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I. PHYSICS

Biophysics

ON THE FORMATION OF THE NUCLEOSOMES *IN VITRO*: AN ELECTRON MICROSCOPIC STUDY

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Chromatin was reconstituted with DNA and an equimolar mixture of the histones H2A, H2B, H3 and H4. The nucleosomes formed at different stages of the reconstitution experiment were examined under the electron microscope and their sizes determined. The subnucleosomes formed at 1.2 to 1.0 M NaCl as a result of binding of the histones H3 and H4, had dimensions of the order of 75 to 80 Å. The histones H2A and H2B started to bind with these core particles when the molarity was reduced to 0.65 M NaCl. The process continued further upto 0.2 M NaCl. These artificial nucleosomes had a dimension of 115 Å. This was in agreement with the size of nucleosomes from natural chromatin.

Keywords : Electron microscopy; Nucleosome dimension; Chromatin structure.

INTRODUCTION

IT is now established that chromatin of all eukaryotic systems consists of a repeating unit, the nucleosomes, composed of eight histone molecules (two each of H2A, H2B, H3 and H4) and 200 base pairs of DNA. The nucleosomes are held together by lengths of extranucleosomal DNA, with which are associated the histones H1 and H5 (Kornberg, 1977). Two lines of experimental work were most helpful in revealing the contribution of different histones in chromatin structure. The first was the progressive depletion of histones from natural chromatin by salt extraction method and study of the resulting changes in conformation (Ohlenbusch *et al.*, 1967; and Burton *et al.*, 1978). The second was the synthesis of artificial chromatin from its fundamental constituents viz., DNA and histones (Germond *et al.*, 1976; Wilhelm *et al.*, 1978; and Oudet *et al.*, 1978). However, the results of reconstitution experiments were not always in agreement with each other and also with those on dissociation of natural chromatin. There are differences of opinion as regards the sizes of the repeating unit at different stages of synthesis and also the exact salt concentration at which they are formed. Further experimental work in this field is necessary.

In the present experiment, artificial nucleosomes were reconstituted from a mixture of DNA and the four histones (H2A, H2B, H3 and H4) in a high salt concentration, which was then gradually reduced. The complexes at different stages of synthesis, were directly observed under the electron microscope. The

formation of the subnucleosome and the final nucleosomal particles were visualised and their exact sizes determined.

Materials and Methods

(*a*) *Materials:*

Chicken erythrocyte histone H_N (a stoichiometric mixture of the histones H2A, H2B, H3 and H4) was obtained as a gift from Dr M. Champagne, IBMC, Strasbourg, France. Calf thymus DNA (D 1501) was purchased from Sigma Chemicals, USA. BAC (Benzyldimethylalkylammonium chloride) used for electron microscopy was a gift from Bayer, Germany. All other chemicals were of Merck analytical grade.

(*b*) *Reconstitution of Artificial Nucleosomes :*

Artificial nucleosomes were prepared by associating chicken erythrocyte histone H_N with calf thymus DNA as follows. The DNA (conc. 1 mg/ml) and the histones H_N (conc. 1 mg/ml) were mixed in 1:1 ratio by volume in buffer A (10 mM Tris-HCl, 0.25 mM EDTA, 5 mM DTT, *p*H 8.0) containing 2M NaCl. After 1 hr. of incubation at 4 °C, the NaCl concentration of the mixture was progressively decreased by dilution at 4 °C with buffer A: 15 min at 1.6 M NaCl, 15 min at 1.2 M NaCl, 30 min at 1.0 M NaCl, 30 min at 0.85 M NaCl, 30 min at 0.8 M NaCl, 30 min at 0.75 M NaCl, 15 min at 0.65 M NaCl, 15 min at 0.5 M and finally 15 min at 0.2 M NaCl. The final DNA concentration in the complex was 50 μg/ml. Next, aliquots were taken from 1.2 M, 1.0 M, 0.65 M, 0.5 M, 0.2 M steps and after final dialysis. They were separated from free histones in hydroxyapatite columns (Faulhaber & Bernardi, 1967) and then diluted in a fixation buffer B (0.1% glutaraldehyde, 5 mM TEA, 10 mM Na- Acetate, *p*H 8.0) to a final concentration of 10 μg/ml and kept overnight at 4 °C for fixation.

