ELECTRON MICROSCOPE STUDIES OF CHROMATIN SUBUNIT PARTICLES

Thesis by

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In Partial Fulfillment of the Requirements for the Degree of

Master of Science

California Institute of Technology
Pasadena, California 91125

1975
(Submitted March 27, 1975)

Acknowledgments

I would like to thank Dr. J.-P. Revel and Pat Koen for advice and instruction on the use of the electron microscope, the preparation of specimens, and the interpretation of results. I would also like to thank Joel Gothesfeld for several preparations of the chromatin subunit particles (ν bodies) and for showing me the details of the preparation.

I would also like to thank my advisor, Bob Stroud, as well as the other people in my research group for many helpful discussions and advice.

Last, I would like to thank the National Research Council of Canada for a graduate fellowship this past year.

Abstract

Nuclease resistant chromatin subunits (ν bodies) were isolated from rat liver chromatin after digestion with DNAase II. Electron microscope studies showed them to be similar in size to particles which have been isolated from calf thymus chromatin and also to those seen in native chromatin. ν bodies prepared from native chromatin (ν(+F1) and histone F1-depleted chromatin (ν(-F1)), as well as formaldehyde-fixed products from both these digestions (ν(FF + F1) and ν(FF-F1)) were similar in size. For ν(+F1) the diameter was 80 ± 17 Å (n = 125); for ν(-F1), 87 ± 19 Å (n = 100); for ν(FF + F1), 96 ± 23 Å (n = 50); and for ν(FF - F1) 81 ± 13 Å (n = 85). Multimer ν-body fractions did not show any obvious dimers, trimers, etc., of monomer ν bodies. Sheared, isolated chromatin from rat liver was also examined using electron microscopy and was observed as networks of long thin strands (about 15 Å wide), interspersed with regions of either coiled single strands or strands covered with protein.

TABLE OF CONTENTS

		Page
I.	ACKNOWLEDGEMENTS	ii
II.	ABSTRACT	iii
III.	TABLE OF CONTENTS	iv
IV.	INTRODUCTION	1
V.	EXPERIMENTAL	5
VI.	RESULTS AND DISCUSSION	17
	A. PREPARATION OF ν BODIES	17
	B. ELECTRON MICROSCOPE STUDIES	20
VII.	FUTURE STUDIES	36
VIII.	ADDENDUM	44
IX.	REFERENCES	52

IV. Introduction

The chromatin of eukaryotic nuclei is a complex of DNA with basic histone proteins and acidic non-histone proteins. There are five types of histones called F1 (lysine-rich), F2B and F2A2 (slightly lysine-rich), and F2A1 and F3 (arginine-rich) whose sequences are well conserved from species to species. The non-histone chromosomal proteins on the other hand show great heterogeneity.

The important question of how the DNA is packaged within the chromatin is still not answered, but it must account for folding of about 100 cm of DNA into a volume of less than 10^{-10} cm^3. In x-ray patterns of native and reconstituted chromatin a series of rings at about 110, 55, 37, 27, and 22 Å have been observed[1,2]. This data have formed the basis of two structural models for chromatin. That of Pardon, Wilkins and Richards is a loose super-coil of pitch 120 Å and outer diameter 130 Å.[3] The Bram and Ris model is a compact non-uniform super-coil of 45-50 Å average pitch and 100 Å outer diameter[4].

A "particles-on-a-string" model of chromatin has been proposed by Olins and Olins[5]. This model is based on the observation of spherical particles (average diameter about 70 Å) connected by thin filaments (about 15 Å wide) in preparations of isolated eukaryotic nuclei swollen in water, centrifuged onto carbon films and stained.

In later work[6] they isolated spheroid chromatin units (ν bodies) from formaldehyde-fixed and sonicated chick erythrocyte nuclei. Each particle contains a DNA fragment of about 210 base pairs. From this number and the molecular weight of the complex (295,000), they estimated that each ν body would contain two of each of the five histones. Bram and Ris, a few years earlier, had noticed protuberances about 100 Å thick projecting 80-200 Å from the fiber axis of chromatin isolated from calf thymus[4]. In a study of the SV-40 "minichromosome" (from monkey cells lytically infected with SV-40) visualized by shadowing with tungsten, Griffith found it to resemble a circle of beads each 110 Å in diameter joined by bridges about 20 Å in diameter and 130 Å long[7]. He estimated that each bead contains 170 base pairs of DNA and each bridge 40 base pairs.

Further support of this model comes from studies by Van Holde <u>et al.</u>[8-11] on the portion of calf thymus chromatin which is resistant to staphylococcal nuclease digestion. This fraction consists of particles ("PS-particles") of molecular weight 180,000 each containing a piece of double stranded DNA of about 110 base pairs and the normal complement of histones except F1. Electron micrograph studies of the PS-particles showed spherical particles of diameter 74 ±20 Å. Some particles had a 20 Å thick "tail" and a few particles were joined by these strands.

Also, Noll has found that digestion by micrococcal nuclease of chromatin in rat liver nuclei leads to formation of a subunit consisting of 205 base pairs of DNA and all five histones[12]. Sucrose gradient analysis of the digested chromatin revealed seven resolved peaks which contained DNA the size of the monomer (205 base pairs), dimer (405 base pairs), and trimer (605 base pairs), thus indicating a regularity in the pattern of degradation. A similar regularity was noticed in the endonuclease digestion of chromatin in rat liver which gase DNA pieces in integral units of about 200 base pairs[13]. The regularity in digestion is thought to reflect regularity in the pattern of protein bound to DNA[13].

These data, along with evidence that in neutral aqueous histones F2A1 and F3 form a tetramer of composition $(F2A1)_2(F3)_2$[14] and that histones F2A1, F3, F2A2, F2B and DNA complex to give the x-ray pattern of chromatin[14], led Kornberg to postulate the following model for chromatin structure[15]. The chromatin fiber is a flexibly jointed chain of repeating units of about 200 base pairs of DNA complexed with two of each of the histones F2A1, F3, F2A2, and F2B. Van Holde then extended this model by proposing an arrangement for the DNA and histone in the units[11]. The histones F2A1, F3, F2A2, and F2B exist as a specific complex formed by the interaction of the hydrophobic C-terminal halves of the histones. The DNA is wrapped around the hydrophobic core, with the N-terminal portions of the

histones then wrapped around the DNA (probably in the major groove).

These models correlate very well with the results of a low-angle neutron scattering study on preparations of chromatin gel[16]. The use of "contrast matching" showed that the low-angle rings of 110, 55, 37, and 27 Å do not come from the spacing of a single structural repeat and its higher orders, but from different spatial arrangements of the histones and DNA. The concentration-dependent ring at 110 Å, which comes from protein scatter, was attributed to the spacing of subunits. The 37 Å ring also is attributed mainly to protein scatter and does not move during hydration. It is postulated to be due to the hydrophobic core of Van Holde's model. The DNA component contributes scatter to the rings at 55 and 27 Å. From their results, Baldwin et al. postulated a model very similar to that of Kornberg's[15] and Van Holde's[11].

Recently, Joel Gottesfeld in Dr. Bonner's laboratory has isolated the DNAase II resistant fraction from rat liver chromatin. This preparation seems to be very similar to the ν bodies of Olins and Olins[6], the PS-particles of Van Holde[8-10], and the chromatin subunit particles of Noll[12] in number of base pairs, amount of histone and density. To further confirm the relationship, I decided to look at the particles by negative staining in the electron microscope. With future studies in mind, I also learned how to prepare the ν bodies. Results of these studies are reported on here.

V. Experimental

A) Preparation of chromatin

Rat livers were obtained from Pelfrius and were stored frozen until needed. Chromatin was prepared by the method of Marushige and Bonner[17], except that 0.01 M Tris buffer (pH 8) was used instead of 0.05 M Tris (pH 8). The gelatinous purified chromatin from the final centrifugation through 1.7 M sucrose was washed once with 0.01 M Tris (pH 8) by centrifugation at 16,000 rpm for 20 minutes in a Servall SS-34 rotor. The chromatin pellet was then homogenized in 10 mM sodium acetate (pH 6.6) and dialyzed overnight against six liters of 10 mM sodium acetate (pH 6.6).

B) Preparation of chromatin subunit particles (ν bodies)[18]

After dialysis, the chromatin was pelleted by centrifugation at 10,000 rpm for five minutes in a Servall SS-34 rotor. The pellet was suspended in 50 ml of 10 mM sodium acetate (pH 6.6). The DNA concentration of this suspension was determined from OD_{260nm} to be 13.5 OD units/ml or 675 μg of DNA/ml (675 total OD units of DNA in 50 ml).

The chromatin suspension (volume 50 ml) was brought to room temperature while being stirred with a magnetic bar. DNAase II

(Worthington) was added to a concentration of 10 units DNAase per 1 OD unit of DNA (or 6750 units DNAase II). At the end of a five-minute digestion the mixture was centrifuged at 15,000 rpm in a Servall SS-34 rotor for fifteen minutes at 4°C.

The supernatant (S_1) was brought to pH 7.5 with 0.1 M Tris (pH 11). Then $MgCl_2$ was added to a final concentration of 2 mM. The solution was stirred for 30 minutes, then centrifuged at 10,000 rpm for ten minutes at 4°C (in a Servall SS-34 rotor). The second supernatant (S_2) (enriched in transcribed sequences) was concentrated with a minicon (Amicon) and saved for future studies by J. Gottesfeld.

