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Reconstitution of chromatin : assembly of the nucleosome

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ABSTRACT

The order of reassociation of the four histones H_{2a}, H_{2b}, H_3 and H_4 to the DNA during the reconstitution of chromatin was determined. At each step of the reconstitution the DNA and associated histones were separated from the free histones by centrifugation in a glycerol gradient. The unbound and reassociated histones were analysed by gel electrophoresis and the histone-DNA complexes characterized by circular dichroïsm and electron microscopy. We show that H_3 and H_4 bind first to the DNA between 1.2 M NaCl and 0.85 M NaCl and impose a nucleosome like structure ; in a second step histones H_{2a} and H_{2b} are placed around this kernel to complete the nucleosome.

INTRODUCTION

Reconstituted histone - DNA complexes obtained by mixing DNA and the four histones H_{2a}, H_{2b}, H_3 and H_4 have been widely used to investigate the structure of chromatin (1-10). Indeed these complexes appear to have similar properties to chromatin with respect to formation of nucleosomes (1-4) or patterns of digestion by DNAse I, micrococcal nuclease and proteolytic enzymes (7-8). In native chromatin as well as in reconstituted complexes the nucleosome core is composed of two each of histones H_{2a}, H_{2b}, H_3 and H_4 (11) but the role of each histone in the formation of the core is not well known. Recent experiments suggest that the arginine-rich histones H_3 and H_4 play a fundamental role in organizing the DNA and the other histones of the nucleosome (7,8,12-15). It has been shown that the DNA reconstituted with the arginine-rich histones retained many features of native chromatin : H_3 and H_4 which are able to form a stable tetramer in solution (16-18) organize DNA segments of the length of the nucleosome core (7) and induce

supercoils in closed circular DNA (12-15) ; moreover spherical particles morphologically similar to nucleosomes (12,15) and low angle X-ray diffraction pattern characteristic of chromatin are observed after association of H_3 and H_4 with DNA (14).

In this report we have determined the order of reassociation of the four histones to the DNA during the reconstitution to understand the role of the various histones in the reconstitution process. We have characterized the complexes formed at each step of the reconstitution by electron microscopy. Using the characteristic circular dichroïsm spectra of chromatin (19 and for a review see 20), we have followed the titration of DNA by histones and determined at which step of the reconstitution process the histones were binding to the DNA. Our results give some insight into the structure of the nucleosome core and indicate how the nucleosomes are generated in vitro. We show that during the reconstitution H_3 and H_4 bind first and selectively to the DNA for salt concentration higher than 0.85 M and we suggest that the reconstitution proceeds in two steps : in the first step the DNA is organized by H_3 and H_4 in a nucleosome like structure and in the second step H_{2a} and H_{2b} are placed around this kernel to form the nucleosome.

MATERIAL AND METHODS

Preparation of nuclei, lysine-rich histone depleted chromatin and "reconstituted chromatin"

Nuclei were prepared from mature chicken erythrocytes according to the procedure of Hewish and Burgoyne (21) with the following exceptions :

a) Erythrocytes were collected in 0.024 M EDTA - 0.075 M NaCl, frozen and thawed to allow lysis of the cells.

b) Nuclei were homogenized in buffer A - 0.34 M sucrose - 2 mM EDTA - 0.5 mM EGTA (buffer A = 15 mM Tris-HCl (pH 7.4), 60 mM KCl, 15 mM NaCl, 15 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine) ; the homogenate was layered on 0.5 volume of buffer A - 1.22 M sucrose - 1.5 mM EDTA - 0.3 mM EGTA and centrifuged for 15 min at 16000 g.

c) The nuclear pellet was resuspended and again pelleted as in step b.

d) The nuclei were washed once in buffer A - 0.34 M sucrose and dispersed in the same buffer.

Histones H_1 and H_5 were depleted from chromatin by salt dissociation in 0.7 M NaCl (22) ; under these conditions only H_1 and H_5 are dissociated and the histone/DNA weight ratio of the remaining complex is comprised between 0.8 and 0.9. After the dissociation, the free histones were separated from the histone-DNA complex as follows : 5 ml of the dissociated chromatin was layered at a concentration of 1 mg/ml over a discontinuous glycerol gradient composed of 2 layers of 20% glycerol (12 ml) and 40% (18 ml) glycerol in 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM sodium bisulfite, 5 mM DTT, 0.1 mM PMSF and 0.7 NaCl (buffer B - 0.7 M NaCl). After a centrifugation of 20 h at 25000 rpm in a Spinco SW 27 rotor, 2 ml fractions were collected. The pooled fractions containing the H_1 and H_5 depleted chromatin were dialysed against buffer B - 2.0 M NaCl and used for the reconstitution experiments.

