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A study of the interaction between ethidium bromide and rye chromatin: comparison with calf thymus chromatin

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ABSTRACT

We studied the interaction of ethidium bromide with rye and calf thymus chromatin. Both types of chromatin have the same dye accessibility, which is about 50% of that of DNA. From this result we conclude that the molecular structure of these two chromatins is similar.

For rye, the extraction of H1 produces no change in the binding of ethidium bromide. The subsequent extraction of H2A and H2B produces a 14% increase in the binding, and the removal of H3 and H4, another 54% increase. At this stage, the number of binding sites is still less than that of DNA. This is presumably due to the presence of some tightly bound non-histones. Thus, the arginine-rich histones and the tightly bound non-histones are most responsible for limiting the binding of ethidium bromide to rye chromatin.

INTRODUCTION

Great progress has been made in recent years in the elucidation of the structure of chromatin. A model of a subunit structure has been proposed which takes into account much of the new data (1, 2, 3). In this model chromatin fibers are constituted of beads joined together by a strand of DNA. These beads are made up of a core of the hydrophobic halves of the histones H2A, H2B, H3, and H4, around which the DNA is wrapped. The model is supported by many experiments, particularly by electron microscopic observations in which strings of beads have been visualized (4) and by nuclease digestion of chromatin or nuclei, which produces fragments of nucleoprotein of discrete size containing a segment of DNA of about 200 base pairs (5, 6). These fragments could be the proposed subunits of chromatin. The experiments have been performed in different animal tissues, in Tetrahymena (7) and in yeast (8) indicating that such a structure could be widespread in eucaryotic nuclei. But up to now, little work has been done on plant material. In just one case has nuclease digestion been done on a plant chromatin (pea). It gave results similar to those already obtained with animal chromatin, pro-

ducing after digestion a monomer of about 170 base pairs (9). In order, therefore, to obtain more information on plant chromatin, we studied the interaction of the intercalating dye ethidium bromide with rye (Secale cereale) chromatin and DNA. We then repeated these experiments with calf thymus chromatin and DNA and compared the results to those obtained with rye. Finally, we evaluated the importance of the different chromosomal proteins in the restriction of the binding of ethidium bromide to DNA in the two chromatins.

MATERIALS AND METHODS

Chromatin and DNA extractions

Calf thymus and rye (Secale cereale) were used for chromatin and DNA extractions. Calf thymus was taken from recently slaughtered animals and frozen at -20° C until used. Rye was grown in the dark for seven days; the leaves were then collected, washed, and used immediately.

Calf thymus and rye chromatin purifications were carried out as previously described (10). The 0.15 M NaCl precipitated chromatin was used in the subsequent steps.

For the preparation of DNA, purified precipitated chromatin was solubilized in 0.5 mM EDTA adjusted to pH 7.5 with Tris and extracted according to the technique of Marmur (11).

Protein extractions

Proteins were selectively extracted from the chromatin using two types of buffer: sodium phosphate buffers containing urea and sodium phosphate buffers containing NaCl (12). All buffers were adjusted to pH 5.5 to limit proteolytic degradation of the histones (13).

Purified chromatin (20-30 mg) was solubilized in about 10 ml of deionized water and dialysed overnight against the appropriate buffer. To separate the extracted histones from the partially deproteinized chromatin, the dialysate was centrifuged at 1000 g for 5 minutes and the supernatant chromatographed on a Bio Gel A15 column (2.5 x 35 cm). Fractions of three ml each were collected and the optical densities read at 230 nm and 260 nm with a Zeiss PMQ II spectrophotometer.

The fractions containing the extracted proteins were pooled and dialysed overnight against 0.4 N H_2SO_4. The dialysates were then centrifuged at 18,000 g for 10 minutes, and the solubilized histones were precip-

itated with 5 volumes of absolute ethanol and stored for 3 days at -20° C. To isolate the residual histones, the fractions containing the partially deproteinized chromatin were collected and treated in the same manner as the extracted histones.