The pellet (P_1) was resuspended in 20 ml of 10 mM sodium acetate (pH 6.6) and brought to room temperature with stirring. There were 600 OD units of DNA in this chromatin suspension. DNAase II (6000 units) was added to the chromatin suspension. After 45 minutes, the digestion was stopped by bringing the pH of the suspension to 7.5 with 0.1 M Tris (pH 11). The suspension was then centrifuged at 10,000 rpm for ten minutes at 4°C in a Servall SS-34 rotor. The supernatant (S_3) was saved and contained 160 OD units of DNA. (This is a low yield since usually 80% of the starting OD units of DNA are found in S_3.)[18]

S_3 (6 ml gradient) was layered on three 25 ml isokinetic sucrose gradient (15.1-26%) prepared by the method of Noll[19]. In making the gradients, the mixing flask contained 31.4 ml (Vm) of

15.1% (Ct) sucrose in 10 mM Tris pH 8. The reservoir contained 34.14 % (Cr) sucrose in 10 mM Tris pH 8. The gradients were allowed to sit for one hour before they were used. The gradients were centirifuged for 41.5 hours at 22,500 rpm and 4°C in a Beckman SW25.1 rotor. Collection of the gradients involved pumping heavy sucrose (60%) through a needle punched into the bottom of the tube, thus forcing the contents of the tube out the top into a length of tubing. The effluent passed through an ISCO UV detector hooked up to an automatic fraction collector. Either 20 or 25 drop fractions were collected. Figure 1 shows a sample ISCO readout for one of the three gradients.

For all fractions OD_{260} was determined and plotted against fraction number (see Figure 2 for an example). Fractions (or corresponding ones for the other two gradients) as indicated in Figure 2 were pooled. The first pool has been shown in previous preparations by J. Gottesfeld to be enriched in monomer ν bodies[18]. The rest of the fractions were pooled as indicated, so that the presence of multimer ν bodies could be checked for in the electron microscope. The monomer ν body pool was dialyzed overnight against 4 l of 10 mM Tris (pH 8) at 4°C. After dialysis the monomer ν body pool had 17.6 OD_{260} units of DNA in 28 ml. This sample was concentrated to 5.6 ml in a minicon (Amicon).

Four isokinetic sucrose gradients (5-20%) were prepared

Figure 1: Isokinetic sucrose gradient of S_3 (see VB) from DNAase II digested rate liver chromatin. The arrow indicates the position of the monomer ν-body fraction (ISCO output).

Figure 2: OD_{260} versus fraction number for the fractions from the sucrose gradient shown in Figure 1. Fractions were pooled as indicated for futher purification.

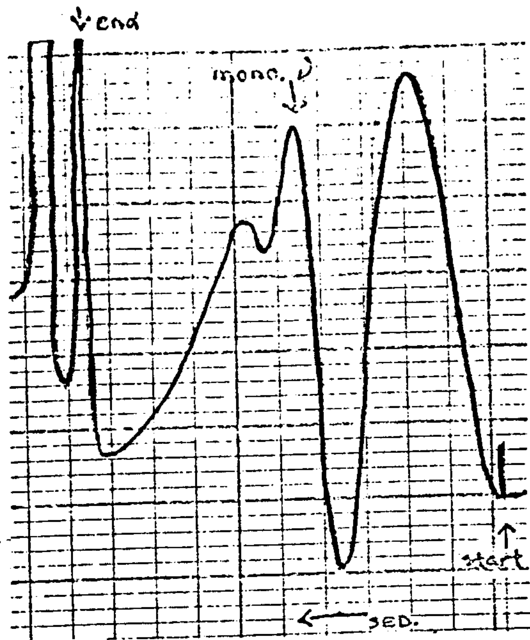


FIGURE 1

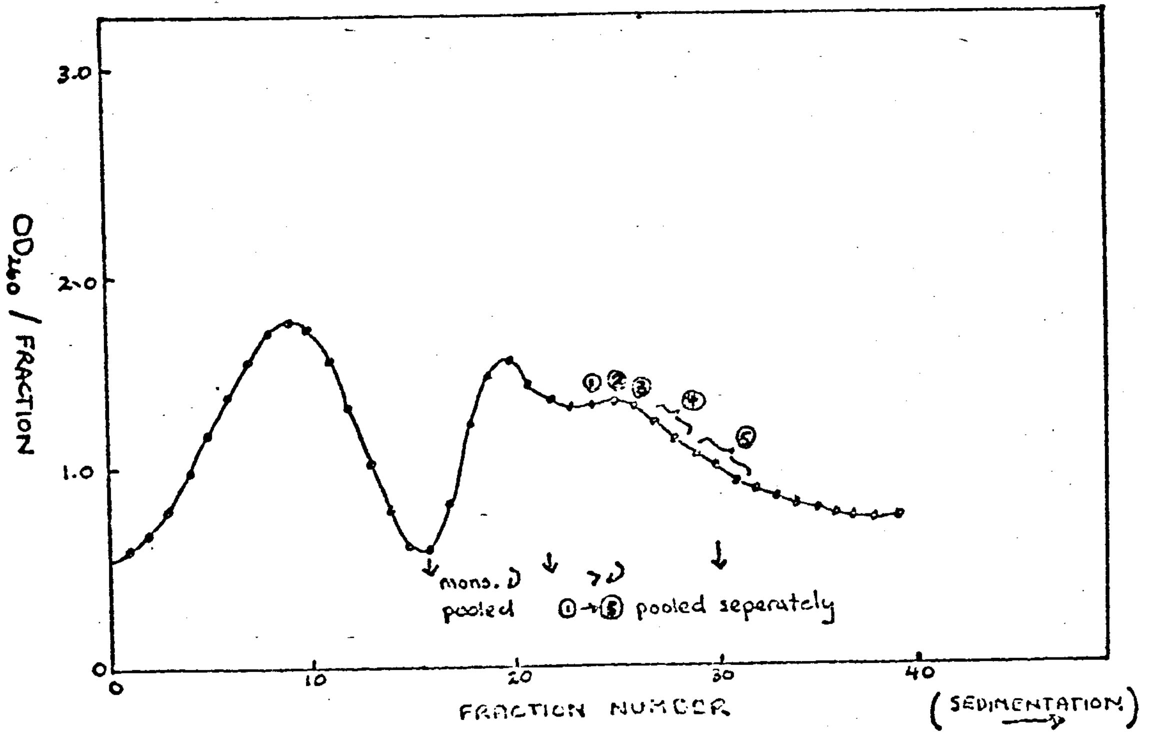


FIGURE 2

by the method of Noll[19] in tubes (filled to 1 cm from the top) for the Beckman SW41 rotor. In this case, Vm = 9.4 ml, Ct = 5.1 % sucrose in 10 mM Tris pH 8, and Cr = 31.4% sucrose in 10 mM Tris pH 8. The concentrated monomer ν body pool (1.4 ml on each gradient) was layered on the top of the gradient, and centrifuged for nineteen hours at 35,000 rpm at 4°C in a Beckman SW41 rotor. Gradients were collected as previously described, collecting 22 drops per fraction and twenty fractions per gradient. A typical ISCO output is given in Figure 3. For each gradient, the OD_{260} per fraction was determined (see Figure 4 for example). The fractions indicated in Figure 4 were pooled (or corresponding fractions in the other three gradients) and were dialyzed overnight against 10 mM ammonium acetate pH 7 (3 x 4 l). (Also dialyzed at this time were pools 1-5 in Figure 2.) The final purified monomer ν body fraction had 4.1 OD_{260} units of DNA in 13 ml and was concentrated in a minicon to 1.56 OD_{260} units DNA/ml. This sample was stored refrigerated and was used as soon as possible for electron microscope studies.

c) Other methods of ν body preparation

Although these preparations were done by J. Gottesfeld, they are mentioned here since ν bodies made by these methods as well as the one previously described were used in the electron microscope studies.

Figure 3: Isokinetic sucrose gradient of the monomer ν-body fractions pooled as shown in Figure 2 (ISCO output).

Figure 4: OD_{260} versus fraction number for the fractions of the sucrose gradient shown in Figure 3. Fractions were pooled as shown, and used as purified ν bodies for electron microscope studies.

