CHAPTER III

RESULTS

AND

DISCUSSION
3.1 STUDIES ON RECONSTITUTED DNA-H₉ COMPLEX

In order to understand the progressive formation of the nucleosomal particles from its constituents DNA and the histone H₉ (an equimolar mixture of H2A, H2B, H3 and H4), the DNA-H₉ complexes were reconstituted in the laboratory, separated from unbound histones through hydroxyapatite columns and examined under the electron microscope.

(a) Chromatographic Behaviour of DNA-H₉ complex

Fig. 5 represents the elution profiles of DNA-H₉ complexes at six consecutive stages of reconstitution namely, 2.0 M, 1.2 M, 1.0 M, 0.65 M, 0.5 M and 0.2 M NaCl. In case of DNA-H₉ complex at 2.0 M NaCl, (Fig. 5A), the O.D₆⁰⁰ peak fraction was just like native DNA (Fig. 4) and most of the histones eluted initially in the free from as observed from O.D₂₃₀ plot. The O.D₂₆⁰ : O.D₂₃₀ ratio of the peak fraction was 2.2 which was exactly the same as that of native DNA. These observations indicated that histones did not bind at all with DNA at this molarity. Electron microscopy of the chromatographic peak fraction also showed naked DNAs. At 1.2 M salt, the O.D₂₆⁰ peak at 0.2 M phosphate decreased a little while the peak at 0.25 M phosphate increased (Fig. 5B).
Fig. 5. Elution profiles of DNA-H$_n$ complexes from (A) 2.0 M NaCl, (B) 1.2 M NaCl, (C) 1.0 M NaCl, (D) 0.65 M NaCl, (E) 0.5 M NaCl, and (F) 0.2 M NaCl step of the gradient dilution method. The solid line indicates O.D$_{260}$ and the dashed line O.D$_{230}$ values. The corresponding phosphate molarities are denoted by arrows at the top.
The O.D_{260} : O.D_{230} ratio of the peak fractions was of the order of 1.80-1.85 which indicated that more than 50% of the total histone were in bound form. In the next molarity of 1.0, the magnitude of the two chromatographic peaks were comparable (Fig. 50), whereas in the next stages, i.e. at 0.65 M, 0.5 M, and 0.2 M salts the magnitude of the 0.2 M phosphate peak decreased gradually while that of 0.25 M phosphate increased (Fig. 5D-F). Also a small peak appeared gradually at 0.3 M phosphate concentration with decreasing NaCl molarity. In each case, the eluent (E) contained the unbound histones, the relative amount of which decreased progressively with decrease in salt molarity as evidenced from its O.D_{230} value and also verified by Microbiurett test. At 0.2-0.3 M phosphate concentrations, histone-bound DNAs eluted as revealed from the electron micrographs of these peak fractions and their O.D_{260} : O.D_{230} ratio. These ratios and the corresponding weight ratio of the bound histones of the peak fractions are given in Table I.

(b) Electron Microscopy of DNA-H_N Complex.

Plates (II-III) showed the electron micrographs of DNA-H_N complexes taken from the most prominent chromatographic peak fractions, corresponding to 1.2 M, 1.0 M,
0.65 M, 0.5 M and 0.2 M steps of the dilution process. Plates IIA and IIB represent reconstituted complexes at 1.2 and 1.0 M NaCl. Spherical particles of approximately uniform size, separated by lengths of DNA could be observed over both the plates. In these two cases, micrographs were made from both the 0.2 M and 0.25 M phosphate peaks, since they were comparable. No difference was observed between the particles corresponding to the two peaks as expected from the more or less identical histone-DNA ratio (Table I). The average size of these particles were \(78 \pm 5 \) Å and \(80 \pm 6 \) Å respectively. The average frequency of the particles were almost the same at these two molarities. The size distribution of these particles at different molarities were shown by histogram in Fig. 6 (A-E). The corrected size of these nucleosomal particles and also the frequency of their formation per micron length of DNA were enlisted in Table I.

In the next molarity of 0.65 M NaCl, particles of different sizes were present (plate IIC). This was also revealed from the broad distribution of the particles in the histogram (Fig. 6C). Some of these particles were uniformly spherical having an average diameter of \(105 \pm 6 \) Å and the other particles were more or less irregular in shape having dimensions between 75-90 Å. However, the frequency of the particles were of the same order.
Fig. 6. Size distribution of the nucleosomal particles in reconstituted DNA-H$_{\text{II}}$ complexes at (A) 1.2 M NaCl, (B) 1.0 M NaCl, (C) 0.65 M NaCl, (D) 0.5 M NaCl and (E) 0.2 M NaCl. Measured number of particles at each molarity=200.
TABLE - I

Different experimental parameters of DNA-Histone (Hg) complex during reconstitution with gradual decrease in NaCl concentration.