Histone electrophoresis

Electrophoresis of the extracted and residual proteins was carried out for 4 hours at 120 volts in 9 cm gels containing 15% polyacrylamide and 2.5 M urea according to the method described by Panyim and Chalkley (14).

Extinction coefficients

The molar extinction coefficients for chromatins were determined according to Tuan and Bonner (15). The optical densities at 260 nm were not corrected for light scattering due to turbidity. For DNA, Tuan and Bonner's value of 6815 M^{-1} cm^{-1} at 260 nm was used.

Ethidium bromide studies

Ethidium bromide was purchased from Sigma Chemical Company. For the binding studies, chromatin and DNA were solubilized in 0.1 mM Tris-HCl, pH 8.0 (16). The partially deproteinized chromatins obtained after chromatography on Bio Gel A15 were dialysed extensively against the same buffer. The native and partially deproteinized chromatin solutions were then sheared in a Virtis 45 homogenizer at full speed for 5 minutes and centrifuged at 30,000 g for 15 minutes. The resulting supernatant was used for the binding studies.

Solutions of native chromatin, partially deproteinized chromatin, or DNA of different concentrations were prepared by dilution with 0.1 mM Tris-HCl pH 8.0. To these solutions an equal volume of an ethidium bromide solution of known concentration (determined spectrophotometrically) was added. Alternatively, 0.1 ml of a concentrated solution of the dye was added to 0.9 ml of chromatin or DNA. Optical densities were then determined at 460 nm with a Zeiss PMQ II spectrophotometer. Using this data and a molar extinction coefficient of 4,800 M^{-1} cm^{-1} at 460 nm for ethidium bromide, Scatchard plots (17) were made according to the method of Peacocke and Skerret (18). The equation for the linear portion of these curves was determined using the method of least squares. From this equation we calculated the number of binding sites for the dye per nucleotide (n) and the association constant of the complex (k).

Protein and DNA assay

In order to determine the protein to DNA ratio of the partially deproteinized chromatins, proteins were assayed according to the method of Lowry (19), and DNA by the diphenylamine method of Burton (20).

RESULTS

Extinction coefficients: The molar extinction coefficients are 7,000 M^{-1} cm^{-1} (SD=200)* and 6,820 M^{-1} cm^{-1} (SD=50) respectively for sheared calf thymus and rye chromatin at 260 nm. Our value for calf thymus is lower than the 7561 M^{-1} cm^{-1} given by Tuan and Bonner (15). This is due to the shearing of our chromatin preparation, which decreased the turbidity of the solution and consequently its absorption. Using unsheared chromatin, we obtained a value of 7,700 M^{-1} cm^{-1}, which is comparable to that of Tuan and Bonner (15). A similar effect was observed with rye chromatin where unsheared chromatin gave an extinction coefficient of 7,440 M^{-1} cm^{-1}.

Since, according to Tuan and Bonner (15), a 0.6 M NaCl extraction of chromatin does not change its extinction coefficient at 260 nm, we used the same coefficients for the native and the H1-depleted chromatins. Furthermore, because of the similarity between the extinction coefficient of DNA and sheared rye chromatin, we used the same value (6815 M^{-1} cm^{-1}) for DNA and for chromatin containing only histones H3 and H4.

Ethidium bromide binding to chromatin and DNA: The first part of this study consisted of a comparison of the binding of ethidium bromide to calf thymus and rye chromatin. The Scatchard plots obtained for these two chromatins are shown in figures 1 and 2. The binding parameters measured are the following: for calf thymus chromatin, $n = 0.152$ and $k = 1.1 \times 10^7$ M^{-1}; and for rye chromatin, $n = 0.156$ and $k = 1.3 \times 10^7$ M^{-1}. Hence, with the techniques employed we found no difference in the binding of ethidium bromide to the two chromatins.