The reconstitution experiments were made according to J.E. Germond et al. (2) by progressively diluting the salt concentration with buffer B. As starting material we have used either the H_1 and H_5 depleted chromatin or a mixture of acid extracted histones and purified DNA at a histone/DNA weight ratio of 0.8. After an incubation in buffer B - 2 M NaCl the complexes were incubated 10 min at 1.6 M NaCl, 1.4 M NaCl, 1.2 M NaCl, 1.0 M NaCl, 0.85 M NaCl, 0.80 M NaCl, 0.75 M NaCl, 0.70 M NaCl, 0.65 M NaCl, 0.50 M NaCl and 0.25 M NaCl.

At each step of the reconstitution the DNA and associated histones were separated from the free histones as described above by centrifugation through a discontinuous glycerol gradient adjusted to the required ionic strength.

Histone isolation and gel electrophoresis

Acid extracted histones were obtained as previously described (23) and contained a stoichiometric amount of H_{2a}, H_{2b}, H_3 and H_4.

After the glycerol gradients the free histones were precipitated overnight by 18% TCA at 4°C. The DNA associated histones were first isolated by extracting the histone-DNA complex with 0.25 N HCl and then precipitated by 18% TCA.

Histones were resolved on SDS polyacrylamide gels (24). The separating gel was made 15% in polyacrylamide with an acrylamide to bisacrylamide ratio of 300:4 in 0.1% sodium dodecyl sulfate - 0.375 M Tris-HCl (pH 8.8). The stacking gel was made 3% in polyacrylamide with an acrylamide to bisacrylamide ratio of 300:8 in 0.1% sodium dodecyl sulfate - 0.125 M Tris-HCl (pH 6.8). Gels were electrophoresed at 100-120 V for 4 h - 6 h using a buffer system of 0.38 M glycine - 0.05 M Tris (pH 8.8) and were run in an 12 cm long slab gel apparatus at room temperature. Gels were stained 2 h with 0.1% coomasie brilliant blue in 50% methanol - 10% acetic acid and destained with 5% methanol - 7% acetic acid.

Circular dichroism

Circular dichroism measurements were performed on a Roussel-Jouan DC III in 1 cm path length thermostated quartz cells.

Electron microscopy

Carbon coated grids (400 mesh) were positively charged according to Dubochet et al. (25). A 50 µl drop of chromatin solution (made 0.5 µg/ml with Tris 10 mM, pH 7.4) was deposited on a parafilm sheet and immediately covered with the grid. After 5 min adsorption, the specimen was positively stained with uranyl acetate (1%, 30 sec.), washed in distilled water (2 min), air dried and finally rotary shadowed with platinium at an angle of 12°. Grids were examined in a Siemens 101 Elmiskop.

RESULTS AND DISCUSSION

Titration of the DNA by histones

When histones interact with DNA the circular dichroism spectrum of the DNA is modified : a decrease of ellipticity at 280 nm is observed. For native unsheared chromatin the measured ellipticity at 280 nm and low ionic strength (< 0.1 M) is about 2000 ± 300 deg cm^2/dmole PO_4 (G. de Murcia et al., to be published) whereas it is 8300 ± 300 deg cm^2/dmole PO_4 for protein free DNA. This variation of ellipticity reflects a change in the secondary structure of the DNA induced by the histones and it was first thought that the histones induced a transition of

the DNA from the B form to the C form but up to now there is no evidence of the presence of DNA in the C conformation in chromatin (26). However the CD results indicate that there must be a deformation of the B structure of DNA in chromatin. Indeed recent X ray diffraction studies of the structure of the nucleosome core suggest that there are 0.4 or 0.7 base pairs less per turn of DNA double helix in chromatin as compared to DNA free in solution (27). Such a variation of the number of base pairs per turn of double helix could well account for the observed variation of circular dichroïsm. We have used the circular dichroïsm properties of chromatin to follow the titration of DNA by histones during the reconstitution. The four histones and the DNA were mixed in 2 M salt and the ionic strength reduced stepwise by progressive dilution as described in Material and Methods. Two sets of reconstitution experiments have been made ; in one set we have reconstituted purified acid extracted histones with highly purified chicken erythrocyte DNA (true artificial complexes) and in a second set we have used H_1 and H_5 depleted chromatin which was dissociated in 2 M salt and then reassociated (natural complexes). The results obtained for the two sets of experiments were qualitatively identical. For the true artificial complexes the reconstitution has been studied at three different temperatures (4°C, 25°C and 37°C) but no influence of the temperature was observed on the titration curve. The reconstitution of the natural complexes was thus always made at 4°C to avoid enzymatic degradation of the chromatin components. In parallel a sample of protein-free DNA was run through all the steps of the reconstitution to measure the variation of CD induced by ionic strength on DNA alone. At each step of the reconstitution the CD spectrum of the histone - DNA mixture and of the free-DNA was measured (figure 1). The titration curve of the DNA by the histone was constructed by substracting the value of the ellipticity at 280 nm of the histone-DNA complex from that of the free DNA. The results shown in figure 2a, indicate that the binding of histones to DNA is not an all and none process but takes place over a wide range of ionic strength : for natural complexes binding starts at 1.4 M NaCl and is complete only at 0.25 M whereas for true artificial complexes the

