STUDIES ON THE ROLE OF HISTONES IN THE STRUCTURE AND FUNCTION OF CHROMATIN

Thesis by

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ABSTRACT

Studies on the dissociation of histones from chromatin by increasing concentrations of sodium deoxycholate (DOC) have shown that histone II is removed at lowest concentrations of DOC, while slightly higher concentrations remove histones III and IV. Still higher concentrations remove histone I.

The complete separation of chromatin and ^{14}C-DOC by sucrose sedimentation indicated that the binding of DOC to chromatin is readily and completely reversible.

The dissociation of histones from chromatin by increasing concentrations of related cholanic acids and some of their conjugated derivatives was studied. The results suggested that the driving force for the interaction between the cholanic acid anion and histones is the lowering of the activity coefficient of the cholanic acid anion which occurs when it is partially removed from solution by interaction with hydrophobic regions of the positively charged histones.

The role of histones in the structure of chromatin has been studied by comparing the effects of selective removal of histones from chromatin by increasing concentrations of DOC with those caused by NaCl (removes histone I at lowest

concentrations, while higher concentrations remove histones II, III, and IV). Properties studied included thermal denaturation, sedimentation velocity, flow dichroism, relaxation times of molecules oriented in a flow field, and the irreversible disruption of a 130 S, cross-linked component of sheared chromatin. The data indicated that none of the structural or chemical parameters with which these properties are correlated show a dependence on the presence of one particular histone fraction.

The template activity (ability to prime a 0.2 M KCl DNA-dependent RNA synthesis system catalysed by E. coli RNA polymerase) increases from that of native chromatin (approximately 25 per cent of that of pure DNA) to that of pure DNA in a fashion which shows a nearly linear relationship to the amount of histone coverage of the template. The precipitability of partially dehistonized chromatin samples in 0.15 M NaCl shows a large dependence on the presence of histone I.

TABLE OF CONTENTS

TABLE OF CONTENTS

GENERAL INTRODUCTION

GENERAL INTRODUCTION

In the nuclei of higher organisms, chromosomal DNA exists in close association with specific proteins. The major portion of these proteins are the histones (Bonner & Ts'o, 1964). Most of the chromosomal material can be separated from other cell components by differential centrifugation (Bonner et al., 1968). Chemically, the preparation of chromatin is mild and work in this field is based upon the concept that this material may serve as worthwhile models for nuclear chromatin. An impression of the type and degree of interaction between chromosomal DNA and the histones can be obtained by finding various conditions for selectively dissociating the chromatin complex and then observing the physical, chemical, and biological effects of doing so.

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Chapter 1

THE SELECTIVE DISSOCIATION OF HISTONES FROM CHROMATIN BY SODIUM DEOXYCHOLATE

Chapter 1

Part I

A NEW ORDER OF REMOVAL

INTRODUCTION

The genetic material in the nucleus of eukaryotic organisms is present as a nucleoprotein complex, chromatin. Chromatin can be isolated as a chemically defined entity of DNA, RNA, histone and nonhistone proteins. The histones, a family of seven major components, comprise the major portion of the proteins (Bonner & Ts'o, 1964; Murray, 1964; H. Busch, 1965). Although the biological functions of the histones are not completely understood, a large body of data supports the hypotheses that histones are involved in repression of genetic activity (Huang & Bonner, 1962; Allfrey, Littau, & Mirsky, 1963; Marushige & Bonner, 1966) and that they confer some structural restraints on chromosomal DNA (Doty, Marmur, Eigner, & Schildkraut, 1960; Samejima & Yang, 1965; Ohba, 1966; Pardon, Wilkins, & Richards, 1967; Tuan & Bonner, 1969).

Several workers have studied the contribution of the various histone fractions to the structure and function of chromatin by biophysical and biochemical analysis of a series of partially dehistonized chromatins (Marushige & Bonner, 1966; Ohba, 1966; Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Tuan & Bonner, 1969). In all cases the partially dehistonized chromatins have been obtained by the selective removal of the various histone fractions by increasing concentrations of ionic dissociating agents, such as sodium chloride,

sodium perchlorate, and guanidine hydrochloride. All present methods, however, yield the same general sequence of histone removal - histones I (lysine-rich) are removed at the lowest concentration of dissociating agent, while higher concentrations remove histones II (slightly lysine-rich), and histones III and IV (arginine-rich).