Next we extracted the DNAs from the chromatins and repeated the binding studies with ethidium bromide. We found no differences in the number of binding sites between rye and calf thymus DNA. This result was expected, since linear DNAs from a variety of sources were found to bind similar amounts of the dye (21). Compared to chromatin, an increase in the number of binding sites was found with n increasing from 0.156 to 0.283 for rye and from 0.152 to 0.284 for calf thymus. The binding constants measured were

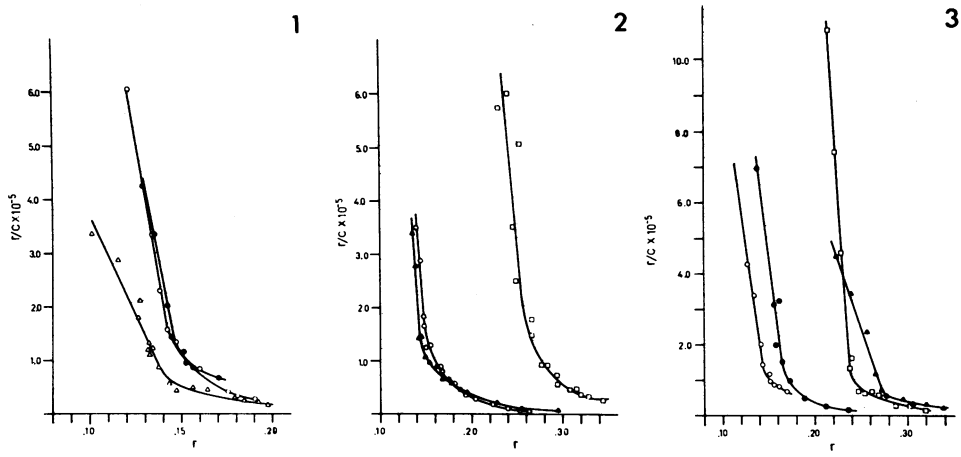

Figure 1: Scatchard plots for the binding of ethidium bromide to rye chromatin ●, 0.5 phosphate 1 M urea-extracted rye chromatin Δ, and 0.6 M NaCl 0.001 M phosphate-extracted rye chromatin o.

Figure 2: Scatchard plots for the binding of ethidium bromide to calf thymus chromatin ▲, 0.5 M phosphate 1 M urea-extracted calf thymus chromatin o, and calf thymus DNA □.

Figure 3: Scatchard plots for the binding of ethidium bromide to rye chromatin o, 0.8 M phosphate 2 M urea-extracted chromatin●, 2.0 M NaCl 0.001 M phosphate-extracted rye chromatin□, and rye DNA ▲.

0.6 x 10^7 M^{-1} for calf thymus and 0.4 x 10^7 M^{-1} for rye DNA (Table 1). We conclude, therefore, that no differences in ethidium bromide binding are detectable when either the DNAs or chromatins of plant and animal tissues are compared.

However, as was demonstrated by Nadeau et al. (10, and Nadeau, unpublished) the rye and calf thymus H1, H2A and H2B** histones show major differences in relative mobilities on polyacrylamide-urea gels, molecular weights, amino acid compositions and tryptic peptide maps. Thus the reduction in the number of binding sites was the same in the two chromatins even though the histones responsible for it were quite different. We, therefore, felt that it would be interesting to study in more detail the effect of chromosomal proteins on the binding of ethidium bromide to plant chromatin. For this reason we undertook the selective extraction of the proteins from rye chromatin.

Table 1 Ethidium bromide binding

Material(*)	Proteins extracted	n	k x 10^{-7}(M^{-1})	% increase of $_n$
Rye chrom. (4)	none	0.156	1.3	---
C.T. chrom. (4)	none	0.152	1.1	---
Rye DNA (3)	all proteins	0.283	0.4	100
C.T. DNA (3)	all proteins	0.284	0.6	100
Rye-A (3)	H1	0.151	2.0	---
Rye-B (4)	H1	0.153	0.7	---
Rye-C (2)	H1, H2A & H2B	0.174	1.8	14
Rye-D (2)	all histones	0.242	3.8	68
C.T. -A (4)	H1	0.2	1.6	36
C.T. -B (2)	H1	0.159	2.4	---

*number of experiments in parentheses

Letters refer to buffers used in chromatin deproteinizations: A(0.6 M NaCl-0.001 M PO_4), B (1.0 M urea - 0.5 M PO_4), C (2.0 M urea - 0.8 M PO_4), D (2.0 M NaCl - 0.001 M PO_4).

