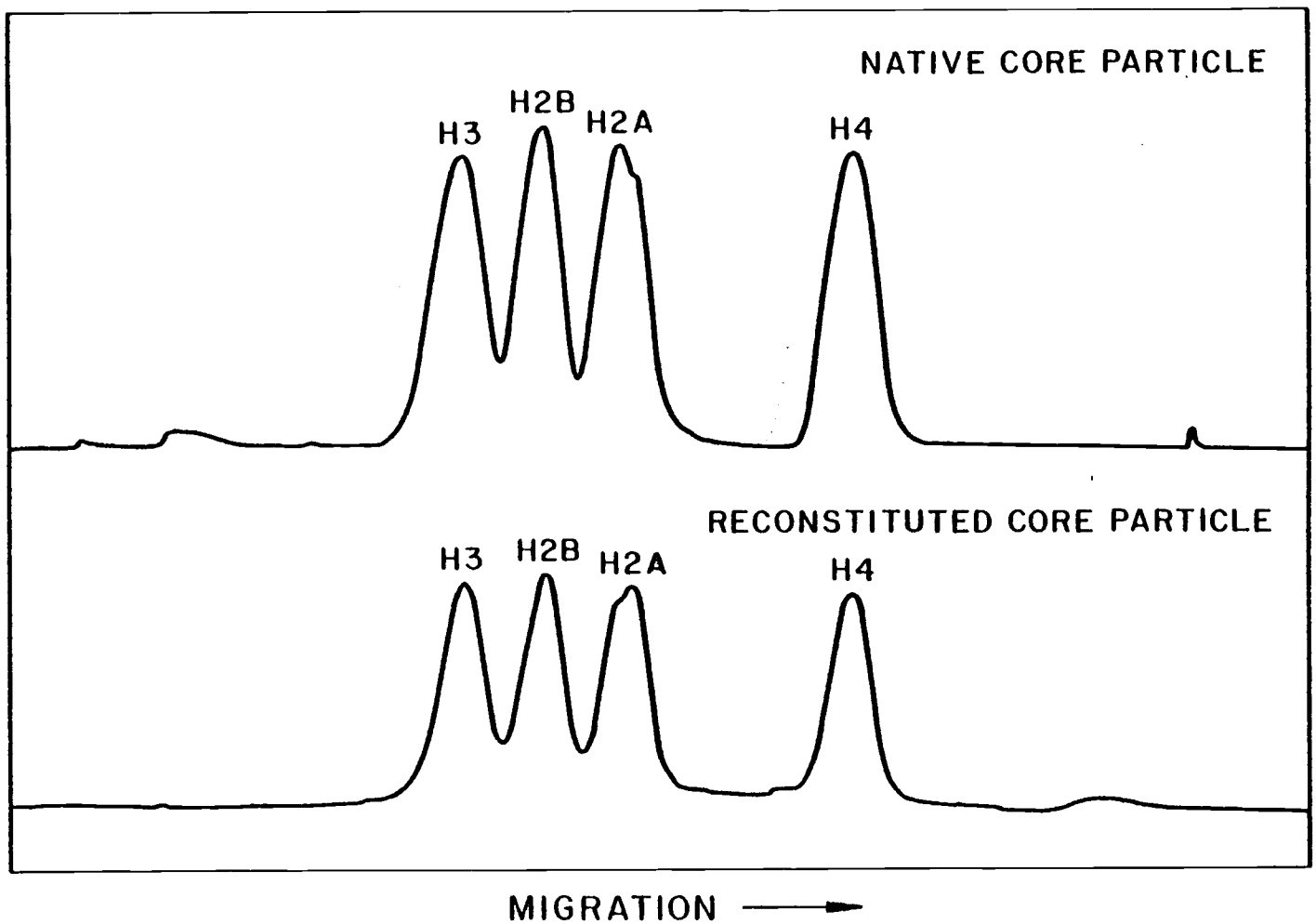

Figure 22. Densitometer tracings of SDS polyacrylamide gel of reconstituted core particles. 15% SDS slab gels were stained with Coomassie blue, destained and densitometer tracing made of each sample. Scans were integrated and the area ratio of the four inner histones were determined for each fraction. The area ratios H3:H2B:H2A:H4 were: native core particle 1.3:1.1:1.1:1.0, reassociated 16S fraction 1.3:1.3:1.2:1.0 (not shown), reconstituted 16S fractions 1.2:1.3:1.3:1.0 (not shown), reassociated 11S core particle 1.3:1.2:1.3:1.0 (not shown), reconstituted 11S core particle 1.3:1.3:1.2:1.0.

appears to be the same, and very likely corresponds to equimolar quantities of each (Joffe et al., 1977; Rall et al., 1977).

CD Spectra. Figure 23 shows the CD spectra, in the range between 310 nm and 250 nm, of native particles, reconstituted particles, and free "core" DNA, under identical conditions. Within the experimental uncertainties of our measurements, the native and reconstituted particles yield identical CD spectra. While we do not understand the reason for the unusual CD spectrum of the DNA in core particles, Figure 22 may be taken as strong evidence that at least the gross conformation of the DNA is identical in native and reconstituted particles. Preliminary results indicate that the CD spectra of native, reassociated and reconstituted particles are also identical between 210-250 nm.

Thermal Denaturation. Native, reassociated and reconstituted core particles were thermally denatured in 0.25 mM EDTA, pH 8.0. Free DNA and core particle samples gave ~35% hyperchromicity upon denaturation. Figure 24 shows the derivative plots of the thermal denaturations. It is quite evident that both native and reconstituted cores melt nearly identically, with a wide premelt centered at 59°C and a main transition at ~74°C.

Nuclease Digestion. The digestion of nuclei or chromatin by pancreatic deoxyribonuclease I (DNase I) produces a series of DNA fragments at 10 b intervals when electrophoresed on denaturing gels

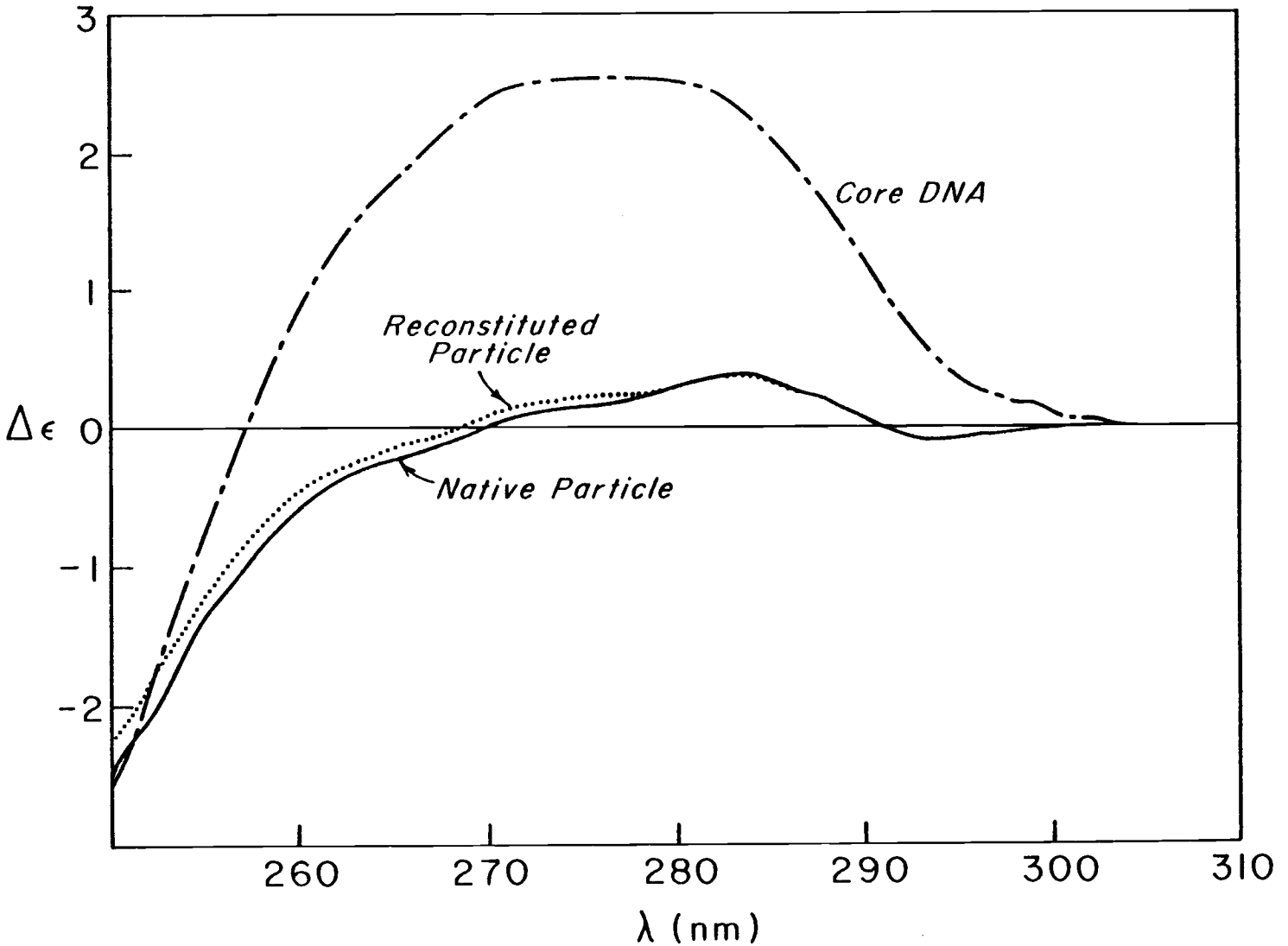

Figure 23. Circular dichroism spectra of native and reconstituted core particles. The CD spectra of native core particles, reconstituted core particles, and core particle DNA were normalized to the DNA concentration assuming the same extinction coefficient for free DNA and core particles (6600 cm^{-1} Mole $base^{-1}$).

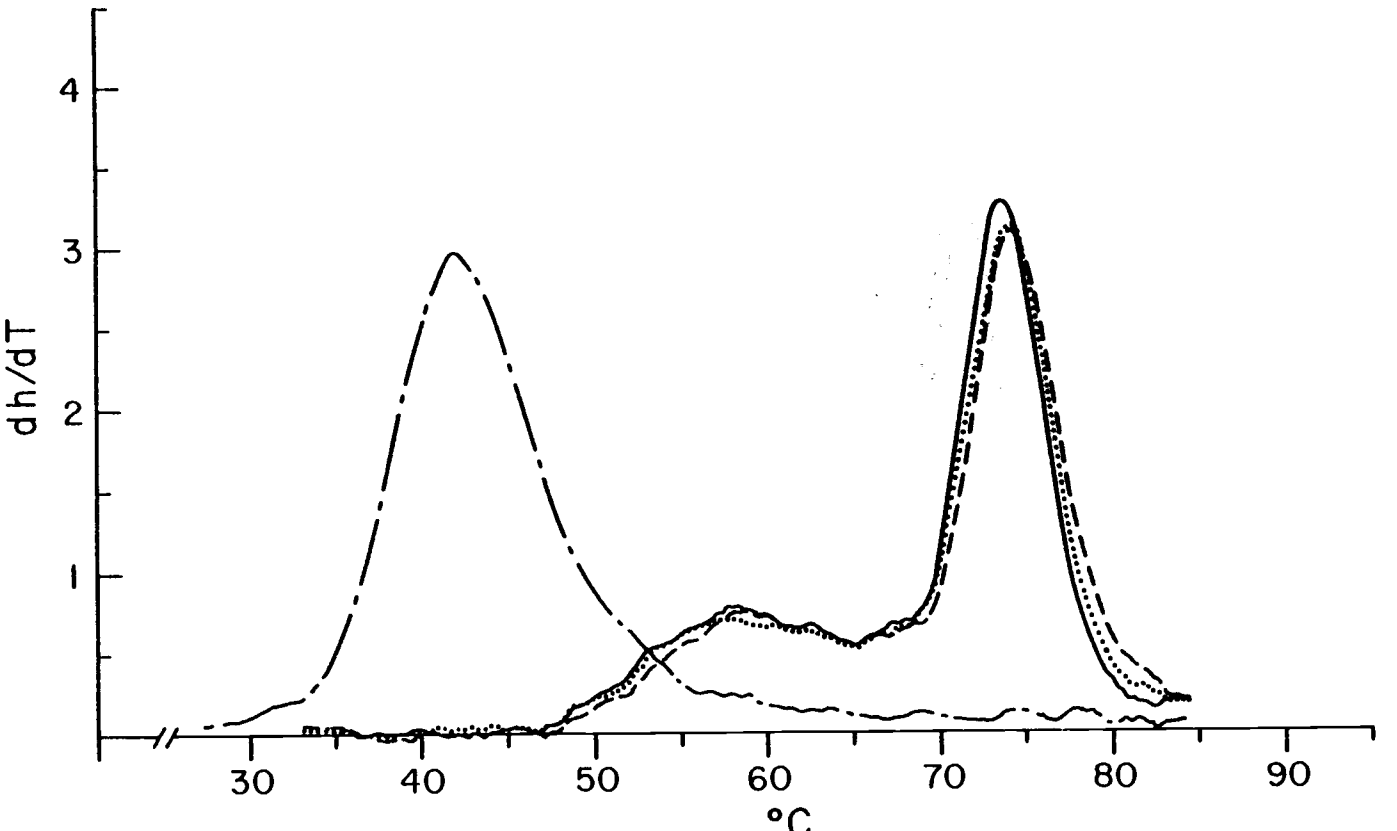

Figure 24. Thermal denaturation of native and reconstituted core particles. Core particle DNA (——·——), reconstituted particles (———), reassociated particles (·····), and native particles (- - - - -) were thermally denatured in 0.25 mM EDTA pH 8.0 and the hyperchromicity followed at 260 nm. The derivative of percent hyperchromicity is plotted as a function of temperature. The total % hyperchromicities for the core particle DNA, reconstituted particle, reassociated particle and native particle were 36.0%, 35.0%, 35.0%, and 34.6% respectively.

(Noll, 1974b). This pattern is also produced upon digestion of native and reconstituted core particles with DNase I (Figure 25). The reconstituted core particles not only give the entire series of fragments from 140 b to 20 b but the relative rate of cleavage seem nearly identical to those obtained from native particles in each case.

Evidence that the reconstituted core particle is nearly identical to the native particle also comes from the digestion of cores with micrococcal nuclease. Digestion with this enzyme also produces specific DNA fragments that reflect in some manner the structure of the core particle. Figure 26 indicates that the native and reconstituted cores have a similar structure with very similar micrococcal nuclease digestion patterns.

Specificity of DNA Placement on the Histone Core

The observation that the DNA bands from a DNase I digestion of reconstituted core particles extends to 144 bp is evidence that the DNA placement on the histone core is correct. A more stringent test of this placement is through the technique of Simpson and Whitlock (1976). Particles containing ^{32}P 5' end labeled DNA are digested with DNase I and the resulting DNA fragments separated by gel electrophoresis and then autoradiographed to detect those fragments containing the end label. Simpson and Whitlock first observed that the familiar 10 base ladder was obtained with the ^{32}P end labeled DNA

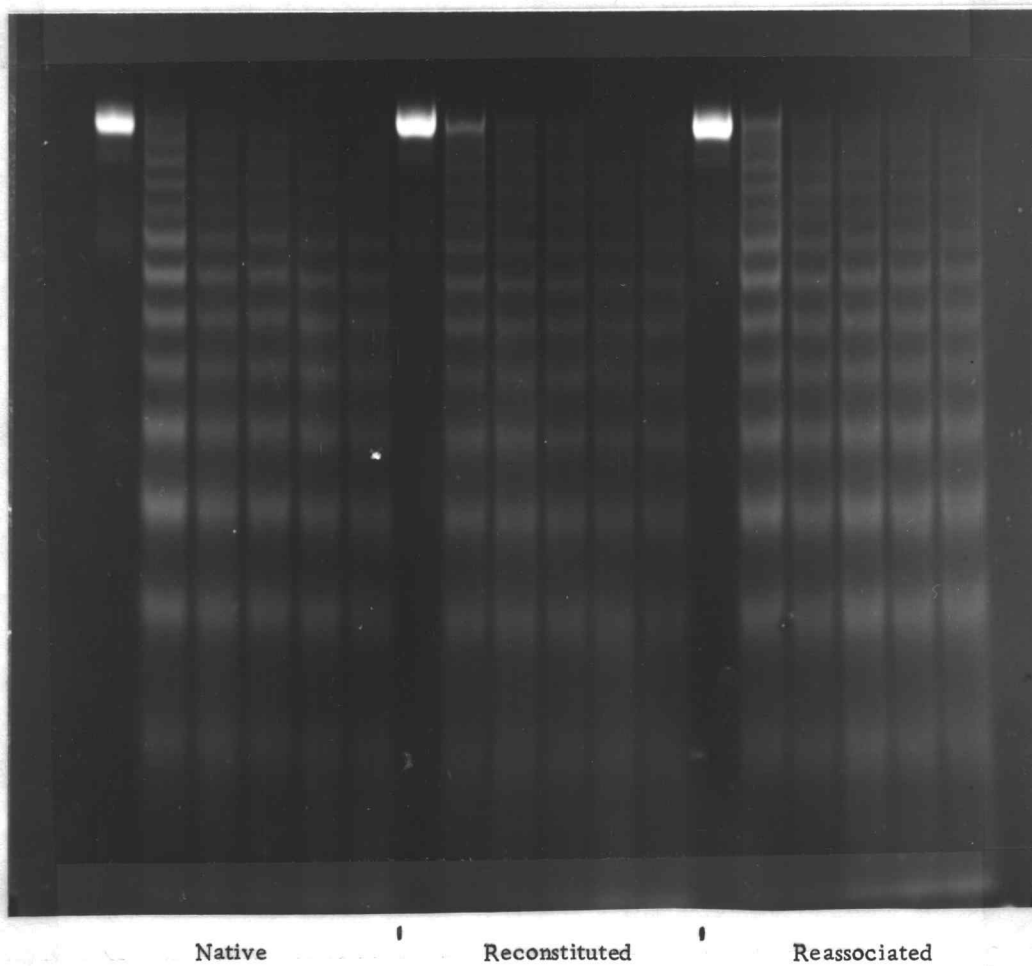

Figure 25. Pancreatic DNase I digests of native and reconstituted core particles. DNA was extracted from a DNase I digestions of native, reconstituted and reassociated core particles and electrophoresced under denaturing conditions on an 8% polyacrylamide-urea slab gel. The times of digestion for each component are, from left to right: 0, 30 seconds, 2 minutes, 5 minutes, 10 minutes and 20 minutes.

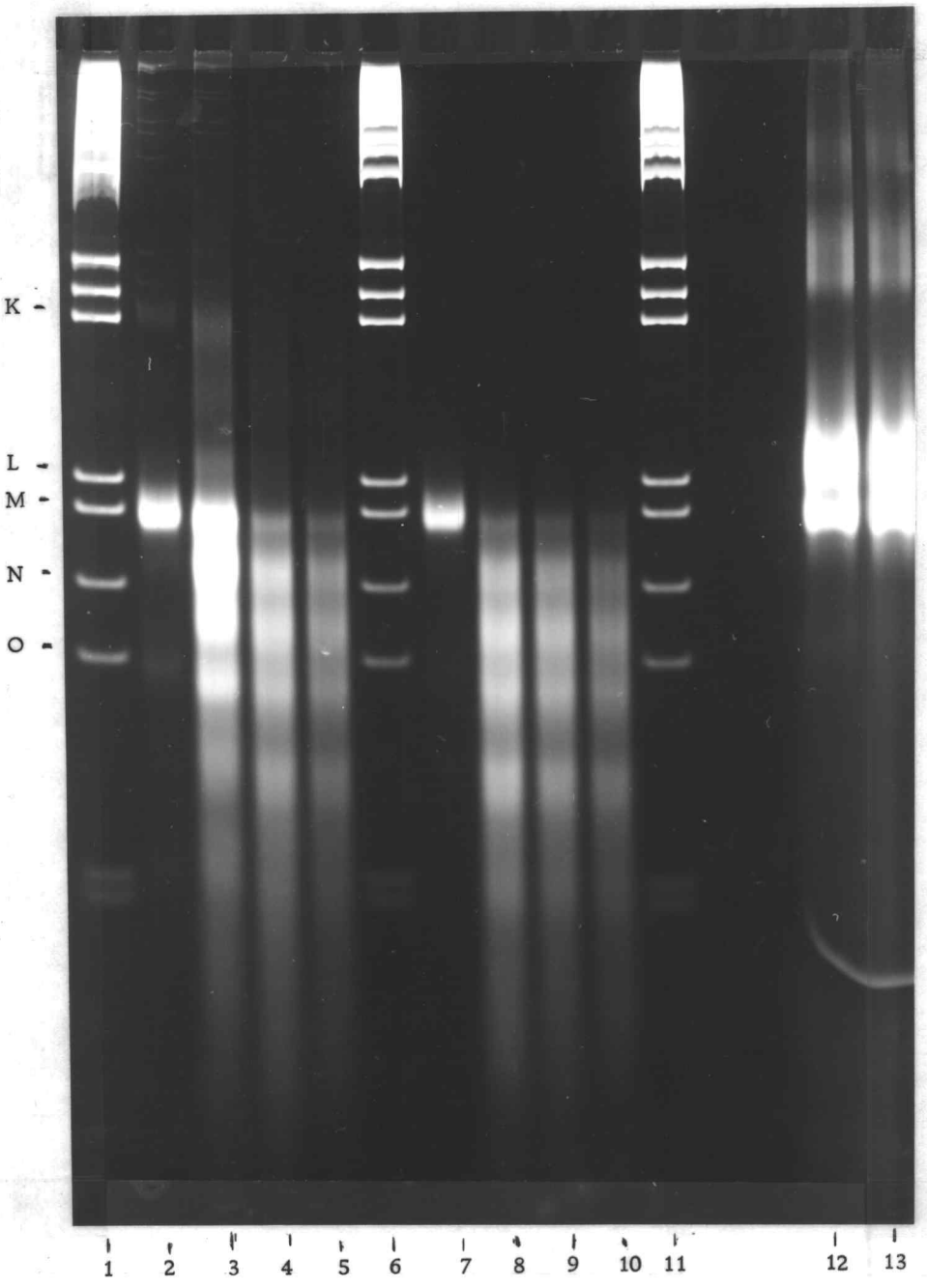

Figure 26. DNA from native and reconstituted core particles and their digestion with micrococcal nuclease. DNA was extracted from particles and electrophoresed on a 6% polyacrylamide gel. Channels 1, 6, and 11 are PM2-Hae III fragments. Channel 2 shows the DNA from core particles isolated from H1 depleted chromatin. Channel 7 shows the DNA from the reconstituted particles. Channels 3, 4, and 5 illustrate the products of digestion of the native particles with 125 units/ml of micrococcal nuclease for 5.15, and 60 minutes respectively. Channels 8, 9, and 10 show comparable digestions of the reconstituted particles. Channels 12 and 13 illustrate the DNA from monomer particles prepared by digestion non H1 depleted nuclei.

fragments but with an important difference; some bands were very intense while others were nearly absent. The absent bands were interpreted as suggesting that specific sites on the histone core are highly protected from nuclease digestion.

If the ends of reconstituted particles are not at the same position on the histone core as in the native particle, the resulting DNase I pattern of labeled DNA fragments should be different from that of the native, even if both give a similar DNase I ladder of total DNA fragments. If the DNA fits randomly with respect to the correct end position, the end-labeled ladder pattern should be lost. If the DNA fits on differently but in 10 base register with the correct end positions the resulting 10 base ladder will be preserved but the intensity pattern should be shifted.