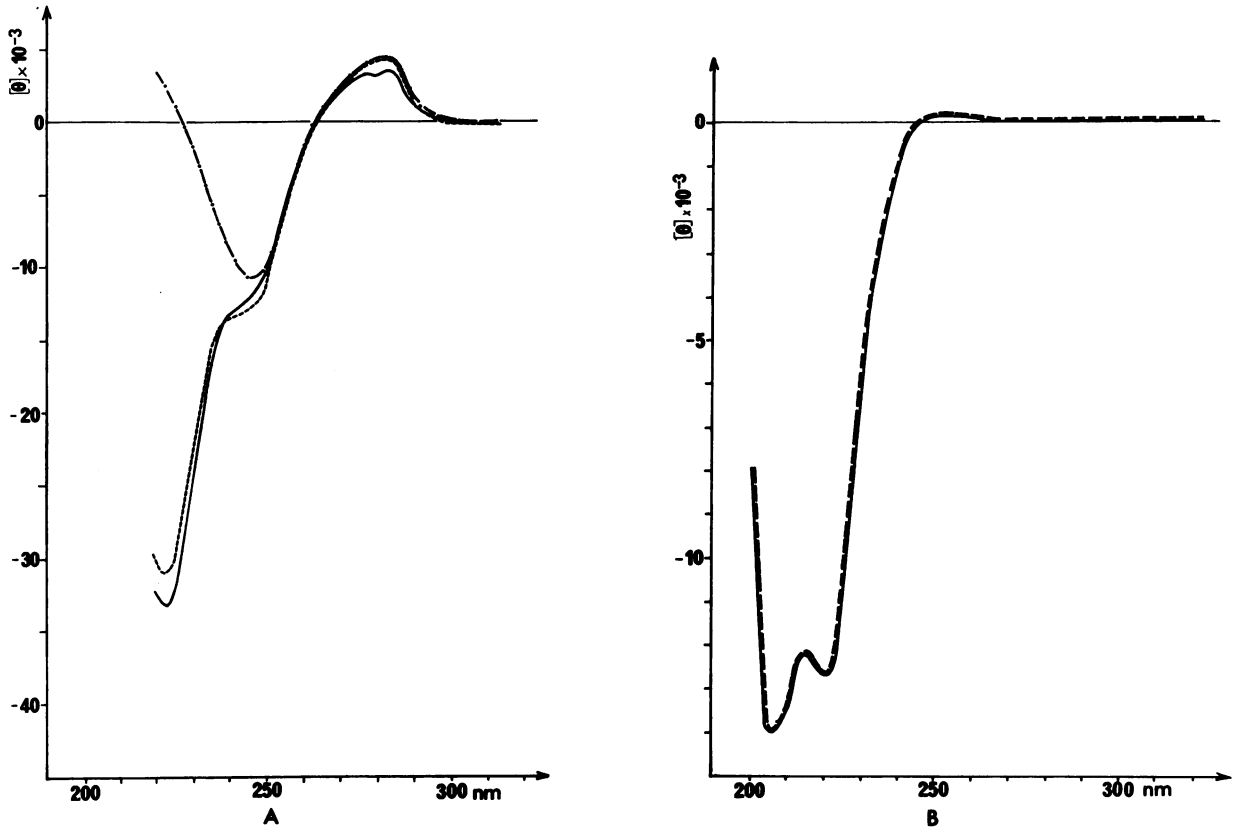

Figure 1 – A. Circular dichroic spectra of the histone – DNA complexes in 2.0 M NaCl (– – –) and 0.25 M NaCl (——) compared to the spectrum of chicken erythrocyte DNA in 2.0 M NaCl (—·—·—). Data are expressed as molecular ellipticity per mole of DNA phosphate (deg cm^2/dmole PO_4).

B. Circular dichroic spectra of the mixture of the four histones H2a, H2b, H3 and H4 in 2.0 M NaCl (——) and 0.25 M NaCl (– – –). Data are expressed as molecular ellipticity per mole of amino acid residue.

titration curve is shifted toward lower ionic strengths. We do not know whether this difference is due to the fact that in the case of true artificial complexes the histones are acid extracted whereas they are salt extracted in the natural complexes or whether some specific factors (free in solution or still bound to the DNA) modify the binding of histones in the natural complexes.