A selective dissociation agent which produced a different order of histone fraction removal would be extremely useful in the interpretation of selective dissociation studies. We have investigated the dissociation of histones from chromatin by increasing concentrations of sodium deoxycholate (DOC). We have found that histone II is removed at the lowest concentrations of DOC, while slightly higher concentrations remove histones III and IV. Still higher concentrations of DOC remove histone I.

METHODS AND MATERIALS

Preparation of Chromatin. Sucrose purified chromatin was prepared from pea buds according to the method of Bonner et al. (1968a) and from calf thymus by the method of Maurer and Chalkley (1967), except that in both procedures the 1.7 M sucrose contained 0.0025 M Tris, pH 8. The chromatin pellets from the sucrose purification step were resuspended to a final $A_{260m\mu}$ of 20 to 30 in 0.0025 M Tris, pH 8, by homog-

enizing in a Potter-Elvehjem homogenizer. The chromatin solution was dialyzed overnight against 0.0025 M Tris, pH 8, sheared in 40 ml aliquots in the Virtis "45" homogenizer at 30 volts for 90 seconds, and then centrifuged at 10,000 x g for 30 minutes. The resulting supernatant was further fractionated by sedimenting the sheared chromatin into a cushion of 1.2 M sucrose - 0.0025 M Tris, pH 8, for the equivalent of 10 hours at 50,000 rpm (Spinco Ti-50 rotor). The supernatant was removed and discarded. The pellet was resuspended as above, and dialyzed extensively against 0.0025 M Tris, pH 8. This solution, referred to as chromatin, constituted the starting material. The chromatin has a final concentration of 20 to 40 $A_{260m\mu}$, a ratio $A_{230m\mu}/A_{260m\mu}$ of approximately 0.75, and a ratio $A_{320m\mu}/A_{260m\mu}$ of less than 0.034. All steps were carried out at 0 to 4° C.

Histone Dissociation Studies. To assure that the chromatin was not subjected to a sodium deoxycholate (DOC) concentration higher than the desired final one, the following procedure was adopted. The chromatin was diluted with 0.0025 M Tris, pH 8, so that a final volume of 10 ml and a final concentration 5 to 7 $A_{260m\mu}$ would be obtained after the addition of the required amount of 0.25 M DOC - 0.0025 M Tris, pH 8. The DOC solution was added dropwise with vigorous stirring on a Vortex mixer. After thorough mixing of the sample, 2 ml of 1.2 M sucrose - 0.0025 M Tris,

pH 8, were gently layered at the bottom of the tube. The partially dehistonized DNA was then separated from the dissociated protein by sedimenting the DNA into the sucrose cushion at the equivalent of 50,000 rpm for 10 hours (Spinco Ti-50 rotor). The top 11 ml of the resulting supernatant were removed and discarded. The remaining loosely packed pellet (contained in 1 ml of sucrose solution) was then removed and resuspended by homogenization in a Teflon homogenizer as above. The samples were exhaustively dialized against 0.0025 M Tris, pH 8, and then centrifuged at 10,000 x g for 10 minutes. The resulting supernatants were then used for further characterization of the partially dehistonized chromatin.

Thermal Denaturation and Ultraviolet Absorption. The partially dehistonized samples were dialyzed exhaustively against 2.5×10^{-4} M EDTA, pH 8, diluted to approximately 1 $A_{260m\mu}$ with dialysate, and melted in a Gilford Model 2000 multiple sample absorbance recording apparatus adapted for the recording of melting profiles. The rate of temperature increase was 0.5 to 1.0 degree/minute. Ultraviolet absorption spectra were determined with a Cary recording spectrophotometer, Model 11.