(*c*) *Preparation of Electron Microscopic Samples by BAC Method:*

For electron microscopy of DNA complexed with histones, a protein-free method for sample preparation was desirable. In the present work, a film formed by BAC instead of cytochrome *c* was used (Vollenweider *et al.*, 1975). In this method, a 0.2% stock solution of BAC in formamide was first prepared. Immediately before use, BAC stock was diluted 100 X in the buffer B so that the BAC concentration was .002%. These fixed DNA-Histone complexes were diluted to a DNA concentration of about 4 μg/ml in buffer B. The diluted BAC solution was then added to the DNA-H_N complex in 1:1 ratio by volume. A 50 μl drop of the final solution was put on a parafilm sheet and covered with a petridish. After 5 minutes, the drop was touched with collodion-coated grid. The grids were then stained with 0.1% uranylacetate and shadowed with 5 Å Platinum-Palladium.

(*d*) *Electron Microscopy and Measurements:*

Micrographs were taken with Siemens Elmiskop 101 B at an electronic magnifi-

cation of 20,000 X. Negatives were enlarged to about 100,000 times and size measurements were carried out with a magnifier fitted with a graticule with 0.1 mm division. For determination of the correct size of the nucleosomes, the increase in dimensions due to BAC film, deposition of stain and shadowing material had to be first estimated. For this purpose, DNA molecule was assumed as the standard. Its diameter was measured simultaneously with that of the nucleosomes in each micrograph. The total correction was estimated from the increase in diameter of the DNA molecule. The dimensions of the nucleosomes reported below, were obtained after correction in this way. Each result was based on the mean of about 50 particles distributed over two micrographs, prepared on two different dates.