In all of the reconstitution procedure described it is necessary to incubate the histone-DNA mixture during a relatively long period of time at each step of the reconstitution. We have tested by CD whether a change of the secondary structure of DNA occured during this period ; we could not detect any change of the CD signal at 280 nm during the incubation period at any stage of the reconstitution. We have thus tested whether the reconstitution could be done more rapidly. Therefore the incubation period at each step of the reconstitution was reduced from 10 min to 30 sec. We found that the yield of nucleosome formation was highly reduced, if the rapid reconstitution procedure was used. The explanation of this result may be that the DNA undergoes a structural rearrangement (i.e. genesis of a supercoil of DNA around the histone core) necessary for the formation of nucleosomes which is not detected by CD. An other possibility may be that the structural rearrangement of histones is the rate limiting step. The very close agreement of the CD spectrum of the histone-DNA complex dissociated in 2 M NaCl with that of the complex completely reassociated in 0.25 M in the region of absorption of the peptide bond (fig. 1a) suggests that little variation in the secondary structure of the histones occurs during the reconstitution in agreement with recent results of Thomas et al. (26). Indeed a constant value of ellipticity at 227 nm was observed during the whole reconstitution (fig. 2b) (At 227 nm the ellipticity value of DNA is close to zero and a minimum is observed in the CD spectrum of histones (fig. 1b), therefore $|\theta|_{227}$ is a good probe for the secondary structure (α helix content) of histones in chromatin).

In any case neither the change of tertiary structure of the histones nor the structural rearrangement of DNA could be detec-

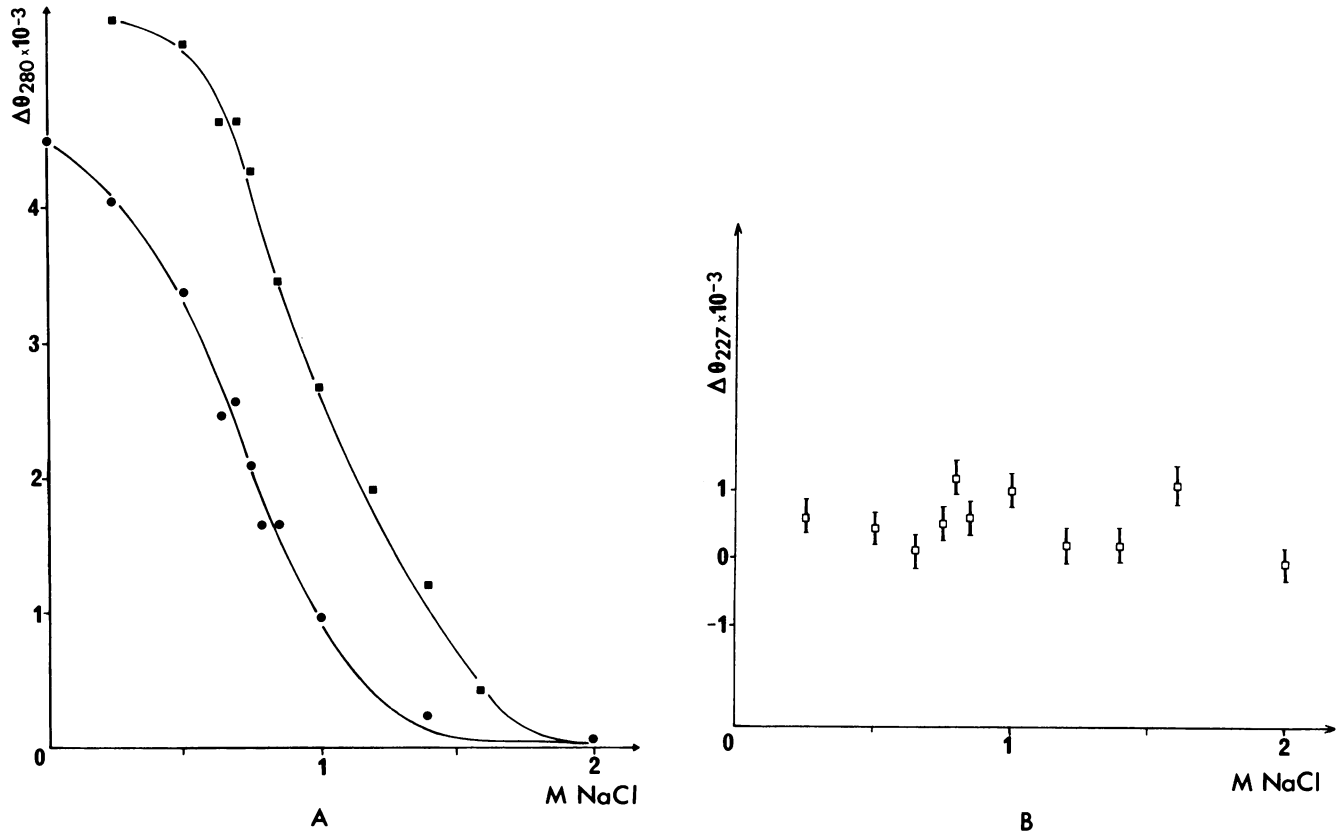

Figure 2 – A. Variation of $\Delta\theta_{280}$ as a function of salt concentration for natural complexes (– – –) and true artificial complexes (– – –). ($\Delta\theta_{280}$ = molecular ellipticity at 280 nm of free DNA minus molecular ellipticity at 280 of the histone – DNA complex)

B. Plot of $\Delta\theta_{227}$ as a function of salt concentration for true artificial complexes ($\Delta\theta_{227}$ = molecular ellipticity at 227 nm of free histones minus molecular ellipticity at 227 nm of the histone – DNA complex).

ted by CD measurements.