Histone extractions: Lysine-rich histone (H1) was selectively extracted from both rye and calf thymus tissues by either a 0.001 M sodium phosphate buffer at pH 5.5 containing 0.6 M NaCl (12), or a 0.5 M sodium phosphate buffer at pH 5.5 containing 1M urea (12, 13).

As higher NaCl concentrations gave no specifity of histone extraction from rye chromatin, we used another sodium phosphate-urea buffer (2 M urea - 0.8 M sodium phosphate, pH 5.5) to extract selectively H1, H2A and H2B leaving only the arginine-rich histones H3 and H4 bound to the DNA (12).

Finally, whole histones were extracted from rye chromatin using a 0.001 M sodium phosphate buffer at pH 5.5 containing 2.0 M NaCl (12).

Photographs of electrophoretic gels of extracted and residual rye histones are shown in Figure 4. It can be seen that no degradation is observable in residual proteins. It can also be seen that the extractions were selective since no cross-contamination was detectable between the extracted and residual proteins. When fractions H1, H2A and H2B were extracted with 2.0 M urea - 0.8 M sodium phosphate, a band having the same mobility as H3 was

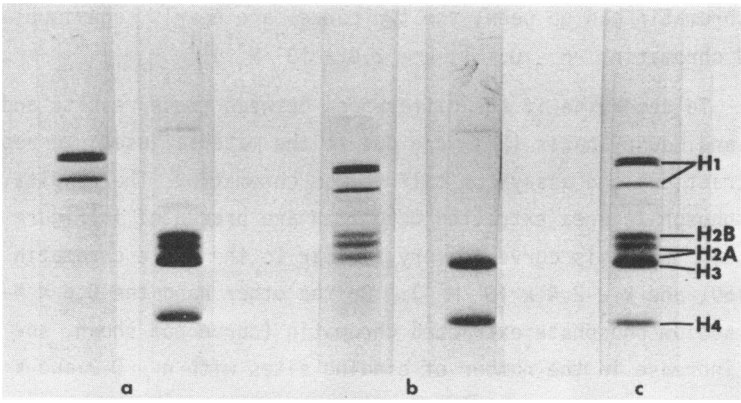

Figure 4: Electrophoretic gels of selectively extracted rye histones.
a) left gel: 0.5 M phosphate 1 M urea-extracted proteins.
right gel: residual proteins. It should be noted that we obtained identical results with a 0.6 M NaCl 0.001 M phosphate-extraction.
b) left gel: 0.8 M phosphate 2 M urea-extracted proteins.
right gel: residual proteins.
c) 2.0 M NaCl 0.001 M phosphate-extracted proteins.

observed. To test for the presence of H3 we attempted to provoke dimer formation in the histones extracted with this buffer. Since in no case did we observe any band corresponding to a dimer, we concluded that the extraction with 2.0 M urea - 0.8 M phosphate is specific for histones H1, H2A and H2B. The band with a mobility identical to H3 most likely contains a fraction of histone H2A. A similar observation was made by Sommer and Chalkley for pea histones (22).

Ethidium bromide binding to partially deproteinized chromatin: H1 was first extracted from rye chromatin using 1 M urea in 0.5 M sodium phosphate buffer at pH 5.5. The Scatchard plot obtained for the binding of ethidium bromide to this H1-depleted chromatin is presented in Figure 1. To our surprise, and contrary to results already published for calf thymus chromatin (23), we found no influence of this protein on the number of binding sites for the dye ($n = 0.153$). The only difference observed with chromatin was that the slope of the curve and thus the binding constant were slightly lower ($k = 0.7 \times 10^7\ M^{-1}$) though the difference was small ($k_{chrom.} = 1.3 \times 10^7\ M^{-1}$).