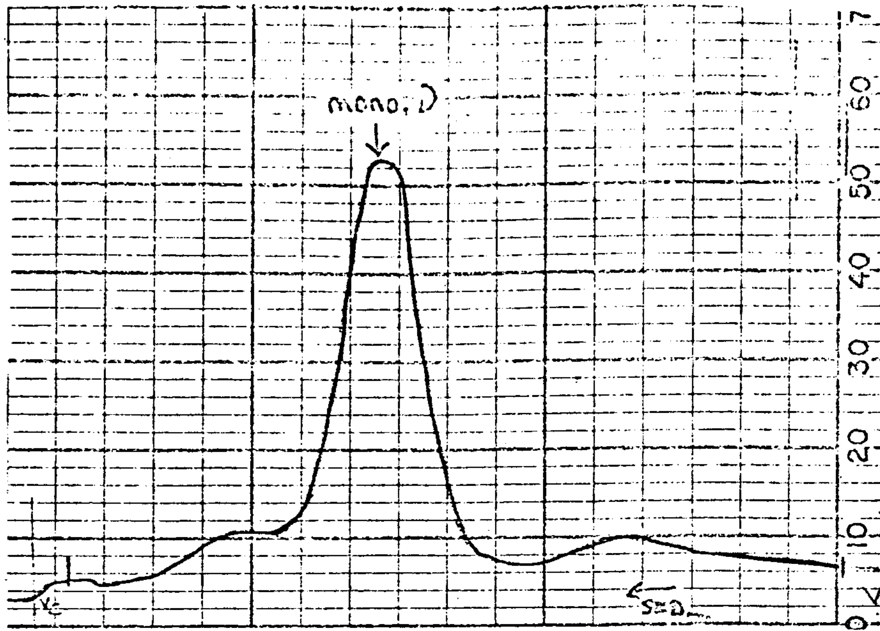


FIGURE 3

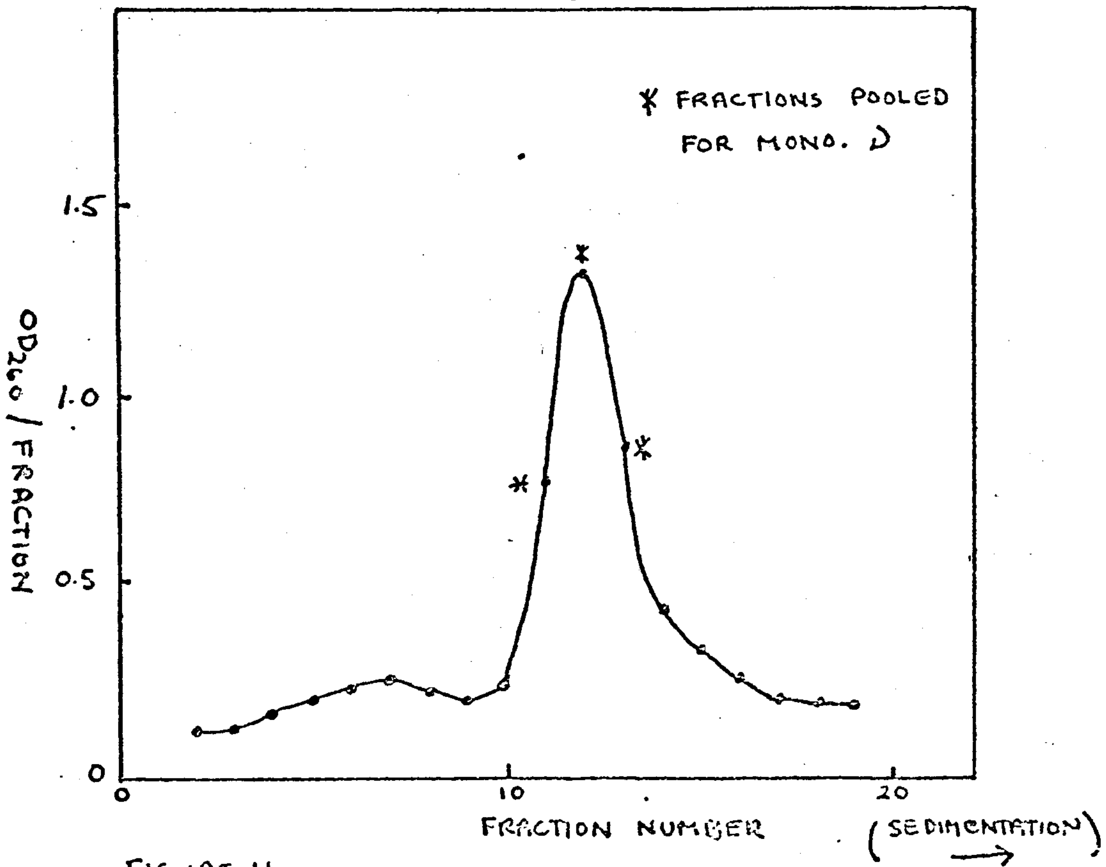


FIGURE 4

1) Preparation from histone F1 depleted chromatin

Chromatin was prepared as in VA). The final chromatin pellet was homogenized (20 strokes) in 0.5 M NaCl. The suspension was stirred at 4°C for 1-2 hours, then centrifuged at 45,000 rpm in a Beckman SW50 (titanium) rotor at 4°C for five hours. The pellet was homogenized in 25 mM sodium acetate pH 6.6 and dialyzed versus 6 l of the same buffer overnight. The treatment of the chromatin with 0.5 M NaCl removes histone F1.

The ν bodies were prepared by digestion of the F1 depleted chromatin by DNAase II. In a typical preparation 160 ml of the chromatin (10 OD units of DNA/ml in 10 mM sodium acetate pH 6.6) was brought to room temperature and DNAase II was added to 10 units per 1 OD unit of DNA. After a 60-minute digestion, the mixture was centrifuged for fifteen minutes at 15,000 rpm (in a Servall SS-34 rotor) after adjusting the pH to 8.0 with 10 mM Tris pH 11. The supernatant (S_1) was concentrated on a minicon devide to a final concentration of 21.4 OD_{260} units of DNA/ml (52 ml total volume). Sucrose gradients as described previously were run.

2) Preparation from histone F1 depleted, formaldehyde-fixed, sheared chromatin

Chromatin was prepared as in VC1). The final chromatin pellet was homogenized in 44 ml 10 mM Tris-HCl, pH 8. The chromatin was then sheared in a Virtis blender (30 volts, 90 sec.), and centrifuged for fifteen minutes at 15,000 rpm (in a Servall SS-34 rotor). The pellet was discarded. To the supernatant was added 6.25 ml 4 M NaCl slowly, while stirring at 4°C. Then 0.5 ml 5 M sodium bisulfite in 10 mM Tris-HCl pH 8 was added. The solution was stirred for one hour at 4°C, then centrifuged at 46,000 prm in the Beckman Ti50 rotor at 4°C for fourteen hours. A 0.5 ml 30% sucrose cushion was used at the bottom of the centrifuge tube. The pellet was suspended in triethanolamine (TEA) pH 7.8 and homogenized, then again centrifuged for fifteen minutes at 15,000 rpm (in a Servall SS-34 rotor). The pellet was discarded. The supernatant was dialyzed against 4 l 0.01 M TEA pH 7.8 for four hours. The total volume of the chromatin solution was adjusted to 40 ml (11 OD_{260} units DNA/ml). 22 ml of this solution was stored at 4°C (call this fraction N-chromatin). To eighteen ml of this solution was added 2 ml of 10% formaldehyde, 0.05 TEA, pH 7.8. The mixture was stirred at 4°C for twenty hours, then dialyzed overnight against 0.01 M TEA, pH 7.8 (3 x 4 l). The formaldehyde-fixed chromatin

(FF-chromatin) was then dialyzed against 6 1 25 mM sodium acetate pH 6.6 for four hours as was the N-chromatin.

DNAase II digestion was carried out as described in VC1) except that the digestion was for 90 minutes.

3) Preparation from chromatin without removal of fraction enriched in transcribed sequences

This was done as in VC1) except that histone F1 was not removed from the chromatin, and the DNAase II digestion was for 90 minutes. Part of the monomer ν body pool from the first isokinetic sucrose gradient was formaldehyde-fixed.

The monomer ν body fraction was dialyzed against 10 mM TEA pH 7.8 (2 x 6 l). 4.5 ml was taken and 10% formaldehyde in 10 mM TEA pH 7.8 (0.5 ml) was added with stirring. After an incubation of 24 hours at 4°C, the mixture was dialyzed against 10 mM TEA pH 7.4 (6 l). A second sucrose gradient was then run as in VB).

D) Electron microscopy

Negative staining was used to visualize the ν bodies. Many preliminary trials were done with various stains to determine which gave the most satisfactory results. Ammonium molybdate (pH 6.9), ethanolic phosphotungstic acid (PTA), uranyl acetate in 90% ethanol,

aqueous uranyl acetate (1%, pH 4.35), and uranyl oxalate (pH 6.9) were all tried with no great success. Of these, however, uranyl acetate and uranyl oxalate were the best but the contrast was poor.

After about six weeks of testing stains, a paper came out by Van Holde et al.[10] on the electron microscopy of chromatin particles from nuclease digestion of calf thymus chromatin. I tried their method of negative staining, which I will describe, and got excellent results.

In the procedure, 5 μl of the ν body preparation (about 1 OD_{260} unit DNA/ml in 10 mM ammonium acetate pH 6.5) was mixed with 20 μl of freshly prepared 1% uranyl oxalate (pH 6.9) (prepared according to Mellama et al.[19]) in a plastic weighing dish on ice. All stains and sample solutions were kept on ice during the grid preparation.

A drop of the mixture was applied to a carbon-coated 400-mesh grid (prepared according to Griffith[20]) using a micropipet and allowed to sit for one minute. The excess solution was then blotted off with a piece of filter, leaving a thin layer. Immediately, a drop of 1% uranyl acetate (pH4.35) was placed on the grid. After one minute the excess stain was removed and the grid allowed to dry. The grid was examined in the electron microscope within a few hours. Control grids, where the sample was just buffer or buffer and latex particles (.109 ± .002 μ, Pelco) were prepared in the same way.

The electron micrographs were taken with a Philips EM301 operating at 60 or 80 KV on sheets (Kodak 4489). The magnification of 59,000 x was calibrated either with negatively stained latex spheres (.109 ±.002 μ) or with a carbon grating replica having 54,800 lines/in. The chromatin subunit particles were measured from the negatives with a Leitz and Wetzlar microscope with a calibrated eyepiece (graduated to 0.1 mm, magnification 12.5 x).

VI. Results and Discussion

A) Preparation of ν bodies

All the preparations of ν bodies used for the electron microscope studies were done by J. Gottesfeld with the exception of the last one (see VA) and B) for experimental details) with which I was also involved. A comparison of the properties of ν bodies prepared from normal (i. e., not F1 depleted) and F1 depleted chromatin is given in Table I. The preparation of ν bodies I was involved with was only characterized by an approximate $S_{20,w}$ value of 13.1 S (from the second sucrose gradient, Figure 3) and by electron microscope studies. It was assumed it would have properties similar to other ν body preparations from normal chromatin. No analysis was done on the fraction thought to be multimer ν bodies.

TABLE I: Properties of ν body preparations from rat liver chromatin used in this study

Type of chromatin ν bodies prepared from	$S_{20,w}$ [a]	Length [b] DNA (base pairs)	Density [c] (CsCl)	Histone/DNA [d] (wt./wt.)	Gel electrophoresis on extracted histones
Native	13.1	210	1.422	1.1 (± .1)	not done
F1-depleted	11.4	175	1.448	.9 (± .1)	F2B, F2A2, F2A1, F3

[a] From the second sucrose gradient. Numbers are approximate since no marker of known $S_{20,w}$ was used.

[b] From alkaline sucrose gradient analysis.

[c] From CsCl gradients on formaldehyde-fixed ν bodies. For native chromatin the ν bodies were fixed after preparation. For F1 depleted chromatin, the chromatin was fixed prior to preparation of the ν bodies.