Order of reassociation of the histones during reconstitution

At each step of the reconstitution the DNA and associated proteins were separated from the free histones by centrifugation as described in Material and Methods. The unbound and reassociated histones were analysed on SDS - polyacrylamide gel electrophoresis (fig. 3). In 2 M NaCl the histones are completely dissociated from DNA. No detectable amount of protein is bound in 1.6 M and 1.4 M NaCl. The first histones to bind to DNA are the histones H_3 and H_4 in 1.2 M NaCl, but the binding is very weak since much H_3 and H_4 are still found in the unbound fraction. In 1.0 M NaCl almost all H_3 and H_4 are bound to the DNA with a little contamination by H_{2a} and H_{2b}. No preferential binding of one of the two histones H_3 or H_4 appears to occur in 1.2 M and 1.0 M, probably because the two histones form a stoichiometric complex before they bind to the DNA (it is certainly a $(H_3)_2(H_4)_2$ tetramer but there is no experimental evidence that this tetramer exists preferentially in 1.2 M or 1.0 M NaCl when the four histones are present). In 0.85 M NaCl H_3 and H_4 are still the predominant histones bound to DNA but bands corresponding to H_{2a} and H_{2b} are clearly visible. The remainder of H_{2a} and H_{2b} binds progressively to the DNA but even in 0.65 M NaCl and 0.5 M NaCl some H_{2a} and H_{2b} is still found in the unbound fraction. Finally, all the histones are completely reassociated in 0.25 M NaCl. We have obtained comparable results with the true artificial complexes and the natural complexes, however the specificity of binding of H_3 and H_4 is not as good in the true artificial complexes. Here again the difference in the method of preparation of histones or the lack of specific factors in the true artificial complexes could account for the observed difference.

As a control experiment we have centrifuged a mixture of the four histones in the absence of DNA to check whether under the conditions of the experiment an aggregate of H_3 and H_4 is present which would migrate together with the DNA. It is clear from the result of this experiment (data not shown) that no histones migrate at the level of the DNA in the absence of DNA at any ionic strength used for the reconstitution.