We next extracted H1 from rye chromatin using the 0.6 M NaCl -

0.001 M sodium phosphate buffer, pH 5.5. This Scatchard plot is given in Figure 1 and here, a still greater similarity between chromatin and H1-depleted chromatin can be seen; the two curves are nearly superimposable (H1-depleted chromatin: $n = 0.151$, $k = 2.0 \times 10^7\ M^{-1}$).

To determine if the differences between these results and those of Angerer and Moudrianakis (23) were due to the material used, we repeated the same extractions and assays on calf thymus chromatin. The results obtained for the phosphate-urea extracted chromatin are presented in Figure 2. It can be seen that this curve is very similar to the whole chromatin curve ($n = 0.159$, and $k = 2.4 \times 10^7\ M^{-1}$). On the other hand the 0.6 M NaCl - 0.001 M sodium phosphate extracted chromatin (curve not shown, see Table 1) gave an increase in the number of binding sites with $n = 0.2$ and $k = 1.6 \times 10^7\ M^{-1}$. This represents an augmentation of 36% in comparison with whole calf thymus chromatin. Thus using a similar H1 histone extraction technique and the same tissue, we obtained results comparable to those of Angerer and Moudrianakis (23). On the other hand, when phosphate-urea was used for extracting H1 from calf thymus chromatin, the results were quite different.

Histones H1, H2A and H2B were extracted from rye chromatin with 2.0 M urea - 0.8 M sodium phosphate leaving a partially deproteinized chromatin containing only histones H3 and H4. This chromatin was used for binding studies with ethidium bromide, and a typical result is shown in Fig. 3. In comparison with native chromatin, only a slight increase in the number of binding sites for the dye was observed, with n increasing from 0.153 to 0.174. The binding constant was $1.8 \times 10^7\ M^{-1}$. Thus the arginine-rich histones H3 and H4 by themselves limited the dye binding to a considerable extent. The extraction of all the histones with 2.0 M NaCl - 0.001 M sodium phosphate caused a large increase in the number of sites, since we obtained $n = 0.242$ with $k = 3.8 \times 10^7\ M^{-1}$. In spite of this increase we did not obtain a number of sites identical to that of DNA. Presumably this was due to the presence of some non-histone proteins.

To obtain information about the amount of protein extracted from the chromatin, the protein to DNA ratios were determined. From these data we calculated that with the extraction of H1, approximately 8% of the total proteins were removed from the chromatin. When H1, H2A and H2B were extracted, 52% of the proteins were removed, and when the chromatin was dialysed against 2 M NaCl to eliminate all the histones, approximately 97% of the proteins

were removed. This last result is interesting in light of the fact that the remaining 3% of the proteins were capable of limiting the binding of ethidium bromide to an appreciable extent.

DISCUSSION

In the first section of this study we compared the binding of the intercalating dye ethidium bromide to rye and to calf thymus chromatin and found a nearly identical number of primary binding sites. This result suggests that in spite of the presence of different histones, the two chromatins have a similar structural organisation at the molecular level. This hypothesis is also in accord with the results of McGhee and Engel (9). They found that after nuclease digestion of pea chromatin more than half of the DNA existed as fragments of 170 base pairs, a result similar to that obtained for animal chromatin.