[d] Calculated from ρ_{CsCl} assuming $\rho_{histone} = 1.245$ g/cc and $\rho_{DNA} = 1.695$ g/cc.

Comparison of the molecular parmeters observed for ν body preparations from several laboratories[6,8,10,12,13] shows some similarities and some differences. Most monomer preparations, including ours, contain from 180-220 base pairs of DNA per particle, except for Van Holde's PS particles[8,10] which have only 110-120 base pairs. The ratio of protein to DNA ranges from 1.1 for our preparation to 1.3 for others, and the buoyant density in CsCl gradients goes from 1.414 g cm^3 for the ν particles from chick erythrocyte isolated by Olins and Olins[6] to 1.45 g cm^3 for the particles isolated by Noll[12]. However, our value for the buoyant density (from which the protein to DNA ratio was calculated) is the result of only one determination and is thus only approximate.

From Table I it is apparent that ν bodies prepared from Fl depleted chromatin contain about 40 base pairs less DNA than ν bodies prepared from normal chromatin. One could postulate that this stretch of base pairs is protected by histone Fl in normal chromatin, however, it is not known yet whether histone Fl is associated with ν bodies prepared from normal chromatin. It is of interest that Noll[12] has found that on digestion of chromatin in rat liver nuclei with micrococcal nuclease the DNA in the monomer ν body fraction consists of a doublet of 205 ± 15 base pairs and 170 ± 10 base pairs. With longer digestion times, the relative intensities of the two bands on

polyacrylamide gels shifts in favor of the lower molecular weight band. His data thus favor a model where the true monomer DNA length is 205 base pairs and the 170 base pairs of DNA is a degradation product of it produced by cleavage about 35 base pairs within the monomer. From Gottesfeld's data it would seen that the 35 base pairs, which are digested away, are somewhat protected by histone F1, but that there is a region between histone F1 and the rest of the complex which is less resistant to nuclease attack.

B) Electron microscope studies

Specimens were prepared by the method given in VD) of ν bodies from normal chromatin (ν (+F1)), histone F1 depleted chromatin (ν(-F1)), histone F1 depleted and formaldehyde-fixed chromatin (ν(FF-F1)), and formaldehyde-fixed ν bodies from normal chromatin (ν(FF+F1)). Electron micrographs are shown in Figures 5 to 8. Two types of areas are usually observed. In the heavily stained areas the particles appear to be aggregated but outside these areas are lightly stained regions where roughly spherically shaped particles can be observed. Similar particles were not observed in control grids. Particles in this type of area were measured for each different specimen. A histogram for each of the four preparations of ν bodies is shown in Figure 9a-9d. For ν(+F1) bodies the average diameter is

Figure 5: Electron micrograph of a field of ν (+Fl) particles prepared by the method given in VB), and stained by the method given in VD). Bar represents 1000 Å.

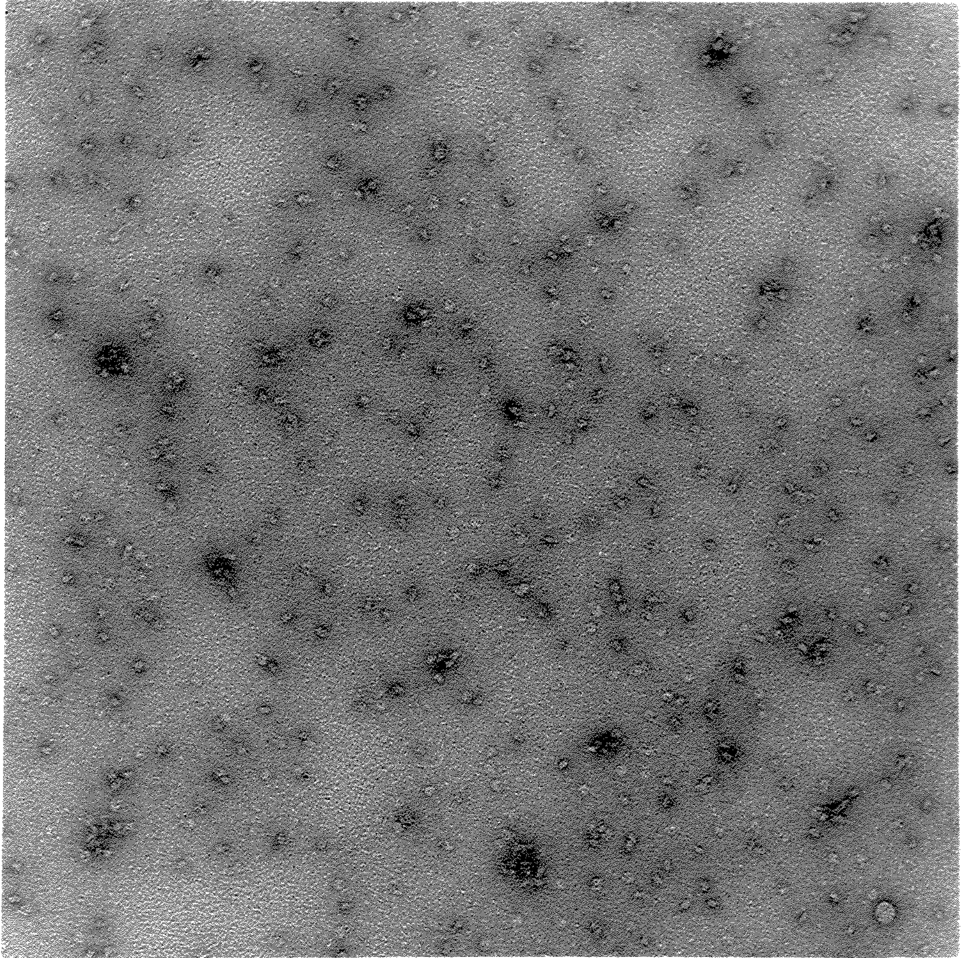


Figure 6: Electron micrograph of a field of ν (-F1) particles prepared by the method given in VC1) and stained by the method given in VD). Bar represents 1000 Å.

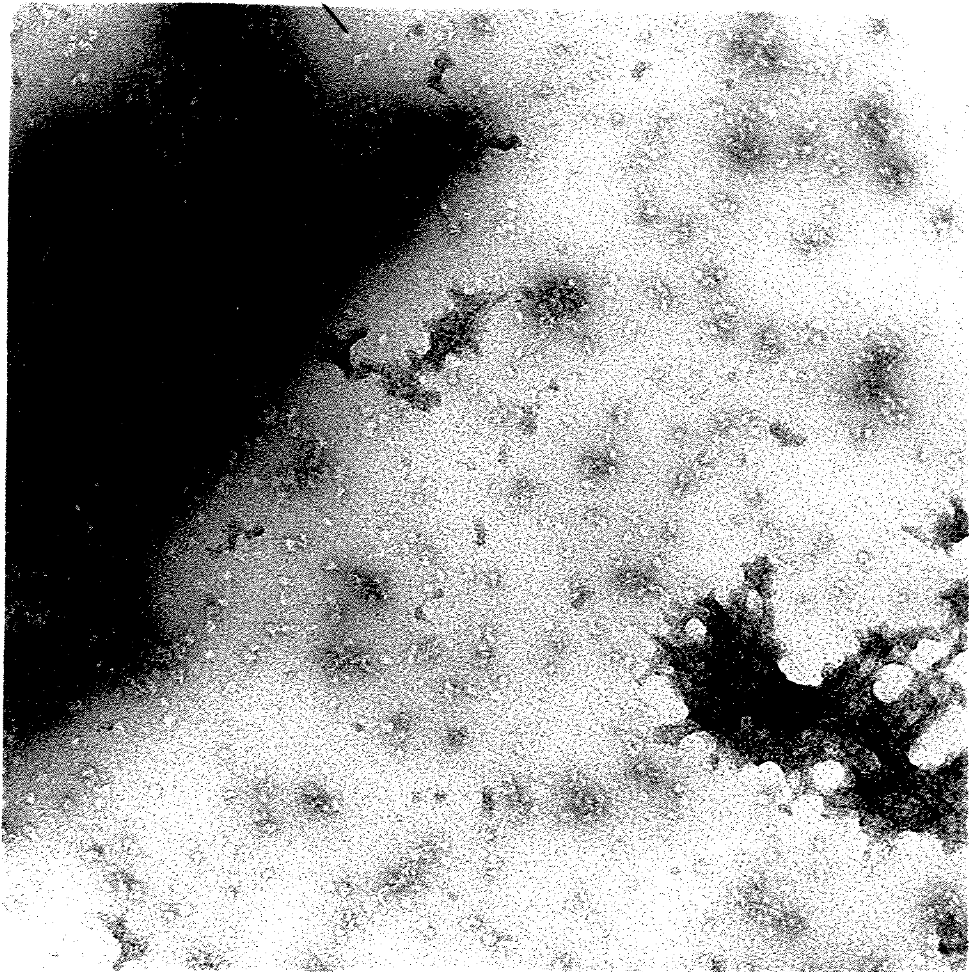


Figure 7: Electron micrographs of a field of ν (FF - F1) particles prepared by the method given in VC2) and stained by the method given in VD). Bar represents 1000 Å.

