Binding of Ethidium Bromide and Quinacrine Hydrochloride to Nucleic Acids and Reconstituted Nucleohistones

By A. V. CHITRE* and K. S. KORGAONKAR†

*Department of Chemistry, Sophia College, Bhulabhai Desai Road, Bombay 400 026, India, and
†Biophysics Division, Tata Memorial Cancer Research Institute, Parel, Bombay 400 012, India

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Studies of binding of ethidium bromide and quinacrine hydrochloride to native DNA at low ionic strength indicate that for both compounds the binding is selective, with about one binding site for about four nucleotides. Annealing of unfraccionated histones to DNA by a salt-gradient dialysis method slightly decreases the binding of the dyes to DNA. Similar observations made with reconstituted preparations by using individual histone fractions reveal that the arginine-rich histones (histones H3 and H4) are most effective in decreasing the binding. The binding studies with ethidium bromide at high ionic strength and with denatured DNA show that strong dye binding to DNA is strongly dependent on the ionic strength and on the secondary structure of DNA. The histones are not effective in decreasing the dye binding under conditions of high ionic strength. The results are consistent with the observations [Oliver & Chalkley (1974) Biochemistry 13, 5093–5098; Axel, Melchoir, Sollner-Web & Felsenfield (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4101–4105] that histones form some kind of surface structures on DNA through non-specific interactions and [Kornberg & Thomas (1974) Science 184, 865–868; Kornberg (1974) Science 184, 868–871; D’Anna & Isenberg (1974) Biochemistry 13, 4992–4997; Vandegrift, Serra, Marve & Wagner (1974) Biochemistry 13, 5087–5092] that the tendency of arginine-rich histones to aggregate may be an important factor in determining the structure of chromatin.

Intercalation has been accepted as the classic mechanism by which dyes interact with DNA. Since this process is highly selective, the dyes can be used as probes to give very precise information about the sites available for binding on the macromolecules; this can be useful in elucidating the structure of the macromolecules.

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) has been used as such a classic intercalator and optical probe for nucleic acids (Waring, 1965; Lepecq & Paoletti, 1966, 1967). Quinacrine hydrochloride [N\(^{4-}\)(6-chloro-2-methoxy-9-acridinyl)-N\(^{5-}\)'diethyl-1,4-pentanediamine hydrochloride] is known as an anti-malarial drug, and many workers have reported its binding to DNA (Irvin & Irvin, 1954; Kurnick & Radcliffe, 1962; O’Brien et al., 1966). We have calculated the binding parameters \(n\) (number of binding sites per dye molecule per nucleotide) and \(k’\) (the association constant for the dye–nucleotide complex) for the interaction between these dyes and nucleic acids by spectrophotometry.

To elucidate nucleohistone structure and the role of histones in chromatin we have further observed the changes in these binding parameters on annealing unfraccionated histones to nucleic acids by using salt-gradient dialysis in urea and EDTA (Shih & Bonner, 1970a). Though reconstituted nucleohistones have been used as simpler models of chromatin structure by some earlier workers (Olins, 1969; Washington et al., 1973) and ethidium bromide has been used as a probe for nucleohistone- or chromatin-binding studies (Olins, 1969; Lurquin & Selegy, 1972; Angerer & Moudrianakis, 1972), the results of such studies have been conflicting, and the salt-gradient-dialysis technique has not been used in these reconstitution studies. Also, the reconstituted preparations obtained by the same method, but with the individual histone fractions, namely the lysine-rich (H1), arginine-rich (H3 and H4) and slightly lysine-rich (H2A and H2B), were studied to obtain information about the role of individual histone fractions in chromatin structure.

The binding parameters between ethidium bromide and DNA and ethidium bromide and nucleohistones at high ionic strength or with denatured DNA were also studied to find the effect of histones on the binding under such conditions.