<table>
<thead>
<tr>
<th>Salt molarity of DNA-H$_N$ complex</th>
<th>0.260:0.230 ratio at phosphate molarities</th>
<th>Histone-DNA weight ratio at phosphate molarities</th>
<th>Diameter of the particles in Å unit.</th>
<th>Frequency of the particles per micron length of DNA.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2.2 2.2</td>
<td>0.02 0.02</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>1.2</td>
<td>1.82 1.80 1.85</td>
<td>0.53 0.55 0.52</td>
<td>78±5</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1.85 1.80 1.80</td>
<td>0.52 0.55 0.55</td>
<td>80±6</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td>0.65</td>
<td>1.70 1.73 1.77</td>
<td>0.82 0.80 0.79</td>
<td>103±6</td>
<td>10.0 ± 0.8</td>
</tr>
<tr>
<td>0.5</td>
<td>1.66 1.65 1.60</td>
<td>0.94 0.94 0.96</td>
<td>112±6</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>0.2</td>
<td>1.62 1.60 1.65</td>
<td>0.95 0.96 0.94</td>
<td>115±5</td>
<td>9.9 ± 0.3</td>
</tr>
</tbody>
</table>
In plate II at 0.5 M NaCl, the particles were well formed, the average diameter and the frequency of the nucleosomes being $112 \pm 6 \AA$ and $10.0 \pm 0.8$ per micron length respectively. Plate IIIB showed the effect of further lowering of NaCl molarity upto 0.2. The diameter and frequency of the nucleosomes remained almost the same as in the previous stage. The reconstituted DNA-H$_N$ complex at 0.5 and 0.2 M salt was similar in appearance with H1-depleted chromatin (Thoma and Koller, 1977). The spacer regions however, showed various irregular features. Relatively long stretches of apparently histone-free DNA and histone association at some sites of the DNA fibre without formation of nucleosome-like particles seem to reflect local failures in the reconstitution process.

3.2 STUDIES ON RECONSTITUTED DNA-H1 COMPLEX

To understand the effect of H1 binding with DNA in relation to the chromatin structure, an electron microscopic study was also made on DNA-H1 complexes reconstituted in the laboratory after separation through hydroxyapatite columns.
a) Chromatographic Behaviour of DNA-H1 Complex.

DNA-H1 complexes were prepared by step gradient dilution as well as slow mixing methods. Both type of complexes were separated from unbound part with the help of HAP column. The elution patterns of the reconstituted DNA-H1 complexes under different experimental conditions were shown in Fig. 7. The first four curves represented the elution patterns of the complexes at four different steps, namely 1.0 M, 0.5 M, 0.2 M and 0.05 M salts of the gradient dilution method (Fig. 7A-D), while the last one showed the profile of the complex formed by slow mixing in presence of 0.2 M salt (Fig. 7E).

The DNA-H1 complex in presence of 1.0 M NaCl eluted mainly at 0.2 M phosphate buffer (Fig. 7A). The $\frac{OD_{260}}{OD_{230}}$ ratio of the peak fraction was 2.1 which indicated that the amount of histone bound was negligible. This was also supported from the observation that almost all the histone eluted previously with the eluent. In the next molarity of 0.5 M salt, the complex was found to elute in the same region as in the previous one (Fig. 7B). But the peak fraction at this stage contained DNA bound with histone H1 in a weight ratio of about 0.55 (W/W). At the next step of 0.2 M NaCl, the fraction eluted at 0.2 M phosphate buffer contained DNA bound with histone H1 in a weight ratio of 0.95 (W/W) (Fig. 7C).
Fig. 7. Elution profiles of DNA-H1 complexes formed at (A) 1.0 M NaCl, (B) 0.5 M NaCl, (C) 0.2 M NaCl, (D) 0.05 M NaCl steps of the gradient dilution method and (E) 0.2 M NaCl by slow mixing method. The solid line indicates $O.D_{260}$ and the dashed line, $O.D_{230}$ values. Arrows at the top denote the corresponding phosphate molarities.
When the molarity was lowered one step more i.e. at 0.05 M NaCl, the elution profile showed two prominent peaks, at 0.2 M (peak c) and 0.25 M (peak h) phosphate buffer (Fig. 7D), the latter peak being higher. The histone bound with DNA eluted with 0.2 M phosphate was greater (0.95 W/W) than that at 0.25 M fraction (0.80 W/W). Moreover, the total amount of bound histone at 0.05 M NaCl was less than that in the molarity of 0.2 M NaCl which indicated that a reverse process (i.e. dissociation) took place when salt molarity was lowered from 0.2 to 0.05 M NaCl. This was further evidenced from the fact that the amount of unbound histone eluted increased at this stage.