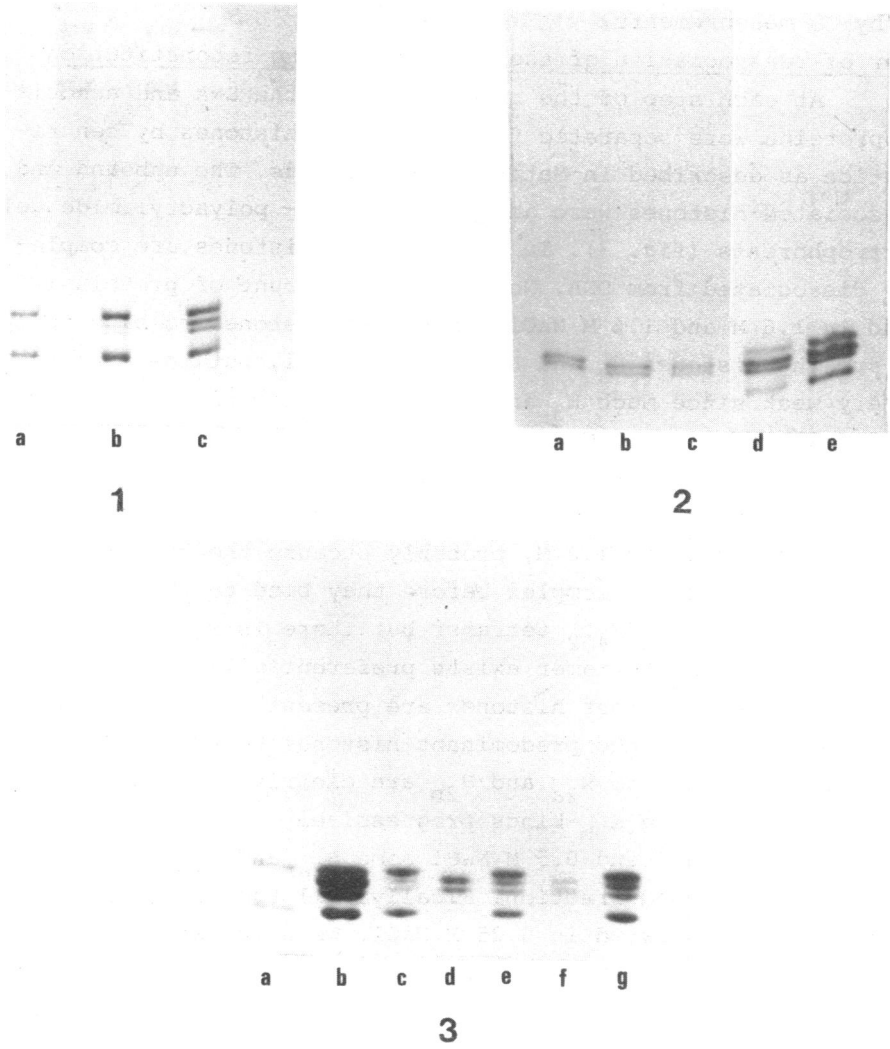

Figure 3 - SDS gel electrophoresis analysis of the unbound and reassociated histones at various stages of the reconstitution.
1) and 2) Experiment n° 1 :
- DNA associated histones in 1.0 M NaCl (1a), 0.85 M NaCl (1b) and 0.65 M NaCl (1c).
- Unbound histones in 0.5 M NaCl (2a), 0.65 M NaCl (2b), 0.85 M NaCl (2c), 1.0 M NaCl (2d), 2.0 M NaCl (2e)
3) Experiment n° 2 :
- DNA associated histones in 1.2 M NaCl (3a), 0.85 M NaCl (3c), 0.70 M NaCl (3e) and 0.5 M NaCl (3g)
- Unbound histones in 1.2 M NaCl (3b), 0.85 M NaCl (3d) and 0.7 M NaCl (3f).

Our results thus indicate that during the reconstitution the histones bind to DNA in two steps and that, although it has been proved that the four histones could form an octamer in solution, this octamer does not bind as such during the reconstitution or, alternatively, that it is very unstable and dissociates when H_3 and H_4 are in contact with the DNA.
The order of reassociation of histone to DNA was previously studied by Gadski et al. (6) but the reconstitution was made in the presence of urea with whole chromatin including the lysine rich histone. It was found that histones H_1 and H_5 were binding first to the DNA and no specificity of binding of the other histones was observed. It has been shown since (5) that urea decreased the number of nucleosomes formed and this could explain the lack of specificity of histone binding observed by Gadski et al. We have thus used whole chromatin to make a reconstitution experiment under our experimental conditions and we show (fig. 4) that the order of reassociation of the four

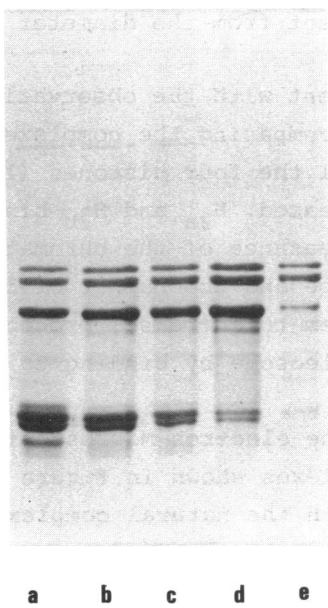

Figure 4 - SDS gel electrophoresis analysis of the unbound histones at various stages of the reconstitution of whole chromatin
a) 1.2 M NaCl b) 1.0 M NaCl c) 0.85 M NaCl
d) 0.75 M NaCl e) 0.50 M NaCl

small histones H_{2a}, H_{2b}, H_3 and H_4 is the same for whole chromatin as compared to H_1 and H_5 depleted chromatin. In contrast to the result of Gadski et al. (6), we find that the lysine rich histones do not bind first to the DNA but only after most of the other histones are bound to the DNA : H_5 binds to the DNA in 0.5 M salt and H_1 is the last histone to bind to the DNA.

Electron microscopy

The complexes formed at each step of the reconstitution and separated by centrifugation were examined by electron microscopy (figures 5 and 6). In 1.2 M NaCl most of the DNA is naked and appears as a fiber about 30 Å in diameter ; a few beads are seen along the DNA molecule.

The density of particles increases markedly when the ionic strength is reduced to 1.0 M. Since we have shown above that the histones bound to DNA at these two ionic strengths are histones H_3 and H_4, the beads seen in the electron microscope are thus the H_3 - H_4 particles which have been recently characterized (12,15). In our hands the diameter of the H_3 - H_4 beads is not very different from the diameter of the nucleosome (110 Å - 130 Å).