These studies have provided very useful information about the nature of association between histones and DNA.
Materials and Methods

Materials

Nucleic acids. Highly polymerized calf thymus DNA (sodium salt; more than 95% pure; from the Biochemicals Unit of Delhi University, New Delhi, India; mol.wt. approx. 8900000) was used for the studies, and highly polymerized calf thymus DNA (sodium salt; BDH Chemicals, Poole, Dorset, U.K.) was used as a standard (ε_{260} of a solution containing 1 g-atom of P/litre was 6000–7000 M⁻¹ cm⁻¹).

The denatured DNA was prepared by heating this DNA at 90°C for about 10 min and rapidly cooling the solution to 0°C.

Histones. The unfractionated histones and various histone fractions were supplied by Dr. J. L. Irvin, Department of Biochemistry, University of North Carolina, Chapel Hill, NC, U.S.A. These were isolated and fractionated by the method of Bellair & Maurizten (1964, 1965).

Dyes. Ethidium bromide (Calbiochem, San Diego, CA 92112, U.S.A.) and quinacrine hydrochloride (Sterling Winthrop Pharmaceutical Co., Rensselaer, NY 12144, U.S.A.) were supplied by Dr. Irvin and were used as such.

Other chemicals. All other chemicals used in this work were A.R.-grade reagents from BDH Chemicals, supplied by Glaxo Laboratories, Bombay, India.

Water. Deionized water, distilled in Pyrex glass over KMnO₄, was used throughout the work. The pH of this water and all other buffer solutions etc. was checked with a Beckman H⁺ pH-meter.

Dialysis tubing. Tubing for reconstitution was from Fisher Scientific, Pittsburgh, PA, U.S.A. (size 5–10mm) or was Visking tubing (30–36mm) from Scientific Instruments Centre, London W.C.1, U.K. The tubes were pretreated by boiling in 0.25 M-EDTA for 10 min, washing in deionized water, soaking in 95% (v/v) ethanol for 10 min and finally rinsing with deionized water. The tubes were kept in deionized water for at least 15 min before use.

Methods

The saturated DNA solution and the dye solutions were prepared by the method of Waring (1965), assuming ε₄₆₀ = 5600 M⁻¹ cm⁻¹. The nucleohistones were reconstituted by salt-gradient dialysis in urea/EDTA (Shih & Bonner, 1970a).

Ethidium bromide–DNA and ethidium bromide–nucleohistone complexes were prepared by the method of Peacock & Skerette (1956). These complexes were prepared and kept at 20°C for at least 30 min before measurement of the absorbance.

The changes in ionic strength were brought about by increasing the NaCl concentration in the buffer. The absorbance measurements were made with a Beckman DU-2 spectrophotometer by using 1 ml cuvettes.

The DNA concentrations in the complexes were expressed as DNA P (assuming that 1 g-atom of DNA P had ε₂₆₀ = 6200 M⁻¹ cm⁻¹ in 0.05 M-NaCl at pH 8.0). The DNA P in nucleohistone preparations was determined by the method of Ogur & Rosen (1950) by assuming ε₂₆₈ = 9946 M⁻¹ cm⁻¹ in 0.5 M-HClO₄. The protein concentration in the nucleohistones was measured by the method of Lowry et al. (1951), with bovine serum albumin (92% pure) supplied by Delhi University as standard. The nucleohistone preparations were expressed in terms of nucleohistone P: protein ratio. The A₄₂₅ and A₄₆₀ values were used for calculating the concentrations of quinacrine hydrochloride and ethidium bromide respectively.