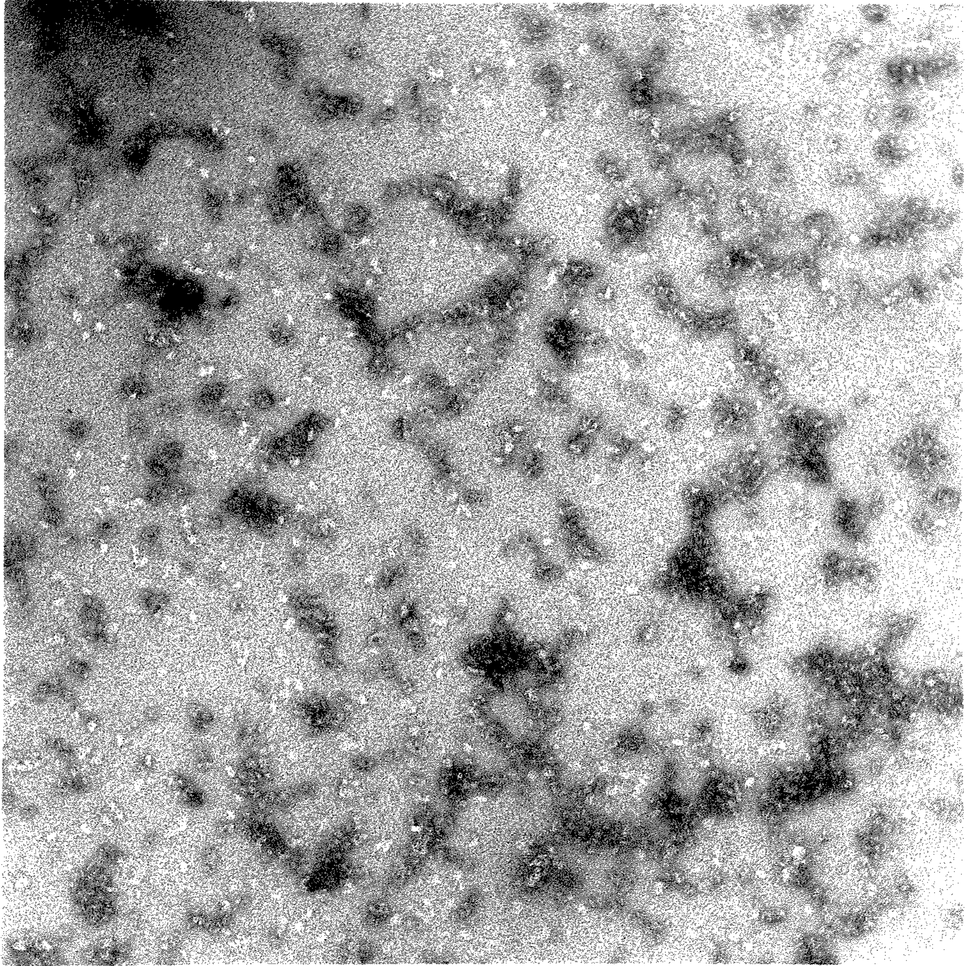


Figure 8: Electron micrograph of a field of ν (FF + F1) particles prepared by the method given in VC3) and stained by the method given in VD). Bar represents 1000 Å.

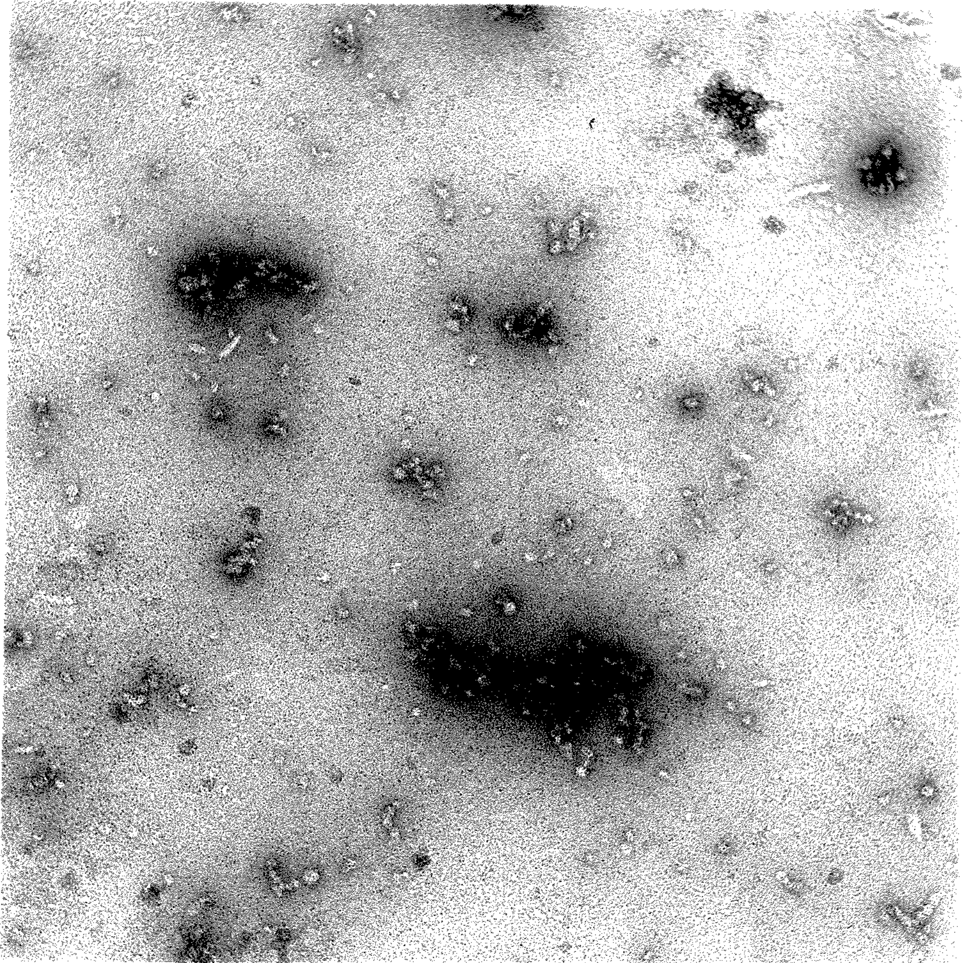


Figure 9: Histograms of diameters of ν bodies from rat liver chromatin.

a) ν (+Fl) particles. 125 particles were measured in a field similar to that shown in Figure 5.

b) ν (−Fl) particles. 100 particles were measured in a field similar to that shown in Figure 6.

c) ν (FF−Fl) particles. 85 particles were measured in a field similar to that shown in Figure 7.

d) ν (FF+Fl) particles. 50 particles were measured in a field similar to that shown in Figure 8.

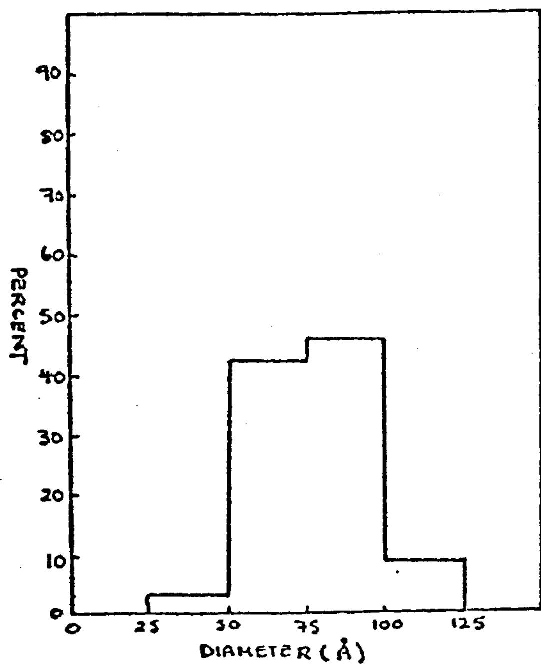


FIGURE 9a)

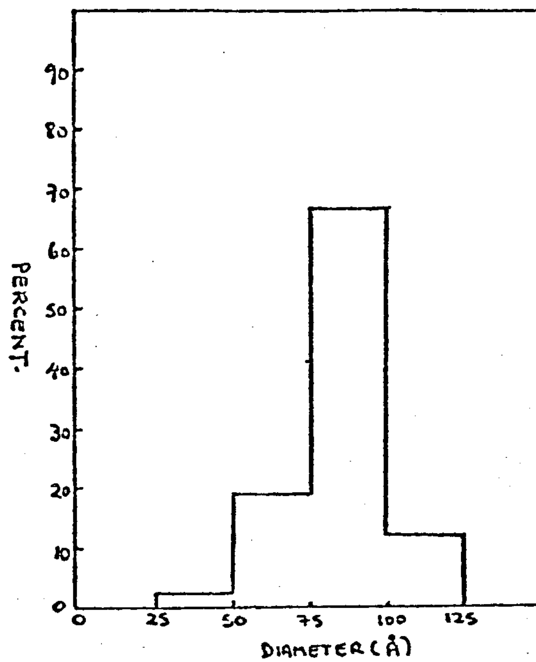


FIGURE 9 b)

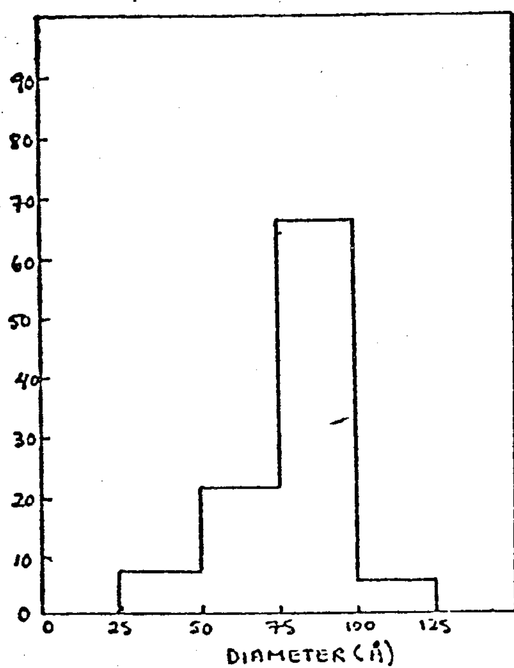


FIGURE 9 c)

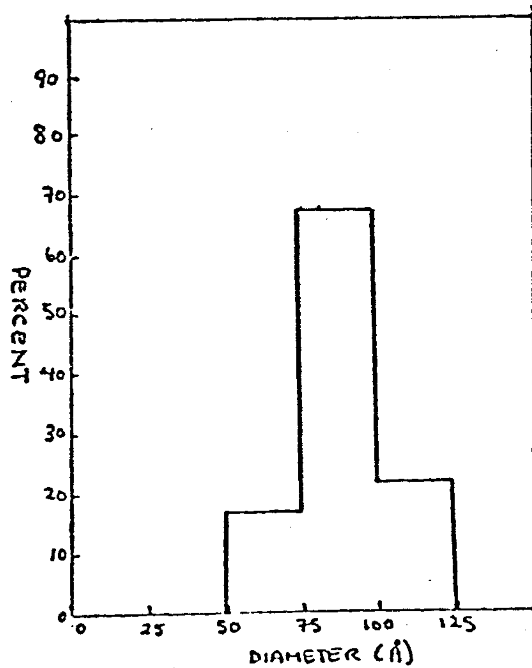


FIGURE 9 d)

80 ± 17 Å ($\pm$ S. D. number of particles measured (n) = 125); for $\nu(-F_1)$ bodies, 87 ± 19 Å (n = 100); for ν(FF−F1) bodies, 81 ± 13 Å (n = 85); and for ν(FF+F1) bodies, 96 ± 23 Å (n = 50). No difference in size or shape of the ν bodies prepared from normal or F1 depleted chromatin can be discerned from the electron micrographs. The presence of F1 is also not necessary for obtaining the characteristic x-ray pattern of chromatin[14].