In addition we found that the rye and calf thymus chromatin bound approximately 45% less dye than did their respective DNAs. This reduced accessibility of the rye chromatin to the dye is consistent with the many experiments suggesting that about half of the DNA in a variety of animal chromatins is much less accessible to various probes (24,25) or to the solvent environment (26) than the rest of the DNA. On the other hand, other workers (16, 23, 27, 28) found that chromatin had one fifth to two thirds of the ethidium bromide sites as compared with DNA. These differences may be caused by the use of chromatin isolated from different sources, prepared by different methods, and in some cases assayed under different ionic conditions. Since all of the studies on ethidium bromide binding were carried out on sheared chromatin, it is also possible that some of the diversity can be explained by differences in the degree of shearing. In this regard it is interesting to note that Remington and Klevecz (29) found that sheared chromatin bound more polylysine than did unsheared chromatin.

It can be seen in Table 1 that the association constants for chromatin, partially deproteinized chromatin, and DNA of rye are similar and vary around a mean value of $1.7 \times 10^7\ M^{-1}$. Using the same spectrophotometric method as we used for detecting ethidium bromide binding, Lurquin and Seligy (16) also found similar association constants for chromatin and DNA. On the other, Angerer *et. al* (28) employing fluorometric techniques were able to distinguish a minimum of two types of intercalation sites in chromatin as compared to only one in DNA. The location of these sites

in chromatin is unknown.

The second part of this work consisted of a study of the chromosomal proteins involved in the restriction of ethidium bromide binding to chromatin. From the results presented in Table 1, it can be seen that for rye chromatin the extractions of H1 with either NaCl-phosphate or urea-phosphate buffers produced no increase in the number of binding sites for ethidium bromide. The extraction of H1 plus the moderately lysine-rich histones H2A and H2B caused a 14% increase, and finally the extraction of all the histones gave a 68% increase in the number of binding sites. The observation concerning the lack of influence of H1 can be interpreted in light of the properties and possible role of this protein. It is the histone which is the least tightly bound to DNA, it is not found associated with the other four histones in isolated nucleosomes (nu bodies) (30), and its presence is not required to obtain the X-ray diffraction pattern characteristic of native chromatin (31). The H1 fraction seems to be involved in crosslinking chromatin fibrils and in the condensation of chromatin (32,33) rather than in the maintenance of the basic subunit structure of chromatin.

Unlike the effects obtained with H1, the extraction of any of the other histones produces structural modification of chromatin detectable by circular dichroism (34) or by X-ray diffraction (35). It is also possible to reconstitute nucleohistone from DNA and histones H2A, H2B, H3 and H4 that possess properties comparable to those of native chromatin (36, 37). Thus these four histones are all involved in the maintenance of the subunit structure of chromatin, and, as we have shown, in the restriction of ethidium bromide binding. But are they equally implicated, or are some of them more important than others? These four histones can be divided in two classes according to their properties: the arginine-rich histones H3 and H4, and the slightly lysine-rich histones H2A and H2B. The arginine-rich histones have been much more conserved during evolution than the two slightly lysine-rich histones, and they are more firmly bound to DNA in both animal (13) and plant tissues (this work). Experiments involving a selective extraction of histones from chromatin, followed by a study of the structure of the residual nucleoprotein by either circular dichroism or X-ray diffraction, demonstrated that the presence of the arginine-rich histones, and even of H4 alone, was enough to maintain the supercoil (38, 39). All these results suggest that the arginine-rich histones play a more important role than the slightly lysine-rich histones in the structural organisation of chromatin. This situation is

reflected in the results we obtained with ethidium bromide where the extraction of H2A and H2B from rye chromatin produced a 14% increase in binding while the extraction of H3 and H4 gave an increase of 54% in the number of primary binding sites for ethidium bromide. On the other hand, we can not absolutely exclude the possibility that the increase in binding in rye is due to the removal of a certain quantity of histone and is, therefore, independent of the type of histone removed. To decide between these possibilities it would be necessary to extract fractions H3 and H4 while leaving histones H1, H2A and H2B complexed to the DNA. In either case we favor the interpretation that the protein-induced folding of the DNA into a more compact structure in chromatin restricts the binding of ethidium bromide and that as long as the folding is totally or partially maintained, the binding remains limited even though a large proportion of the protein is absent.