The amounts of bound (fb) and free (b) dye were calculated as described by Parker & Irvin (1952), by

![Fig. 1. Effect of histones on binding of ethidium bromide to DNA (Table 1)](image)

DNA–ethidium bromide and nucleohistone (DNA+ various amounts of unfractionated histones)–ethidium bromide complexes were studied in 0.01 M-Tris/HCl buffer (pH 7.9)/0.01 M-NaCl. A was measured at 460 nm. Ethidium bromide concentration was constant at 40 μM. DNA-P or nucleohistone-P concentrations varied from 4 to 40 μM. Complexes: ○, DNA–ethidium bromide; △, nucleohistone (DNA+ unfractionated histones, 1:0.26)–ethidium bromide; □, nucleohistone (DNA+ unfractionated histones, 1:0.36)–ethidium bromide; ●, nucleohistone (DNA+ unfractionated histones, 1:0.46)–ethidium bromide. r, mole of nucleotide bound/mol of dye; c, mol of free dye.
assuming the values of \( \varepsilon_t = 5130 \text{M}^{-1} \cdot \text{cm}^{-1} \) and \( \varepsilon_{tb} = 14600 \text{M}^{-1} \cdot \text{cm}^{-1} \) for ethidium bromide (Waring, 1965) and \( \varepsilon_t = 9000 \text{M}^{-1} \cdot \text{cm}^{-1} \) and \( \varepsilon_{tb} = 3800 \text{M}^{-1} \cdot \text{cm}^{-1} \) for quinacrine hydrochloride (Irvin & Irvin, 1954). The values of the binding parameters \( n \) and \( k' \) were calculated from Scatchard (1949) plots. The values were corrected by a least-squares method if necessary.

### Results

The annealing of histones to DNA (Figs. 1 and 2) seems to decrease progressively the values of both \( n \) and \( k' \) for binding of ethidium bromide and quinacrine hydrochloride to native DNA. There is 1 binding site for the drugs per about 4 nucleotides to native DNA, and in the presence of unfractionated histones in a DNA/histone ratio of 1:0.46 in the reconstituted preparations there is 1 drug-binding site per about 5–6 nucleotides (Table 1).

The results (Table 1) with the nucleohistones obtained by using the individual histone fractions show that the arginine-rich histones (H3 and H4) when present in a DNA/histone ratio of 1:0.32 cause the maximum decrease in binding, and decrease the binding sites to 1 site per about 6 nucleotides for DNA–ethidium bromide binding and to 1 site per slightly more than 4 nucleotides for DNA–quinacrine hydrochloride binding. The decrease in association constant is much more pronounced for DNA–ethidium bromide binding, i.e. from \( 112 \times 10^4 \) to \( 2.2 \times 10^4 \) for DNA–quinacrine hydrochloride binding.

![Fig. 2. Effect of histones on binding of quinacrine hydrochloride to DNA (Table 1)](image)

DNA–quinacrine hydrochloride and nucleohistone (DNA+ various amounts of unfractionated histones)–quinacrine hydrochloride complexes were studied in 0.01 M-phosphate buffer, pH 6.5. \( A \) was measured at 425 nm. Quinacrine hydrochloride concentration was constant at 0.4 mM. DNA-P or nucleohistone-P concentrations varied from 4 to 40 mM. Complexes: \( \circ \), DNA–quinacrine hydrochloride; \( \triangle \), nucleohistone (DNA+ unfractionated histones, 1:0.26)–quinacrine hydrochloride; \( \square \), nucleohistone (DNA+ unfractionated histones, 1:0.36)–quinacrine hydrochloride; \( \bullet \), nucleohistone (DNA+ unfractionated histones, 1:0.46)–quinacrine hydrochloride.