The average diameter of the ν bodies from our preparations is in good agreement with that reported by Van Holde et al.[10] for PS-particles obtained by partial nuclease digestion of calf thymus chromatin. The number-average diameter for the PS-particles was 74 ± 20 Å. The ν bodies are also similar in size to linear arrays of spheroid particles observed by Olins and Olins[5] in preparations of isolated nuclei swollen in water, centrifuged onto carbon films, and stained. For rat thymus, rat liver, and chick erythrocyte nuclei the average diameters of the particles were 83 ± 23 Å, 60 ± 16 Å, and 63 ± 19 Å, respectively. A similar bead-like structure was observed in an SV-40 minichromosome (formed when monkey-cell cultures were infected with SV-40), where each "bead" had a diameter of about 100 Å (and contained about 170 base pairs of DNA), and the bridge between the beads was about 40 base pairs of DNA[7].

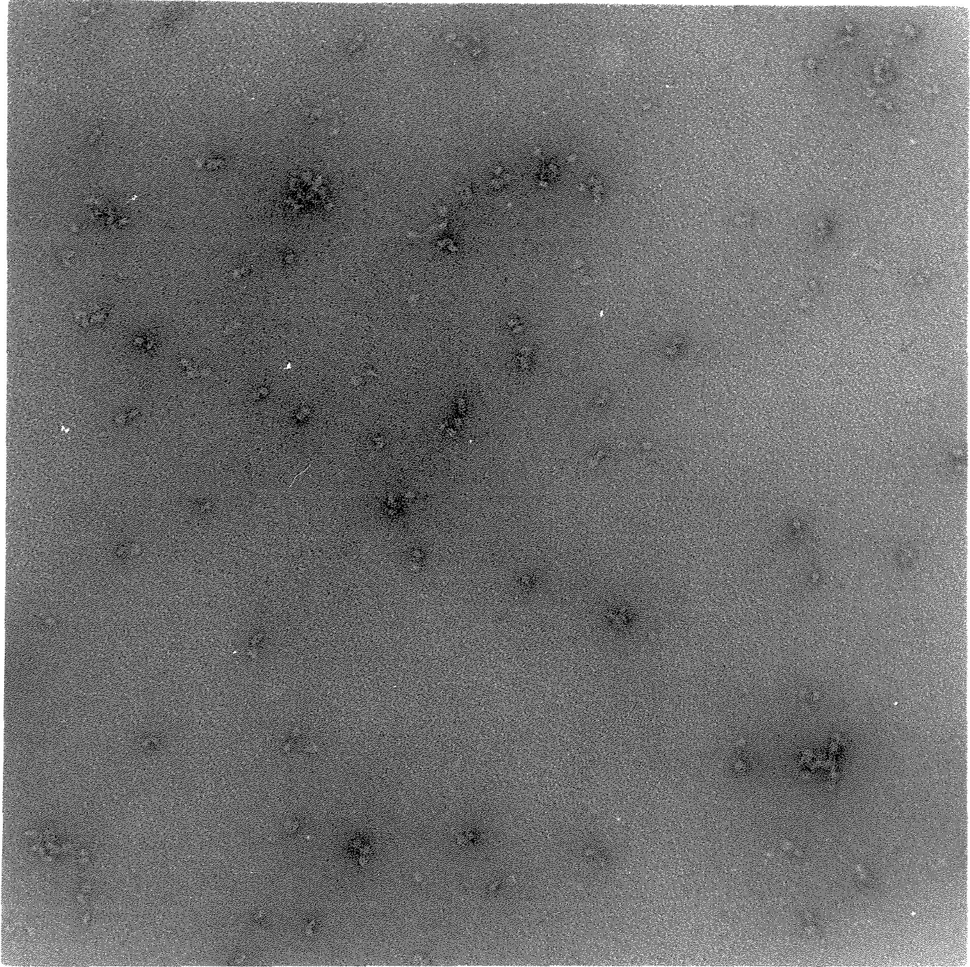
Few if any doublet particles or particles with tails (i. e., thin strands of base DNA) that have been reported by Van Holde et al.

were observed in the electron micrographs. The presence of multimers (and monomers) has also been detected in fragments of chromatin isolated from formaldehyde-fixed and sonicated chick erythrocyte nuclei[6], although no pictures are available.

Also, when the pools thought to be enriched in multimer ν bodies (see VB)) were examined in the electron microscope, no easily identifiable doublets, triplets, etc., of monomer ν bodies were seen. In some micrographs (see Figure 10) some monomers seem to be touching edge to edge, but too few of these were observed to draw any conclusions. However, an increase in the number of large heavily stained aggregates of particles was noticed in these micrographs. Within these areas it is impossible to see any fine structure of the particles.

The absence of doublets or higher multimers in the micrographs of the monomer fraction could be due to several things. First from Van Holde's study it seems that doublets occur very infrequently as he only found 70 in "all available micrographs". Many of my micrographs are somewhat out of focus and visualizing a 20 Å wide filament joining two monomers under these conditions is impossible, thus diminishing the chance of noticing dimers. Second, and more likely, it could be due to the different methods in preparation of the ν bodies. After purification on two sucrose gradients the monomer

Figure 10: Electron micrograph of a field of a multimer ν-body fraction (④) (see VB) and Figure 2). Note the presence of some edge-to-edge monomer ν particles. Bar represents 1000 Å.



fraction from our preparation is quite cleanly separated from the multimer fraction. Van Holde's[8,9] method of preparation of PS particles, however, would select for both monomer and multimer fractions.

This, unfortunately, still does not explain why no definite dimers, trimers, etc., were observed in the multimer fraction of our preparation. Since an increase in aggregation was noticed in these fractions, they could be just non-specific aggregates of monomer ν bodies. Since no analysis on the size of the DNA in this fraction has been done, this possibility cannot be ruled out. However, it is contrary to what M. Noll[12] observed for the digestion of rat liver chromatin with micrococcal nuclease. Sucrose gradient analysis of the products of the digestion shows a regular series of seven resolved peaks which were shown to contain DNA of sizes 205 ± 15 base pairs for the monomer, 405 ± 31 base pairs for the dimer, and 605 ± 40 base pairs for the trimer.

From the electron micrographs and the molecular parameters of the monomer ν bodies from our preparation, the tentative conclusion may be drawn that they are related to the ν bodies of Olins and Olins[5,6], Van Holde's PS particles[6,10], and the chromatin subunits reported by Noll[12]. Thus our data add to a growing amount of evidence that supports a model for chromatin in which much of the DNA is tightly folded in small globular complexes with histone (and

perhaps some non-histone protein). The complexes are separated by regions susceptible to nuclease attack. Furthermore, J. Gottesfeld has shown by sucrose gradient analysis that in the isolated fraction (15-20%) of the chromatin which is enriched in template active sequences (see VB)) for preparation details) there are no particles which have the sedimentation properties of ν bodies[18]. Thus, it seems the histone-DNA interaction is modified on going from inactive to active chromatin, but the sequence of events is not known.

VII. Future studies

A) Rearrangement of histones

During the preparation of the ν bodies, histones may become bound to new sites on the DNA to make the relationship between the structure of chromatin *in vitro* to *in vivo* ambiguous. Under certain conditions, such as high calcium ion concentration or high sodium chloride concentration, bound histones have been shown to be in labile equilibrium with unbound histones[22]. To check to see if this is the case under the reaction conditions used to prepare ν bodies in our studies, an exchange experiment similar to that of Clark and Felsenfeld[22] should be done. This would involve mixing radioactive DNA (prepared from rat liver chromatin by the method of Bernardi

and Felsenfeld[23]) with the chromatin and carrying out the digestion procedure. The ν-body fraction would then be analyzed for radioactivity, the presence of which would indicate that exchange had occurred. Of course, it could be that after exchange the histones and DNA are arranged in precisely the same way as before the exchange, but this is not known.

B) Analysis of the components of ν bodies

1) DNA

We have heard[24] that Noll has done a prolonged digestion with DNAase I on chromatin in rat liver nuclei, followed by gel analysis of the DNA produced. The gels showed bands corresponding to 10 to 210 base pairs of DNA with spacings of 10 base pairs between the bands. This would imply that in the ν body structure every tenth base pair is open to attack by DNAase. It would be nice to confirm this observation by doing a prolonged digestion with DNAase II on our isolated ν-body preparation, followed by analysis by gel electrophoresis of the DNA produced.