In order to test these possibilities 144 bp DNA was 5' end labeled with ^{32}P, reconstituted with histones and the resulting product analyzed as described above. These results are presented in Figure 27 as densitometer tracings of the total DNA ladder and the corresponding end labeled pattern for native and reconstituted core particles. The observation that the reconstituted core particles give a clear end labeled pattern indicates that the DNA placement on the histone core is exacting. The reconstituted pattern, like the native pattern, has weak bands at 30, 60, 80 and 110 bases. A quantitative comparison between the reconstituted and native end labeled pattern is not possible

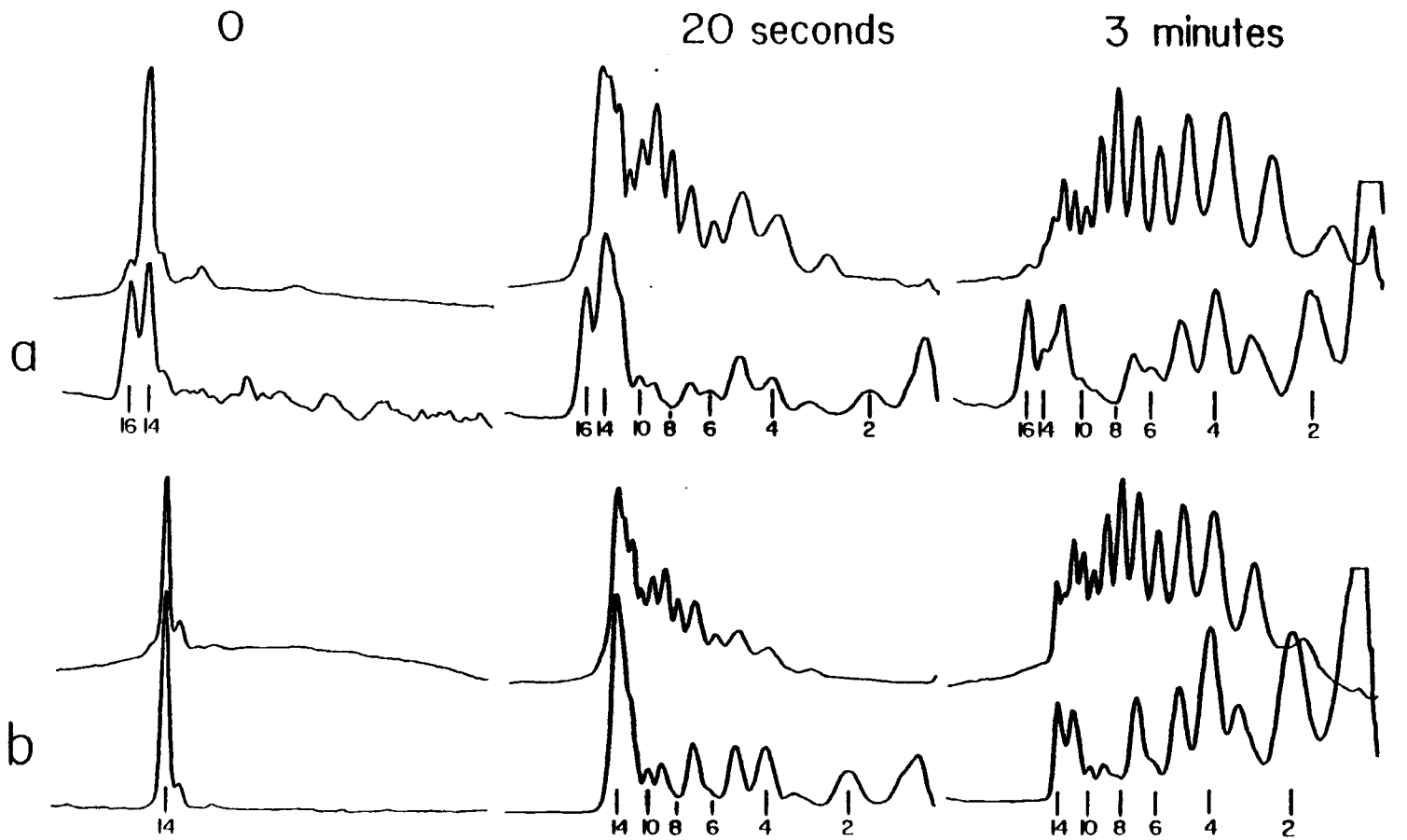

Figure 27. DNase I digestion of ^{32}P end labeled native and reconstituted core particles. Native (a) and reconstituted (b) core particles which contained ^{32}P on the 5' ends of the DNA were digested with DNase I for 20 seconds and 3 minutes (5 minutes in the case of the native particle). The DNA was isolated and electrophoresed on 8% polyacrylamide-urea slab gels. The gels were stained with ethidium bromide, photographed and then autoradiographed. Scans of the ethidium bromide stain are over the autoradiogram scans. The numbers below each band refer to band number. The molecular weight (in bp) of the DNA in each band is approximately 10n where n is the band number.

however, due to an intensely labeled 170 base peak in the native core particle. The core particle preparations used for these experiments contained a small quantity of 170 bp particles which were preferentially labeled in the ^{32}P kinase reaction. Presumably, the DNA ends in the 170 bp particles were more accessible to the kinase enzyme. While the 170 bp DNA made up only a few percent of the DNA in the core particle preparation, the differential labeling caused the 170 bp DNA to make up nearly 50% of the end labeled DNA. This preferential labeling was not observed when kinasing free DNA. Sollner-Webb et al. (1978) have observed a similar phenomena. Interestingly this 170 b particle was more resistant to DNase I digestion than the 144 bp end labeled particle; during the DNase I digestion the 170 bp DNA band becomes much more intense than the 144 bp band. This result complicates the direct comparison of native and reconstituted 144 bp particles. Nevertheless, the end labeling indicates the reconstitution is exacting and the DNA placement is probably the same as on the native particle.

Trypsin Digestion. Further evidence for the similarity between native and reconstituted cores comes from trypsin digestion studies. Sahasrabuddhe and Van Holde (1974) have shown that mild trypsin digestion of nucleosome particles leads to a more extended structure with lower S value. Weintraub and Van Lente (1974) have shown that trypsin cleaves the N-terminal ends of the inner histones leaving a

more resistant core protein particle made up of the C terminal portions of the histones. Electrophoresis of these particles on SDS polyacrylamide gels reveals a specific pattern of specific histone cleavage products. Weintraub et al. (1975) have shown that these cleavage products are not produced with histones alone at low ionic strength while Sollner-Webb et al. (1976) have indicated that reconstitution with all four inner histones plus DNA gives the specific cleavage products. I have trypsin treated both native and reconstituted core particles and find an identical pattern of protein cleavage (Figure 28). This provides further evidence for the specificity of reconstitution.

In summary, I have provided evidence that a core particle nearly if not not wholly identical to the native core can be reconstituted from its protein and DNA components, with at least 80% yield.

Reconstitutions at Different Histone/DNA Ratios

I investigated the significance of faster and slower sedimenting components in reconstituted or reassociated core particles by varying the histone/DNA stoichiometry in reconstitutions. Salt-extracted histone and 144 bp DNA were mixed in various proportions ranging from one mole histone tetramer per mole DNA to four moles histone tetramers per mole DNA; these were reconstituted in the same manner as above and the resultant products analyzed with the analytical ultracentrifuge. Figure 29 illustrates the results of the experiment. The

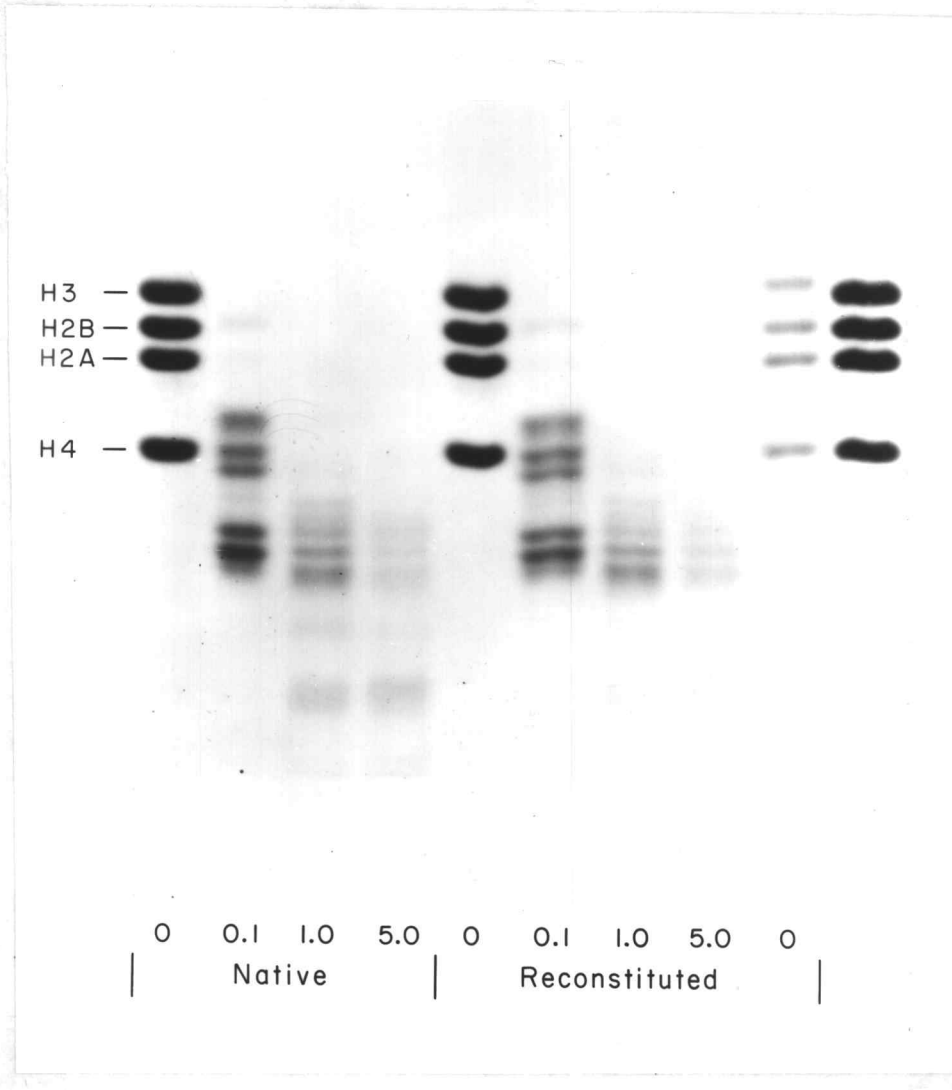

Figure 28. Trypsin digestion of native and reconstituted core particles. Core particles were digested for 16 hours with various concentrations of trypsin (in μg/ml) at 4°C. The reaction was stopped with the addition of TLCK, samples were then lyophilized and run on a 15% polyacrylamide-SDS slab gel. The numbers under each channel refer to concentrations of trypsin in μg/ml.

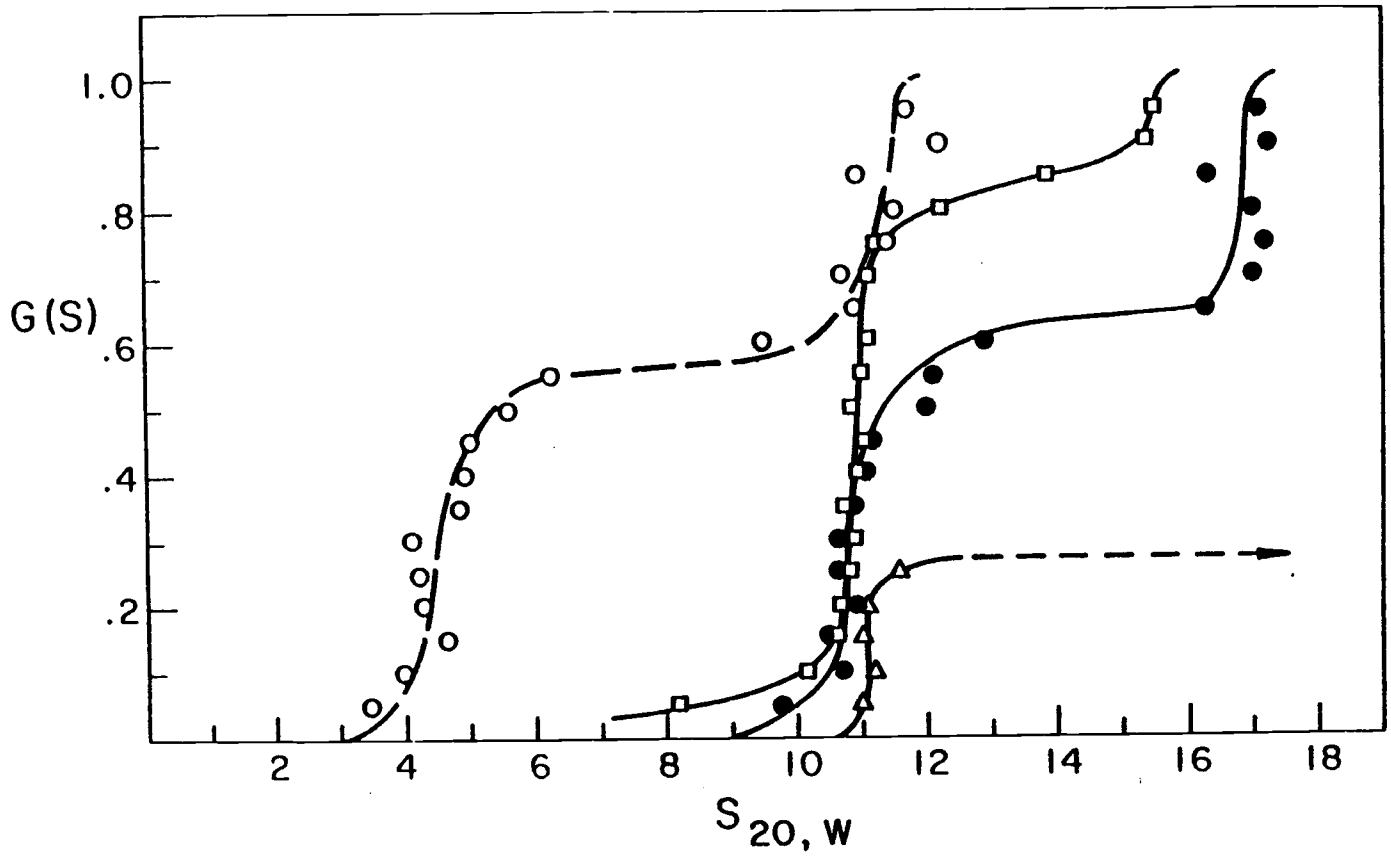

Figure 29. Integral distribution of S (G(S)) for reconstitutions at various histone/DNA ratios. Salt extracted histones and DNA were reconstituted at various histone tetramer:140 bp DNA ratios and the resultant products analyzed by analytical sedimentation. The integral distribution of S (G(S)) is plotted here. 1:1/histone tetramer:DNA (O), 2:1 (□), 2.5:1 (●) and 3.2:1 (Δ). The dashed line and arrow in the 3.2:1 sample is used to indicate aggregation, in this case 70%. A histone tetramer: DNA ratio of 4:1 resulted in 100% aggregation.

1/1 (histone tetramer/144 bp DNA) reconstitution appears just as would be expected if two tetramers combine with one DNA molecule to form a core particle. The 4.5S and 11S boundaries correspond to free DNA and core particles respectively; roughly half of the DNA remains free. Also of importance is the fact that no DNA sediments between the 4.5S and the 11S boundaries, indicating that a "half-core" particle made up of a core DNA fragment and only one histone tetramer is not a stable product in the reconstitution process. When the histone/DNA ratio is raised to 2/1 (histone tetramer/140 bp DNA) almost all the DNA sediments as an 11S boundary with little of the DNA left free in solution. Again it is important to mention that the faster and slower sedimenting components in the 2/1 reconstitution are not the result of the wrong stoichiometry because a simple reassociation of native core particles produces the same components to the same extent.

The 16S component in the 2/1 reconstitution was analyzed further after purification on sucrose gradients. It sedimented in the ultracentrifuge as a homogeneous boundary at 15.5S, the same sedimentation coefficient observed for dimer particles, although it contained mostly core particle length DNA (see Figure 21). The protein/DNA ratio of the 16S component was identical to the 11S reconstituted component (see Table 3).

The 16S material isolated from a reconstitution containing ^{32}P 5'

Table 3. Histone/DNA ratios for native and reconstituted core particles

Sample	Histone/DNA (gm/gm)	Ave.
Native 11S particle	1.20, 1.16, 1.02	1.13
Reconst. 11S particle	1.19, 1.02, 1.06	1.09
Reconst. 16S particle	1.14, 1.21	1.18
Aggregated material	1.86, 1.86	1.86

Protein concentrations were determined by Lowry analysis (Hartree, 1972), calibrated against calf thymus histone H4. DNA concentrations were determined from absorbance at 260 nm.

end label DNA was digested with DNase I , the DNA fragments electrophoresced on polyacrylamide gels, and the resulting end labeled bands observed by autoradiography. Densitometer tracing of the autoradiogram and ethidium bromide stain are shown in Figure 30. The autoradiogram clearly shows a specific pattern of DNA fragments and not just a smear of DNA. This is strong evidence that the ends of the DNA are still phased with respect to the cutting site of DNase I.

Reconstitution at a 2.5/1 ratio leads to further increase in the amount of material sedimenting at $S_{20,w} > 11S$ (see Figure 29). This complex has a sedimentation coefficient significantly greater than 15.5S (~17S). Raising the histone tetramer/144 bp DNA ratio to 3.2/1 leads to considerable aggregation. The solution is visibly turbid with 70% of the DNA pelleting out of solution before the sedimentation run is up to speed. The non-aggregating material sediments as a homogeneous boundary at 11S. A further increase in the histone tetramer/144 bp DNA ratio leads to greater aggregation; at a ratio of 4/1 all the DNA aggregates and no 11S material is left in solution. The histone/DNA ratio (w/w) of this aggregated material is 1.86 (see Table 3). Possible mechanisms will be discussed below.

Method of Reconstitution

Reconstitution by either gradient dialysis or step dialysis (see

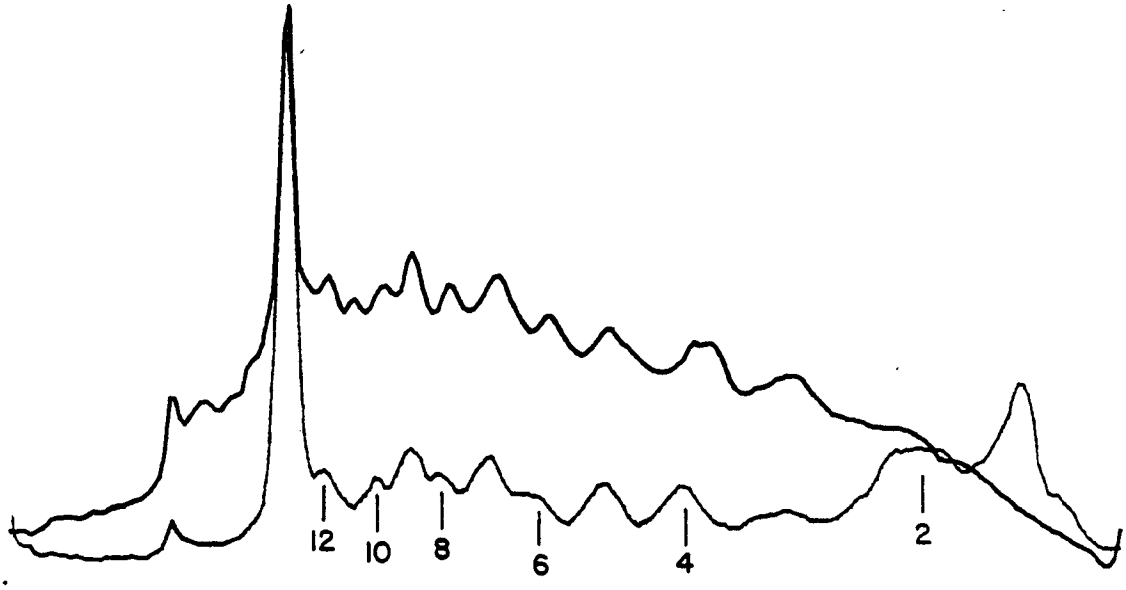

Figure 30. DNase I digestion of the 16 S reconstituted component end labeled with ^{32}P. The 16 S component was digested for 1 minute with DNase I. The DNA was isolated, electrophoresed on an 8% polyacrylamide-urea slab gel, stained with ethidium bromide and then autoradiographed. Scans of the ethidium bromide photograph (Top) and autoradiogram (Bottom) are presented here. The numbers below the peaks refer to band number.

appendix) gave the same product, as judged by analytical sedimentation. To test the necessity for these 24 hour protocols, 144 bp DNA and histones were mixed in 2 M NaCl and diluted quickly to 0.6 M NaCl with 10 mM Tris. The ionic strength was then dropped to 10 mM Tris by dialysis. The comparison of the products from the "slow" and "fast" reconstitution schemes is presented in Figure 31 as G(S) distributions. The "fast" method resulted in greater 16S material and relatively less 11 S. It is still surprising that nearly 50% of the DNA was reconstituted by the fast method in 11S particles. It is likely that the reconstitution protocol could be shortened to just a few hours with no loss of yield but this possibility was never studied in detail.

Discussion

The nearly quantitative reconstitution that can be obtained from either dissociated core particles or mixtures of core DNA and histone tetramers argues that formation of the core particles is a spontaneous process in low salt media. The virtual identity of the physical properties and DNase sensitivity of the native and reconstituted core particles strongly supports this contention.

It is of importance to know the state of the histones during reconstitution from 2 M NaCl. On the basis of crosslinking studies Thomas and Kornberg (1975) have suggested that the structure is a

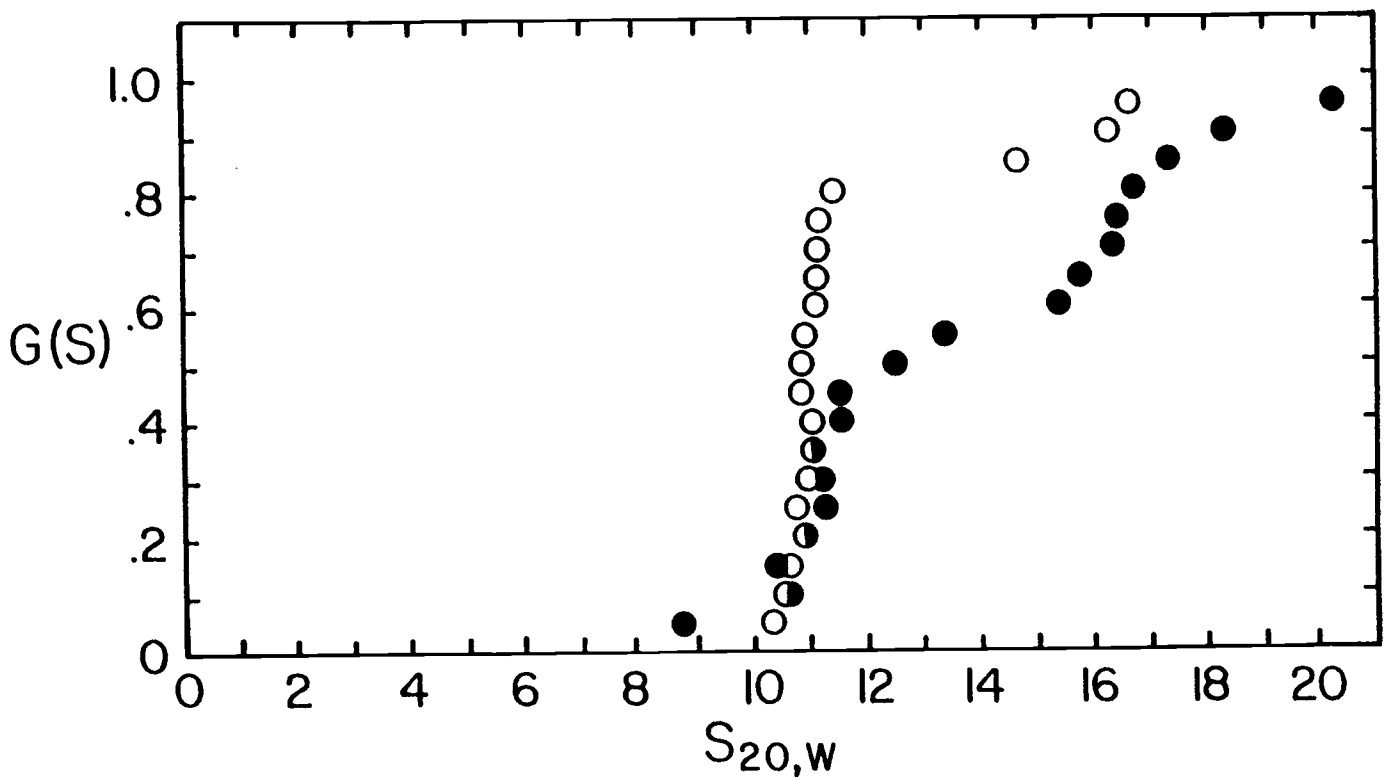

Figure 31. G(S) distribution comparing results of different methods of core particle reconstitution. Open circles (O) indicate the standard 24 hour reconstitution method while filled circles (●) indicate the "fast" dilution method of reconstitution.

histone octomer. Weintraub et al. (1975) have argued that a heterotypic tetramer (histone tetramer made up of one each of the four core histones H2A, H2B, H3, H4) is the dominant species in 2 M NaCl. Recently, using sedimentation equilibrium, Chung et al. (1978) have found strong evidence that salt extracted chicken erythrocyte core histones are in a tetramer-octomer equilibrium in 2 M NaCl. At protein concentrations below 1 mg/ml however, the complexes dissociate. It has also been demonstrated that the complexes present in high salt disproportionate into H3-H4 and H2A-H2B complexes similar to those studied at low ionic strengths by others (D'Anna and Isenberg, 1974c; Kornberg and Thomas, 1974; Roark et al., 1974; Sperling and Bustin, 1975; Martinson and McCarthy, 1975; Alder et al., 1975; Lilley et al., 1976; and Moss et al., 1976a, b) when the salt concentration is lowered below 1 M (Weintraub et al., 1975). This evidence suggests that prior to histone-DNA association, at about 1.2 M NaCl (Wilhelm et al., 1978), the histones may be in complex equilibrium between many states of association.

Recently Wilhelm et al. (1978) have demonstrated that the histones do not all associate with the DNA at the same ionic strength as the ionic strength is decreased. H3 and H4 associate with the DNA at about 1.2 M NaCl while H2A and H2B do not associate until about 0.8-1.0 M NaCl is reached. Differential histone-DNA binding has also been observed by Kleiman and Huang (1972) and Gadski and

Chae (1976) in salt and urea. These results indicate that a histone octomer or heterotypic tetramer does not simply bind to the DNA as the ionic strength is lowered from 2 M NaCl but the histone tetramer-octomer dissociates into H3-H4 and H2A-H2B complexes that then associate with the DNA. Nevertheless there is very strong cooperativity in the binding of all four core histones together to form the core particle. This is dramatically demonstrated by the experiments in which an excess of DNA was used. A conceivable result would have been the association of histones with all the DNA molecules to form structures with $5\ S < S_{20,w} < 11\ S$. This was not observed. Instead, about half of the DNA remained free; half associated with the histones to form 11 S core particles (see Figure 29).

A fundamental question concerns the fidelity with which the DNA is placed upon the set of binding sites on the protein core. Are all of the DNA molecules wholly bound, or are there projecting "tails"? Of course, if the "re-entrant path" model proposed by Cantor (1976) is correct, one would expect that the DNA, wherever it first attached, would lie completely in the path. However, this model has not been demonstrated. If the path is not re-entrant, it would seem possible that DNA molecules could irreversibly attach at an "incorrect" point, and leave a projecting region. The evidence from these experiments is that this either does not happen frequently, or represents an unstable intermediate, at least in reconstitution or reassociation

experiments where the DNA:histone ratio is close to that of the native particles. The fact that the sedimentation coefficient and CD of such particles are very close to those of the native particle is evidence of a sort. More convincing is the evidence from DNase I digestion. The reconstituted or reassociated particles show the same pattern, including the higher (> 100 b) bands, and do not exhibit significant "background" of randomly cleaved fragments, suggesting that in most cases there must be very little "projecting" DNA. It is also noted that the melting curves for the reconstituted and reassociated particles are nearly identical to those for native particles.

The best evidence for the fidelity of DNA placement though is the ^{32}P and label-digestion experiments. The clear banding pattern in the autoradiogram attests that the DNA placement is extremely exacting.