<table>
<thead>
<tr>
<th>DNA or nucleohistone present</th>
<th>Histone present</th>
<th>DNA/histone ratio</th>
<th>Dye present</th>
<th>( n )</th>
<th>( k' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>—</td>
<td>—</td>
<td>Ethidium bromide</td>
<td>0.24</td>
<td><em>112 \times 10^4</em></td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.26</td>
<td>Ethidium bromide</td>
<td>0.21</td>
<td><em>52.8 \times 10^4</em></td>
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<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.36</td>
<td>Ethidium bromide</td>
<td>0.20</td>
<td><em>34.8 \times 10^4</em></td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.46</td>
<td>Ethidium bromide</td>
<td>0.19</td>
<td><em>6.22 \times 10^4</em></td>
</tr>
<tr>
<td>DNA</td>
<td>—</td>
<td>Quinacrine hydrochloride</td>
<td>0.26</td>
<td><em>13.7 \times 10^4</em></td>
<td></td>
</tr>
<tr>
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<td>Unfractionated</td>
<td>1:0.26</td>
<td>Quinacrine hydrochloride</td>
<td>0.25</td>
<td><em>13.5 \times 10^4</em></td>
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<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.36</td>
<td>Quinacrine hydrochloride</td>
<td>0.23</td>
<td><em>11.4 \times 10^4</em></td>
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<td>Unfractionated</td>
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<td>Quinacrine hydrochloride</td>
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<td><em>7.92 \times 10^4</em></td>
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<tr>
<td>Nucleohistone</td>
<td>Histone H1</td>
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<td>Ethidium bromide</td>
<td>0.22</td>
<td><em>220 \times 10^4</em></td>
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<tr>
<td>Nucleohistone</td>
<td>Histones H3 and H4</td>
<td>1:0.32</td>
<td>Ethidium bromide</td>
<td>0.18</td>
<td><em>17.7 \times 10^4</em></td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Histones H2A and H2B</td>
<td>1:0.36</td>
<td>Ethidium bromide</td>
<td>0.24</td>
<td><em>60.3 \times 10^4</em></td>
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<tr>
<td>Nucleohistone</td>
<td>Histone H1</td>
<td>1:0.23</td>
<td>Quinacrine hydrochloride</td>
<td>0.26</td>
<td><em>11.0 \times 10^4</em></td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Histones H3 and H4</td>
<td>1:0.32</td>
<td>Quinacrine hydrochloride</td>
<td>0.24</td>
<td><em>9.53 \times 10^4</em></td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Histones H2A and H2B</td>
<td>1:0.36</td>
<td>Quinacrine hydrochloride</td>
<td>0.25</td>
<td><em>8.0 \times 10^4</em></td>
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</tbody>
</table>

Table 1. Effect of histones on binding of dyes to DNA and nucleohistones

For experimental details see the text and legends to Figs. 1 and 2.
17.7 x 10^4, compared with DNA-quinacrine hydrochloride binding, i.e. from 13.7 x 10^4 to 9.53 x 10^4.

The results of the experiments with DNA-ethidium bromide binding at high ionic strengths (Figs. 3 and 4) show that when I is changed from 0.02 to 1.01 the number of binding sites is decreased to 1 binding site for about 7 nucleotides and the association constant is decreased to 14.1 x 10^4 from 112 x 10^4 (Table 2). At high ionic strengths (Figs. 3 and 4) and with denatured DNA (Fig. 5) the histones in the reconstituted preparations seem to have very little influence on the ethidium bromide-DNA interaction, as shown in Tables 2 and 3.

Discussion

The results indicate that increasing the proportion of histones to DNA in the reconstituted nucleohistone complexes decreases the strength of binding, and there is a small but definite decrease in the number of binding sites available for dye binding. Olins (1969) observed similar decreases in the binding sites in his work on histone HI-DNA complexes with ethidium bromide, but did not observe significant differences in the slopes of the Scatchard (1949) plots. Lurquin & Selegy (1972) also reported that selective deproteinization of chromatin produces a large increase in ethidium bromide-binding sites, but no appreciable difference in the association constant was seen. Our results are more in agreement with those of Angerer & Moudrianakis (1972), who reported decreases in both n and k' values. Washington et al. (1973) also observed in their studies on binding of anti-malarial anti-quinolines to nucleohistone complexes that the decrease in binding is due to masking of certain potential
Table 2. Effect of ionic strength on binding of ethidium bromide to DNA and nucleohistone
For experimental details see the text and legends to Figs. 1, 3 and 4.