This result is in agreement with the observation of Bina-Stein and Simpson obtained by comparing the complexes of SV 40 DNA with H_3 - H_4 and with all the four histones (12). As the ionic strength is further decreased, H_{2a} and H_{2b} bind progressively to the DNA, the same appearance of the chromatin is observed in the electron microscope and the number of beads along the DNA molecule does not seem to increase, probably because H_{2a} and H_{2b} complete the nucleosome by binding to the preexisting H_3 - H_4 particle.

The results of the electron microscopic observation of the true artificial complexes shown in figure 6 are very similar to those obtained with the natural complexes.

CONCLUSION

In the methods currently used to reconstitute chromatin, the DNA and the histones are mixed in high salt (2 M NaCl) and the ionic strength of the mixture is decreased stepwise to allow progressive binding of the histone to the DNA. It has

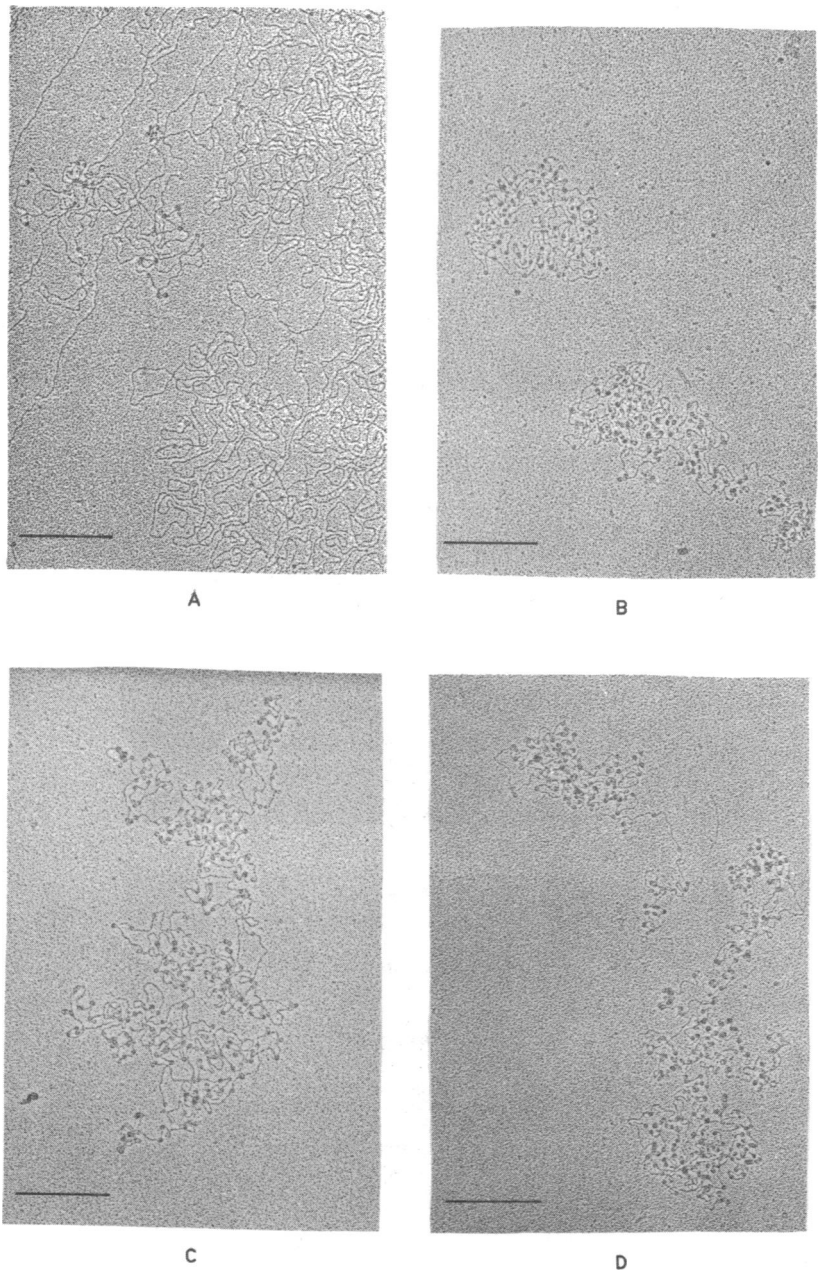

Figure 5 - Electron micrographs of the histone - DNA complexes obtained after centrifugation of natural complexes through a glycerol gradient at various ionic strengths
A) 1.2 M NaCl B) 1.0 M NaCl C) 0.85 M NaCl D) 0.50 M NaCl
Bar = 0.25 µm