As mentioned in the previous section, no analysis has been done on the DNA within the multimer ν-body fraction. This fraction could be deproteinized and the lengths of DNA examined on polyacrylamide gels.

2) Histone

a) Relative amounts of histone per ν body

No studies have been done on the relative amounts of each of the histones in ν bodies, although many people have postulated that there are two of each of the histones F2A1, F3, F2A2, and F2B, and perhaps one histone F1 per ν body. Quantitative gel electrophoresis[25,26] on histones extracted from the ν-body fraction would give the average of the relative amounts. However, the possibility could exist that there are different subfractions of ν bodies, not separated by the purification procedure, with different relative amounts of histones.

Nonetheless, the information from quantitative electrophoresis would be useful. The procedure would involve extraction of the histones from the ν bodies. The histone fraction would then be run on 15% polyacrylamide gels following the procedure of Fambrough et al.[25] or Johns[26]. The histones would also be fractionated by column chromatography on Amberlite LG-50, followed by preparative electrophoresis[25,26]. The purified fractions would be used to relate the intensity of a stained band on a gel to the amount of the fraction present. This would be done for each histone fraction since the stain-binding capacities could be different.

b) Modification of histone structure

Histone structure can be modified by a variety of enzymatic reactions such as methylation, acetylation, and phosphorylation. Various studies[27] have shown that methylation of histones (usually lysine residues of F2A1 and F3) can be correlated with a condensation of the chromatin and a loss of RNA synthetic activity. On the other hand, acetylation of lysine residues of F2A1 and F3, and phosphorylation of F1, F2B, and F3 have been correlated with the diffuse state of chromatin and an increased activity in transcription[16,27]. Since the DNA in ν bodies is condensed and thought to be inactive in transcription, it would be of interest to compare the level of histone acetylation, phosphorylation, and methylation in ν bodies with that of whole chromatin (or that of the template active chromatin fraction).

The experiment could be done by incubating cultures of rat liver cells with the appropriate radioactive precursor (methionine-(methyl-^{3}H) for methylation, sodium acetate-^{3}H or -^{14}C for acetylation, and ^{32}P-orthorphosphate for phosphorylation). The chromatin would then be isolated and ν bodies prepared by methods given previously. The histones would then be extracted, fractionated, and the specific activity determined. The results would then be compared to those for whole chromatin or the template active fraction of chromatin.

One would expect a greater degree of methylation and a lesser degree of acetylation and phosphorylation for the histones of the ν bodies.

Microheterogeneity in the enzymatic modifications of histones from whole chromatin has been noted[28]. For instance, from sequence analysis of calf thymus histone F3 about one quarter of the peptide molecules subject to methylation contain unmodified lysine[28]. Sequence analysis of each of the histones from the ν body fraction should be done to ascertain whether such microheterogeneity exists within this fraction. Existence of microheterogeneity could mean that there is more than one structure of ν bodies. It could also mean that within one ν body, there are differences between two of the same histone fraction.

Since the ν bodies are folded complexes of DNA and histone, it would seem that further modification of the histones should cause the unfolding of the structure. To test this hypothesis, the ν body fraction could be phosphorylated or acetylated using isolated kinases[27] or acetyl-transferases[29]. Following the enzymatic reaction, the ν body fraction would be examined on sucrose gradients to see if this modification had altered sedimentation properties. Also the melting behavior as well as the susceptibility to DNAase and appearance in the electron microscope could be checked. If it turned out that the structure does unfold, then this could be part of the mechanism for conversion of inactive to active chromatin.

3) Non-histone chromosomal proteins

It is not clear whether the non-histone proteins are involved in the structure of the ν bodies which makes them resistant to nuclease attack. From rough composition studies of this fraction, it seems that at most 10% of the total protein is non-histone. Thus at most there could be one non-histone protein (mol. wt. ~15,000) per ν body. Careful gel analysis of the extracted protein from ν bodies may reveal the presence of these proteins but they will be in such low quantities (since it is not likely that the same non-histone chromosomal protein is associated with all ν bodies) the analysis would be difficult.

A second approach to the problem would be reconstitution studies. The ideal way to do these studies would be to grow cells (rat liver cells) in the presence of radioactively labelled amino acids. The chromatin would be isolated, and the non-histone proteins separated under non-denaturing conditions. Then these radioactively labelled proteins would be mixed with histones and DNA (from non-radioactively labelled chromatin) and the chromatin reconstituted. Then ν bodies would be prepared and assayed for radioactivity. Unfortunately, the isolation of non-histone proteins under non-denaturing conditions is very difficult.

Another way would be to reconstitute chromatin with just histones and DNA. If ν bodies could be prepared from this

reconstituted chromatin which had the same properties as those prepared from native chromatin, this would imply that non-histone proteins are not needed for the structure of the ν bodies. This has been found in x-ray studies[14].

C) Optical or electron diffraction studies

If the ν bodies represent a homogeneous group of particles, then it may be possible to crystallize them or to get them to form an ordered two-dimensional array on an electron microscope grid. Studies by J. Gottesfeld using light scattering have shown that certain concentrations of ethanol (~50%) or divalent cations (Cu^{2+}, Ca^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+} ~10 mM) cause the ν bodies to aggregate[18]. This does not necessarily mean that the ν bodies will crystallize under these conditions, but it presents a good place to start. Attempts to grow microcrystals will involve incubating various concentrations of ν bodies with various concentrations of ethanol, and divalent cations. For two-dimensional arrays of particles the first method to be tried will involve placing a drop of the ν-body preparation on a Parlodion covered em grid. The grid will then be floated on a solution containing various concentrations of ethanol or divalent cations. The Parlodion will act as a dialysis membrane. The grids would be floated on the solution for various periods of time and then would be negatively

stained and checked in the electron microscope for two-dimensional arrays (or crystals) of ν bodies.

From the neutron scattering studies of Baldwin et al.[16], it seems that histone F1 plays a cross-linking role between subunits either intra- or inter-chain. Thus it would be interesting to take ν bodies prepared from F1-depleted chromatin and add varying amounts of purified histone F1 to see if this would induce either an ordered two-dimensional array or crystallization of ν bodies.

If such crystals or two-dimensional arrays could be made from ν bodies, then either electron or optical diffraction studies (both of which have been applied to various biological molecules[29-38]) could be done. From such studies information on the overall shape and symmetry of the νbody should be obtained. It may also be possible to discern the position of the DNA relative to the histones, if the resolution is good enough. However, such studies depend entirely on the ν bodies being a homogeneous mixture of particles, and this has not yet been shown.

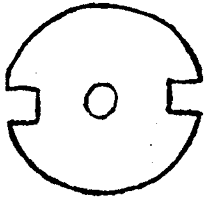
VIII. Addendum

Although ν bodies have been observed on chromatin from nuclei lysed on the electron microscope grid and then stained, they have never been observed clearly as "beads-on-a-string" in isolated chromatin. Thus, we have done some preliminary studies using a modification of the technique used by Miller and Beatty[39,40] to see if a "beads-on-a-string" structure could be seen with isolated sheared chromatin.

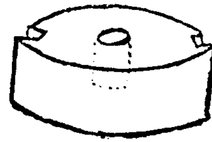
A carbon-coated grid was placed in the bottom of the well in the plexiglas holder shown in Figure 11. Sheared chromatin (50 λ of a 1OD_{260}/ml solution in 10 mM ammonium acetate, see VA) for preparation details) was then placed in the well. At this point care was taken that the grid did not flip over and that no air bubble formed below the grid. After the addition of the sample, the well was covered with a moist cover-slip and placed in a centrifuge tube for the Beckman SW25-1 rotor. The samples were centrifuged at 22,000 rpm for 1 to 1.5 hours. After centrifugation, the grid was removed by turning the grid holder over (without removing the supernatant) and allowing the grid to float to the top. It was then removed with fine point forceps and allowed to dry. The grid was stained by the method given in VD), and then visualized in the electron microscope. Control grids, where the sample was just 10 mM ammonium acetate, were also prepared.

Examples of the electron micrograph obtained are given in

Figure 11: Grid holder used for centrifuging samples onto grids. a) gives a top view, and b) a side view. The diameter of the holder is 2.4 cm while the diameter of the central well is .4 cm. The volume of the central well is about 50 λ. The height of the holder is 1 cm.



(a)



(b)

Figures 12 and 13. As can be seen in these figures, the sheared chromatin prepared by this method consists of regions of presumably bare DNA (strand width about 15 Å) interspersed by regions of either coiled DNA or DNA covered by proteins. No definite "beads-on-a-string" type structure is observed. Thus, it would seem that under these conditions of preparation of the sample for the electron microscope studies, this type of structure is disrupted. However, under almost identical conditions, the isolated monomer ν bodies seem not to be disrupted. The difference in the two procedures is the centrifugation step. Attempts were made to visualize the sheared chromatin using the method given in B4), but so far this has failed.

While this thesis was in preparation, Olins et al.[41] reported visualizing ν bodies in preparations of isolated chromatin and in reconstructed chromatin from chick erythrocytes. The difference between our procedure and theirs is that the solution of chromatin was made 1% in formaldehyde for at least 30 minutes before centrifugation. The chromatin was then centrifuged through 10% formaldehyde (pH 7). After centrifugation, the grids were washed with dilute Kodak Photo-Flo, dried, and then stained with ethanolic phosphotungstic acid or ammonium molybdate. It would thus seem that it is important to formaldehyde-fix the chromatin before preparing the electron microscope grids in order to visualize ν bodies as "beads-on-a-string", although it is not clear why this should be so.

Figure 12: Electron micrograph of sheared rat liver chromatin prepared by procedure given in the ext. Bar represents 1000 Å.