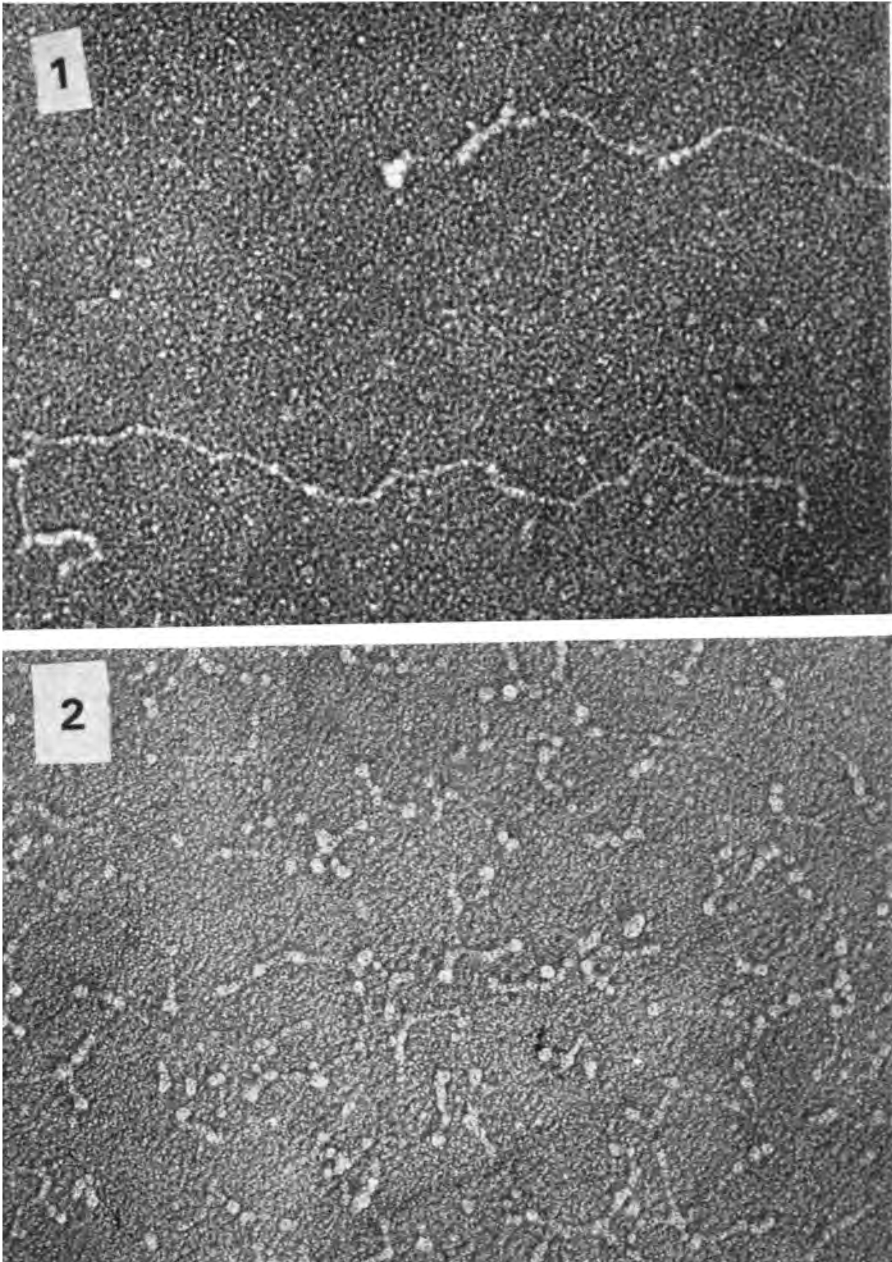
RESULTS AND DISCUSSIONS

Fig. 1 shows an electron micrograph of calf thymus DNA made with the BAC technique after staining and shadowing. The width of a DNA molecule in this micrograph was 100 ± 3 Å. DNA molecules micrographed with the protein monolayer technique (Kleinschmidt, 1968), stained and shadowed as above, measured about 200 Å in diameter. The extra width of 100 Å in the protein monolayer technique, was due to the layer of protein surrounding the DNA molecules. Owing to the low molecular weight of BAC (330), compared to that of cytochrome c (13000), the distortion in dimension was much less with the BAC technique. This made this technique more suitable for binding studies of DNA with proteins.

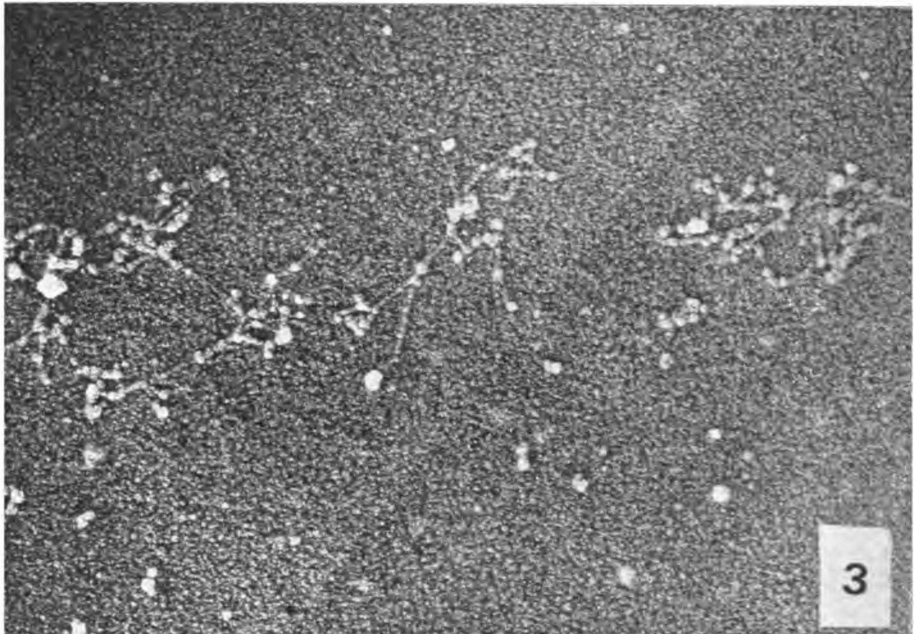
Figs. 2-6 are electron micrographs of DNA-histone complexes taken from different steps of the dilution experiment. They revealed clearly the preliminary formation of the subnucleosomal particles and the gradual changes in their dimensions until the completed nucleosomes were finally produced.