However, especially at higher histone:DNA ratios, there is evidence for poorer fidelity in DNA placement. This comes from the existence of higher aggregates, which increase in both size and amount as the histone:DNA ratio is increased. It is difficult to explain the formation of such structures other than by the formation of DNA crosslinks between octamers. A tentative model for the reassociation process is shown in Figure 32. It presumes that at some intermediate stage in the gradient dialysis, the salt concentration will be such that DNA-histone complexes are only marginally stable. At low

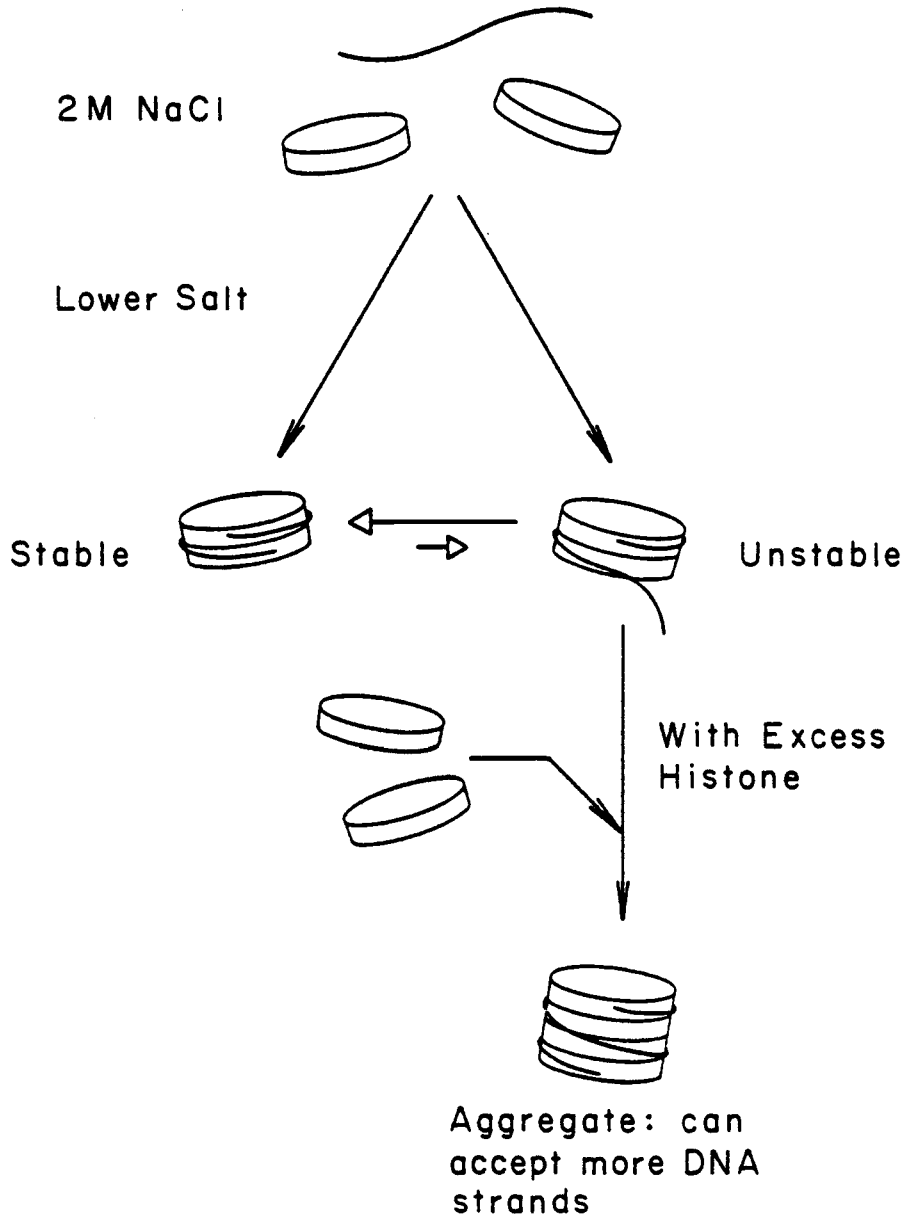

Figure 32. A schematic diagram of the model for the reconstituted process. In 2 M NaCl, the components are postulated to be free DNA and histones, mainly present as heterotypic tetramers. As the salt concentration is reduced, association of DNA and histone takes place to form core particles. Both "correct" (left) and "incorrect" (right) alignment of the DNA can initially occur; however, the latter structures are relatively unstable, and tend to rearrange to form "correct" particles. In the presence of excess histone, however, the incorrectly aligned DNA will tend to serve as a "crosslink" between adjacent octamers. These structures can in turn add more DNA to form large aggregates.

histone:DNA ratios, incorrect placement of a DNA on an octamer will be corrected by exchange, for a partially attached DNA will be relatively unstable, compared to a "fully" attached DNA. However, at high histone:DNA ratio, a poorly attached DNA may be stabilized if another protein core is immediately available to "stack" on the first. This generates an "out of phase" structure which can grow to a larger aggregate by successive attachment of DNA fragments and protein cores. This "out of phase" structure is not due to random placement of 144 bp DNA on the histone core since the end labeled DNase I pattern is a definite banding pattern and not just a smear (Figure 30). A possible structure for the "out of phase" particle is a core particle in which the DNA is displaced asymmetrically 10 or 20 base pairs from the preferred DNA binding location. Such a structure would still give the end labeled DNase I banding pattern but with different peak intensities since the DNA ends would now be at different distances (multiples of about 10 base pairs) from the strong DNase I protection sites.

As might be expected, this "out of phase" process can occur to some degree even when the histone/DNA stoichiometry is exact, as in the case of reassociation experiments (Figure 19). It is also not surprising that more 16 S and higher molecular weight material form upon very rapid reconstitution, as in the case of the simple dilution experiment (Figure 31). These experiments argue that the

reconstitution process requires a finite period of time for correct reconstitution. It may be possible to force all the 16 S particles into 11 S core particles by annealing the reconstitution mixture at the right salt concentration. It is noteworthy that the 16 S component cannot be accounted for by dimer-size DNA (there is only 10% dimer DNA in the isolated dimer peak (Figure 21), nor is sufficient H1 or H5 present to account for the dimer found. Only in cases where the histone tetramer/144 bp DNA ratio is less than 2/1 do we see a complete absence of structures larger than the core particles.

The techniques described here provide a way for making large quantities of core particles, using specifically modified histones or a mixture of histones. Chromatin reconstitution experiments with individually purified histones (Camerini-Otero et al., 1976; Sollner-Webb et al., 1976) and even acid purified histones (Germond et al., 1976) indicate that reconstitution need not be done with histone complexes as a starting material. Even if a small fraction of dimers or larger structures is formed, these can easily be separated. The 11 S particles so produced appear to be, in every way tested so far, identical to the native particles.

V. COMPACT OLIOMERS FROM H1 DEPLETED CHROMATIN

Introduction

That the nucleosome is a fundamental unit of eukaryotic chromatin seems now to be well established. The only exceptions include protamine containing sperm chromatins (Honda et al., 1974) and possibly some dinoflagellates that do not contain histones (Van Holde et al., 1978). Furthermore, it has recently become clear that while the size of the DNA fragments observed as structural repeats by nuclease digestion may vary from one cell type to another, the nucleosomal core particle containing about 145 bp of DNA and 8 histone molecules (Van Holde et al., 1975; Sollner-Webb and Felsenfeld, 1975; Axel, 1975; Shaw et al., 1976; Noll, 1976b; Morris, 1976a; Compton et al., 1976; Lohr et al., 1977b) is truly ubiquitous. The general structure appears to involve nearly identical core particles containing two copies each of H2A, H2B, H3 and H4 connected by spacer DNA segments which carry H1 (and H5 in avian erythrocytes) (Shaw et al., 1976; Noll and Kornberg, 1977).

The average spacer length varies from one organism and even one tissue or cell type to another (Compton et al., 1976; Spadafora et al., 1976; Morris, 1976b; Lohr et al., 1977b; Thomas and Thompson, 1977). The spacer may even vary between nucleosomal regions of the same cell (Lohr et al., 1977a; Martin et al., 1977; Prunell and

Kornberg, 1978. The length varies from about 100 bp in sea urchin (240 bp repeat) (Spadafora et al., 1976) to only 20 bp in yeast and other fungi (154-170 bp repeat) (Morris, 1976a; Noll, 1976b; Thomas and Furber, 1976; Lohr et al., 1977a). A complete tabulation of presently available data can be found in Van Holde and Weischet (1978a).

Two lines of evidence indicate that the commonly held chromatin model, consisting of the 144 bp core particle with a variable spacer, may be over simplified. Todd and Garrard (1977) have isolated nucleosome fractions containing 160 bp DNA not containing H1. Under some conditions the core histones are thought to interact with more than 144 bp of DNA in chromatin depleted of H1 (Christiansen and Griffith, 1977; Weischet, Allen, and Van Holde, manuscript in preparation). The second line of evidence comes from the protein crosslinking experiments of Hardison et al. (1977) and Bonner (1978). They find histone H1, previously thought to bind to the spacer region, crosslinked to core histones.

A more realistic view of the nucleosome is therefore a complex structure with core histones interacting in some way with at least part of the previously designated spacer, and histone H1 interacting with core DNA and histones as well as spacer DNA. The variability of the spacer length allows chromatin to acquire many different conformations, at least in theory. In this chapter I will examine one

extreme example of internucleosomal conformation. This is a class of nucleosome oligomers probably containing no spacer at all. The dimer of this series contains only 265 bp of DNA, the trimer only 390 bp and the tetramer only 520 bp. Digestion of these with pancreatic DNase I yields DNA fragments that are multiples of ten bases extending to 270 bp in the dimer and to at least 340 bp in the trimer (Lohr et al., 1977c).

Although there is no direct evidence for the existence of these particles in vivo they represent a most interesting type of possible chromatin structure. A model proposed for their structure might explain some of the "extended DNase I ladder" observed in vivo in nuclei by Lohr et al. (1977c). Most of the results in this chapter have been presented by Tatchell and Van Holde (1978). A proposed compact oligomer model (Figure 42) has also been mentioned previously in Van Holde and Weischet (1976a). Klevan and Crothers (1977) have also reported the isolation and characterization of similar compact dimers containing only 240 bp of DNA.

Results

The compact oligomers were isolated from a micrococcal nuclease digestion of H1 depleted chromatin prepared as described in Chapter II. After digestion to 20%-30% acid soluble DNA, over 90% of the remaining chromatin exists as 144 base pair core particles.

The remaining small fraction of soluble chromatin exists as a set of stable particles containing DNA in two main size classes: a 260-270 base pair class and a 380-400 base pair class. The particles containing 260-280 and 380-400 bp DNA are called "compact dimer" and "compact trimer" respectively. In some digests I have seen a DNA band at about 520 bp that would correspond to a compact tetramer. I have obtained small quantities of this oligomer, allowing partial characterization. It should be emphasized that this class of oligomers represent structures with very much smaller DNA than those normally found on brief micrococcal nuclease digestion of chicken erythrocyte nuclei. A normal dinucleosome from chicken erythrocyte nuclei contains 350 bp of DNA.

On high resolution polyacrylamide gels, the compact dimer DNA is a band centered at 265 base pairs ± 10 bp, the trimer DNA at 390 bp ± 20 bp (Figure 33a). A less accurate value for the tetramer DNA is 520 ± 40 bp. When compared with the homogeneous restriction fragments it is obvious that while there is some heterogeneity in the DNA lengths of the compact oligomers, they are far more homogeneous than are oligomers prepared by the usual technique of digestion of chromatin in nuclei. On denaturing gels the compact dimer DNA is resolved into two sharp bands at 260 b and 270 b. The trimer DNA seems to be resolved into bands at 380, 390 and 400 b, although the resolution is poor for the trimer. I have not so far been able to

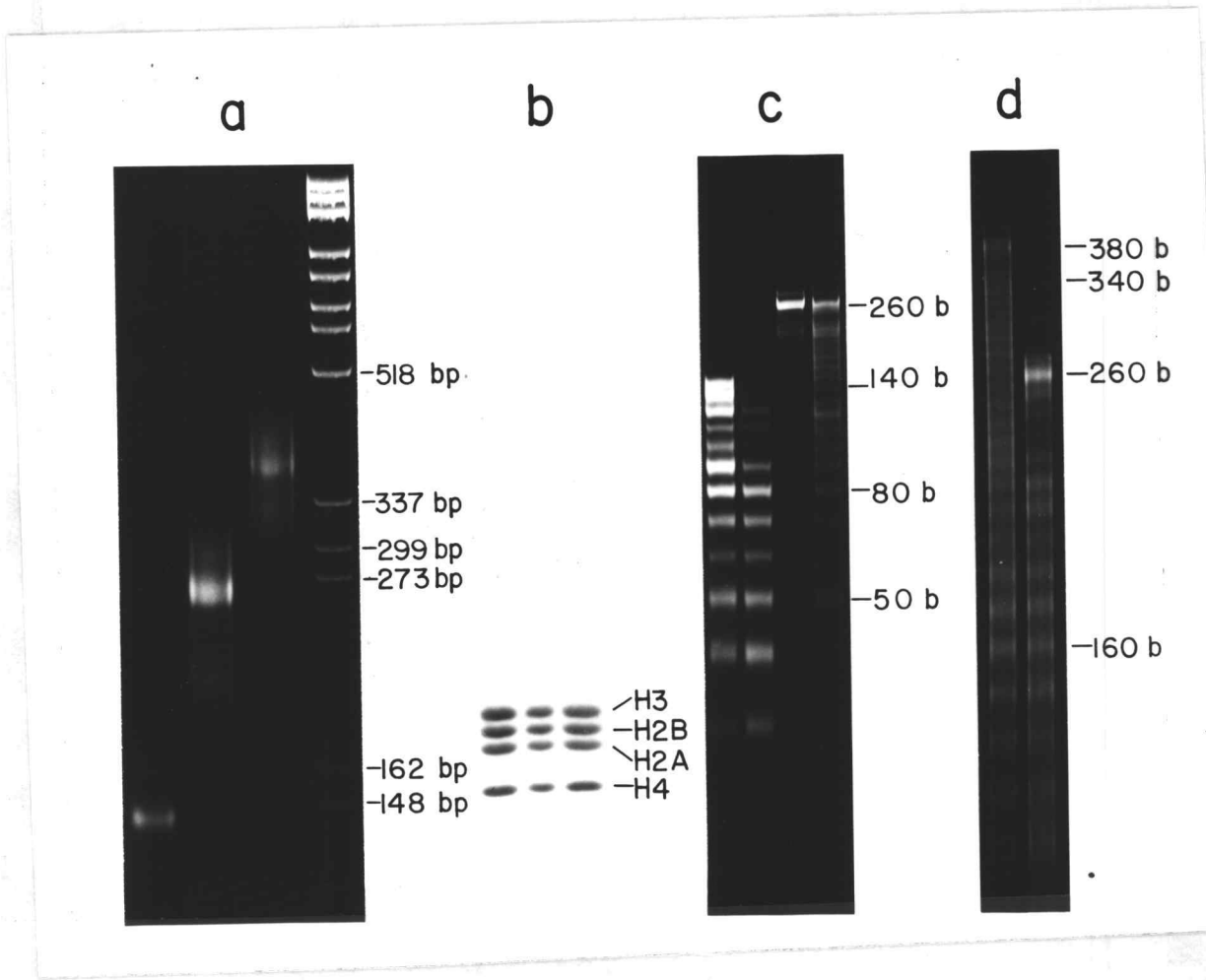

Figure 33. Electrophoretic characterization of compact oligomers. (a) A 4.5% polyacrylamide non-denaturing gel showing DNA isolated from (left to right): core particles, compact dimers, compact trimers and PM2-Hae III restriction fragments. Electrophoresis is from top to bottom. (b) 15% polyacrylamide-SDS gel showing proteins from (left to right): core particles, compact dimers and compact trimers (c) An 8% polyacrylamide-urea denaturing gel of DNase I digested core particles and compact dimers. From left to right: 1 minute and 5 minute digestions of core particles; 0 digestion and 20 second digestion of compact dimers. (d) An 8% polacrylamide-urea denaturing gel of DNase I digested compact trimer (20 second digestion) and compact dimer (1 minute digestion). The DNA smaller than 110 bp was run off the gel to allow better resolution of the higher molecular weight bands.

observe such resolution of the oligomer DNA into bands on non-denaturing gels. A possible explanation is that some oligomers contain duplex DNA with different length strands, i.e., a compact dimer with one 270 base DNA strand and one 260 base strand. Another possible explanation is that base sequence and composition influences the migration of double-strand DNA on polyacrylamide gels. Evidence for this comes from the abnormal migration of certain sequenced restriction fragments on non-denaturing polyacrylamide gels (Reddy et al., 1978).

When compact oligomer particles are isolated from preparative sucrose gradients, each sediments with a homogeneous boundary in the analytical centrifuge. The $S_{20,w}$ values are presented in Table 4. The value for the compact tetramer may be in error due to slight proteolysis in just this sample. Compact oligomers have the same histone stoichiometry as nucleosomes or core particles; roughly equal amounts of the histones H3, H2B, H2A and H4 are found, but no H1 or H5 (Figure 33b) and no modified histones are indicated by electrophoresis on acid-urea-triton polyacrylamide gels (data not shown) (Dr. B. Shaw, personal communication). Thus so far as we can see, there is nothing unusual about the protein composition. The protein/DNA ratios presented in Table 4 indicate the dimer contains two octamers of histones (four each of the inner histones), while the trimer contains three octamers of histones.

Table 4. Properties of compact oligomers

	Properties of Compact Oligomers					
	DNA size (bp)[a]	protein/DNA (w/v)[b]	$S_{20,w}$(obs)[c]	$S_{20,w}$(pred)[d]	% premelt	bp DNA in premelt
core particle	144± 5	1.1±0.1	10.9±0.1	(10.9)	29	42
compact dimer	265±10	1.2±0.1	15.4±0.3	15.8	20	53
compact trimer	390±20	1.2	19.4±0.4	19.1	14	55
compact tetramer	520±40		22.8	22.9		

a) (±) indicates approximate half-width of main peak.

b) Averages of multiple samples from two preparations for core and dimer, one for trimer.

c) Average of 3 experiments for core, dimer, and trimer; one measurement for tetramer. All values in svedbergs.

d) Assuming $S_{20,w}$ for core to be 10.9S. See text.

The compact oligomers show a higher melting temperature than the core particles when thermally denatured. A first derivative plot of the denaturation is shown in Figure 34. While the melting profiles are complex, it is interesting that the T_m values (at the major transition) of the compact oligomers are higher than for the cores and the fraction of DNA associated with the lower melting transition is decreased.

The circular dichroism spectra of core particles, compact dimers and compact trimers are presented in Figure 35. The compact oligomers have a greater negative ellipticity at 295 nm, diminished positive ellipticity at 280 nm, and increased positive ellipticity at about 270 nm, as compared to the core particles. A preliminary experiment with compact tetramers indicates (not shown) that this trend continues; the increase at 270 nm being most marked.

Digestion of compact oligomers by pancreatic nuclease gives the "extended ladder" of DNA fragments as shown in Figures 33c, 33d and 36. With high resolution gels the bands can easily be resolved to up to 270 bases in the dimer and to 340 bases in the trimer; in all likelihood they continue to 380 or 390 bases in the latter case (Figure 33d). Just as in the digestion of nuclei <u>in situ</u> with pancreatic nuclease, the DNA bands in the compact oligomers are of unequal intensity with the 80 base band being most intense. Higher molecular weight bands also show a reproducible variation in intensity.

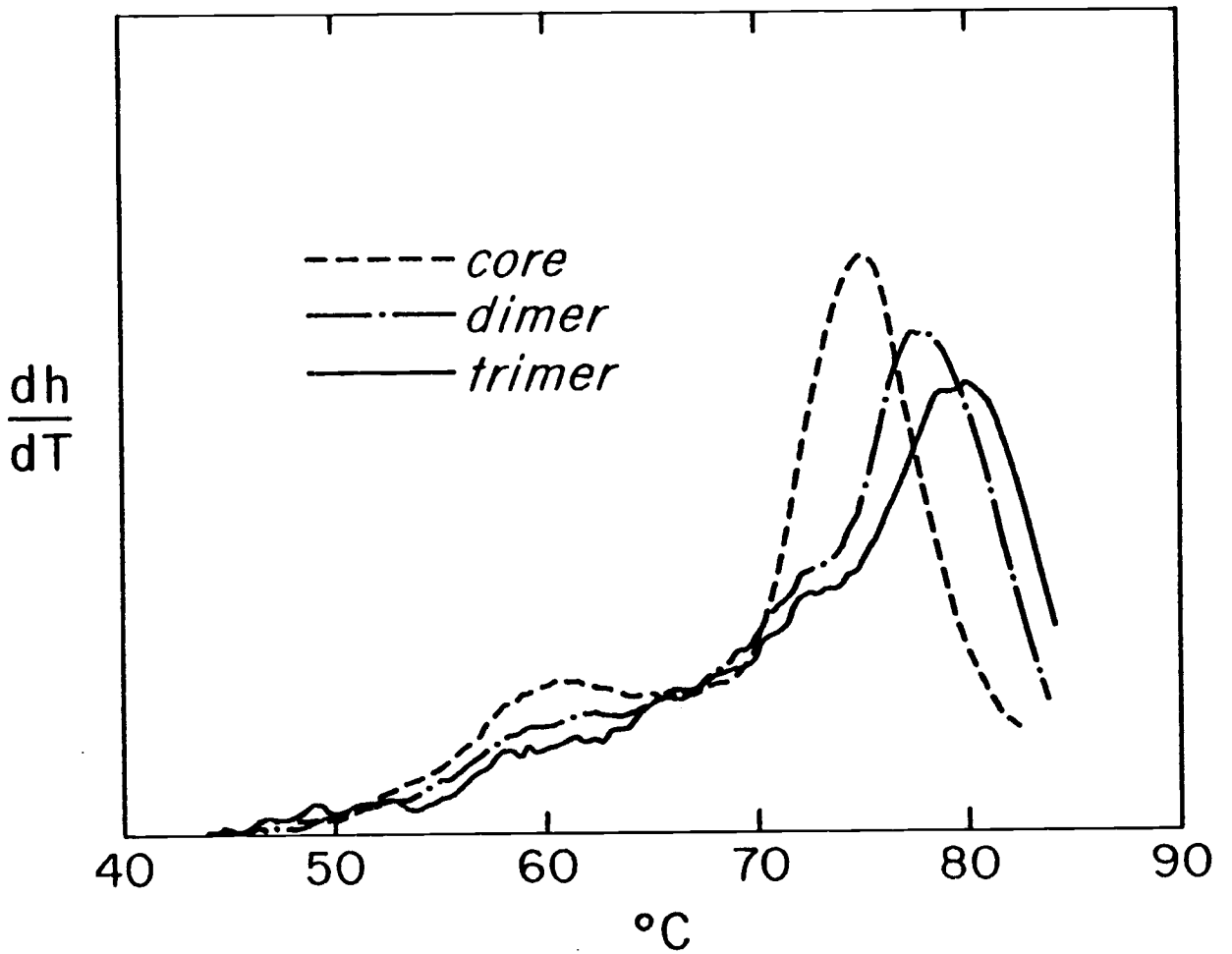

Figure 34. Thermal denaturation of core particles and compact oligomers. Shown are derivative hyperchromicity curves (dh/dT) for the core particle, compact dimer, and compact trimer, in 1.0 mM cacodylic acid pH 7.2.

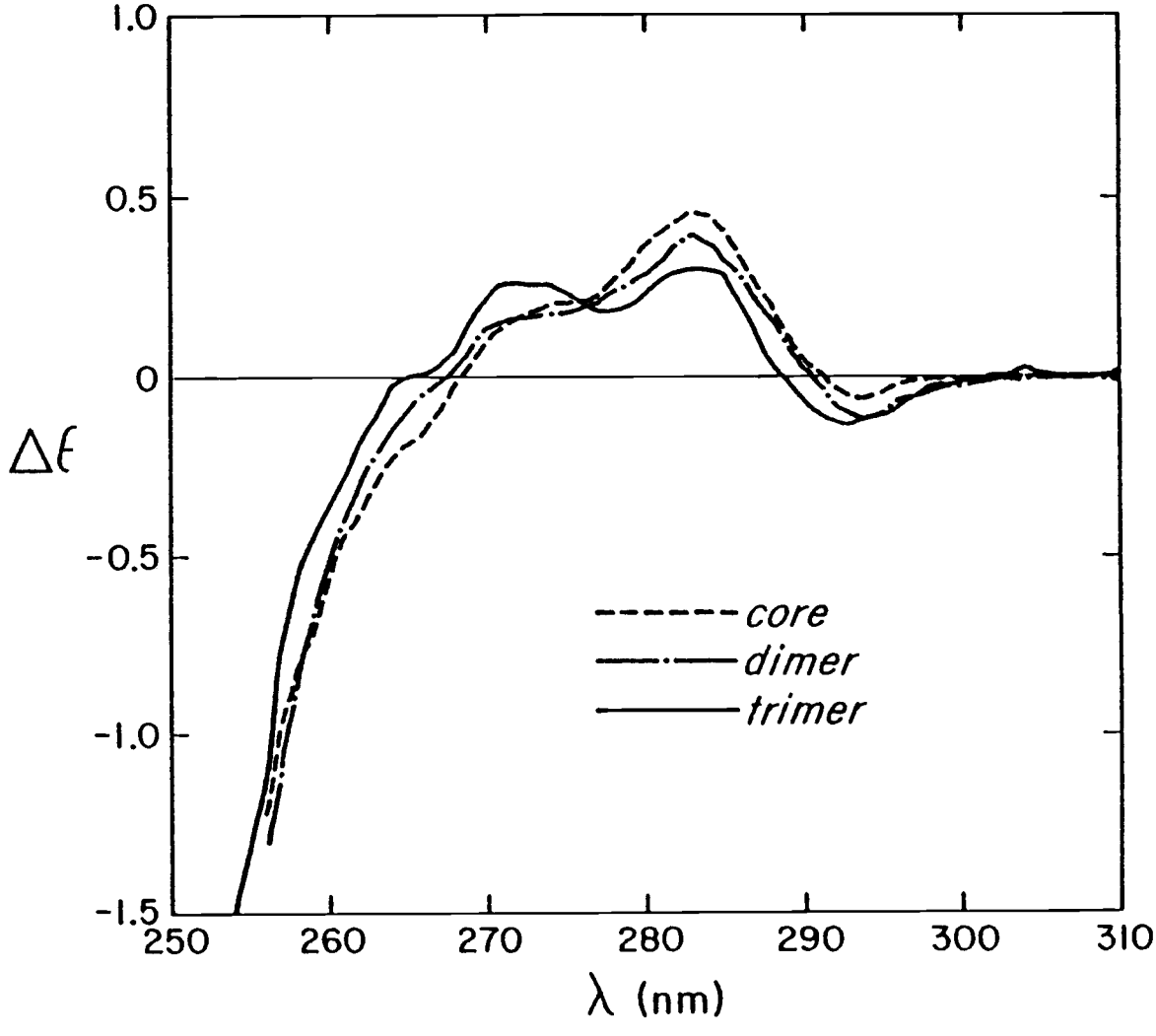

Figure 35. Circular dichroism spectra in the near UV of core particles, compact dimers, and compact trimers.