The last profile (Fig. 7E) which was that of DNA-H1 complex reconstituted by slow mixing in presence of 0.2 M NaCl, was similar to that of the complex prepared by gradient dilution upto 0.2 M salt. The amount of bound histone was also of the same order (0.95 W/W). From the elution profile of DNA-H1 complexes, it was remarkably noticed that there was no shift of the main peak with the binding of H1 molecules, as was observed previously in case of DNA-H complex.

(b) Electron Microscopy of DNA-H1 Complex.

Plates (UV–W) are the electron micrographs of the chromatographic peak fractions corresponding to the Fig. 7(A–D)
The micrograph of DNA-H1 complex in presence of 0.5 M NaCl showed thick fibres, formed by multiple folding of DNA over itself (Plate IVA). In the next stage when the molarity was lowered to 0.2 M salt, the reconstituted complex showed large doughnut-like structures (Plate IVB). From a number of electron micrographs taken from different specimens made on different dates, the diameter of these doughnuts were found to lie between 900–1100 Å. As a result of further dilution up to 0.05 M, two prominent peaks were observed in the elution profile (Fig. 7D). To detect the structural changes occurred, electron micrographs were taken separately from the two peaks (a and b). Plates IV (A and B) are the corresponding electron micrographs. A few doughnuts of small and irregular size could be observed in plate IVA, i.e. at 0.2 M phosphate peak while thick fibres were seen all over the two plates. It seemed that dissociation of a few histone molecules had resulted in the straightening of the doughnut structure (a loss in compactness) which was previously indicated by the shift of the elution peak towards higher molarity of phosphate (Fig. 7D) and a decreased histone-DNA weight ratio.

Plate VC represented DNA-H1 complex formed by slow mixing at 0.2 M NaCl corresponding to the peak fraction of Fig. 7B. Doughnuts of same dimension as in plate IVB were
present in this micrograph. However, the doughnut formation was more regular when dilution method was followed. Previously Von Mickwitz et al. (1979) found that double fibres seen in their DNA-H1 complexes at 0.02 M NaCl were also produced when slow mixing method was followed instead of gradient dialysis, but with a lower frequency. Hence one may safely infer that a salt gradient is preferable but not so critical (as in the case of nucleosomal histone binding) for the binding of H1 with DNA.

3.3 STUDIES ON NATURAL CHROMATIN

In order to compare the conformation and the chromatographic behaviour of the reconstituted DNA-histone complexes with those of natural chromatin, similar experiments were also performed on native chicken erythrocyte chromatin.

The chromatographic elution pattern of chicken erythrocyte chromatin was shown in Fig. 8. The profile was very similar to that of DNA-H$_N$ complex at 0.2 M NaCl. The prominent peak at 0.25 M phosphate was followed by a minor one at 0.3 M phosphate. The histone/DNA weight ratio of the peak fraction was 1.2 corresponding to $O.D_{260} : O.D_{230}$ ratio of 1.45. The observed weight ratio was in
accordance with that found in chromatins from different eukaryotic cells (Elgin and Weintraub, 1975). The absorbance of the eluent at 230 nm was due to the presence of small amounts of contaminating proteins.

Plate VI was an electron micrograph of the native chromatin corresponding to the chromatographic peak fraction. Closely packed nucleosomes of diameter 125 ± 2 Å were seen all over the plate. Oudet et al. (1975) also observed nucleosomes of same dimension in electron micrographs of chromatin in the native compact state.

3.4 DISCUSSION

The preliminary formation of the subnucleosomal particles and the gradual changes in their dimensions until complete nucleosomal particles were finally produced, could be visualised from plates (II-III). The size and the frequency of the particles were almost conserved between the molarities 1.2 to 1.0 M which indicated that there was no additional binding of histones between these two molarities. This was confirmed from the chromatographic elution pattern (Fig. 3B,C) and the histone-DNA weight ratio (Table I), which was also the same at these two molarities (approximately half of the
Burton et al. (1978) showed that in the molarity range of 2.0 to 1.2 M NaCl, the histones H3 and H4 bound reversibly with DNA. On the other hand, Wilhelm et al. (1978) found that the density of the particles increased markedly when the ionic strength was reduced to 1.0 M, from which they concluded that H3 and H4 combined in the molarity range of 1.2 to 0.85 M NaCl. The present results confirmed those of Burton et al. (1978) showing that the binding of H3 and H4 (out of HN) was complete at 1.2 M NaCl. Simon and Felsenfeld (1979) also found that H3 and H4 were dissociated from native chromatin only above 1.2 M NaCl.