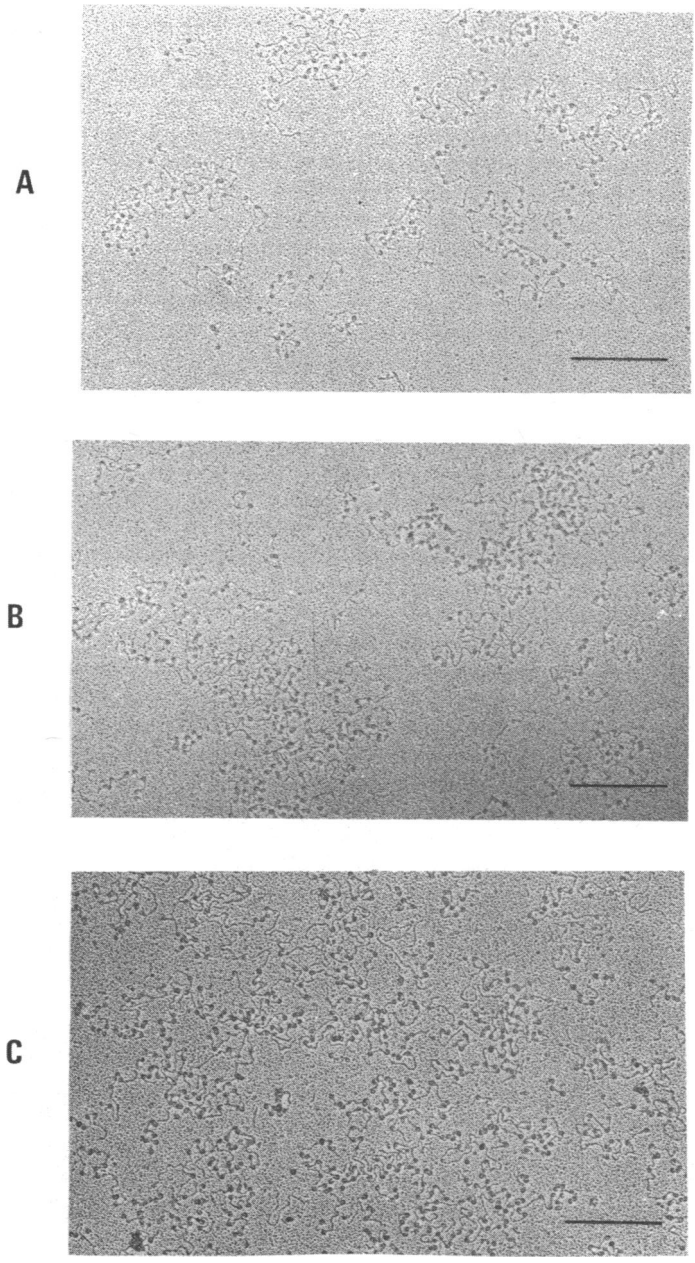

Figure 6 - Electron micrographs of the histone - DNA complexes obtained after centrifugation of true artificial complexes through a glycerol gradient at various ionic strengths
A) 1.0 M NaCl B) 0.70 M NaCl C) 0.25 M NaCl
Bar = 0.25 µm.

been shown that histones in the presence of DNA in 2 M NaCl were able to form specific complexes akin to those obtained when the histones are crosslinked in chromatin (11,24). Particularly the octamer found in the solution of high ionic strength is similar to the octamer bound to the nucleosome core. In the present study we have investigated whether this octamer was binding as such to the DNA during the reconstitution process. Our results demonstrate that this is not the case but that H_3 and H_4 bind first to the DNA probably as a $(H_3)_2(H_4)_2$ tetramer. H_{2a} and H_{2b} bind to the DNA only after H_3 and H_4 are completely bound. This result is in good agreement with previous reports on the removal of histones by salt from native chromatin (28 and for a review see 29) showing that H_{2a} and H_{2b} are dissociated first from chromatin. However the dissociation experiments did not say whether the backward reassociation, starting from a mixture of histones and DNA completely dissociated, worked in the reverse order. There is an apparent discrepancy between our present results and the report of J.E. Germond et al. (2) concerning the stability of nucleosomes which shows that the histones move in the form of an octamer from one molecule of DNA to the molecule of a competitor DNA in 0.88 M NaCl. However, it should be recalled that the binding of histones to DNA is an equilibrium process and if the binding constant of the histones is greater for the competitor DNA the equilibrium can well be displaced in favor of the competitor DNA. Thus, our results cannot be directly compared to those of J.E. Germond et al.

In conclusion, our results together with a growing body of evidence (7,8,12,13,15) show that the histone H_3 and H_4 play the fundamental role in the architecture of the nucleosome. During the reconstitution H_3 and H_4 bind first to the DNA and impose a nucleosome like structure ; in a second step histones H_{2a} and H_{2b} are placed around this kernel but can be removed or reassociated to the DNA without affecting the gross structure of the nucleosome. Our results have stressed the importance of time in the reconstitution of chromatin ; the sensitivity to time seems even greater for the association of the arginine rich histones with DNA (12). Studies are now in

progress in our laboratory to understand the dependence of reconstitution on time and to investigate the changes which occur in the tertiary structure of histones or in the histone-histone interactions during the reconstitution.

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