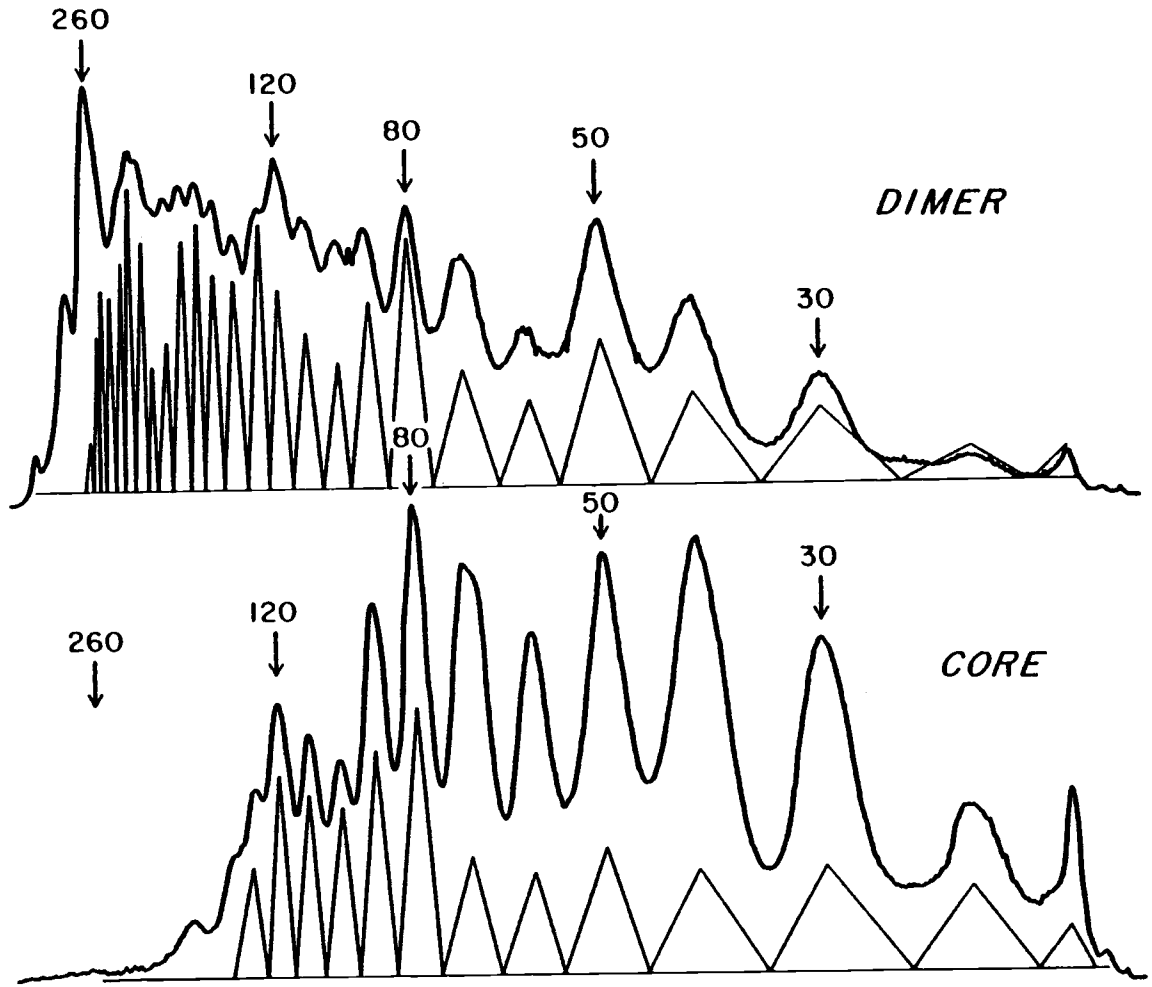

Figure 36. Gel electrophoresis of single-strand DNA fragments from DNase I digestion of compact dimers and core particles. The data are shown as scans of ethidium bromide stained gels. Electrophoresis conditions as described in text. The triangles represent (by height) weight amplitudes of different bands as predicted by the model calculation described in Discussion. The numbers refer to the approximate molecular weight in base pairs.

I have labeled the 5' ends of the DNA in the compact oligomers with ^{32}P and followed these ends in the pancreatic nuclease digestion by autoradiography of the DNA gels in a manner similar to that used by Simpson and Whitlock (1976). Scans of the autoradiographs are represented by dotted lines in Figure 37. The distribution of ^{32}P in DNA bands below 140 bases (Figure 38) supports Simpson and Whitlock's results and is nearly the same in core, compact dimer and compact trimer digests. The pattern of labeled bands above 140 bases in the dimer and trimer will be discussed below.

I am concerned about the actual sizes of the DNA bands produced by DNase I. They are generally considered to be multiples of ten bases. But the DNA in the core particle is not 140 base pairs! I now obtain a value around 144 bp, as do others (Mirzabekov et al., 1978). If the DNase I bands extend at a constant multiple, from zero bases to the full length of the core particle, the interval between bands is 10.3 bases rather than 10.0 bases. Another possibility is that the bands are not all evenly spaced; the interval near the DNA ends in the core particle may be larger than the interval in the center of the DNA. I do not have hard evidence to support either possibility but one observation is relevant to this question. When DNase I DNA digest of core particles and compact oligomers are electrophoresed in adjacent wells, the largest band of the core particle (band 14) does not co-electrophorese with band 14 of the compact oligomer (Figure 33c).

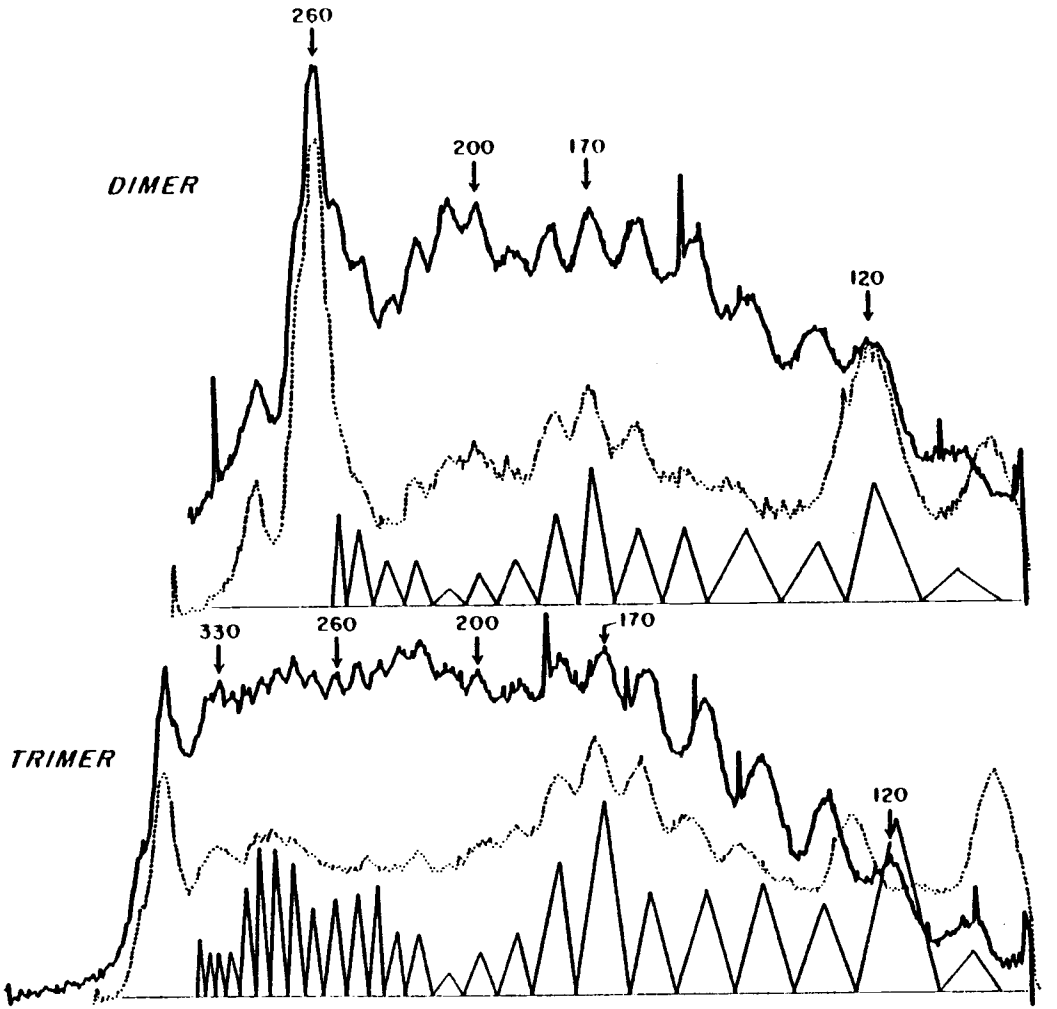

Figure 37. Gel electrophoresis of single-strand DNA fragments from a DNase I digestion of compact dimers and trimers end-labeled with ^{32}P prior to the digestion. The data are shown as scans of ethidium bromide stained gels (solid line) or scans of the autoradiogram (dotted line).

The triangles represent (by height) amplitudes of different ^{32}P containing bands as predicted by the model calculations described in Discussion. The numbers refer to the approximate molecular weight in bp.

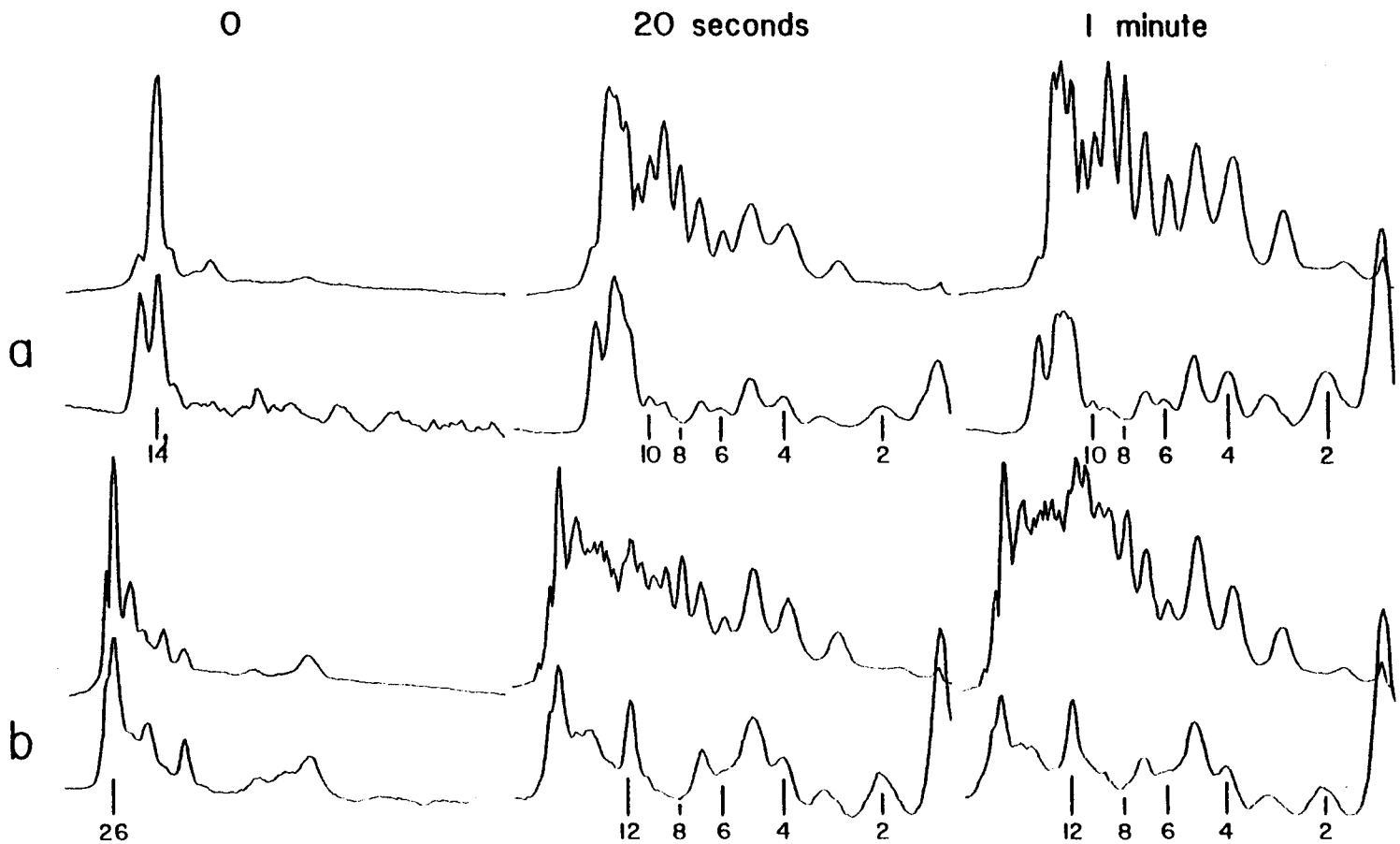

Figure 38. Gel electrophoresis of DNA fragments from a DNase I digest of core particles and compact dimers end labeled with ^{32}P prior to digestion. Core particles (a) and compact dimers (b) were digested for 20 seconds (middle) and 1 minute (right) with DNase I. The top scan of each set is the ethidium bromide stained gel while the lower scan is of the autoradiogram.

This tends to support the "non-equal" model over a "non-integral" model but at this time I will refer to each DNase I DNA band by its number (from zero bases) and not by its absolute size.

DNA was isolated from compact dimers and reconstituted with salt extracted histones as described in Chapter IV. The initial protein/DNA ratio was varied between 1 histone octomer/DNA molecule to 2.5 histone octomers/DNA molecule. A G(S) distribution profile for the resulting products is illustrated in Figure 39. In each experiment the amount of DNA unreconstituted or reconstituted with one or two histone octomers can be determined from the G(S) distribution. Free DNA sediments at roughly 5-6 S, reconstitutes with one histone octomer at 9-11 S and reconstitutes with two octomers at 14-16 S. It was surprising that the putative single nucleosome with 265 bp of DNA sedimented significantly slower than the 144 bp core particle. A most likely explanation is that the extra DNA increases the frictional coefficient of the particle, thereby lowering the sedimentation coefficient. Dr. K. E. Van Holde (personal communication) has calculated the theoretical sedimentation coefficients of particles with 160-265 bp DNA. The calculations assume the extra DNA (DNA beyond 144 bp) extends as a rigid rod from one end of the particle. Theoretical calculations for general "lollypop" structures have been made by Garcia de la Torre and Bloomfield (1977). Such calculations predict a sedimentation coefficient of 9.4 S for the 265 bp particle; a value

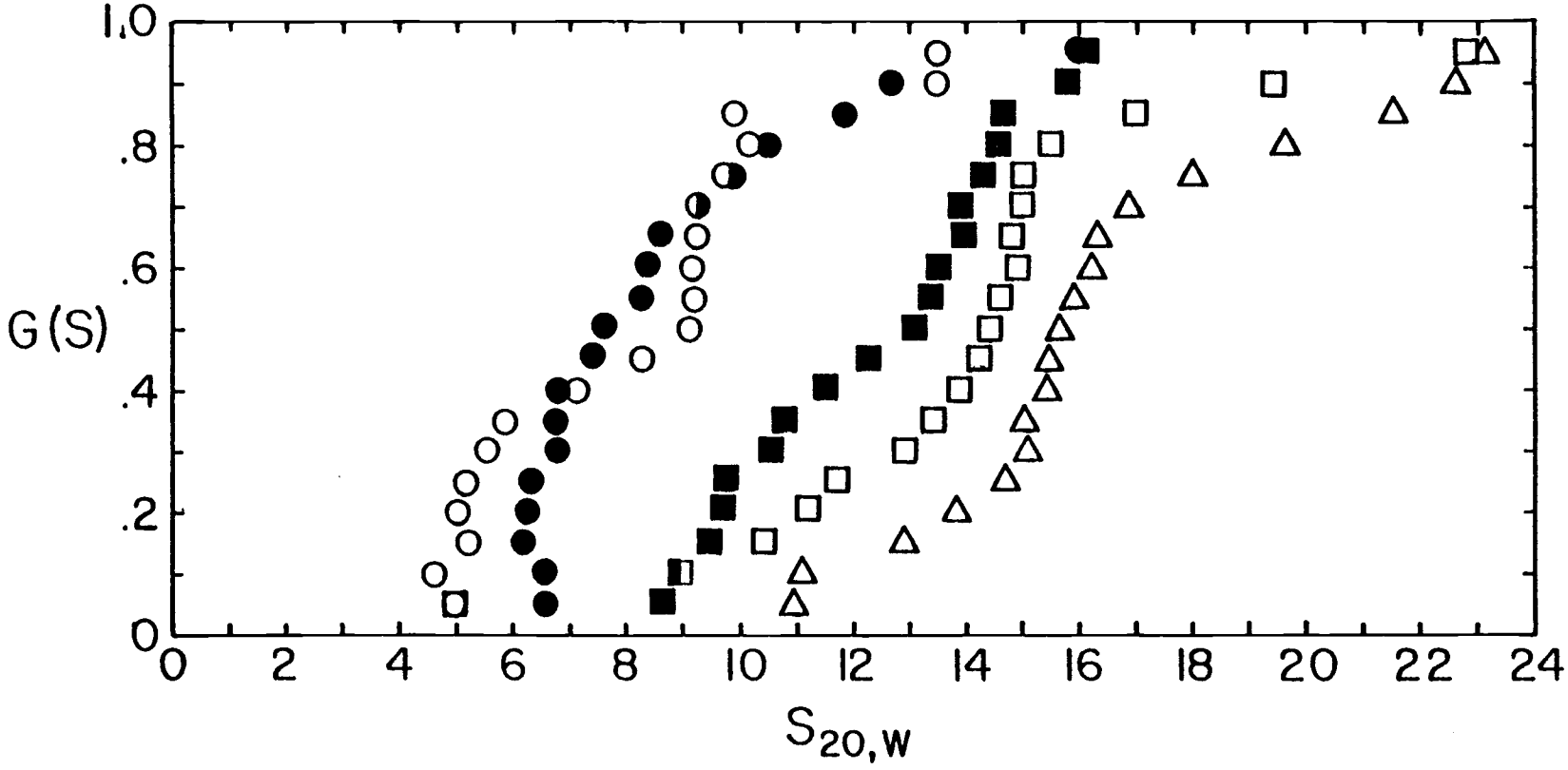

Figure 39. G(S) distribution for reconstitutions with 265 bp and 360 bp DNA at different histone/DNA ratios. The various symbols indicate: (○), 265 bp DNA reconstituted with 1 histone octomer/DNA fragment; (●), 360 bp DNA reconstituted with 1 histone octomer/DNA fragment; (□), 265 bp DNA reconstituted with 2 histone octomers/DNA fragment; (■), 360 bp DNA reconstituted with 2 histone octomers/DNA fragment; (△), 265 bp DNA reconstituted with 2.5 histone octomers/DNA fragment.

very close to the experimental.

The sedimentation coefficient for the free 265 bp DNA was also lower than that expected from published values. Sedimentation coefficients for the restriction fragments of PM2 (Kovacic and Van Holde, 1977) indicate a 265 bp DNA molecule should sediment at 6.2 S. The discrepancy is most likely due to the Donnan effect of the highly charged DNA in the low ionic strength buffer used in these experiments. This effect will cause any highly charged macromolecule to sediment more slowly through low ionic strength medium.

Nucleosome dimers were isolated from nuclease digests of whole nuclei . The DNA from these particles, significantly larger than the compact dimer DNA (360 bp vs. 265 bp), was isolated and reconstituted with core histones. The G(S) profile for these reconstitutions, also included in Figure 39, are similar to the compact dimer reconstitutions.

The 265 bp and the 360 bp dimer reconstitutions were digested with pancreatic DNase I and the resulting DNA fragments electrophoresed under denaturing conditions. The results are presented in Figure 40. While all digestions produced the now familiar 10 base "ladder" of DNA fragments below 140 bases, differences are observed above 140 bases. The 265 bp reconstitute, with an initial histone/DNA ratio of two histone octomers/DNA molecule, gave the "ladder" of DNA fragments above band 14 when digested with DNase I.

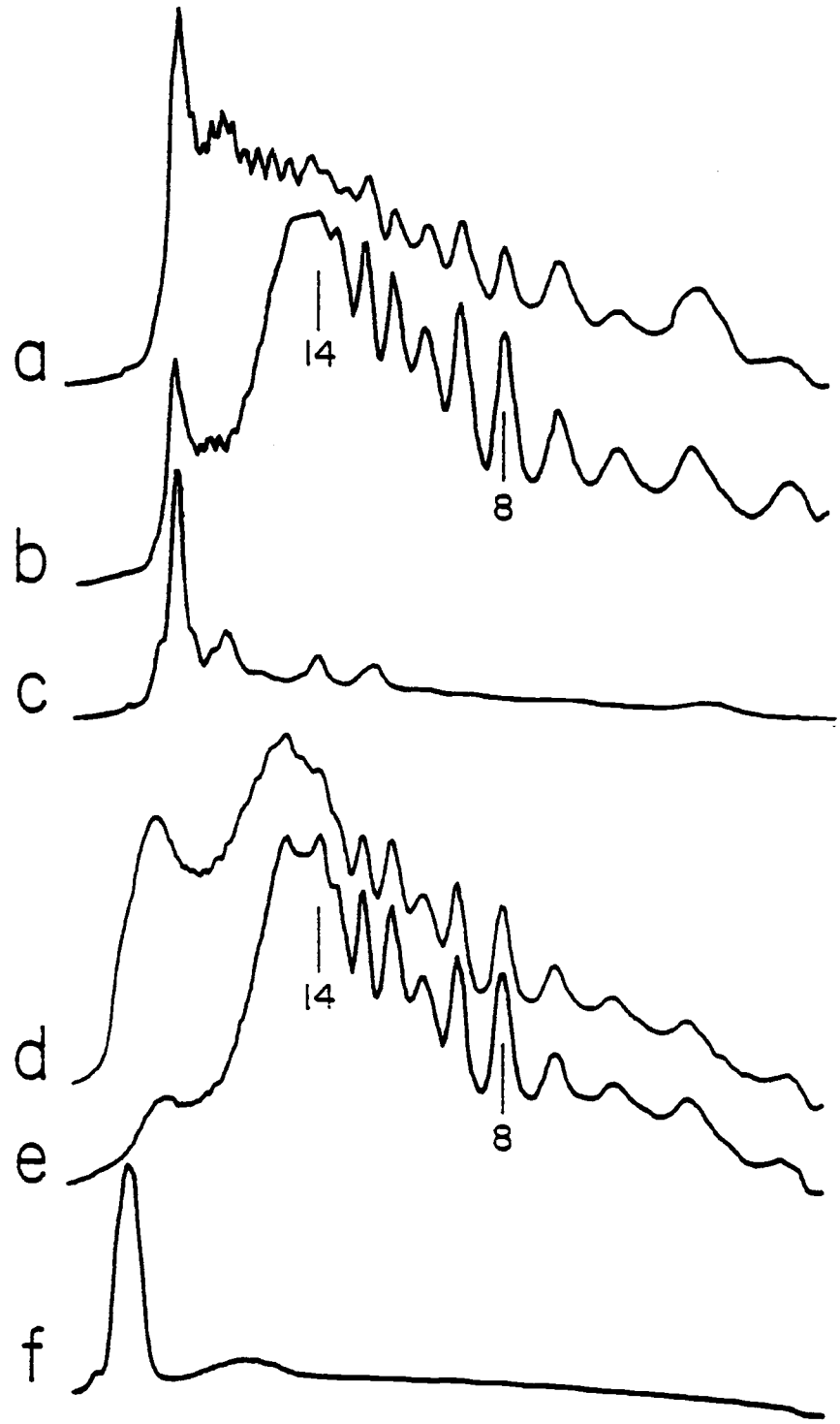

Figure 40. Gel electrophoresis of single strand DNA fragments from a DNase I digestion of reconstituted 265 bp and 360 bp DNA. DNA was reconstituted at different ratios of histone/DNA and then digested for 30 seconds with DNase I. The data are shown as scans of an ethidium bromide stained slab gel. The scans are: a) 265 bp reconstitute, 2/1, mole histone octomer/mole DNA; b) 265 bp reconstitute, 1/1; c) undigested 265 bp DNA; d) 360 bp reconstitute, 2/1; e) 360 bp reconstitute, 1/1; f) undigested 360 bp DNA.

The corresponding 360 bp reconstitute gave little of the DNA bands above band 14 (Figure 40d). DNase I digest of reconstitutes containing only one histone octomer per DNA molecule also produced little of the "ladder bands" above band 14 but did contain an intense peak of DNA at 140-160 bases (Figure 40).

Compact dimers reconstituted with ^{32}P end labeled DNA also gave an autoradiogram pattern similar to the native compact dinucleosome pattern (Figure 41).

Discussion

At first it seemed remarkable that such particles as these compact oligomers could exist at all. If the 144 bp core particle is in fact the basic unit of chromatin structure, the smallest dimer and trimer might be expected to contain 288 bp and 432 bp of DNA, respectively. However, after carefully considering the model proposed by Finch et al. (1977), for the core particle, there is a simple explanation, consistent with all of the available data. This model was proposed first by Dr. K. E. Van Holde in Van Holde and Weischet (1978a).