The present study also showed that the subnucleosomal particles, formed at 1.2 to 1.0 M NaCl, had dimensions of the order of 78-80 Å. Oudet et al. (1973) observed similar 80 Å subnucleosomal particles by combining H3 and H4 with DNA. Bina-stein and Simpson (1977) also showed particles of 75 Å diameter in SV40 DNA complexed with H3 and H4.

From the wide variation of the size of the particles at 0.65 M NaCl (Fig. 6C), it seemed that the remaining histone fractions i.e. H2A and H2B began to bind near about this molarity. The increased histone-DNA weight ratio...
at this stage also supported additional binding. But the frequency of the nucleosomal particles remained unchanged which showed that no new particles were formed at this stage.

Plates A and B showed well formed nucleosomes. There was no significant difference in size or frequency of the particles in the two plates corresponding to 0.5 M and 0.2 M NaCl. Also the elution patterns (Fig. 5B, F) and the histone-DNA weight ratio (Table I) were almost the same at these two molarities. All these indicated that the binding of the histones H2A and H2B was almost complete at 0.5 M NaCl. Burton et al. (1978) previously showed that these two histones bound reversibly between 1.2 to 0.7 M NaCl, whereas Wilhelm et al. (1978) on the basis of gel electrophoretic analysis, showed that H2A and H2B combined with DNA only between 0.85 to 0.5 M NaCl. Simon and Felsenfeld (1979) found that these histones began to dissociate from chromatin above 0.6 M NaCl and were completely stripped off at 1.2 M NaCl. The present electron microscopic observations revealed that H2A and H2B did not combine with DNA even at 1.0 M NaCl. Also there was evidence that binding of these two histones were not complete at 0.65 M NaCl. Thus the present studies agreed partly with those of Wilhelm et al. (1978) with the conclusion that the binding of H2A and H2B took place below 1.0 M NaCl and continued at least up to 0.5 M NaCl.
It was interesting to note that the frequency of the particles formed did not increase at all below 1.0 M NaCl (Table I). This confirmed that only H3 and H4 could initiate the formation of the nucleosome core and the role of other histones H2A and H2B was only to complete the structure. The broad distribution in size of the particles at 0.65 M NaCl indicated that H2A and H2B might bind with DNA both in dimeric and in tetrameric forms. The fully formed nucleosomes were about 35 Å larger in size than the subnucleosomal particles. The artificial nucleosomes were slightly smaller than the dimension of the natural nucleosomes as observed in the present work (Plate VI) and also found previously by Oudet et al. (1975). The slightly higher dimension might be due to compactness of the native chromatin which was possibly due to presence of the histone H1.

A gradual shift of the chromatographic peak towards higher phosphate molarity was observed as a result of $H^{	ext{H}}_N$ binding (Fig. 5) whereas no such shift of the main peak could be detected for DNA-H1 binding (Fig. 7). Also the elution profile of native chromatin (Fig. 3) was similar to the reconstituted DNA-$H^N$ complex at 0.2 M NaCl, (Fig. 5F) although the histone H1 was present in the former case. Previously Li et al. (1977) found that the removal of histone H1 from chromatin had only a minor effect on the hydrodynamic properties of chromatin while removal of other
histones caused drastic changes in these properties. Also Bradbury et al. (1972) showed that dissociation of H1 produced no effect on the low angle X-ray diffraction pattern of chromatin.

The thick stem and doughnut-like structures found in DNA-H1 complexes at 0.5 M and 0.2 M salt (Plates IVA and IVB) resembled the structures observed by Olins and Olins (1971) and Hsiang and Cole (1977) at comparable salt conditions. The fibre-like appearances present in plate XV (A and B) when the molarity was lowered to 0.05, were similar to the back folded structures of DNA-H1 complexes observed by Von Mickwitz et al. (1979) at a NaCl molarity of 0.02. The structures of DNA-H1 complexes observed under different experimental conditions in the present work have been compared with those of previous workers in Table II.