Other laboratories (16, 23, 27, 28) have studied the binding of ethidium bromide to partially deproteinized chromatin. Lurquin and Seligy (16) and Williams et. al (27) found that the removal of H1 plus H5, but not H1 alone, from gander erythrocyte chromatin led to a large increase in the number of ethidium bromide binding sites. Also, Angerer and Moudrianakis (23) and Angerer et. al (28) working with calf thymus chromatin, noted a substantial increase in the number of binding sites following the extraction of H1 with 0.5 M NaCl. It can be seen in Table 1 that we obtained results comparable to those of Angerer and coworkers (23, 28) in that the extraction of H1 from calf thymus chromatin with 0.6 M NaCl led to a 36% increase in the number of binding sites. On the other hand, when we extracted calf H1 with 0.5 M phosphate-1 M urea, no increase in binding was observed. Obviously the two H1-depleted chromatins were different. Our experiments illustrate that analyses performed with partially deproteinized chromatin should be interpreted with caution since the results obtained may vary with the method of histone extraction employed.

On the other hand, as we have already mentioned, a different result was obtained with rye chromatin, where the histones H3, H2A, H2B, and H4 inhibited the ethidium bromide binding to the same extent as did the complete histone complement. The extraction of H1 with either NaCl or with phosphate-urea solutions did not lead to an increase in binding. This difference in behaviour between identically prepared H1-depleted rye and calf thymus chromatin is most easily explained by the presence of different histone fractions. Although rye and calf H2A and H2B fractions have similar

affinities for DNA (12),the rye H2A and H2B histones are larger with molecular weights of 15000 and 17000 compared with 12500 and 14000 for the corresponding calf fractions; they also have different amino acid compositions and tryptic maps (P. Nadeau, Ph.D. thesis). Why these proteins do not afford the same protection to rye and calf chromatin depleted of H1 by NaCl remains unknown, although their susceptibility to redistribution during the H1 extraction may be implicated.

The results found in Table 1 indicate that rye chromatin extracted with 2.0 M NaCl bound less ethidium bromide than did DNA. We have shown (Fig. 4) that this concentration of NaCl removed all of the histones. In another experiment (results not presented), native rye chromatin was treated with 2 M NaCl, and the resulting residual chromatin separated from the solubilized proteins. This residual chromatin was then extracted with 0.4 N H_2SO_4 in an attempt to isolate any remaining histones, but none were detected. It thus appears that the proteins which remained bound to the DNA after 2.0 M NaCl treatment were non-histone proteins.

We have determined that approximately 30% of the non-histones remain attached to DNA after the extraction with 2.0 M NaCl. Pederson and Bhorjee (41) have also described a class of non-histone proteins which remained bound to HeLa cell DNA after extraction of the chromatin with 2.5 M NaCl - 5.0 M urea. It is possible that these tightly bound rye proteins are responsible for the observed differences in dye binding between histone-depleted chromatin and DNA. Similar results were obtained by other laboratories for the binding of ethidium bromide to avian erythrocyte chromatin (16) as well as for the binding of proflavine and toluidine blue to calf thymus chromatin (42, 43). Thus, it seems that some tightly bound non-histones are able to mask a relatively high proportion of DNA.