In the model of Finch et al. the 140 bp DNA makes 1.75 turns about a 70 Å diameter protein octamer. The DNA is assumed to complete one turn per 80 bp, with a pitch of 28 Å. Pardon et al. (1977) have proposed that the histone octamer core is a squat cylinder, 70 Å in diameter by 35-40 Å high. Putting these data together, one

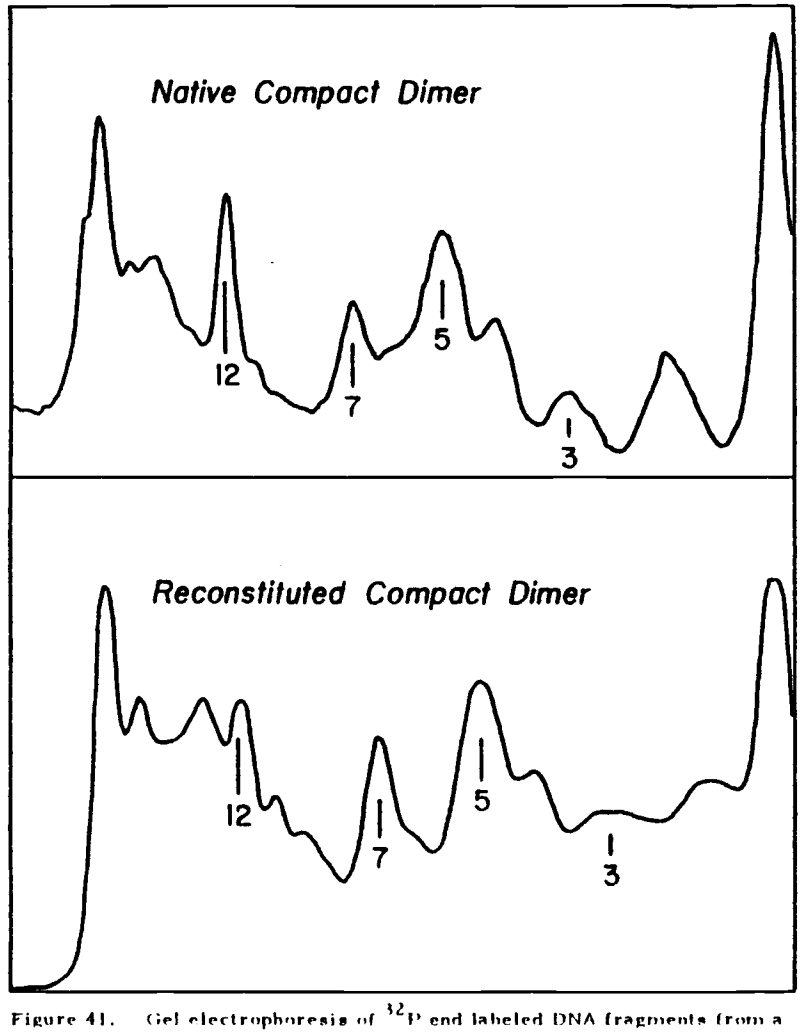

Figure 41. Gel electrophoresis of ^{32}P end labeled DNA fragments from a DNase I digestion of reconstituted compact dimers. Scans of autoradiograms are presented here. The band pattern below band 12 (~120 bp) is similar for the native and reconstituted particles but differences are observed above band 12. The two samples were run on separate gels so the peaks are not coincident.

arrives at the model of the core particle shown in Figure 42a. The dimensions of this particle immediately suggest a model for our compact oligomers. The compact (260 bp) dimer can be visualized by stacking cores about a (pseudo) dyad axis, as shown in Figure 42b. In such a particle, the stacked cores are practically in contact, and are rotated 180° with respect to one another about the superhelix axis. Such a particle, if the DNA ends are trimmed to the same extent as in the core particle, will contain just 260 bp of DNA. Furthermore, the corresponding trimer will have 380 bp of DNA. In fact, these particles are members of a series, of which each member (n) contains [120n + 20] bp of DNA coiled in a uniform superhelix of pitch 28 Å. This model requires each histone octamer to interact with less than the usual 140 bp of DNA; that is, some DNA is shared between octamers. This assumption is reasonable in light of evidence that core particles are stable with a DNA size as small as 120 bp (Noll and Kornberg, 1977; Bakayev et al., 1977) (Chapter VI) and the strong DNA/histone interactions start 20 bases in from the ends of the DNA (Weischet et al., 1978; Whitloch and Simpson, 1977) (Chapter III).

It is clear, however, that this series represents only one class (the most compact) of a number of kinds of particles in which protein cores are closely stacked. The next series can be generated by inserting DNA symmetrically about the new dyad axis. But if the

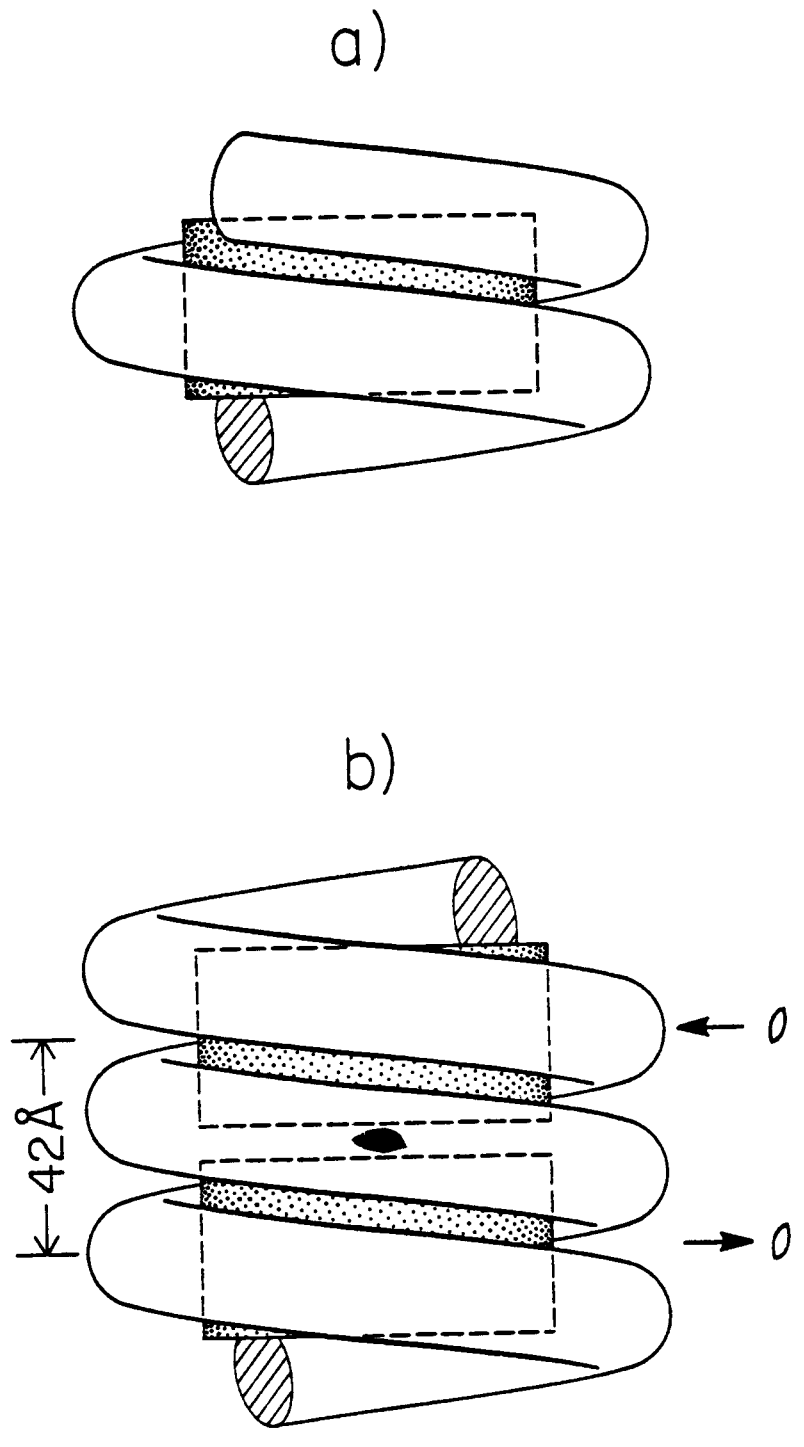

Figure 42. Model of core particle (a) and compact dimer (b) based on the data of Finch et al. (1977) and Pardon et al. (1977). The protein core is shown as a cylinder (stippled) about 70 Å in diameter and 35 Å high. The 140-bp DNA is wound about this in 1.75 turns in a 28-Å pitch helix. (b) Model of compact dimer. The 280 bp DNA are wound about two protein cores with coincident cylinder axes. The (pseudo) dyad axes (0) of two core particles are rotated 180° with respect to one another about the cylinder axis. The particle thus created has a (pseudo) dyad axis (●) perpendicular to both of the above axes. Other models with the protein cores offset with respect to each other cannot be excluded.

protein cores are to remain stacked in a parallel array, this extra DNA can be inserted only in 10 bp increments. This is required because the ends of the DNA strands in the inserted segment must match. Any other length (say 7 bp, for example) will lead to either a twisting of one core with respect to the next or a deformation of the DNA if the histone cores remain parallel. Thus, the next such dimer should have 270 bp, the next 280, etc. This requirement could explain some or most of the observed "extended ladder," or "nucleosome phasing" found in chromatin from a variety of sources (Ponder and Crawford, 1977; Lohr et al., 1977c). The explanation is consistent with the observation that the extended ladder is best resolved with chromatins (like yeast) which display a short average repeat (Lohr et al., 1977c).

A number of experimental results strongly support this model. I have isolated representatives of the first two series, i.e. (120n+20) and (130n+10). These particles should be expected to be stable to long nuclease digestion, for there is no spacer region susceptible to nuclease attack. The next series, 140n, was not obtained, possibly because it most likely would possess a region or locus susceptible to micrococcal nuclease and not be stable through the long periods of nuclease digestion used to prepare the particles.

If the (120n+20) series represents the most compact oligomers in which core stacking is possible then further shortening of the DNA

should lead to steric hindrance by overlap of protein cores. Recently Klevan and Crothers (1977) have isolated a "compact dinucleosome" with 240 bp of DNA. Evidence from electric dichroism studies indicates that these dimer particles are somewhat asymmetirc, and a "slipped disk" structure has been proposed (Klevan et al., 1978). Rotational diffusion data is not available for my particles. However, expected sedimentation coefficients for my oligomer particles have been calculated using a linear "contacting bead" model (see Van Holde, 1975, pp. 234) and correcting for the fact that the molecular weights are not exact multiples of the monomer weight. The results are given in Table 4. While such calculations are both approximate and rather insensitive, the values are certainly not in contradiction to this model. However, an asymmetric structure cannot be dismissed for these particles with the available data.

I now turn to interpretation of the melting and CD data. In Chapter III it was found that 40 bp of the DNA in the core particle thermally denatured in a melting transition 15°C below the main transition. I proposed that the ends of the DNA were involved in the premelt. If this is applied to the model of compact oligomers the percentage of the premelt would be expected to decrease, since the 40 bp of DNA at the ends would make a smaller fraction of the total. A tabulation of the data in Table 4 indicates the percent of the premelt does go down, although not to the exact amount predicted.

The increase in temperature of the main transition can also be explained in light of the finding that the denaturation of the protein in the core particle precedes the main DNA transition by a few degrees (Chapter III). If the denaturation of the protein core initiates the denaturation of the DNA then stabilization of the protein should increase the T_m of the DNA. In the model of the compact oligomers, the protein cores are in close proximity and could possibly stabilize one another, thereby raising the T_m of the DNA. The compact trimer would be further stabilized by virtue of the fact that the center histone octamer has octamers on either side.

The changes seen in the circular dichroism spectra (Figure 34) can be interpreted in a number of ways. For example, the differences between core and compact dimer and trimer could be interpreted as a greater amount of overwound DNA in the latter particles (Hanlon et al., 1972; Wolf et al., 1977). An argument similar to the one proposed to explain the thermal denaturation could be used here if it were assumed that the DNA ends were more like the solution form of DNA. The compact oligomers, with proportionally less "end" DNA would have a reduced ellipticity.

Another possibility is that the supercoils of duplex DNA are close enough in the nucleosome to interact and perturb the CD spectra (Fasman and Cowman, 1978). Such an interaction would have an even greater effect in the large oligomers due to the greater

amount of tight coiling. However, at this time there is no conclusive evidence for this or any other model.

I have attempted to predict the DNase I cutting pattern of the compact dimer and trimer by using the model of Finch et al. (1977) to predict nuclease susceptible and resistant sites. Attacks at 10, 20, 40, 50, 90, 100, 120 and 130 base pairs along the 140 base pair core DNA are assumed to occur with equal frequency while no cutting is allowed at the other sites. Since the histone octamer dictates the sites of nuclease cutting I have extended this cutting model to 260 or 270 bp for the dimer and 280 to 400 bp in the trimer. The cutting is assumed to be symmetrical from the two ends, in agreement with the postulated dyad symmetry.

By allowing only one or two DNase I cuts per particle, and by tallying up all possible combinations of products I have generated the expected frequency distributions for the fragments from the core particle, dimer, and trimer. In Figure 36 the theoretical weight average distributions for the core particle and dimer are presented with the experimental data. It is interesting that my theoretical ladder, with all its simple and perhaps naive assumptions fits the data so well. The only serious discrepancy is prediction of a major band at 130 b instead of 120 b as observed.

Similar comparisons for the trimer (not shown) indicate that I have correctly predicted the basic curve shape but not necessarily

the exact peaks of maximum intensity. With the large particles the envelope of peaks is relatively uniform, and strong conclusions are hard to make.

I have also derived a theoretical pattern for the DNA fragments containing the 5' end label. Instead of using an "all or nothing" cutting model as I did to predict the total DNase I ladder pattern, I have assigned DNase I cutting frequencies to each 10 bp site starting from the 5' end of the DNA. The results from the core particle, which were the same as obtained by Simpson and Whitlock (1976) are used to predict the cutting frequency along the 260 or 270 bp of DNA in the dimer and the 380-400 bp in the trimer (Figure 37). I have no explanation for the inexact alignment of the ethidium bromide scan and autoradiogram at 120 b in the trimer. Again, the theory bears marked likeness to the scan of the autoradiogram, lending credibility to the model. These results are of special importance, for they virtually rule out models for the compact oligomers which involve closely spaced 140 bp core particles with additional end trimming. Such particles should show prominent bands at other positions.

Analytical sedimentation and DNase I digestion studies indicate that compact dimers can be efficiently reconstituted by dialysis from high salt. Furthermore, no special modified histones or other co-factors are necessary for histones to associate into the compact oligomer conformation. Reconstitutions with longer DNA (360 bp)

and histones also form dinucleosomes, as evidenced by sedimentation coefficients of the particles (15-16 S). However, these dimers do not clearly produce the 10 base "ladder" of DNA fragments when digested with DNase I. Instead, a peak of DNA at 140-160 bases is produced upon digestion with DNase I. This would suggest that in such dimers cuts in the spacer region are favored over cuts in the core DNA, and that such spacer cuts are irregularly spaced. Nevertheless, the DNA above 140 bases is not entirely without structure; some DNase I bands are present above 140 bases although they are clearly not as well resolved as in the case of the reconstitution with 265 bp DNA (Figure 40). The fact that well resolved bands above 160 bp are not seen indicates that the DNA between most histone octomers is not an integral of 10 bases, as in the compact dinucleosome. The high fidelity of compact dimer reconstitution may be due to the restricted DNA length used. The shorter DNA may force two histone octomers into the compact mode, a conformation that would otherwise not be very probable. Steinmetz et al. (1978) have found that core histones reconstituted onto high molecular weight DNA form nucleosomes at a 140-150 bp repeat. This is a short repeat but nevertheless not the compact conformation studied in this chapter.

Weischet, Allen and Van Holde (manuscript in preparation) have evidence showing that compact oligomers obtained in chromatin digestion may be a product of nucleosome sliding in the 0.6 M NaCl used

to prepare the H1 depleted chromatin. They deplete calf thymus nuclei of histone H1 by incubating the nuclei at low pH (Cole et al., 1978). This procedure effectively removes all the H1 without removing the core histones. Subsequent micrococcal nuclease digestion of chromatin made from these nuclei does not yield DNA fragments the size of compact oligomer DNA. The DNA distribution instead, is very similar to that of native chromatin. However, incubation of the depleted chromatin overnight in 0.5 M NaCl prior to micrococcal nuclease digestion (at low ionic strength) did result in the production of DNA bands the size of compact oligomer DNA! If Steinmetz et al. (1978) do not find nucleosomes in the compact mode when reconstituting with long DNA, why are they found in a small fraction of the salt treated chromatin? One possibility is that some torsional constraint in the DNA of chromatin may force nucleosomes into the compact mode. The salt treatment allows the core histones to slide on, but not to be released from the DNA. Compact oligomers therefore will not form on completely relaxed DNA, as in the case of reconstitutions, unless the DNA is small enough to force the core histones together.

Another possible explanation of the lack of compact oligomers in the reconstituted chromatin is that a relatively long incubation in 0.6 M NaCl may be necessary for compact oligomer formation. The protocol for H1 depletion requires incubation in 0.65 M NaCl for

two days while chromatin is in 0.6-0.7 M NaCl only a few hours during the reconstitution. Thus, compact oligomers may just not have enough time to form during the reconstitution. It may be possible to form compact oligomers by reconstitution if the reconstitute is annealed in 0.6 M NaCl for an extended time.

Although the compact oligomers studied in this chapter are probably the product of nucleosome sliding, the possibility still exists for at least their transient existence in vivo. There is some recent evidence that nucleosomes present on newly replicated chromatin have shorter DNA spacers than bulk chromatin (Seale, 1978). Martin et al. (1978) also have evidence for a very short nucleosome repeat in a subset of calf thymus chromatin. Nevertheless, it remains to be seen if these examples represent the compact structures investigated in this chapter.

Even if the most compact structures are not observed in chromatin in vivo, the symmetry arguments for quantization of inserted DNA are still valid. In relatively compact chromatin, parallel stacking of cores may be important, and this will impose restrictions on DNA insertion lengths.

VI. RECONSTITUTION WITH DNA MOLECULES SMALLER THAN 140 BASE PAIRS

Introduction

As was shown in Chapter IV, nucleosomes can be reconstituted from isolated DNA and histones, reproducing most or all of the structural features found *in vivo*. Using physical and enzymatic probes as criteria for reconstitution, I have shown that nucleosomal core particles reconstituted from 144 bp DNA and histones are very similar, if not identical to the native particle. In this chapter I describe reconstitutions using core histones together with DNA molecules shorter than that usually associated with the core particle. The resultant products have been characterized by a number of techniques.

The core histones in eukaryotic chromatin protect 140-160 bp DNA fragments from hydrolysis by micrococcal nuclease but there is evidence that DNA-histone interactions responsible for this protection are not uniform along the DNA (Simpson and Whitlock, 1976; Mirzabekov *et al.*, 1978; and Weischet *et al.*, 1978) (Chapter III). Pancreatic DNase I digestion of core particles containing DNA end labeled with ^{32}P indicate that some positions along the DNA are highly protected while others are much more susceptible to hydrolysis (Simpson and Whitlock, 1976). Mirzabekov *et al.* (1978) have mapped the sites of histone-DNA interaction by protein-DNA

crosslinking and found that the 5' end of the DNA in the core particle is devoid of major crosslinks. Thermal denaturation studies of core particles indicate about 40 bp out of the 144 bp DNA are less resistant to thermal denaturation (Chapter III, Weischet et al., 1978). The results presented here further define the positions of histone-DNA interaction and provide a method of studying conformational changes in nucleosomes and association reactions between such particles.

While nucleosomes can be reconstituted onto DNA from many sources, eukaryotic and procaryotic alike, the possibility still exists for some sequence specificity of histone binding. Ponder and Crawford (1977) have observed a partial phasing of nucleosomes on the polyoma virus chromosome that could be the result of sequence recognition by histones. Reconstitution studies with specific sequences are important to understanding the mode of DNA histone interaction but care must be taken in generalizing from reconstitutions with sequence specific DNA's to reconstitutions with random sequence DNA. Therefore I have sought to study DNA length dependence on the reconstitution process using random sequence DNA. A micrococcal nuclease "limit digest" of nuclei has provided the random sequence DNA molecules necessary for these experiments.

The DNA in chromatin or whole nuclei can be hydrolyzed by micrococcal nuclease until about 50% of the DNA is acid soluble (Clark and Felsenfeld, 1971). At this point a digestion plateau is

reached since additional enzyme or digestion time does not increase the amount of acid soluble DNA. The DNA at this limit is found to be a series of short double stranded fragments ranging from 160 bp to 30 bp (Axel, 1975). Although micrococcal nuclease has a slight preference for A-T rich DNA (Roberts et al., 1962) no sequences in chromatin are preferentially cleaved by micrococcal nuclease (Lacy and Axel, 1975). Fractionation of this limit digest into different DNA sizes should therefore provide DNA molecules that resemble the entire genome in sequence complexity.

Results

The DNA molecules used in these reconstitution studies were obtained by electrophoretic separation of DNA fragments produced by extended digestion of nuclei with micrococcal nuclease. While not completely homogeneous in length, DNA fractions were obtained in large enough quantities to physically characterize reconstituted products. Figure 43 shows the size distribution of a number of these DNA fractions as demonstrated by polyacrylamide gel electrophoresis. DNA molecules of roughly 145, 120, 110, 100, 90 and 70 bp were use used in most reconstitution studies. The half width of the fractions at peak half height varied between 5 bp and 14 bp. Reconstitution was accomplished by simply mixing the appropriate DNA with salt extracted core histones in 2 M NaCl and slowly lowering the ionic

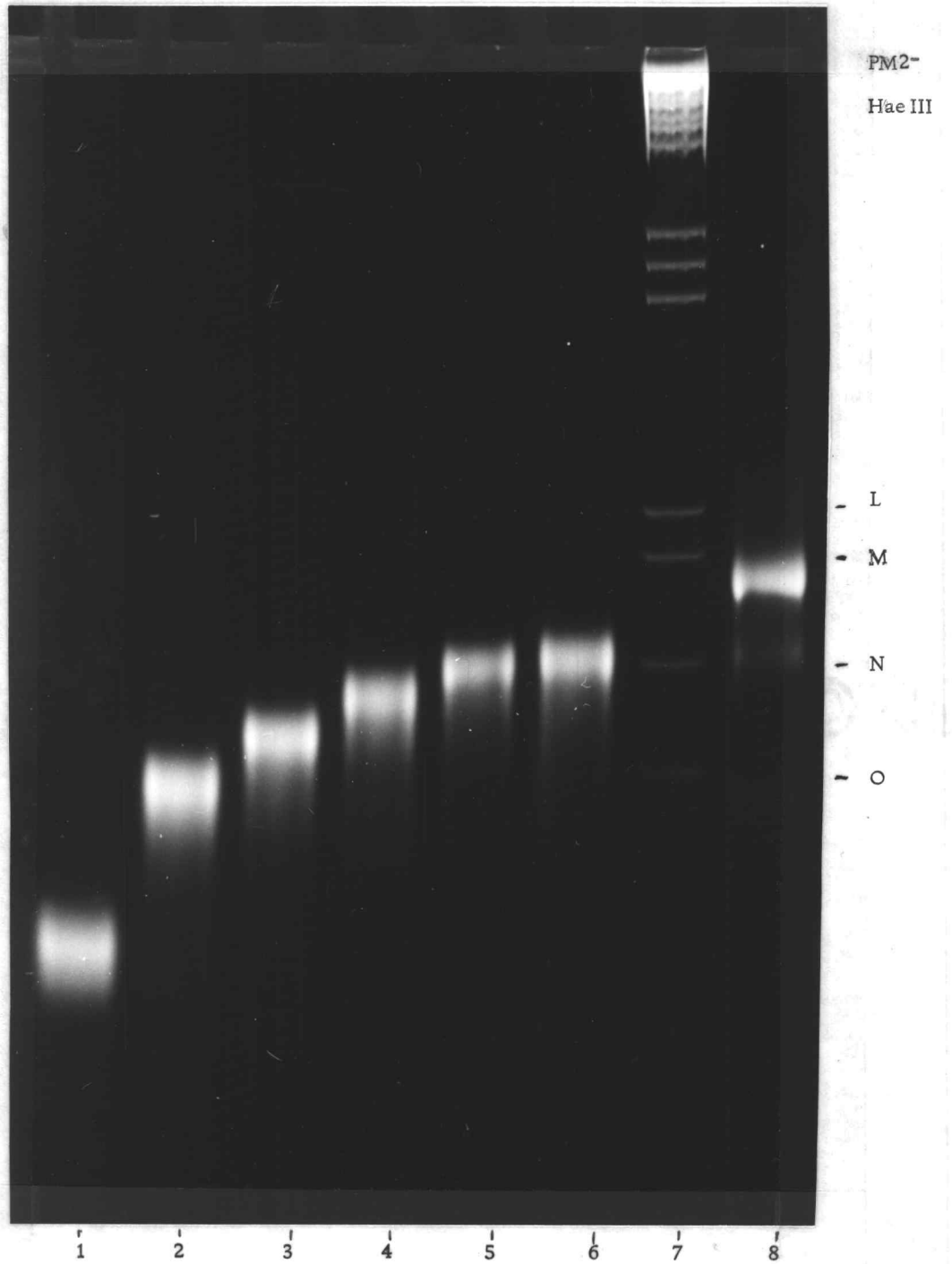

Figure 43. Low molecular weight DNA fractionated by preparative electrophoresis. Selected fractions from an electrophoretic fractionation of the micrococcal nuclease "limit digest" were electrophoresed on a 6% polyacrylamide slab gel. The channels from left to right are: 1, 69 bp DNA; 2, 94 bp; 3, 105 bp; 4, 114 bp; 5, 120 bp; 6, 123 bp; 7, PM2-Hae III fragments; 8, 144 bp DNA from core particles.

strength by gradient dialysis. The results of these reconstitutions are discussed below.

Reconstitutions with DNA Fragments Shorter than 145 Base Pairs Form Stable Complexes Very Similar to Core Particles

In the first set of experiments, histones and DNA were mixed at a molar ratio of one histone octamer (two each of the four core histones H2A, H2B, H3, and H4) per DNA fragment. As Table 5 indicates, each DNA size down to 100 bp could combine with core histones to form particles with a 1:1, mole histone octamer:mole DNA ratio. In the case of 90 bp and 65 bp DNA, complete precipitation of the complex occurred and no attempt was made to characterize the product. Nevertheless, soluble, well characterized particles could be obtained with 70 bp DNA if the DNA:histone ratio was increased (see below).