Free-Zone Electrophoresis. Free-zone electrophoresis was performed as described by Olivera, Baine, and Davidson (1964) in 0.01 M NaCl - 0.001 M Tris, pH 7.5, using their

apparatus. Data were fitted to a straight line using a least squares computer program.

Sedimentation Analysis. Sedimentation velocity was studied using band-sedimentation techniques on preformed sucrose gradients in a Spinco Model L2-65 ultracentrifuge. All steps were performed at 0 to 4° C.

Chromatography. Chromatographic separation of chromatin from DOC was accomplished as follows. The appropriate concentration of sodium deoxycholate was added to the chromatin solution. The mixture was then applied to a jacketed, 2 cm x 30 cm Biogel P-150 column, which had been equilibrated with 0.0025 M Tris, pH 8. The column was eluted with 0.0025 M Tris, pH 8, and the fractions analyzed for $A_{260 m\mu}$ and C^{14} cts./min. All steps were performed at 0 to 4° C.

Chemical Analysis. RNA was separated from DNA by the modified Schmidt-Tannhauser procedure of Ts'o and Sato (1959). DNA concentration was determined by the diphenylamine assay of Burton (1956) and by ultraviolet absorption. RNA concentration was determined by the orcinol reaction of Dische and Schwartz (1937). Purified calf thymus DNA (Worthington Biochemical Corp.) and yeast RNA (Sigma) were used as standards.

Protein concentration was determined by ultraviolet absorption and by the procedure of Lowry et al. (1951) after separation of whole protein into histone and nonhistone

components. The histone samples were prepared by adding 0.25 ml of 2 N H_2SO_4 per ml of chromatin solution. The solution was vigorously mixed, allowed to stand at 0° C. for 30 minutes with occasional mixing, and then centrifuged at 24,000 x g for 20 minutes. The supernatant was removed by pipetting and analyzed for protein content by ultraviolet absorption. Three volumes of 95 per cent ethanol were then added and the proteins precipitated at -20° C. for 24 hours. They were pelleted by centrifugation at 24,000 x g for 20 minutes, and washed twice with cold 95 per cent ethanol. The pellet was air-dried and dissolved in the proper amount of 8 M urea to make the final solution approximately 1 mg protein/ml solution. Acid-insoluble material was washed once with 95 per cent ethanol, air-dried, and then dissolved in 1 N NaOH. Calf thymus histones and bovine serum albumin (Sigma) were used as standards.

The absorptivity of total histone at 230 mu is 4.15 l/cm g (Jensen, 1966); for DNA contained in chromatin it is 22 l/cm g at 260 mu (Tuan, 1966); for RNA contained in chromatin it is assumed to be 25 l/cm g at 260 mu.

Disc Gel Electrophoresis of Histones. Acrylamide disc gel electrophoresis of isolated histones was performed by the method of Bonner et al. (1968a). The quantity of each electrophoretic component was determined by densitometry after Fambrough (1967).

Chemicals. Deoxycholic acid-24-C^{14} (Nuclear Equipment Chemical Corp.), 3.6 mC/mM, was dissolved in 0.5 N NaOH and then adjusted to pH 8.

All cholanic acids and their conjugated derivatives were obtained from Mann Research Laboratories, Inc.