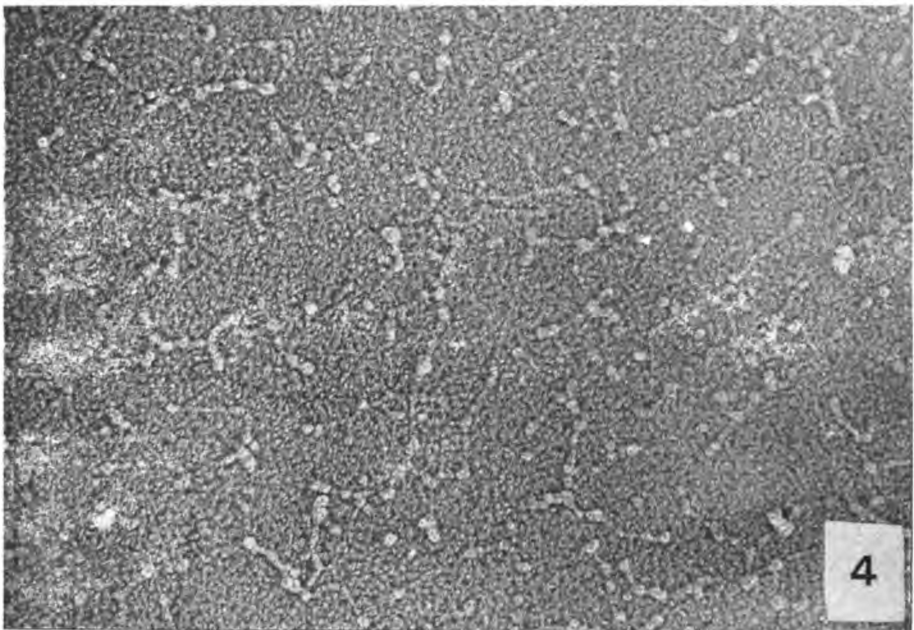
Figs. 2 and 3 represent reconstituted complexes formed at 1.2 and 1.0 M NaCl. Spherical particles of uniform size, distributed along the DNA molecules could be observed in both the figures. The average sizes of the particles in these two micrographs were 75 ± 5 Å and 80 ± 6 Å respectively. The frequency of the particles per unit micron length of DNA was almost the same in both cases.

Burton *et al.* (1978) showed that in the molarity range 2.0 to 1.2 M NaCl, H3 and H4 bound reversibly with DNA. On the other hand Wilhelm *et al.* (1978) reported that H3 and H4 combined in the molarity range of 1.2 to 0.85 M NaCl. There is a discrepancy between these two results. The present observations show that the size and the frequency of the particles were conserved between the molarities 1.2 to 1.0 M. Hence, it appears that there was no additional binding of histones between these two molarities. Our results confirm those of Burton *et al.* (1978) showing that the binding of H3 and H4 (out of H_N) was complete at 1.2 M NaCl. We have also shown that the subnucleosomal particles formed, as a result of binding of H3 and H4, have dimensions of the order of 75–80 Å. The observed size of the particles agreed with measurements of Oudet *et al.* (1978) who combined only H3 and H4 with DNA.