Particles reconstituted from ~120 bp DNA resemble very closely the 144 bp reconstitutes. Both sediment at 10-11 S in the analytical ultracentrifuge and the measured protein/DNA ratio indicates both have one molecule of DNA associated with each histone octamer (see Table 5). Identical values for the main phase of the thermal denaturation (Figure 44) also attest to the fidelity of reconstitution. A point of interest is the smaller premelt found for the 123 bp reconstitute. This is consistent with the suggestion that the premelt in the core

Table 5. Reconstitutions with one mole DNA/mole histone octomer

DNA size (bp)[a]	Protein/DNA (w/w)		$S_{20,w}$		
	observed[b]	predicted[c]	2.5×10^{-4} M[d]	0.01 M[e]	0.15 M[f]
144± 5	1.2±0.1	1.15	10.0, 10.0	10.9±0.1	11.1
125± 9	1.40, 1.41	1.38	9.0	10.4±0.3	11.3±0.8
111± 6	1.38, 1.42	1.51	8.7	10,0	14.0
102±14	1.6±0.1	1.66	9.0, 9.5	16.5	➢ 20
90± 6	-	1.85	aggregation		
65± 5	-	2.56	aggregation		

a) ± indicates half width of DNA at peak half height.

b) ± indicates standard deviation of ≥ 3 samples. This also applies to $S_{20,w}$ values.

c) Assuming 108,000 Daltons and 650 Daltons/bp for the molecular weight of the histone octomer and DNA respectively.

d) 2.5×10^{-4} M EDTA, pH 7.2.

e) 0.01 M Tris-Cac., 7×10^{-4} M EDTA, pH 7.2.

f) 0.15 M NaCl, 0.01 M Tris-Cac., 7×10^{-4} M EDTA, pH 7.2.

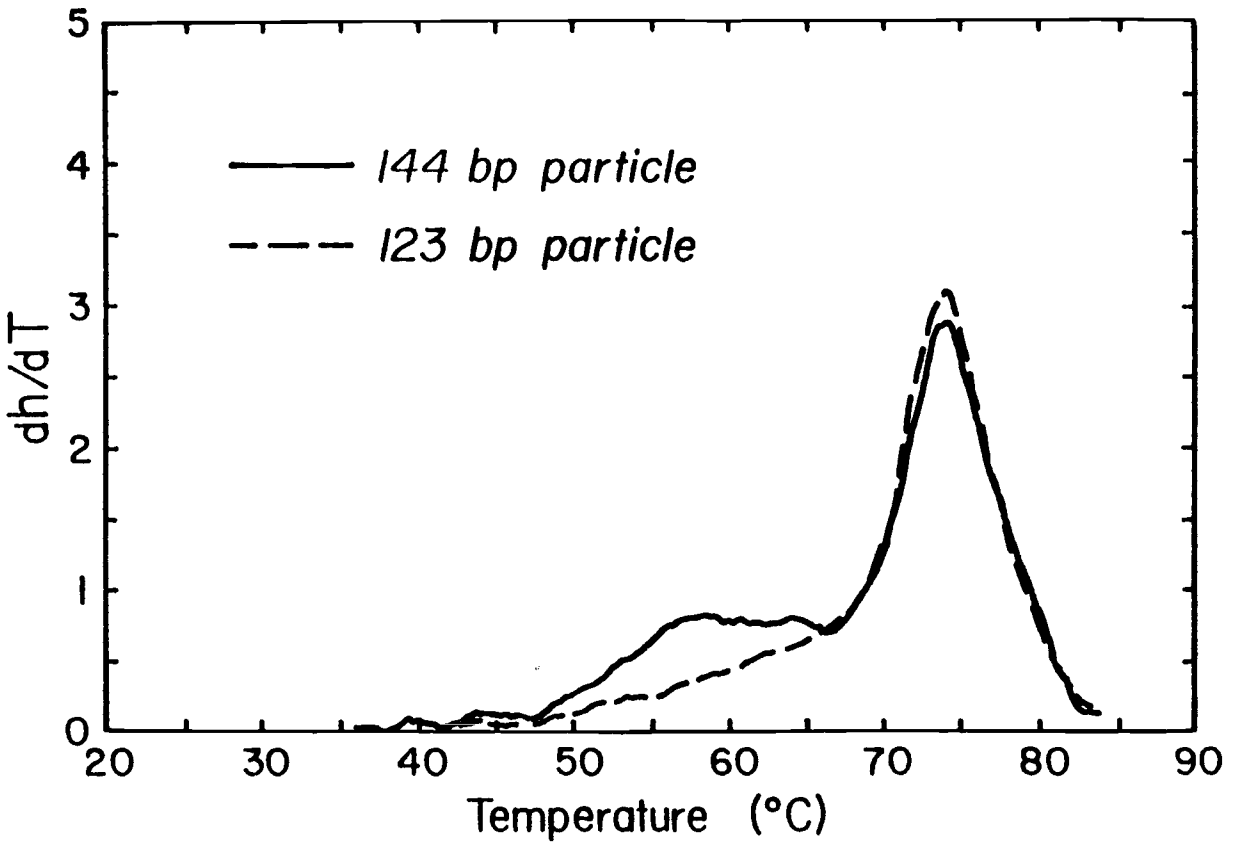

Figure 44. Thermal denaturation of reconstituted particles containing 144 bp and 123 bp DNA.

particle involves approximately 40 bp at the ends of the DNA (see Chapter III). It seems reasonable that if 20 bp of this "end" DNA were removed, as in the 120 bp reconstitution, the premelt might be smaller without necessarily effecting the main melting transition.

Particles with Less than 120 bp of DNA Undergo Ionic Strength Dependent Aggregation

As the DNA length in the reconstitution is decreased, keeping the molar ratio of DNA fragment to histone octamer at unity, particles are obtained which undergo a reversible aggregation. This phenomena depends on the ionic environment as well as the DNA length. Particles containing 125 bp DNA undergo no association reactions, even at 0.15 M NaCl, while the 102 bp particle aggregates at this ionic strength to form very large complexes which precipitate out of solution. Particles with intermediate lengths of DNA (110 bp) associate to form smaller complexes with an $S_{20,w}$ = 15-18 S. As the ionic strength is decreased the average sedimentation coefficient decreases to a minimum of 9-10 S at very low ionic strengths. The results of these experiments are plotted in Figure 45, as G(S) vs. $S_{20,w}$ (Van Holde and Weischet (1978b). These results confirm the results of Noll and Kornberg (1977) and Bakayev et al. (1977) who found that chromatin fragments containing less than 120 bp of DNA precipitate from solution during a micrococcal nuclease digest of

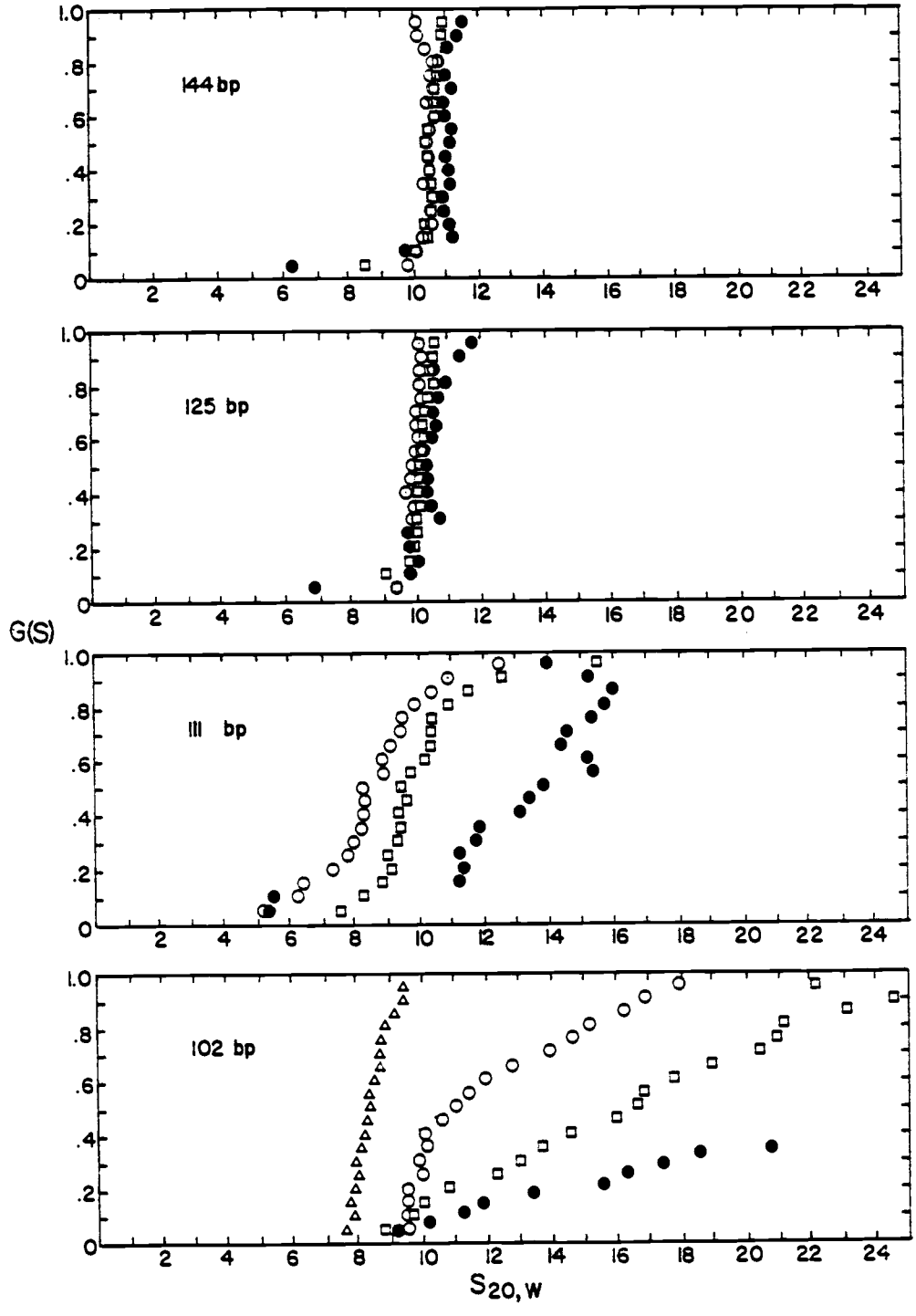

Figure 45. G(S) distribution for particles with various lengths of DNA. The DNA sizes used are indicated in each portion of the figure. The buffer solutions employed are as follows: (○) 5 mM Tris-cacodylate, 0.35 mM EDTA; (□) 10 mM Tris-cacodylate, 0.7 mM EDTA; (●) 0.15 M NaCl, 10 mM Tris-cacodylate, 0.7 mM EDTA; (△) 0.25 mM EDTA. All are at pH 7.2.

chromatin. It should be emphasized that the histone stoichiometry in all the reconstitution products tested appear to be identical to that of the native core particle; the four core histones present in equimolar amounts.

The Histone Core will Accept More than One DNA Molecule

I observed that only one 144 bp DNA molecule would reconstitute with the core histones, even in the presence of excess DNA (Chapter IV). Similar experiments with shorter DNA molecules did not give this result but provided evidence that the histone octamer could bind more than one DNA fragment, if the fragment is appreciably shorter than 144 bp.

In these experiments, core histones and DNA were mixed together at a molar ratio of 1:2, histone octamer:DNA fragment. Unlike the case with 144 bp DNA, where half the DNA in such reconstitutes would remain free in solution, sedimenting in the analytical ultracentrifuge with a sedimentation coefficient identical to that of free DNA, less than half of the shorter DNA would sediment as free DNA. In the case of 65 bp DNA, no free DNA boundary existed; each histone core was binding exactly two DNA molecules.

The particles made at these high DNA:protein input ratios were fractionated from any free DNA by centrifugation through sucrose gradients and studied in the analytical ultracentrifuge. A summary

of these experiments is presented in Table 6. Unlike the particles with one DNA molecule per histone core, these particles show no signs of aggregation at the higher ionic strengths. Judging from the observed protein:DNA ratio, the 105 bp particles did not all contain two DNA molecules per octamer, whereas the 65 bp and possibly the 94 bp particles each contain two pieces of DNA. The 65 bp particle with the same protein:DNA ratio as the native core particle contains a total of 130 bp of DNA, in two pieces instead of one. This particle co-sedimented in the ultracentrifuge with the 144 bp core particle in 10 mM Tris-cacodylate and 0.15 M NaCl. Surprisingly, at extremely low ionic strength the 65 bp particle sediments very differently. Whereas particles containing one piece of DNA (100-145 bp) sediment as a homogeneous boundary in the analytical ultracentrifuge with ($S_{20,w}$ = 8 - 10 S) in 0.25 mM EDTA the 65 bp particle sediments at only 7.5 S and exhibits a more heterogeneous boundary. G(S) vs. S plots of 144 bp and 65 bp particles illustrating this change are presented in Figure 46. Such a change is consistent with either a dissociation reaction or drastic conformational change.

The DNA does not Bind Randomly on the Histone Core

Assuming the 144 base pairs of DNA in the core particle wrap symmetrically around the histone octamer, the placement of the smaller DNA molecules can be ascertained using the method of

Table 6. Reconstitutions with two moles DNA/mole histone octomer

D	DNA size (bp) [a]	Protein/DNA (w/w)		$S_{20,w}$ at:		
		observed [b]	predicted [c]	$2.5X10^{-4}$ M [d]	0.01 M [e]	0.15 M [f]
	105 ±7	1.15, 1.20	0.76	7.5	9.0, 9.0	11.0, 11.4
	94 ±6	0.83, 1.00	0.83	6.3	10.0, 10.5	10.5, 11.3
	65± 5	1.20, 1.40	1.28	7.5±0.8	10.3, 10.6	11.0

a) ± indicates half width of DNA at peak half height

b) ± indicates standard deviation of ≥ 3 samples. This also applies to $S_{20,w}$ values

c) Assuming 108,000 Daltons and 650 Daltons/bp for the molecular weight of the histone octomer and DNA respectively

d) 2.5×10^{-4} M EDTA, pH 7.2

e) 0.01 M Tris-Cac., 7×10^{-4} M EDTA, pH 7.2

f) 0.15 M NaCl, 0.01 M Tris-Cac., 7×10^{-4} M EDTA, pH 7.2

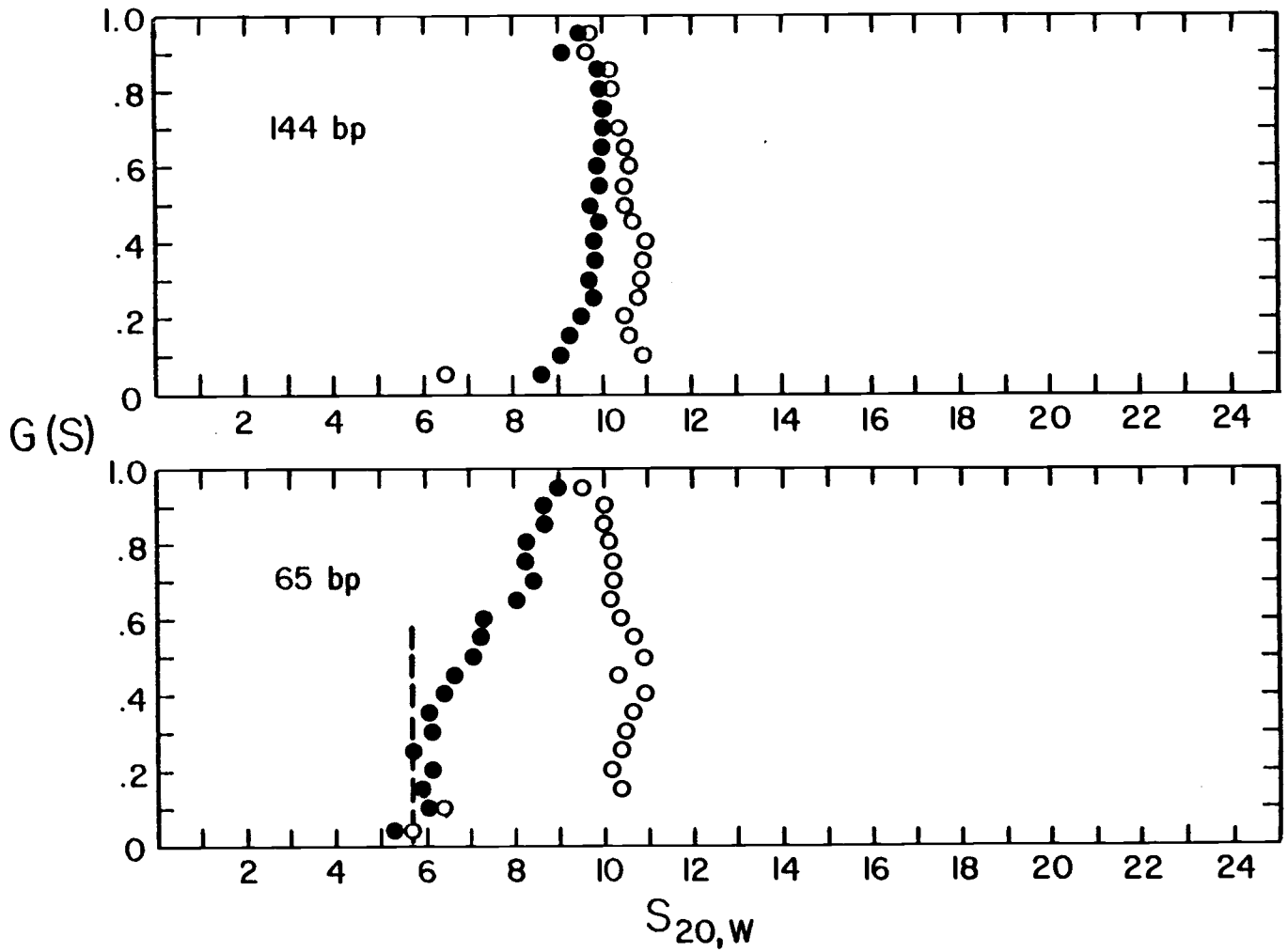

Figure 46. G(S) distribution for core particles reconstituted with 144 bp and 65 bp DNA. Solution conditions are indicated as follows: (O) 10 mM Tris-cacodylate, 0.7 mM EDTA; (●) 0.25 mM EDTA. The dashed line indicates the predicted sedimentation coefficient of the hemisome, referred to in the Discussion.

Simpson and Whitlock (1976). The DNA is labeled on the 5' end with ^{32}P, reconstituted with histones, then digested with pancreatic DNase I and the resulting DNA fragments run on denaturing polyacrylamide gels and autoradiographed. Simpson and Whitlock (1976) showed that certain sites on the nucleosome were very resistant to DNase I digestion while other sites were easily cleaved. Using this same method of analysis on reconstituted particles we can accurately define the position of the DNA with respect to these protected and exposed sites.

If the DNA of reconstituted particles goes on the histone core randomly, the resulting autoradiogram of the DNase I digestion should be a smear. If, on the other hand, the placement of DNA is not random but phased with respect to the internal protection sites, a series of bands should appear on the autoradiogram. The stringency of this test should be emphasized. Suppose, for example, there were an equal probability of phasing the DNA anywhere ± 5 bp from the required frame. This would obliterate the ^{32}P banding pattern.

The results from such experiments, presented in Figure 47 indicate the placement of the DNA is not random. As observed in Chapter IV, the 144 bp reconstitution gives a banding pattern very similar to that observed by Simpson and Whitlock (1976), with sites at bands 3, 6, 8, and 11 highly protected. The virtual non-existence of a peak at band 8 (about 80 bases) and at band 6 (about 60 bases)

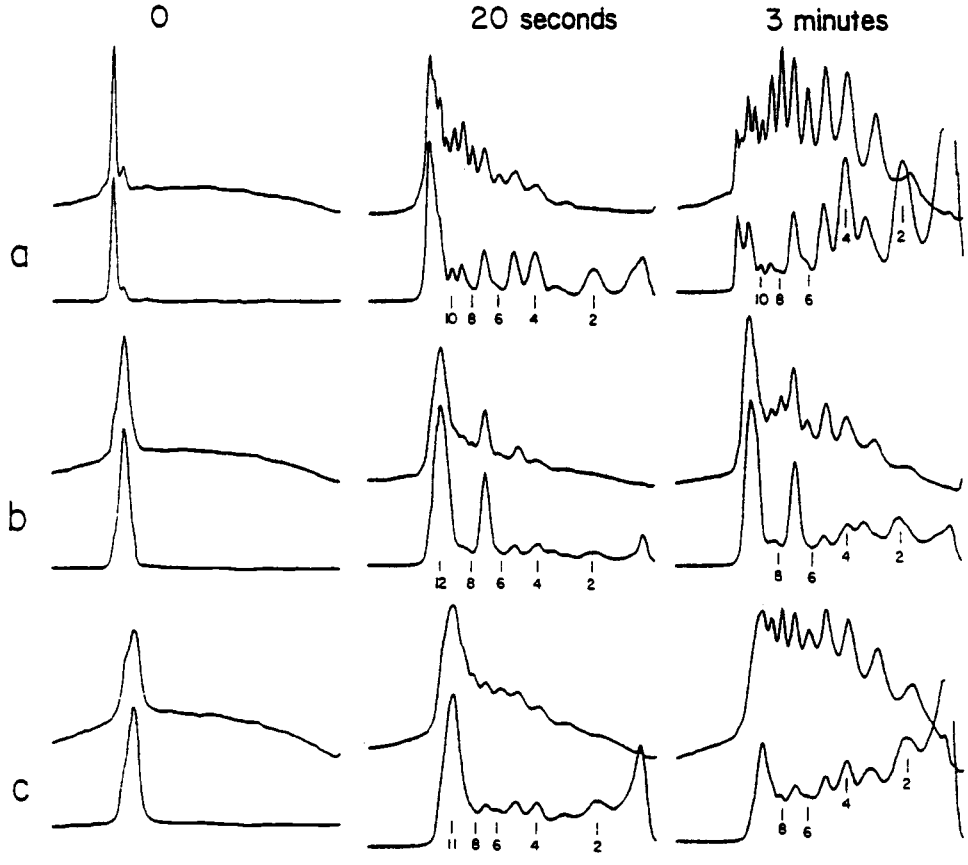

Figure 47. Gel electrophoresis of DNA fragments from a DNase I digestion of particles containing various lengths of DNA. Particles were reconstituted with 144 bp DNA (a), 125 bp DNA (b), and 111 bp DNA (c). Each reconstitute, containing ^{32}P end labeled DNA, was digested with DNase I for 20 seconds and 3 minutes. The DNA was isolated and electrophoresed under denaturing conditions. The ethidium bromide staining pattern is shown as the top scan in each set while the autoradiogram scan is directly underneath.

attests to the fidelity of reconstitution. The 125 bp and 111 bp reconstitutes also show a clearly discernible series of bands on the autoradiogram (Figure 47b, c). In the 125 bp reconstitution (Figure 47b), the peaks in the autoradiogram at bands 8 and 6 are again almost at background, indicating a remarkable degree of fidelity in the DNA placement. In all the DNase I digestion experiments with ^{32}P labeled DNA, the bands below 4 in the autoradiogram do not coincide with their respective ethidium bromide bands. In particular, band 3 in the autoradiogram is consistently larger than band 3 of the ethidium bromide scan. The ethidium bromide scan represents the average of all fragments roughly 30 bases long, while the autoradiogram band represents a specific fragment from the 5' end of the DNA. I do not have an explanation for this discrepancy but it could possibly reflect the specific binding of histones at a site roughly 35 bases from the 5' end of the DNA.

The existence of such well defined bands in the autoradiogram of the 125 bp and 111 bp DNase I digests, remarkably sharp considering the broad size distribution of the DNA, attests to a striking fidelity in DNA alignment of the histone core. Assuming that most of the DNA is not randomly reconstituted onto the histone core, I have used the autoradiogram profile to determine where the 125 bp and 111 bp DNA is placed on the histone core. In the case of the 125 bp particle, is it symmetrically or asymmetrically placed with respect to the 144 bp

DNA placement? The symmetric mode would result if the 125 bp DNA were displaced about 10 bp in on each end, whereas the asymmetric mode would result from placement of one DNA end at the site of the 144 bp end and the other end recessed by about 25 bp. These two different modes are illustrated in Figure 48 using a model for the nucleosome proposed by Finch et al. (1977). Either of these arrangements should give sharp DNase 1 patterns with end labeled DNA. However, the two different modes should give clearly distinguishable banding patterns upon digestion with DNase I, assuming the bands are defined by histone binding.

The banding pattern for the symmetric mode should be identical to the pattern with 144 bp DNA but with each ^{32}P labeled band shortened by about 10 bases. The highly protected site at band 8 and band 6 from the core should now be found at band 7 and band 5 respectively in the symmetric particle. The banding pattern predicted for the asymmetric mode is more complicated because the ends of the DNA are not in identical histone environments. Cutting from one 5' end would result in a 144 base pattern down-shifted by about 25 bases while cutting from the other 5' end would be identical to the 144 base pair pattern. Model cutting patterns for these two modes are represented in Figure 49 for the 125 bp particle along with a densitometer tracing of the autoradiogram. While the actual autoradiogram does not correspond exactly to either of the two simple models presented

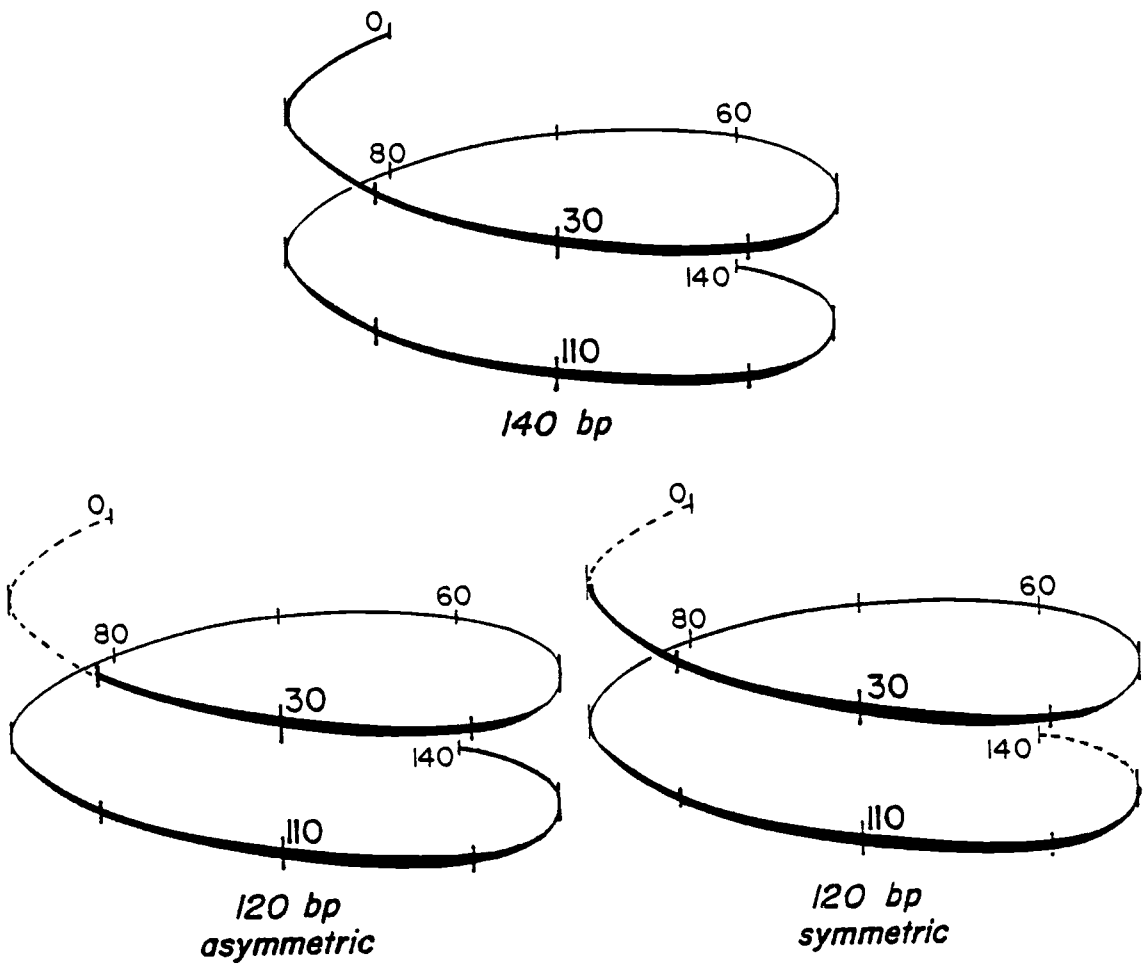

Figure 48. Symmetric vs. asymmetric model for DNA placement on the histone core. The solid helical lines represent the paths of duplex DNA. The model is taken from Finch et al. (1977) where the core particle is considered to have just 140 bp of DNA and equally spaced DNase I cutting sites every ten base pairs.