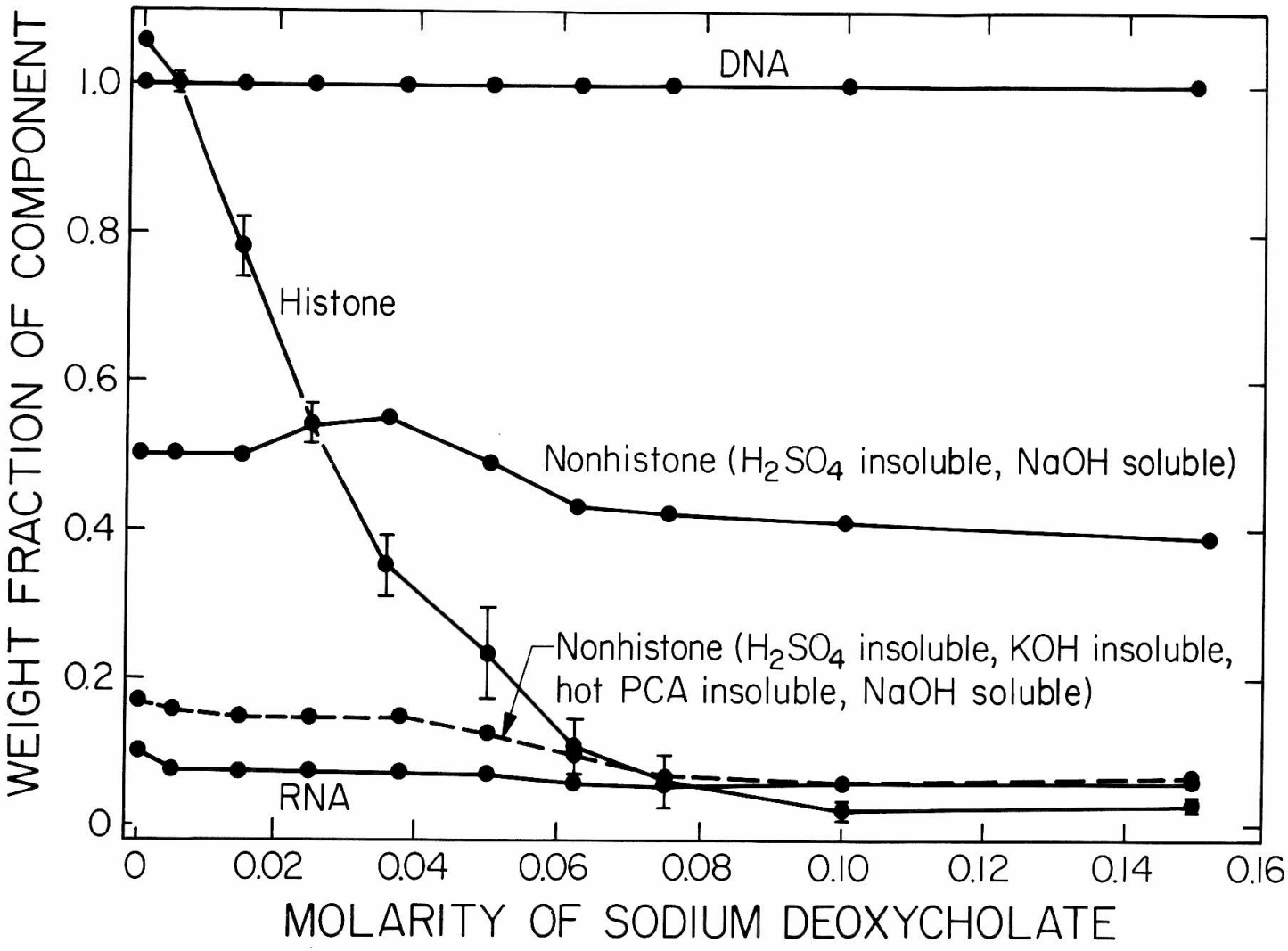
Note: All assays were performed within one week of the preparation of chromatin.

RESULTS

The weight fractions of total histone protein, nonhistone protein, and RNA remaining bound to the DNA of pea bud chromatin as a function of the molarity of sodium deoxycholate (DOC) used for dissociation are shown in Figure 1. As is the case with other agents that have been used to selectively dissociate the nucleoprotein complex, increasing concentrations of DOC principally dissociate histone protein. Relatively little nonhistone protein or RNA, amounting to no more than 20 per cent of initial amounts, is extracted by DOC over the concentration range studied.

Figure 1 presents data on the removal of two types of nonhistone protein. Nonhistone protein, as defined in methods, is that fraction of chromatin-bound protein which is insoluble in 0.4 N H_2SO_4 and subsequently soluble in 1 N NaOH. This type of nonhistone protein amounts to approximately 0.5 weight fraction of the DNA contained in the

Figure 1. The weight fraction of components of chromatin remaining bound to DNA after extraction of chromatin with increasing concentrations of DOC. Each point for histone protein is the average value from five experiments. Each point for RNA is the average value from three experiments. Each point for both types of nonhistone protein is the average value from two experiments.