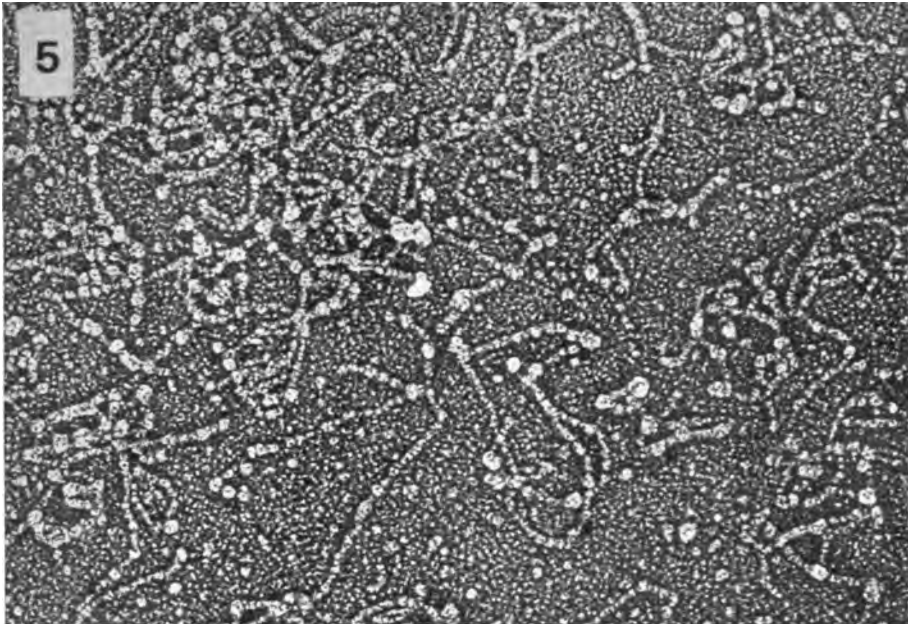


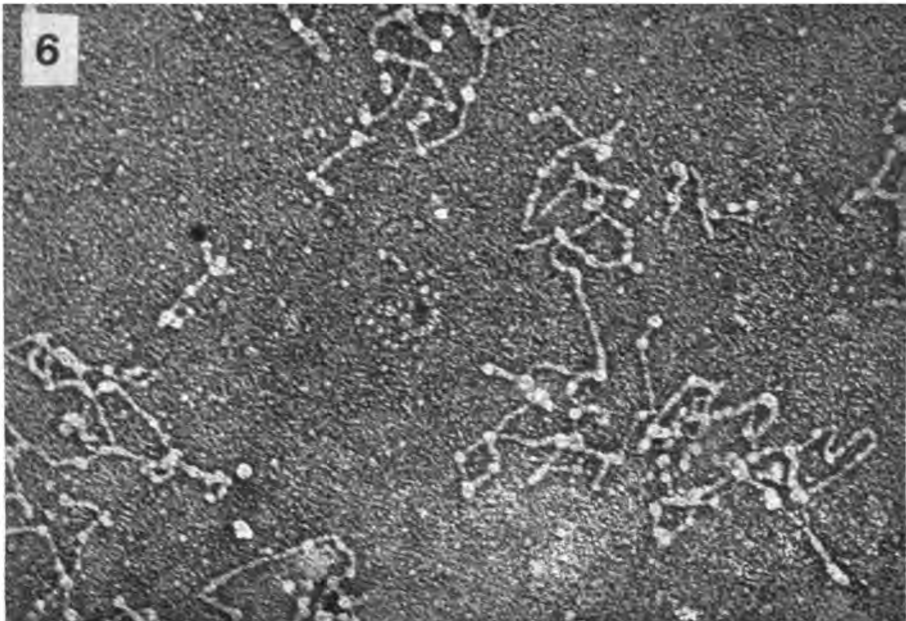
FIGS. 1 & 2 : (1) Calf Tnymus DNA micrographed with the BAC Technique : × 73,000; (2) Reconstituted DNA-Histone Complex at 1.2M NaCl : × 82,000.





FIGS. 3 & 4 : (3) Subnucleosomal particles at 1.0M NaCl : × 80,000; (4) Reconstituted DNA-Histone complex at 0.65M NaCl showing particles of different sizes : × 80,000.