<table>
<thead>
<tr>
<th>DNA or nucleohistone present</th>
<th>Histone present</th>
<th>DNA/histone ratio</th>
<th>I</th>
<th>n</th>
<th>k'</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>—</td>
<td>1:0.46</td>
<td>0.02</td>
<td>0.24</td>
<td>112 x 10^4</td>
</tr>
<tr>
<td>DNA</td>
<td>—</td>
<td>1:0.46</td>
<td>0.07</td>
<td>0.21</td>
<td>101 x 10^4</td>
</tr>
<tr>
<td>DNA</td>
<td>—</td>
<td>1:0.46</td>
<td>0.61</td>
<td>0.16</td>
<td>21 x 10^4</td>
</tr>
<tr>
<td>DNA</td>
<td>—</td>
<td>1:0.46</td>
<td>1.01</td>
<td>0.15</td>
<td>14.1 x 10^4</td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.46</td>
<td>0.02</td>
<td>0.19</td>
<td>6.22 x 10^4</td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.46</td>
<td>0.07</td>
<td>0.20</td>
<td>11.8 x 10^4</td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.46</td>
<td>0.61</td>
<td>0.15</td>
<td>14.1 x 10^4</td>
</tr>
<tr>
<td>Nucleohistone</td>
<td>Unfractionated</td>
<td>1:0.46</td>
<td>1.01</td>
<td>0.14</td>
<td>10.1 x 10^4</td>
</tr>
</tbody>
</table>

Table 3. Effect of denaturation on binding of ethidium bromide to DNA and nucleohistones at low ionic strength (I 0.02)
For experimental details see the text and legends to Figs. 1, 3 and 5.

<table>
<thead>
<tr>
<th>DNA or nucleohistone present</th>
<th>Histone present</th>
<th>DNA/histone ratio</th>
<th>n</th>
<th>k'</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>—</td>
<td>1:0.46</td>
<td>0.24</td>
<td>112 x 10^4</td>
</tr>
<tr>
<td>Denatured DNA</td>
<td>—</td>
<td>1:0.46</td>
<td>0.17</td>
<td>96.3 x 10^4</td>
</tr>
<tr>
<td>Nucleohistone (native DNA)</td>
<td>Unfractionated</td>
<td>1:0.46</td>
<td>0.19</td>
<td>6.22 x 10^4</td>
</tr>
<tr>
<td>Nucleohistone (denatured DNA)</td>
<td>Unfractionated</td>
<td>1:0.36</td>
<td>0.168</td>
<td>73.8 x 10^4</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of denaturation on binding of ethidium bromide to DNA and nucleohistones (Table 3)
Denatured DNA—ethidium bromide and nucleohistone (denatured DNA + unfractionated histones, 1:0.36)–ethidium bromide complexes were studied in 0.01 M-Tris/Cl buffer (pH 7.9)/0.01 M-NaCl. A was measured at 460 nm. Ethidium bromide concentration was constant at 40 μM. DNA–P or nucleohistone-P concentrations varied from 4 to 40 μM. Complexes: ○, denatured DNA–ethidium bromide; △, nucleohistone–ethidium bromide.

binding sites by histones. They, however, used much higher histone/DNA ratios in their studies.

It is noteworthy that even when histones are present in a DNA/histone ratio of 1:0.46 in the nucleohistone complexes the decrease in n is not significantly large. This suggests that either the dye molecules are able to compete successfully with the histones by penetrating through the protein layer or that most of the dye-binding sites on DNA are not directly occupied by histones. Thus it seems probable that histones interact with DNA, through mostly non-specific interaction, and may not occupy fixed sites on DNA, but just form some kind of surface structures that stabilize the DNA double helix, thereby giving less opportunity for the drug molecules to intercalate. The stabilization of DNA double helix by histones has been reported previously (Shih & Bonner, 1970a; Lesko et al., 1968). Observations of Oliver & Chalkley (1974) that histones act asymmetrically with DNA along the entire site of the molecule and that about half of the DNA is unprotected by proteins also support our observation. Axel et al. (1974) also observed that the binding of histone to DNA in chromatin involves a very limited number of specific and well-defined contacts between proteins and DNA which arise from the structural properties of histones. There are no previous reports on the binding of quinacrine hydrochloride to nucleohistone complexes, but the similarity in its behaviour to that of ethidium bromide suggests that a similar mechanism of binding is involved in both the cases. Histones alone do not show direct binding
with ethidium bromide or quinacrine hydrochloride and as such have no influence on the spectra of the dyes.