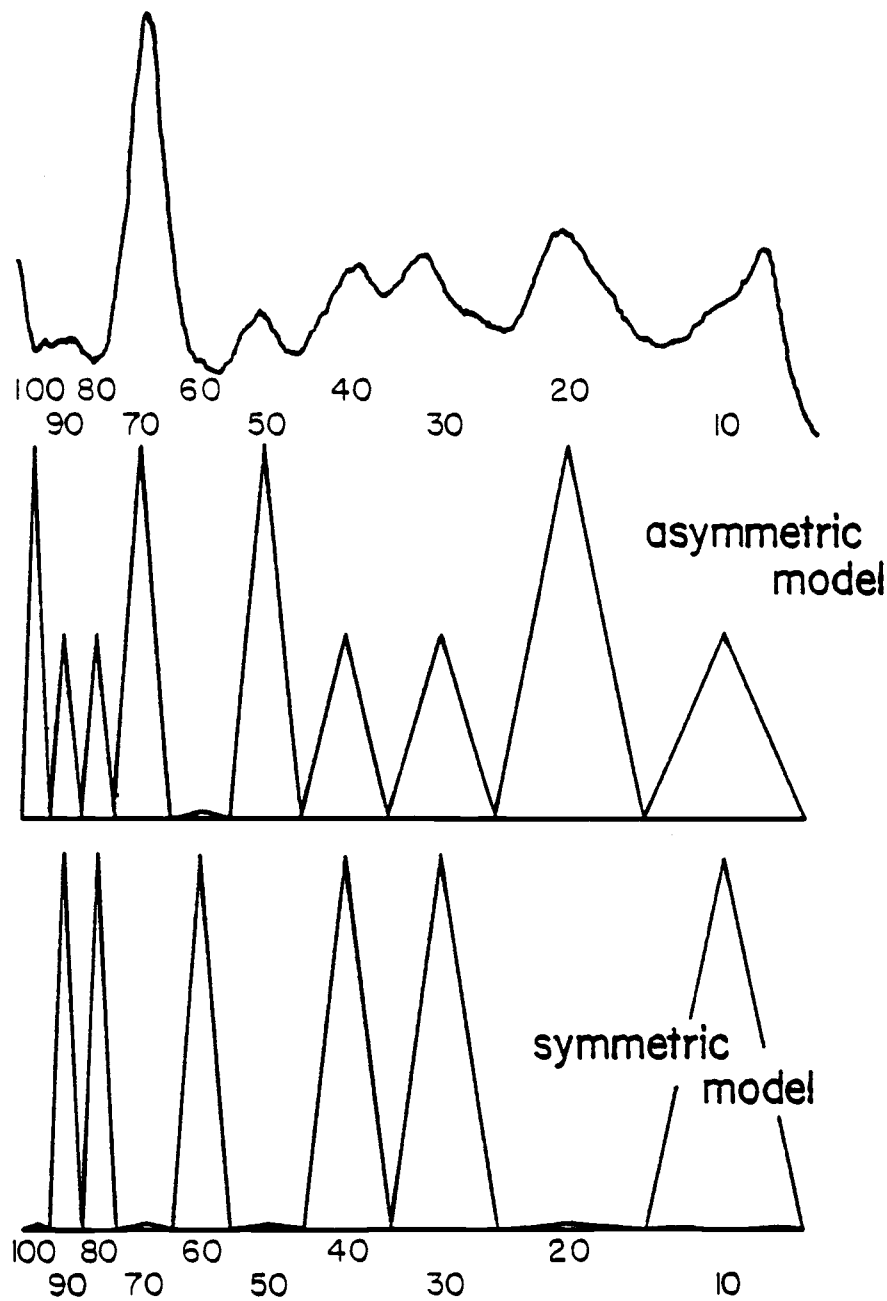

Figure 49. Theoretical DNase I cutting pattern for an asymmetric and symmetric placement of the 125 bp DNA on the histone core. A region of the 125 bp autoradiogram is reproduced from Figure 47 along with two possible models for the DNA placement. The models are calculated assuming cuts cannot occur at sites roughly 30, 50, 80, and 110 bases from the 5' end along the core particle but can occur with equal probability at other sites. The peak heights in the two models represent the number average cutting frequency.

it very strongly supports the asymmetric mode of DNA placement. Strong peaks in the autoradiogram at bands 5 and 7 and weak ones at band 6 and 8 are predicted by the asymmetric model but virtually rule out the symmetric model. Band 7 is consistently higher than would be predicted in all the recobstitutions. I have no explanation for this at this time.

The placement of 111 bp DNA cannot be easily modeled as either symmetric or asymmetric since the DNA is approximately three DNase I bands (30 bp) shorter than the core particles. Nevertheless, the similarity in the autoradiogram of the 111 bp pattern and the core particle pattern suggests an asymmetric model. The reasons for this similarity will be discussed below.

Discussion

It is now clear that stable nucleosomal particles can exist with less than the usual quantity of DNA. The 125 bp reconstituted particle with 20 bp less DNA than the core particle is still very stable to thermal denaturation and has a sedimentation coefficient nearly identical to that of the core particle. However, as the length of the DNA is decreased further, particles still form with the usual histone stoichiometry but they tend to associate at higher ionic strengths. This aggregation phenomenon collaborates the observations of Noll and Kornberg (1977) and Bakayev et al. (1977) who find that chromatin

digested to DNA fragments shorter than 120 bp tends to precipitate during a micrococcal nuclease digestion.

The fidelity of reconstitution with ^{32}P end labeled DNA is surprising. The DNA size classes used were far from homogeneous, with a half width at half height of 5-14 bp, yet particles made from these fragments gave a sharp banding pattern upon digestion with DNase I. I have calculated that a variability of only ± 5 bp in the distance between the labeled DNA end and the DNase I cutting site should obliterate the banding pattern. These results seem highly improbable unless one end of the DNA is always phased with respect to the DNase I cutting sites. The results indicating an asymmetric mode for reconstitution of the smaller DNA fragments makes this possibility a reasonable one. During the reconstitution with DNA molecules shorter than the usual 144 bp, one end of the DNA will always bind at the 144-0 bp site while the other will be recessed. This would assure that one ^{32}P labeled DNA end would always be phased with respect to the DNase I cutting site, no matter how heterogeneous the DNA population was, and give a definite banding pattern upon digestion with DNase I. Of course, the other end of the DNA will be randomly placed and the high background in the autoradiogram of the 111 bp reconstitution can be accounted for by this.

The asymmetric mode of DNA placement also seems reasonable from an energetic standpoint. If specific sites on the histone core

bind DNA more strongly than others, the most stable state is that in which all the strong binding interactions are filled. In the case of reconstitutions with 144 bp DNA, the most favorable state is identical to the native particle, with both sites (0 bp and 144 bp) complexed with DNA. With shorter DNA however, it is not possible to fulfill all the binding requirements. One alternative, of course, is to bind more than one fragment of DNA to the histone core. This does seem to occur if excess DNA is present during the reconstitution. If no excess DNA is present the DNA molecule will fit onto the histone core in such a way as to satisfy as many strong protein-DNA interactions as possible. If we assume the sites of strong interaction correspond to the sites of DNase I protection, these strong interactions occur at roughly 140 b, 110 b, 80 b, 60 b, 30 b, and 0 bases from each 5' end in a symmetric fashion (Simpson and Whitlock, 1976). Now the proposed symmetric model for the binding of 125 bp DNA, with each DNA end set in 10 bp with respect to the 144 bp binding site, would leave two strong binding sites unfilled, at 0 and 144 bases. The asymmetric model, on the other hand, would leave only one strong binding site unfilled. Hence, from such an argument the asymmetric binding mode would be predicted for the 125 bp particle.

If the strong interactions determine the placement of small DNA molecules on the histone core, the placement of two 65 bp DNA molecules should be quite specific. One end of each DNA will bind

at the site of the 144 bp DNA binding while the other end will sit at the proposed dyad axis of the core particle. This particle should be nearly identical to the 144 bp particle under most conditions and indeed the 65 bp reconstitute does act similarly to the 144 bp particle, as judged by analytical sedimentation, under most conditions. Differences are seen however between the 144 bp and the 65 bp particles at extremely low ionic strength (Figure 46). This is the same ionic strength that Gordon et al. (1978) have observed a small reproducible change in the sedimentation coefficient of core particles. Oudet et al. (1978b) and Poon and Seligy (1978) have both observed a partial unfolding of nucleosomes at low ionic strength. Oudet et al. (1978b) find that the 20 or so nucleosomes on the SV-40 chromosome unfold into about 40 slightly smaller particles. Poon and Seligy (1978) have found a similar phenomena in isolated mono and dinucleosomes.

If the nucleosome does dissociate into half-nucleosomes or "hemisomes" at low ionic strength the 65 bp reconstitute should be a good candidate for observing this conformational change. The continuous strand of DNA across the two halves of the core particle is broken in the 65 bp particle, allowing the conformational change observed with the 65 bp particle at low ionic strength. The predicted ratio of sedimentation coefficients for the hemisome and core particle have been calculated by Dr. K. E. Van Holde, assuming the shape of both particles to oblate ellipsoids of 27.5 Å x 110 Å and 55 Å x 110 Å

respectively. Assuming no change in partial specific volume, the ratio of S(hemisome)/S(coreparticle) is 0.57. Since, at low ionic strength, the sedimentation coefficient for the core particle is about 10.0 S, the predicted sedimentation coefficient for the hemisome is 5.7 S. This value is close to the minimum sedimentation coefficient for the 65 bp reconstitution (Figure 45) and is significantly larger than the sedimentation coefficient of free 65 bp DNA ($S_{20,w} \simeq 4$). I do not know why the G(S) profile for the 65 bp reconstitution is a broad boundary between 5.7 S and 9 S but such a boundary would be predicted if the 65 bp particle is in rapid monomer-dimer equilibrium at very low ionic strength. The minimum sedimentation coefficient at 5.7 S would represent the monomer (hemisome) while the maximum sedimentation coefficient would represent that of the dimer (core particle). Unfortunately I have no evidence that the 65 base particle dissociates into homologous pieces, the possibility still exists that the dissociation may produce heterologous particles, i.e., one particle containing only H3 and H4 and one particle containing only H2A and H2B.

Reconstitutions with DNA molecules shorter than 144 bp help illustrate the non-random nature of the protein-DNA interactions within the nucleosome. The placement of these short DNA molecules seems to be directed by these interactions. The reconstituted products illustrate a number of interesting association reactions and

conformational changes, the significance of which are not known at the present. However these phenomena might be used as indicators in the study of histone modification or non-histone protein influence on nucleosome structure.

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APPENDIX

MATERIALS AND METHODS

Isolation of Chicken Erythrocyte Nuclei

Blood was obtained from White Leghorn chickens by heart puncture or bleeding through the neck vein in the presence of 1/10 volume 6% sodium citrate. The vein exsanguination procedure required anesthetizing the birds with diethyl ether, removing skin and feathers from the neck, then cutting the two prominent veins over a beaker. The heart puncture procedure required turning an unanesthetized bird on its back with its body elevated and then forcing an 18 gauge 1.5-2.0 inch needle into the heart from above the breast bone. All birds were decapitated after exsanguination. 50-80 ml. of blood could be obtained from each bird. Sodium citrate was used as an anticoagulant in favor of heparin since heparin has been found to strip histones from chromatin (Hildebrand et al., 1977).

All manipulations after the exsanguination were done on ice. The blood was centrifuged at 2000 x g (3000 rpm) in a GSA (Sorval) rotor for ten minutes to pellet the blood cells. The pellet was washed three to four times with three volumes of 0.15 M NaCl, 0.015 M Sodium citrate (SSC), with centrifugation at 2000 g between each wash. The final centrifugation was done in a swinging bucket rotor to facilitate the removal of the buffy coat (the layer of white cells that form a dense layer over the red blood cells after centrifugation). Packed red blood cells (erythrocytes) were frozen at -70°C in 2-6 ml. portions. The erythrocytes could be stored in this form for up to one year, although most experiments were carried out with samples days to weeks old.

Erythrocyte nuclei were prepared following a procedure of Shaw et al. (1976) with a few modifications. One to two volumes of 0.15 M NaCl, 0.015 M Na cacodylate, pH 7.2, 0.1 mM phenylmethane sulfonyl fluoride (PMSF) (nuclei buffer) was added to a tube of frozen erythrocytes which were thawed at 37°C with vigorous mixing through a pasteur pipet. Although avian erythrocytes have few if any nuclear proteases the serine protease inhibitor, PMSF was present during all the nuclei and chromatin preparations. The PMSF stock (0.1 M PMSF in isopropanol) was added to the nuclei buffer immediately before use with mixing because the PMSF has a half life of only a few hours in aqueous solution (Gold, 1967).

The thawed erythrocytes, which were lysed by this freeze-thaw procedure, were diluted with 6-8 volumes of cold nuclei buffer and centrifuged at 2000 g (4000 rpm) in a SS 34 (Sorval) rotor for 10 minutes. The pellet was washed, once with 10 volumes of nuclei buffer, once with 10 volumes of nuclei buffer containing 0.25% NP-40 (nonionic detergent) and once or twice more without the NP-40. Nuclei counts were made with a Neubauer hemacytometer. The centrifugation steps after the NP-40 step were at 750 g (2500 rpm) and care was taken in resuspending the nuclei in order to prevent premature nuclear lysis in the sucrose digestion buffer.

Preparation of Chromatin Depleted of Histones H1 and H5

Freshly prepared nuclei were lysed in 10 mM Tris-cacodylate, pH 7.2, 0.7 mM Na_2 EDTA, 0.1 mM PMSF, (lysis buffer) by pipeting the nuclei into ice cold lysis buffer. Approximately 50 ml of lysis buffer was used with the nuclei from each ml of packed erythrocytes. The osmotically lysed nuclei, in lysis buffer, were made 0.6 M NaCl by slowly adding solid NaCl to the stirring solution. This material was allowed to stand overnight at 4°.

The solution was centrifuged at 10,000 g (8000 rpm) for 30 min. in a GSA rotor to pellet the chromatin gel. This gel was transferred to lysis buffer containing 0.65 M NaCl and allowed to stand overnight again. The chromatin was given one more wash in lysis buffer containing 0.65 M NaCl, but usually for only 4 hours instead of overnight. The gel electrophoresis profile of the proteins solubilized in the 0.6 M and 0.65 M NaCl washes and those proteins in the pellet solubilized after the wash by 2 M NaCl are shown in Figure 50. This salt depletion procedure effectively removed nearly all the histones H1 and H5 and most of the nonhistone proteins. Figure 50 indicates that a small amount of H5 was left in the chromatin pellet. The second 0.65 M NaCl wash removes most of the remaining H5. Small amounts of core histones or other proteins co-migrating with the histones were also found in the 0.65 M NaCl wash but no attempt was made to quantitate this observation. H1 and H5 depleted chromatin at this stage was used to prepare either core particles by nuclease digestion or core histones by further salt washes.

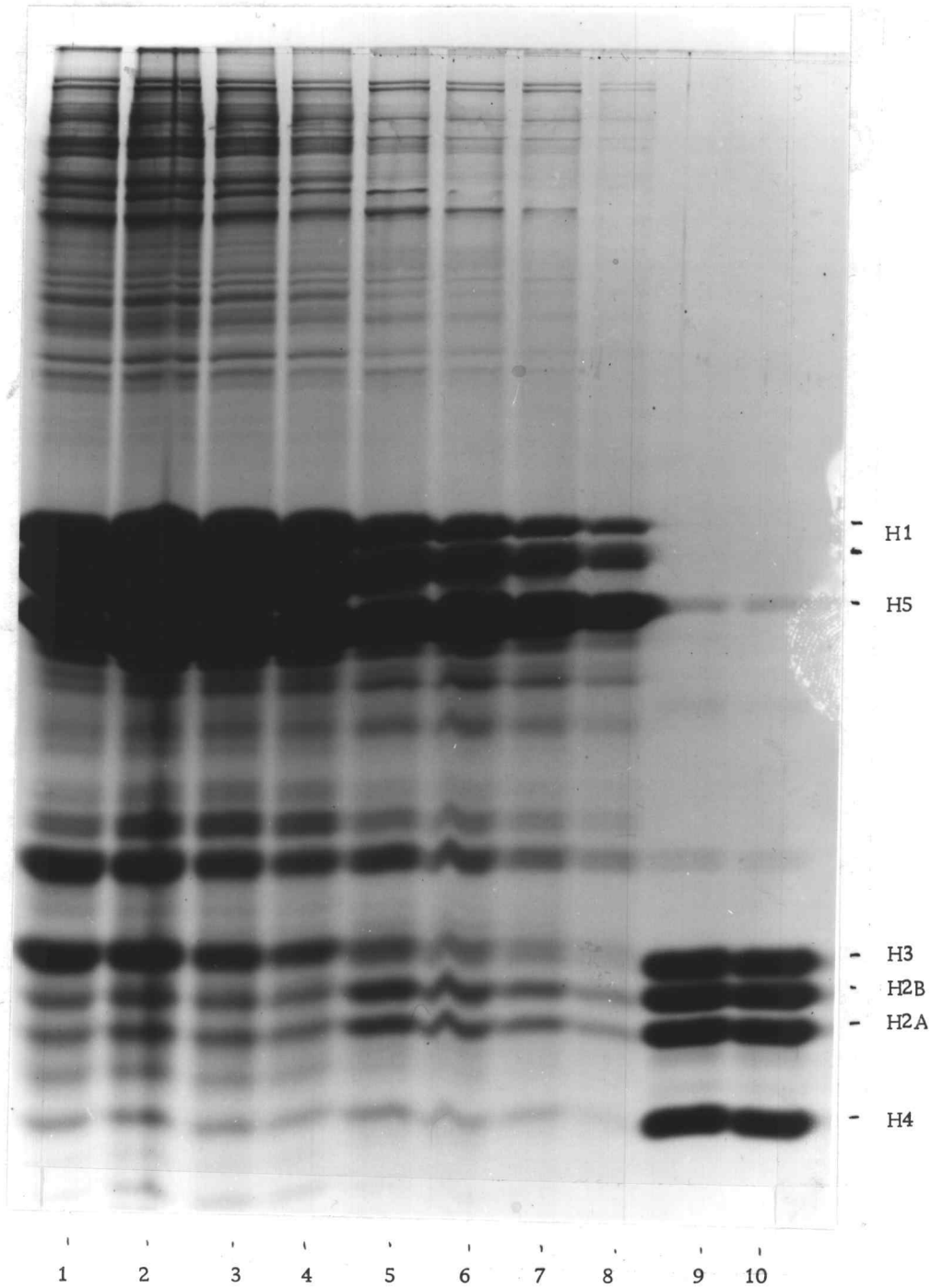

Figure 50. Chromatin proteins solubilized by NaCl treatment. Solutions of proteins solubilized by 0.6 M NaCl (channels 1-4), 0.65 M NaCl (channels 5-8), and 2 M NaCl (channels 9, 10) were dialyzed against distilled water, lyophilized and electrophoresed on a 15% polyacrylamide-SDS gel.

Preparation of Core Particles from H1 and H5 Depleted Chromatin

H1 and H5 salt depleted chromatin from 6-10 ml of packed erythrocytes was used for a large (10 mg) core particle preparation. The chromatin gel, still in 0.65 M NaCl, was transferred to lysis buffer without NaCl and stirred to remove the salt. The chromatin was observed to undergo a condensation during this procedure, becoming more opaque as the NaCl was removed. The chromatin was centrifuged at 12,000 g for 10 minutes after stirring in the lysis buffer for 2 hours. The chromatin pellet was washed three more times in the lysis buffer as above over an eight hour period to insure the ionic strength of chromatin was down to 10 mM. The chromatin pellet was now sheared with a few strokes of a loose fitting dounce homogenizer at a DNA concentration of 5 mg/ml DNA, made 1.5 mM in Ca^{++} with a 100 mM CaCl stock solution, warmed to 37°, and incubated with 125 units/ml micrococcal nuclease (Worthington). The nuclease reaction was terminated by making the solution 5-10 mM EDTA with a 100 mM Na_2 EDTA stock solution and cooling on ice. The exact time of digestion to prepare core particles was determined from a small analytical digestion time course study. A small aliquot of chromatin was incubated with micrococcal nuclease; at different times of digestion portions containing 25μg of DNA were stopped with EDTA as above and the DNA analyzed by gel electrophoresis. A typical digestion time course is shown in Figure 8. Short digestion times produced some core particles with DNA larger than 144 bp while very long digestion produced particles with smaller (120-50 bp) DNA fragments. The best digestion times in my hands were about one hour.

Purification of DNA

DNA from various nucleosomal preparations was extracted by a modified Marmur procedure, involving phenol-chloroform extraction of the DNA (Britten *et al.*, 1974). In cases where the DNA was to be used for reconstitution experiments, the DNA solution was extracted with ether 3-4 times in addition to the Marmur extractions. The DNA was precipitated with 2.5 volumes of 95% ethanol, stored overnight at -20°, then centrifuged at 17,000 g for 30 minutes to pellet the DNA. The pellet was dried under vacuum and then dissolved in the appropriate buffer.

Preparation of Histones for Reconstitution

In most cases salt extracted histones were used for the reconstitution experiments. The few experiments with acid extracted histones used proteins kindly provided by Dr. I. Isenberg. H1 and H5 depleted chromatin, prepared as described above, was made 2 M NaCl with solid salt. The viscous solution, which contained about 5 mg/ml DNA was centrifuged at 170,000 g for 24 hours in a Ti50 rotor (Beckman) to pellet the DNA away from the histones. The bottom 1/4 of the nonviscous supernatant, containing most of the core histones was chromatographed through a Sephadex G-100 column or a Sephracryl S-200 (Pharmacia) column equilibrated with lysis buffer containing 2 M NaCl. One ml of a 20 mg/ml protein solution was chromatographed through a 2.5 cm x 90 cm column. The peak fraction, containing the histone core complex was well resolved from any residual H1 and H5. A small peak containing only H2A and H2B also separated from the main peak. This peak was generally small if the initial concentration of protein was kept above 10 mg/ml but if the column was run at a low protein concentration (2 mg/ml) the H2A-H2B peak became significant (private communication from C. T. Wu) indicating the core histone complex probably dissociates at low concentration. Figure 51 shows typical column profile. Peak fractions of the core protein peak were pooled and stored at -70° for many months.

Fractionation of Chromatin by Gel Chromatography

Nucleosomes, or core particles were fractionated on Biogel A5M (Biorad) by the method of Shaw et al. (1975, 1976). Amounts of 500-600 A_{260} units were fractionated on a 2.5 cm x 90 cm column run in lysis buffer. As in previous studies the core particles or nucleosomes were obtained in a well resolved peak in the included volume.