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* SD = standard deviation

** Histones H2B and H2A were previously called PH_I and PH_{II} (see ref. 10)

REFERENCES

1. Kornberg, R.D. (1974) Science 184, 868-871
2. Baldwin, J.P., Bosely, P.G., Bradbury, E.M. and Ibel, K. (1975) Nature 253, 245-249
3. Van Holde, K.E., Sahasrabuddhe, C.G. and Shaw, B.R. (1974) Nucl. Acids Res. 1, 1579-1586
4. Olins, A.L. and Olins, D.E. (1974) Science 183, 330-332
5. Sollner-Webb, B. and Felsenfeld, G. (1975) Biochemistry 14, 2915-2920
6. Noll, M. (1974) Nature 251, 249-250
7. Gorovsky, M.A. and Keevert, J.B. (1975) Proc. Nat. Acad. Sci. (USA) 72, 3536,3540
8. Lohr, D. and Van Holde, K.E. (1975) Science 188, 165-166
9. McGhee, J.D. and Engel, J.D. (1975) Nature 254, 449-450
10. Nadeau, P., Pallotta, D. and Lafontaine, J.-G. (1974) Arch. Biochem. Biophys. 161, 171-177
11. Marmur, J. (1961) J. Mol. Biol. 3, 208-218
12. LaRue, H. and Pallotta, D. (1976) Can. J. Biochem. in press.
13. Bartley, J.A. and Chalkley, R. (1972) J. Biol. Chem. 247, 3647-3655
14. Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-346
15. Tuan, D.Y.H. and Bonner, J. (1969) J. Mol. Biol. 45, 59-76
16. Lurquin, P.F. and Seligy, V.L. (1972) Biochem. Biophys. Res. Commun. 46, 1399-1404
17. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672
18. Peacocke, A.R. and Skerret, J.M.H. (1956) Trans. Far. Soc. 52, 261-279
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
20. Burton, K. (1956) Biochem. J. 62, 315-323
21. Waring, M.J. (1965) J. Mol. Biol. 13, 269-282
22. Sommer, K.R. and Chalkley, R. (1974) Biochemistry 13, 1022-1027
23. Angerer, L. M. and Moudrianakis, E.N. (1972) J. Mol. Biol. 63, 505-521
24. Clark, R.J. and Felsenfeld, G. (1971) Nature New Biol. 229, 101-106
25. Itzhaki, R.F. (1971) Biochem. J. 122, 583-592
26. Hanlon, S., Johnson, R.S., Wolf, B. and Chan, A. (1972) Proc. Nat. Acad. Sci. USA 69, 3263-3267
27. Williams, R.E., Lurquin, P.F. and Seligy, V.L. (1972) Eur. J. Biochem. 29, 426-432
28. Angerer, L.M., Georghiou, S. and Moudrianakis, E.N. (1974) Biochemistry 13, 1075-1082
29. Remington, J.A. and Klevecz, R.R. (1973) Biochim. Biophys. Acta 308, 422-425
30. Honda, B.M., Baillie, D.L. and Candido, E.P.M. (1975) J. Biol. Chem. 250-4643-4647
31. Bradbury, E.M., Molgaard, H.V., Stephens, R.M., Johns, E.W. and Bolund, L.A. (1972) Eur. J. Biochem. 31, 474-482.
32. Bradbury, E.M., Carpenter, B.G., Rattle, H.W.E. (1973) Nature 241, 123-126
33. Littau, V.C., Burdick, C.J., Allfrey, V.G. and Mirsky, A.E. (1965) Proc. Nat. Acad. Sci. (USA) 54, 1204-1213
34. Hjelm, R.P. Jr. and Huang, R.C.C. (1974) Biochemistry 13, 5275-5283
35. Murray, K., Bradbury, E.M., Crane-Robinson, C., Stephens, C., Haydon, A.J. and Peacocke, A.R. (1970) Biochem. J. 120, 859-871
36. Oudet, P., Gross-Bellard, M., and Chambon, P. (1975) Cell 4, 281-300
37. Weintraub, H., Palter, K. and Van Lente, F. (1975) Cell 6, 85-110
38. Vandegrift, V., Serra, M., Moore, D.S. and Wagner, T.E. (1974) Biochemistry 13, 5087-5092

39. Skidmore, C., Walker, I.O., Pardon, J.R. and Richards, B.M. (1973) FEBS Letters 32, 175-178
40. Pederson, T. and J.S. Bhorjee (1975) Biochemistry 14, 3238-3242
41. Lawrence, J.-J. and Louis, M. (1972) Biochim. Biophys. Acta 272, 231-237
42. More, I.A.R. and Paul, J. (1973) Expl. Cell Res. 76, 79-86