The histone H3 and H4 fraction seems to be most effective in decreasing the binding of dyes to DNA. These results are consistent with the observations of various other workers. Akinrimisi et al. (1965) reported that at neutral pH the binding of arginine-rich histones is greater than that of lysine-rich histone fractions. Lurquin & Selegy (1972) also found that removal of arginine-rich fractions from the chromatin causes significant increase in binding of ethidium bromide to chromatin. The observations of Shih & Bonner (1970a,b) also suggest that arginine-rich histones cause more stabilizations of DNA and give maximum half-melting-temperature values for the DNA–histone-H4 complex. The results are inadequate to determine the precise role of the various histone fractions in chromatin structure and function, but this effect of the arginine-rich histone fraction seems to be consistent with the observations of Kornberg (1974) and Kornberg & Thomas (1974) that there is less of histone H1 in histone–DNA complexes and that the arginine-rich fraction has a tendency to aggregate. This effect might have an important bearing on the structural characters of chromatin and is probably more involved in histone binding to DNA. D’Anna & Isenberg (1974) have also reported the aggregation of arginine-rich histone, and supercooling of DNA in chromatin by these histones has been reported by Vandegrift et al. (1974). The results of the experiments at higher ionic strengths indicate that even the strong primary binding of ethidium bromide to DNA is also strongly dependent on ionic strength. This decrease in the binding of ethidium bromide to DNA can be attributed to the stabilization of the DNA double helix observed by Marmur et al. (1963) and partly to the decrease in electrostatic factors consequent on the large increase in salt concentration. The presence of histones has practically no influence on DNA–ethidium bromide interaction at high ionic strength. This smaller effect at higher ionic strength can be attributed to progressive dissociation of histones at higher ionic strength.

The decrease in dye binding to denatured DNA is in agreement with the previous studies (Parker & Irvin, 1952; Irvin & Irvin, 1954; Gersch & Jordan, 1965; Lurquin & Buchet-Mahieu, 1971) and reveals that loss of double-helical structure of DNA provides less opportunity for the dye to intercalate and to bind strongly.

These results further show that when reconstitution is attempted with denatured DNA the amount of histones that can be annealed to DNA without the precipitation of the nucleohistone complexes during the dialysis is decreased by about 20%. Similar decrease in binding of proteins to DNA has been previously observed by Akinrimisi et al. (1965) and Ascoli et al. (1961). Bonner & T’so (1964) have similarly observed that substances such as steroids or carcinogens, which facilitate uncoiling of DNA, will also probably decrease the binding of histones to DNA. Thus histone–DNA interaction is facilitated by secondary structure with some ordered geometry and consequent increase in the charge density, though specific binding sites may not be necessary. Further, the histones seem to have weaker influence on the binding of ethidium bromide to denatured DNA as compared with binding to native DNA. This may be due to the smaller quantities of histones present in such reconstituted preparations, which may not be capable of causing the necessary stabilization of already denatured DNA structure.

It is not certain whether these reconstituted preparations behave as the natural chromatins. Obviously the non-histone proteins are absent from these preparations. However, the salt-gradient-dialysis procedure used here should minimize the histone–histone aggregates and histone–nucleohistone complex interactions and the reconstituted nucleohistone complexes should show characters very nearly comparable with those of the natural chromatins.

We thank Dr. J. L. Irvin, Department of Biochemistry, University of North Carolina, Chapel Hill, NC, U.S.A., for his gift of histones, dyes and other chemicals and for his valuable suggestions. We also acknowledge the financial assistance to A. V. C. by the University of Bombay, the University Grants Commission, New Delhi, and the Maharashtra State Government, without which this work would not have been possible.

References


1979