Sucrose Gradient Sedimentation

Some chromatin digests and reconstituted nucleosome preparations were fractionated by sucrose gradient sedimentation through 5% to 20% sucrose gradients at 4° either in a SW40 or SW27 (Beckman) rotor. Less than 0.5 ml of sample was loaded on the SW40 gradients

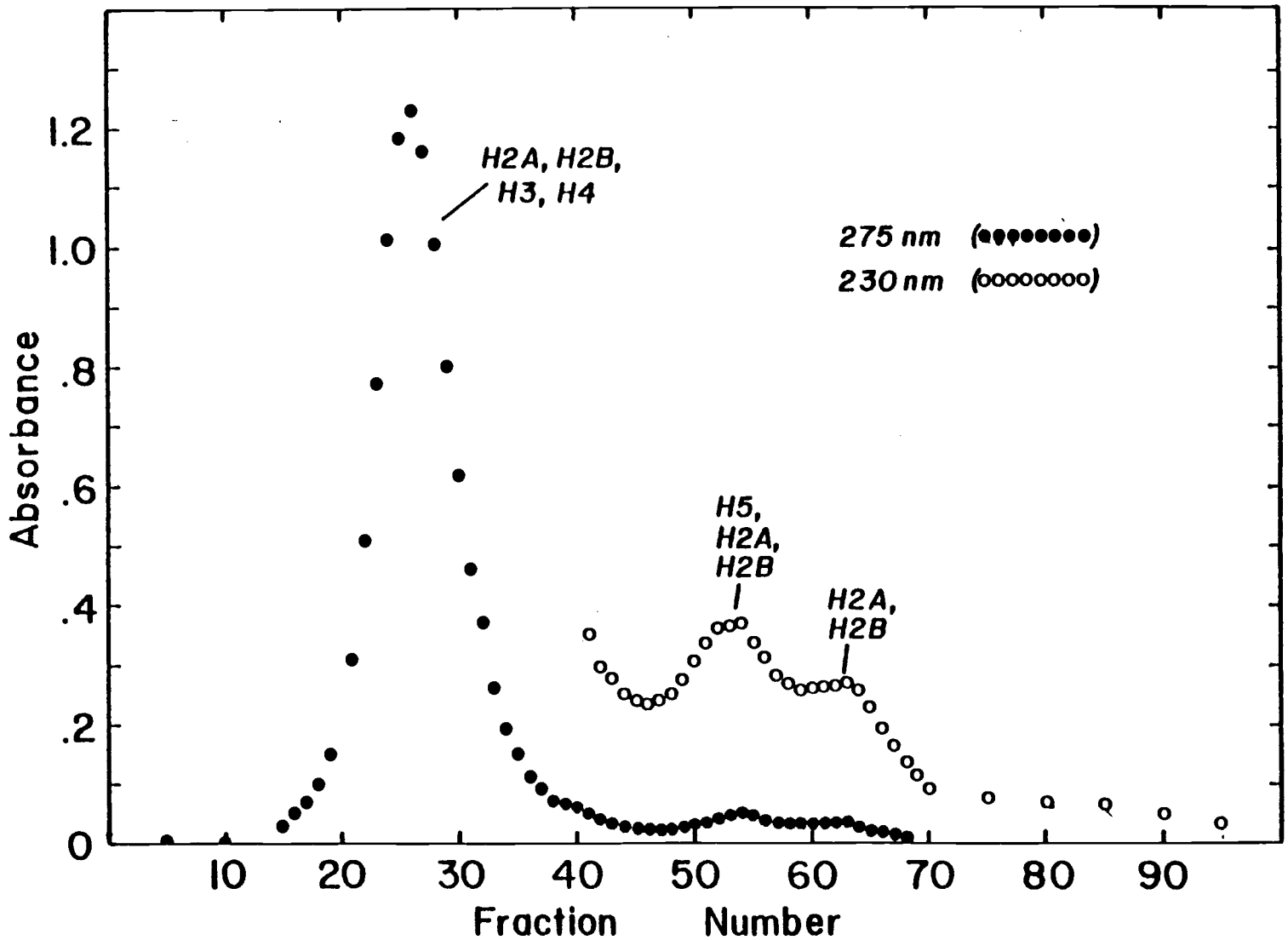

Figure 51. Elution of core histones in 2 M NaCl from a Sephracryl S-200 column.

while up to 3 ml was loaded on the SW27 gradients for large scale preparations of core particles and compact oligomers. Isokinetic sucrose gradients (5% sucrose at the top) prepared according to the procedure of McCarty et al. (1971), were used in the SW40 while 5%-20% linear gradients were run in the SW27. Centrifugations of 15 hours and 24 hours put the 11 S nucleosome half way down the gradients of the SW40 and SW27 respectively at full speed. The gradients were fractionated by dripping from the bottom or, in the case of the large SW27 gradients, by pumping the gradient out through a glass 50 μl pipet inserted down through the gradient. In most cases the gradients were run in lysis buffer.

Preparation of Nucleosomes and Oligomers by Digestion in Intact Nuclei

Chicken erythrocyte nuclei, prepared as described above, were pelleted at 750 g and the nuclei brought up in 0.3 M sucrose, 10 mM Tris, pH 7.2, 0.75 mM $CaCl_2$ to a concentration of 2×10^9 nuclei/ml. The nuclei had a tendency to lyse prematurely in this buffer if they had been centrifuged too hard or treated roughly during the nuclear isolation. The nuclei were digested with 125 units/ml micrococcal nuclease at 37° for 10-15 minutes; the longer the digestion the more nucleosome monomer was produced and correspondingly less oligomer. The nuclease digestion was terminated by making the solution 5-10 mM EDTA and cooling on ice, then centrifuged at 17,000 g for 20 minutes. The nuclear pellet, still containing 80-90% of the OD_{260} absorbing material, was brought up in lysis buffer and sheared in a Virtis "45" homogenizer for 90 seconds at half speed. The lysed nuclei were centrifuged again at 17,000 g for 20 minutes. The supernatant contained most of the nucleosomes and was further fractionated by gel chromatography or sucrose gradient centrifugation.

Hydroxylapatite Chromatography of Chromatin

DNA-Grade Bio-Gel HTP hydroxylapatite (HAP) (Biorad) was equilibrated in 1.2 mM Na PO_4 pH 6.8, 0.7 mM EDTA. Stock phosphate solutions were made from equimolar mono and dibasic sodium phosphate, HA

The equilibrated HAP was defined by allowing a solution to settle for a few minutes and then aspirating off the overlaying solution

still containing fine HAP particles. 2 ml of equilibrated HAP was then packed onto a bed of glass beads in a 1 cm i.d. glass column. The packed column was allowed to flow under gravity.

Chicken erythrocyte nuclei, digested with micrococcal nuclease were lysed and the digested chromatin dialyzed against 1.2 mM $NaPO_4$, pH 6.8, 0.7 mM EDTA (1.2 mM PB). Chromatin containing 1 mg DNA was layered on the 2 ml HAP column and diluted through the column. Nearly all the A_{260} nm absorbing material adhered to the column. In some experiments the chromatin was eluted from the column with 3-4 ml steps of increasing ionic strength phosphate buffer (PB); 0.05 M PB, 0.1 M PB, 0.2 M PB, 0.3 M PB, 0.4 M PB, and 0.6 M PB steps were used. In latter experiments the chromatin was eluted with a 50 ml linear gradient of PB (0.05 M-0.4 M) and a final step of 0.6 M PB. The column profile of such a run is illustrated in Figure 1. Column fractions were dialyzed against 10 mM Tris-HCl, pH 7.2, 0.7 mM EDTA for sedimentation velocity analysis.

Salt Fractionation of Nucleosomes

Nucleosomes in lysis buffer at a DNA concentration of 100 μg/ml were made between 0.02 and 0.5 M NaCl by direct addition of 4 M NaCl with rapid mixing. The sample was allowed to sit on ice for 4 hours to insure complete precipitation, then centrifuged at 17,000 g for 30 min in an SS 34 rotor. The pellet was redissolved in lysis buffer and both pellet and supernatant analyzed by gel electrophoresis. In some cases the fractions were further purified by sedimentation through isokinetic sucrose gradients as described above.
H1 and H5 stripped nucleosomes were prepared by sedimentation of the pellet through isokinetic sucrose gradients in the presence of 0.6 M NaCl.

The 0.12 M NaCl soluble nucleosome fraction, containing mostly 144 bp DNA, and free of lysine rich histones, was mixed with acid extracted calf thymus histone H1 in a molar ratios between 1:4 and 3:1, mole H1:Mole nucleosome. The concentration of H1 was determined by optical density at 275 nm using an extinction coefficient of 1345 M^{-1} cm^{-1}. The H1 nucleosome mixture, in 10 mM Tris-HCl, pH 7.2, 0.7 mM EDTA, was precipitated with NaCl as above. Percent solubility was determined from the percentage A_{260} in the supernatant after the 17,000 g spin.

Isolation of Compact Oligomers

Compact oligomers were obtained as biproducts of the micrococcal nuclease digestion of H1 and H5 depleted chromatin. Those fractions running ahead of the large core particle peak of the agarose A5 M column or sucrose gradient contained the compact oligomers. These fractions were pooled and rerun on sucrose gradients. The individual compact oligomers, dimer through tetramer, were resolved on the second gradient.

Core Particle Reconstitution

Two types of reconstitution experiments were carried out: In the first, which may be better described as "reversible dissociation" or "reassociation," a solution of core particles (in lysis buffer) was made 2 M in NaCl and the salt was then removed by stepwise dialysis (as below). In the second technique, salt extracted histones and purified DNA, in 2 M NaCl, were mixed together in the desired ratio and dialyzed against dialysates of successive lower ionic strengths. The total reconstitution time was approximately 24 hours at 4°C with 4 hour steps of 1.5 M NaCl, 1.0 M NaCl, 0.75 M NaCl, 0.5 M NaCl, and finally two changes of lysis buffer. 0.1 mM PMSF was present in the first step (2 M NaCl).

In some later experiments the successive step dialyses were replaced by an exponential salt gradient using a constant volume mixing chamber. The ionic strength in the mixing chamber (containing the samples) could be determined at any time from the following equation:

$$C = C_{mix}\, e^{-b/v} + C_{res}\,(1-e^{-b/v})$$

where,

v = volume of mixing chamber (ml)
b = volume delivered (ml)
C_{mix} = NaCl concentration (M) in the mixing chamber (initial)
C_{res} = NaCl concentration (M) in the reservoir
C = NaCl in mixing chamber after b ml has been delivered

In most cases the parameters used were: v, 200; C_{mix}, 2; C_{res}, 0.5; with a flow rate of reservoir buffer into the mixing chamber of 30 ml/hour. This gave a change from 2 M NaCl to 0.6 M NaCl in about 16-17 hours with the flattest portion of the gradient in the region (1.2-0.8 M NaCl) where histone-DNA associations occur (Wilhelm et al. (1978). One mixing chamber volume of lysis buffer was then pumped into the mixing chamber and finally the samples were dialyzed against two or more changes of lysis buffer. The total reconstitution time was again about 24 hours. In some experiments, the ionic strength was dropped from 2 M NaCl to 0.6 M NaCl simply by rapid dilution and then dialyzed further in dialysis membranes. The concentration of DNA in the reconstitution mixture was 50-100 μg/ml, except as otherwise specified. All reconstitution dialyses were done in treated Union Carbide dialysis membrane.

When reconstituted particles were to be fractionated from any DNA or protein that had not reconstituted or from aggregated material, the sample was first concentrated and then run on sucrose gradients in a SW40 rotor. Vacuum dialysis or Amicon ultrafiltration cells (Model 3 and Model 12 with PM 30 or PM 10 membranes) were used to concentrate the samples with little loss of material. In a control, 90% of the 260 nm absorbing material was recovered out of an Amicon model 12 with PM 10 membrane after concentration. The PM 30 membrane also gave good recovery but with a much faster flow rate. Core particle preparations have been concentrated to 20 mg/ml and down to as little as 0.2 ml in the Amicon Model 3 with no visible aggregation or damage (as determined by velocity sedimentation after redilution).

All native and reconstituted core particle preparations were stored at 4°C. Samples could be stored at 4°C for up to two months without any observable change in the physical structure, as determined by sedimentation, thermal denaturation, or gel electrophoresis. No change in the sedimentation coefficient of one nucleosome sample was observed after storing the sample at room temperature for 7 days. Nevertheless all studies on reconstituted and native particles were done as soon as possible after preparation.

Protein Electrophoresis

Proteins were electrophoresced on sodium dodecylsulfate (SDS) 15% polyacrylamide, with 6% polyacrylamide stacker (acrylamide:bis, 30:1) slab or tube gels according to the procedure of Laemmli (1970).

Large slab gels (0.1 cm x 20 cm) were run for about 22 hours at 95 volts or until the bromophenol blue tracking dye had migrated to the bottom of the gel. Mini slab gels (0.05 cm x 8 cm), designed by Matsudaira and Burgess (1978), were run for 1.5 to 2 hours at 25 mA constant current at 4°C. Some protein samples were run on 15% polyacrylamide (acrylamide:bis, 30:1) acetic acid urea 8 cm tube gels according to Panyim and Chalkley (1969). Gels were run at 120 volts for 6-7 hours and then stained in 0.5% amido black, 7% acetic acid then destained in 7% acetic acid. Gels were either photographed with Polaroid Type 55 P/N film or scanned in a Gilford gel scanner at 600 nm.

When the protein composition of chromatin was to be studied in low ionic strength buffer, the particles were lyophilized, dissolved in sample buffer containing 2.0% SDS, 5% B-mercaptoethanol, 0.16 M Tris-HCl pH 6.8, 0.001-0.1% bromophenol blue, and 10% glycerol, and then heated at 100°C for 1 minute prior to electrophoresis. 1-2 μg protein/band were electrophoresced on the 0.1 x 20 cm slabs while 0.25 μg protein/band was electrophoresced on the Matsudaira mini slabs. Gels were stained in 0.05% coomassie blue R-250 (Sigma), 25% isopropanol, 10% acetic acid, and destained in 10% acetic acid. The destained gels were either photographed with Polaroid Type 55 P/N film (1/8 sec, f8-11, yellow wratten filter, trans illumination) or scanned on a Gilford gel scanner at 550 nm or an Ortec densitometer.

Protein samples in high ionic strength buffer were precipitated in 30% TCA for 15 minutes on ice, centrifuged 10 min at 12,000 g, washed twice in acetone (centrifugation at 12,000 g after washing), then dried and brought up in electrophoresis sample buffer.

Analytical Protein Determination

Lowry protein determinations according to Hartree (1972) were done using calf thymus H4 (gift from Dr. I. Isenberg) as the primary standard. This protein was chosen since it was available in a highly purified form, of known extinction coefficient, and is representative of the inner histones. A standard curve using Bovine serum albumin was calibrated against an H4 standard curve so that bovine serum albumin could be used as the secondary standard.

Analytical DNA Polyacrylamide Gel Electrophoresis

1. Gel electrophoresis, under non-denaturing condition.

DNA prepared as above was redissolved in 1/10 strength electrophoresis buffer plus 10% glycerol. The DNA solution was then layered on polyacrylamide gels prepared and run following the procedure of Loening (1967) using a Tris-acetate EDTA buffer system. The acrylamide:bis ratio was kept at 20:1. Twenty cm long cylindrical gels were run at 5 mA/tube while 20 cm long slab gels were run at 50-60 mA. 3% to 5% slab gels were usually 0.3 cm thick while 6% to 10% gels were 0.2 cm thick; the extra thickness on the lower percentage acrylamide gels was necessary for stability during handling. Bromophenol blue and xylene cyanol FF were run as tracking dyes. The molecular weight of the DNA co-migrating with the dyes varied with polyacrylamide percentage but approximate values were obtained from Maniatis et al. (1975).

Gels were stained 45-60 minutes in electrophoresis buffer containing 0.5-1.0 μg/ml ethidium bromide and photographed on black plexiglas trays under long wavelength ultraviolet light using Polaroid Type 107 or Type 55 P/N film. Exposure times of 10 seconds and 10 minutes were generally needed for the two types of film respectively, using a yellow wratten filter. Gels were sometimes stained with 40 μg/ml toluidine blue (MCB) in distilled water or 0.005% Stains-All (Eastman) in 50% formamide, pH 7.3 overnight then destained in distilled water. Care was taken not to over-destain the gels; a slight background stain was left in the gels for subsequent scanning or photography.

Photograph negatives, and toluidine blue or Stains-All stained slab gels were scanned with an Ortec or Joyce Lobel densitometer. Cylindrical gels were scanned in a Gilford gel scanner at 546 nm for toluidine blue stained gels and 600 nm for Stains All stained gels.

Hae III endonuclease fragments of bacteriophage PM2 were electrophoresed on wells adjacent to chromatin DNA samples on slab gels or on separate tube gels of the same run. These fragments have been calibrated against sequenced restriction fragments by Dr. R. T. Kovacic. The sizes used for the PM2-Hae III fragments are: H, 518 bp; I, 337 bp; J, 299 bp; K, 273 bp; L, 162 bp; M, 148 bp; N, 120 bp; O, 97 bp. The sizes for fragments P and Q are not known as accurately, but tentative values 50 bp and 47 bp were used for these respectively.

2. Electrophoresis under Denaturing Conditions.

DNA for electrophoresis was dissolved in 10 M urea, 50 mM Tris-borate, pH 8.3, 1 mM EDTA, 10% glycerol. The DNA sample solution was made 0.1 N KOH (to denature the DNA) before loading on 8% polyacrylamide (30:1, acrylamide:bis), 7 M urea slab gels and run according to Maniatis et al. (1975) in 50 mM Tris-borate, pH 8.3, 1 mM EDTA. 20 cm x 0.2 cm slab gels were run for 6-16 hours at 300 volts and then stained in 1 mM EDTA pH 7.2, 1 μg/ml ethidium bromide for 1 hour prior to photographing.

In cases where ^{32}P labeled DNA was electrophoresed, 25-30 μg of DNA carrying 10^5 cpm of ^{32}P was loaded on each well so that the gel could be both stained with ethidium bromide and then autoradiographed. Gels to be autoradiographed were soaked in 5% trichloroacetic acid (TCA), after ethidium bromide staining and photography, for 15 minutes at 4°C to fix the DNA in the gel. Excess TCA was removed by two 1 minute rinses in cold water followed by one 5 minute rinse. The gel was then covered with plastic wrap and autoradiographed using Kodak X-omat R X-ray film and a Kodak intensifying screen. Polaroid Type 55 negatives of the ethidium bromide stained gel and the autoradiograms were scanned with an Ortec or Joyce Lobel densitometer. ^{32}P end labeled PM2-Hae III fragments were run as DNA markers on the gels. Complimentary strands of restriction fragments run with different mobilities on the urea denaturing gels described here. Running gels with lower acrylamide:bis ratios (6:1 instead of 30:1, acrylamide:bis) and at higher temperatures (50°C, by running at higher voltages) has been reported to eliminate this base composition or base sequence artifact (Dr. L. Lutter, personal communication).

Digestion of Native and Reconstituted Core Particles by Pancreatic DNase 1, Micrococcal Nuclease, and Trypsin

Chromatin material, at a concentration of 50 μg/ml DNA, was made 4 mM in Mg^{++} and digested with 100 units/ml pancreatic DNase 1 (Worthington) at 37°C. Digestion was terminated by making the solution 10 mM in EDTA and cooling on ice.

Chromatin material was digested similarly with 100 units/ml micrococcal nuclease, except that 3 mM Ca^{++} replaced the Mg^{++}. The high levels of Mg^{++} and Ca^{++} used in these experiments were in part to compensate for the presence of EDTA in the chromatin

preparations. Repetition of the experiments with 1.5 mM Mg^{++} or Ca^{++} gave identical results. The lower divalent ion concentrations are preferable because some precipitation does occur at the higher concentrations.

Core particles in 10 mM Tris-HCl, pH 8, 0.7 mM EDTA were incubated with various concentrations of trypsin (Sigma) at 4°C for 16 hours similarly to Lilley and Tatchell (1977). The reaction was stopped by making the reaction mixture 10 μg/ml N-p-tosyl-l-lysine chloromethyl ketone (TLCK); the mixture was immediately lyophilized to dryness and then dissolved in electrophoresis sample buffer in preparation for gel electrophoresis.

Circular Dichroism

The circular dichroism (CD) spectra of nucleosome preparations were recorded with either a Jasco J-41A or a Jasco J-SD specto-polarimeter calibrated against camphor-sulfonic acid.

The CD spectra from the melting experiments were all recorded with the Jasco J-41A. A jacketed cylindrical cuvett with a 1 cm optical pathlength was used for all measurements. Temperatures were recorded with a thermister (Yellow Springs Instrument Company) directly immersed in the sample. The cell was stoppered to prevent evaporation. Samples were prepared as for thermal denaturation. Two types of experiments were performed: In the first, spectra were recorded between 200 and 260 nm and 250 and 330 nm at a constant temperature, followed by baseline scans with buffer at the same temperature. In the second, the melting of a sample was monitored at a fixed wavelength (λ =223 nm, λ =273 nm) while the temperature was linearly increased by about 0.2 degrees/minute. In these cases the buffer baselines were recorded at the initial and final temperatures only. The recorder graphs were digitized and converted to mean residue ellipticities, $[\theta]$, or changes thereof, $\Delta(\theta)/\Delta T$, using an extinction coefficient of 6600 per mole of nucleotide residue at room temperature.

Thermal Denaturation

Most melting experiments were done in either 0.25 mM EDTA, pH 8.0 or 1 mM Na cacodylate, pH 7.2. Since EDTA has been reported to alter chromatin structure (Williams et al., 1972; Johnson

et al., 1972; and Reeck, 1976), melting experiments in the most commonly used buffer (0.25 mM EDTA) were compared with melting experiments in 1 mM cacodylate. The two curves for core particle melting were identical in shape but the cacodylate curve was 1° higher, probably an ionic strength effect.

Core particles and other chromatin samples for thermal denaturation studies were dialyzed extensively against the thermal denaturation buffer. Samples with 0.5-1 A_{260} were placed in quartz cuvettes with 1 cm pathlength, bubbled with helium to de-gas the solution, then overlayered with Dow Corning 200 silicone fluid and sealed with ground glass stoppers. Absorbances and temperatures were recorded directly on punch tape from a Beckman Acta III spectrophotometer. The temperature readings of the ACTA III probe were calibrated against a thermister probe placed directly in the sample cuvette. The rate of temperature increase was 0.25°/min. and one set of data (absorbance, reference absorbance, and temperature) was collected every minute. The data were processed with a Hewlett Packard 9521 minicomputer, correcting for thermal expansion, to give percent hyperchromicity (h) vs. temperature (T) or the first derivative of h with respect to T. The percent hyperchromicity at temperature T is defined as:

$$h = \frac{A_{260} - A^{o}_{260}}{A^{o}_{260}} \times 100$$

where A_{260} is the absorbance of T, (corrected for thermal expansion) and A^{o}_{260} is the absorbance at the base temperature (usually 20°). A linear least squares fit in an interval around each point in the curve was used to obtain the derivative dh/dT. The number of points in the interval was varied between 7 and 20 points and was found to have no effect on the melting peaks, which were defined as melting temperatures. A quadratic least-squares fit was tried but it was unsatisfactory.

Preparative Polyacrylamide Gel Electrophoresis

Chicken erythrocyte nuclei were digested with 200 units/ml micrococcal nuclease for 4-8 hours at 37°C in 10 mM Tris-cacodylate, pH 7.2, 1 mM CaCl. A limit of digestion is reached when about 50% of the DNA becomes acid soluble (Clark and Felsenfeld, 1971) so there is no danger of overdigesting the nuclei. The DNA from this limit digest was purified and fractionated into size classes by preparative gel electrophoresis. Double stranded DNA fractions were obtained

from 160 bp to 50 bp in roughly 10 b intervals. The width of each size class at peak half height was 10-18 bp.

Two methods of preparative electrophoresis were used to fractionate the "limit" DNA digest: In the first, a large 10% polyacrylamide slab 0.6 x 20 cm x 20 cm was loaded with 15-20 mg of DNA, electrophoresced and then sliced into horizontal strips. The gel was stained briefly in ethidium bromide to observe any curvature in the banding pattern The DNA from each slice was eluted from the gel by the procedure of Maxam and Gilbert (1977). A 1.0-1.5 ml gel slice was placed in a 5 ml Pipetman (Gilson) pipet tip that had been plugged with a piece of siliconized glass wool and heat sealed at the tip. The gel slice was ground to a paste in elution buffer (0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% SDS, 0.1 mM Na_2EDTA). Another ml of elution buffer was added and mixed with the paste. The pipet tip was covered with parafilm and incubated overnight at 37°C. The sealed pipet tip was then cut allowing the elution buffer and solubilized DNA to flow through the glass wool and out the tip. The pipet tip was washed with an additional ml of elution buffer and the DNA contained in the combined elutants was precipitated with 2.5 volumes of ethanol. The precipitated DNA was redissolved in buffer, extracted with isoamylalcohol to remove any residual ethidium bromide, and dialyzed extensively against lysis buffer. Small pieces of polyacrylamide were sometimes not filtered out by the glass wool. These pieces were removed by centrifugation prior to dialysis.

The second technique for preparative fractionation required electrophorescing DNA through a large preparative gel and eluting the DNA from the bottom of the gel as it electrophoresed off. Two apparatuses were used: An apparatus containing a 1.7 cm cylindrical gel (Savant) and an apparatus containing a 7.7 cm cylindrical gel constructed according to Hagen (1978). 1-2 mg of DNA was electrophoresed through the small gel while 20-25 mg was loaded on the large 7.7 cm model. In each case an 8 cm long 10% polyacrylamide (20:1, acrylamide:bis) gel was electrophoresed at 40-50 volts at room temperature. The elution buffer flow rate was 0.1-0.3 ml/minute. The slower flow rate gave less homogeneous fractions while faster flow rates gave more homogeneous fractions but at a lower concentration of DNA. In most cases adjacent 2 ml fractions were pooled in order to obtain enough DNA of one size class but not without some loss in size homogeneity.

End Labeling DNA with ^{32}P

T4 polynucleotide kinase was prepared from T4 lysates according to Richardson (1972) and purified by DEAE cellulose chromatography. This enzyme was kindly provided by Dr. C. Tahourdin. Nucleosome preparations were labeled with ^{32}P at the 5' ends of the DNA as described by Simpson and Whitlock (1976) to a specific activity of 10^5-10^6 cpm/μg of DNA using ^{32}P labeled ATP (provided by Dr. R. McFarland). The 20-50 μl incubation was done in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM BMCE, and at least 1 μM ATP containing 1-2 μgDNA. The reaction mixture was incubated at 37°C with 2 units T4 kinase for one hour then terminated with excess EDTA and cooling on ice. At times the incubation mixture was diluted with an excess on cold sample then immediately digested with pancreatic DNase I as above.

Core particles containing DNA molecules longer than 144 bp majority were found to be preferentially labeled. The ^{32}P end labeled material was therefore not always identical to the total preparation. This preferential labeling was not found when free DNA was labeled.

The DNA ^{32}P and labelings were done as above but with 50 mM Tris-HCl instead of the 10 mM Tris-HCl. When this DNA was to be reconstituted with histones, and labeled sample was diluted with an excess of cold DNA and reconstituted immediately as above. The unreacted ^{32}P ATP and $^{32}PO_4$ was removed during the reconstitution dialysis. DNA samples prepared from micrococcal nuclease digests could be kinased without pretreatment with bacterial alkaline phosphotase to remove the 5' phosphate because micrococcal nuclease mediated hydrolysis leaves a free 5' OH group, the necessary substrate for the T4 kinase.

Sedimentation Experiments

All sedimentation experiments were performed with a Beckman Model E analytical ultracentrifuge equipped with scanner optics. A wavelength of 265 nm was used in most experiments. The linearity and accuracy of the scanner had been checked with nucleotide solutions of known absorbance. The accuracy of temperature recording and control had been checked by diphenyl ether melting, using the technique of Gropper and Boyd (1965).

Sedimentation velocities were determined from the midpoints of the sedimenting boundaries, using a calculation program that automatically compensates for the change in scanner transit time to the boundary. All data were recorded at or near 4° and corrected for temperatures and buffer effects to yield $S_{20,w}$ values.

In most cases, the integral distribution of sedimentation coefficients was calculated, using a modification of the method of Gralen and Lagermaln (1952). Details of this method have been described by Van Holde and Weischet (1978b).