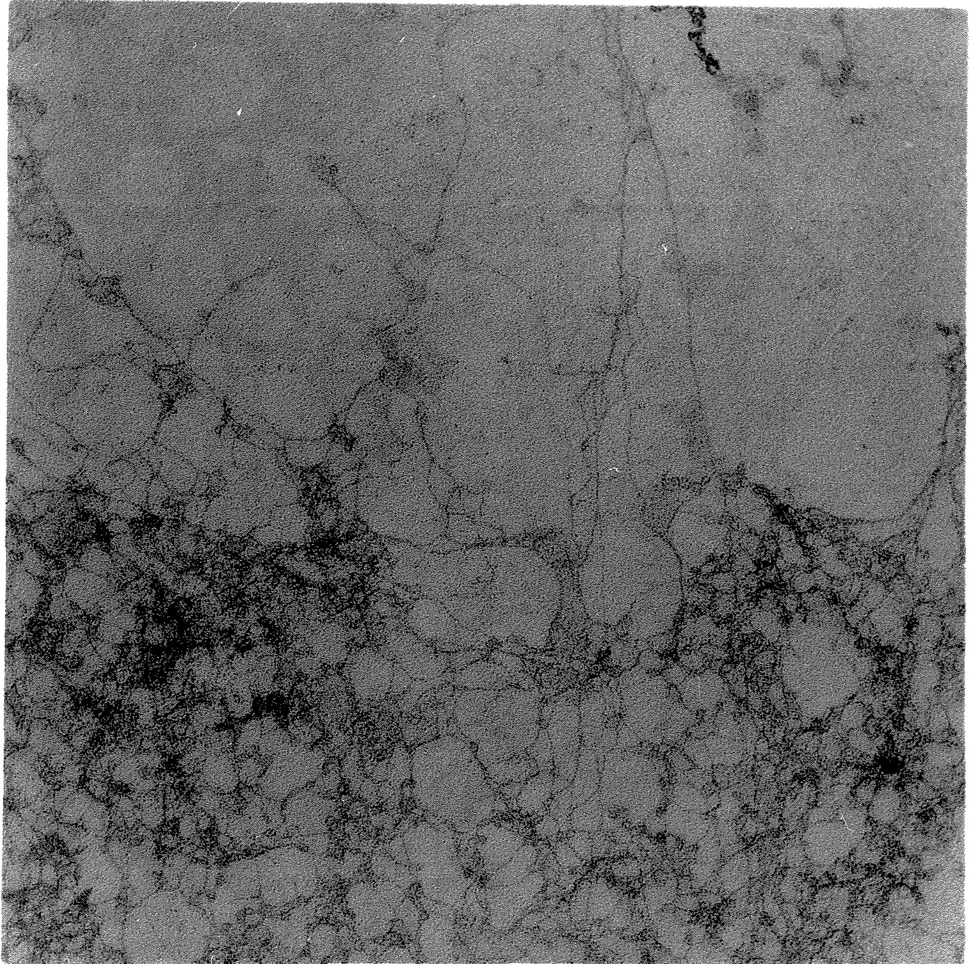
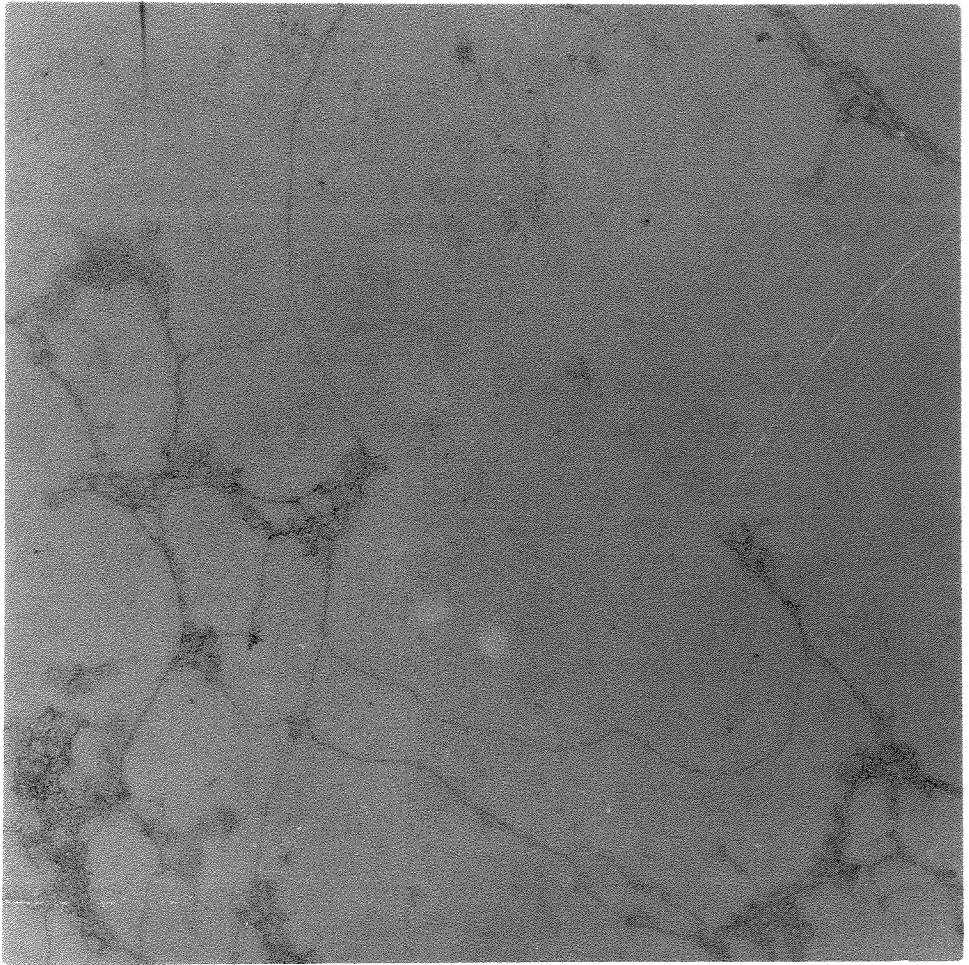


Figure 13: Electron micrograph of sheared rat liver chromatin prepared by procedure given in the text. Bar represents 1000 Å.



IX. References

1. M.H.F. Wilkins, G. Zubay, and H.R. Wilson, J. Mol. Biol. 1, 179 (1959).
2. V. Luzzati and A. Nicolaieff, J. Mol. Biol. 7, 142 (1963).
3. J.F. Pardon, M.H.F. Wilkins, and B.M. Richards, Nature 215, 508 (1967).
4. S. Bram and H. Ris, J. Mol. Biol. 55, 325 (1971).
5. A.L. Olins and D.E. Olins, Science 183, 330 (1974).
6. A.M.B. Senior, A.L. Olins, and D.E. Olins, Science 187, 173 (1975).
7. J. Griffith, Science--in press, 1975.
8. R. Rill and K.E. Van Holde, J. Biol. Chem. 248, 1080 (1973).
9. C.G. Sahasrabuddhe and K.E. Van Holde, J. Biol. Chem. 249, 152 (1974).
10. K.E. Van Holde, C.G. Sahasrabuddhe, and B.R. Shaw, Bioch. Bioph. Res. Comm. 60, 1365 (1974).
11. K.E. Van Holde, C.G. Sahasrabuddhe, and B.R. Shaw, Bioch. Bioph. Res. Comm.--in press (1975).
12. M. Noll, Nature 251, 249 (1974).
13. L.A. Burgoyne, D.R. Havish, and J. Mobbs, Bioch. J. 143, 67 (1974).

14. R.D. Kornberg and J.O. Thomas, Science 184, 865 (1974).

15. R.D. Kornberg, Science 184, 868 (1974).

16. J.P. Baldwin, P.G. Boseley, E.M. Bradbury, and K. Ibel, Nature 253, 245 (1975).

17. K. Marushige and J. Bonner, J. Mol. Biol. 15, 160 (1966).

18. J. Gottesfeld, private communication.

19. H. Noll, Nature 215, 360 (1967).

20. J.E. Mellema, E.F.J. Van Bruggen and M. Gruber, Biochim. Biophys. Acta 140, 180 (1967).

21. J. Griffith in "Methods in Cell Biology" (D.M. Prescott, ed.) Vol. VII, p. 129, 1973.

22. R.J. Clark and G. Felsenfelt, Nature New Biology 229, 101 (1971).

23. F. Doppler-Bernardi and G. Felsenfeld, Biopolymers 8, 733 (1969).

24. R. Henderson, personal communication of M. Noll's results.

25. D. Fambrough, F. Fujimura, and J. Bonner, Biochemistry 7, 575 (1968).

26. E.W. Johns, Bioch. J. 104, 78 (1967).

27. V.G. Allfrey in "Histones and Nucleohistones" (D.M.P. Phillips, ed.) pp. 260-281. Plenum Publishing Co., New York, 1971.

28. R. De Lange, E. Smith, and J. Bonner, Bioch. Bioph. Res. Comm. 40, 989 (1970).

29. D. Gallwitz and I. Jures, Bioch. Bioph. Acta 263, 315 (1972).

30. R. Murray and R.P. Ferrier, J. Ultrastruc. Res. **21**, 361 (1968).

31. R. Glaeser and G. Thomas, Biophys. J. **9**, 1071 (1969).

32. A. Klug, Phil. Trans. Roy. Soc. London **B261**, 173 (1971).

33. M. Moody, ibid. **261**, 181 (1971).

34. A. Elliot, ibid. **261**, 197 (1971).

35. E. O'Brien, P. Bennett and J. Hanson, ibid. **261**, 201 (1971).

36. D. DeRosier, ibid. **261**, 209 (1971).

37. J. Finch and A. Klug, ibid. **261**, 211 (1971).

38. R. Growther, ibid. **261**, 221 (1971).

39. O.L. Miller, Jr., B.R. Beatty, J. Cell Physiol. **74** (Supplement 1) 225 (1969).

40. O.L. Miller, Jr., B.R. Beatty, Science **164**, 955 (1969).

41. A.L. Olins, R.D. Carlson and D.E. Olins, J. Cell Biol. **64**, 528 (1975).