FIGS. 5 & 6 : (5) Artificial nucleosomes at 0.5M NaCl : × 80,000; (6) Completely formed nucleosomes at 0.2M NaCl : × 80,000.

In Fig. 4 (0.65 M NaCl), particles of different sizes are present, some of which have the same dimensions as in the previous micrographs; others are comparatively larger, but not spherical. However, a few larger spherical particles were also present, the average size of which was 105 ± 6 Å. It seemed that H2A and H2B began to bind near about 0.65 M NaCl.

In Fig. 5 (0.5 M NaCl), the particles are well formed and their average diameter is 106 ± 6 Å. Fig. 6 shows the effect of further lowering of molarity to 0.2 M NaCl. The average size of the nucleosomes at this stage was about 115 ± 5 Å. This slight increase in size seemed to indicate that the binding of H2A and H2B continued even beyond 0.5 M NaCl. In some experiments, the artificial nucleosomes were micrographed after complete dialysis of NaCl. In this case, the size did not increase further. Hence, the binding of H2A and H2B was completed at 0.2 M NaCl.

Burton *et al.* (1978) concluded that H2A and H2B bound reversibly between 1.2 to 0.7 M NaCl, while Wilhelm *et al.* (1978) on the basis of gel electrophoretic analysis showed that these two histones combined with DNA only between 0.85 to 0.25 M NaCl. Our electron microscopic studies show that H2A and H2B did not combine with DNA even at 1.0 M NaCl. On the other hand, there was evidence that these two histones started binding at 0.65 M NaCl and the process continued upto 0.2 M NaCl. The fully formed nucleosomes were about 35 Å larger in size than the subnucleosomal particles. The observed size of the artificial nucleosomes at 0.2 M NaCl was in good agreement with the dimensions 120 Å for the natural nucleosomes (Oudet *et al.*, 1975).

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References

Burton, M. J., Hyde, J. E., Phillips, D., Skidmore, C. J., and Walker, I. O. (1978) The interactions of core histones with DNA-Equilibrium binding studies. *Nucleic Acids Res.*, **5**, 3643–3655.

Faulhaber, I., and Bernardi, G. (1967) Chromatography of calf thymus nucleoprotein on hydroxyapatite column. *Biochim. Biophys. Acta*, **140**, 561–564.

Germond, J. E., Bellard, M., Oudet, P., and Chambon, P. (1976) Stability of nucleosomes in native and reconstituted chromatin. *Nucleic Acids Res.*, **3**, 3173–3191.

Kleinschmidt, A. K. (1968) Monolayer techniques in electron microscopy of nucleic acid molecules : *Methods in Enzymology*, **12**, Part B (*Eds.* L. Grossman & K. Moldave). Academic Press, New York pp. 361–377.

Kornberg, R. D. (1977) Structure of chromatin. *Ann. Rev. Biochem.*, **46**, 931–954.

Ohlenbusch, H. H., Olivera, B. D., Tuan, D., and Davidson, N. (1967) Selective dissociation of histones from calf thymus nucleoprotein. *J. mol. Biol.*, **25**, 299–315.

Oudet, P., Gross-Bellard, M., and Chambon, P. (1975) Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. *Cell*, **4**, 281–300.

Oudet, P., Germond, J. E., Sures, M., Gallawitz, D., Bellard, M., and Chambon, P. (1978) Nucleosome structure I : All four histones H2A, H2B, H3 and H4 are required for formation of subnucleosomal particles and nucleosomes *in vitro* : Electron microscopic evidence. *Cold Spring Harb. Symp. Quantitative Biology*, **42**, 287–300.

Vollenweider, H. J., Sogo, J. M., and Koller, Th. (1975) A routine method for protein-free spreading of double and single stranded nucleic acid molecules. *Proc. natn. Acad. Sci. (US)*. **72**, 83–87.

Wilhelm, F. X., Wilhelm, M. L., Erard, M., and Daune, M. P. (1978) Reconstitution of chromatin: Assembly of the nucleosome. *Nucleic Acids Res.*, **5**, 505–521.