From the present results it was revealed that the binding of H1 started a little above 0.5 M NaCl and it was complete at about 0.2 M NaCl. From dissociation experiments, Frisman et al. (1974) concluded that the histone H1 was definitely dissociated in 0.7 M NaCl, Simon and Felsenfeld (1979) also obtained the total amount of H1 in dissociated form at 0.63 M NaCl. From fluorescence polarisation studies, Glotov et al. (1977) showed that at low ionic strength, all the segments of the H1 molecule were immobilised on binding to DNA. The gradually increasing NaCl concentration in the
<table>
<thead>
<tr>
<th>Reference</th>
<th>Reconstitution Process</th>
<th>Salt Molarity</th>
<th>H1/DNA input weight ratio</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olins and Olins</td>
<td>Step gradient dialysis in presence of urea</td>
<td>(i) 0.10</td>
<td>0.95*</td>
<td>Doughnuts of diameter 1000-3000 Å and stem-like structures.</td>
</tr>
<tr>
<td>(1971)</td>
<td></td>
<td>(ii) 0.001</td>
<td>0.95*</td>
<td>Loose network of DNA-like fibers.</td>
</tr>
<tr>
<td>Heisang and Cole</td>
<td>Slow mixing.</td>
<td>0.17</td>
<td>1.3</td>
<td>Doughnuts of diameter 1600-3600 Å.</td>
</tr>
<tr>
<td>(1977)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Von Mickwitz et al.</td>
<td>Step gradient dialysis.</td>
<td>(i) 0.06</td>
<td>0.9</td>
<td>Dense stem-like structure.</td>
</tr>
<tr>
<td>(1979)</td>
<td></td>
<td>(ii) 0.02</td>
<td>0.9</td>
<td>Loop-like back folded structures.</td>
</tr>
<tr>
<td>Present workers</td>
<td>Step gradient dialution.</td>
<td>(i) 0.50</td>
<td>1.0</td>
<td>Stem-like structures of 250-300 Å with (Plate IV A).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) 0.20</td>
<td>1.0</td>
<td>Doughnuts of diameter 900-1100 Å (Plate IV B).</td>
</tr>
<tr>
<td></td>
<td>Slow mixing.</td>
<td>(iii) 0.05</td>
<td>1.0</td>
<td>A few small doughnuts and thick fibers formed by multiple folding of DNA over itself (Plates VA and VB).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.20</td>
<td>1.0</td>
<td>Doughnuts of diameter 900-1100 Å and thick fibers (Plate VB).</td>
</tr>
</tbody>
</table>

* The weight ratio was calculated from the charge ratio cited in the literature.
H1-DNA complex was accompanied by additional retardation of the histone mobility in the complex and then by progressive release of histone H1 from the complex which was complete at 0.5 - 0.6 M NaCl.

The doughnuts observed in the present work were more uniform with a comparatively smaller size variation. The doughnut structure was lost when salt molarity was lowered below 0.05 in the present experiment. Previously, Olins and Olins (1971) observed similar structural changes, e.g., loose network of DNA-fiber in place of large doughnuts when the salt molarity was lowered from 0.10 to 0.001. Von Mickwitz et al. (1979) also reported similar structural variation in their DNA-H1 complexes. Olins and Olins (1971) concluded that the inability of doughnut and stem formation at decreased ionic strength may result from the high proline content at the basic portion of H1, consequent disruptions of lysine-phosphate interactions, and increased susceptibility of polyelectrolyte expansion.

Combining the present results with the previous data, it can be concluded that the binding of H1 with DNA is strongest in the range 0.1-0.2 M NaCl and results in a characteristic doughnut shaped structure. The formation of the doughnut structure at a particular salt molarity and their much larger dimension compared to that of the
nucleosomes definitely proves that H1 must be involved in higher order folding of the chromatin fibre and plays some fundamental role during chromatin condensation at different stages of the cell cycle.

3.5 CONCLUSION

(i) Reconstitution experiments showed that the core histones bind with DNA in two distinct steps. The arginine-rich histones bind around 1.2 - 1.0 M NaCl producing nucleosome-like particles of dimension 78-80 Å. The remaining histones bind near about 0.65-0.5 M NaCl completing the nucleosome structure and imposing a size of 112-115 Å.

(ii) The lysine-rich histone H1 binds with DNA within the range of 0.5-0.2 M NaCl producing thick stem-like structures and finally large doughnuts of dimension 900-1100 Å. The structure is not maintained when the salt molarity is lowered beyond 0.05.
REFERENCES