WEIGHT FRACTION OF COMPONENT
1.0
0.8
0.6
0.4
0.2
0
DNA
Histone
Nonhistone (H_2SO_4 insoluble, NaOH soluble)
Nonhistone (H_2SO_4 insoluble, KOH insoluble, hot PCA insoluble, NaOH soluble)
RNA
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
MOLARITY OF SODIUM DEOXYCHOLATE

nucleoprotein complex. Nonhistone protein has previously been defined as that fraction of chromatin-bound protein which remains insoluble upon subjecting the chromatin to extraction with 0.4 N H_2SO_4, followed by the modified Schmidt-Tannhauser procedure of Ts'o and Sato (1959) (0.3 M KOH, 37° C. for 18 hours; 0.5 M $HClO_4$, 100° C. for 10 minutes), and which is subsequently soluble in 1 N NaOH (Bonner et al., 1968). As is shown in Figure 1, this type of nonhistone protein amounts to approximately 0.17 weight fraction of the DNA in chromatin. The discrepancy in the yields obtained by the two methods can be explained by assuming that a step(s) in the modified Schmidt-Tannhauser procedure causes a loss of some nonhistone protein, and/or a change in some nonhistone protein which renders it insoluble in 1 N NaOH. That the second explanation is apparently correct is supported by the repeated observation that after hydrolysis of the 0.4 N H_2SO_4 insoluble material of pea bud chromatin in hot 0.5 M $HClO_4$ a significant amount of 1 N NaOH insoluble material remains. Fambrough (1967) found that this material was almost totally soluble in 1 per cent sodium dodecylsulfate - 8 M urea, and could be purified by precipitation with ammonium sulfate, a method developed for the study of the nonhistone protein of rat liver chromatin (Marushige, Brutlag, & Bonner, 1969). This material was estimated to amount to 0.25 to 0.33 weight fraction of the

DNA from which it was isolated. Studies on the dissociation of chromatin with increasing concentrations of NaCl indicate that a large portion of this nonhistone protein is not dissociated from DNA by extraction of chromatin with 2 M NaCl (Fambrough, 1967).

An increase in the weight fraction of nonhistone protein above control levels can be seen in the chromatin samples previously extracted with 0.025 and 0.0375 M DOC. This increase appears to be due to histone and nonhistone protein aggregation (Levinson, Smart, & Bonner, 1969) and/or to histone-nonhistone-DOC micelle formation.*

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*Extraction of chromatin with concentrations of sodium deoxycholate between 0.0625 and 0.1 molar forms a small amount of white precipitate which remains insoluble after extensive dialysis against 0.0025 M Tris, pH 8, or 2.5×10^{-4} M EDTA, pH 8. The precipitate is also insoluble in 0.4 N H_2SO_4, but is soluble in 1 N NaOH or 1 per cent sodium dodecylsulfate (SDS). Examination of the proteins contained in the precipitate by SDS disc gel electrophoresis shows a significant amount of proteins which have the same mobilities as the histone fractions (Elgin, 1969). Although this precipitate pellets upon centrifugation at 10,000 x g for 10 minutes, it appears that it is not possible to remove

Data on the fraction of each histone component dissociated from chromatin by increasing concentrations of sodium deoxycholate are presented in Figure 2. Histone II (slightly lysine-rich) is most readily dissociated by DOC. Histones III and IV (arginine-rich) are extracted by slightly higher concentrations of DOC, while histone I (lysine-rich) is least readily dissociated by DOC. The same general pattern of histone removal has been found with calf thymus chromatin. Consequently, this method for the selective dissociation of histones produces a pattern of histone removal which is different from that produced by increasing concentrations of NaCl, $NaClO_4$, and guanidine hydrochloride (GuCl). Sodium deoxycholate extraction of histones is particularly useful because it selectively removes histones II, III, and IV at low concentrations of dissociating agent,

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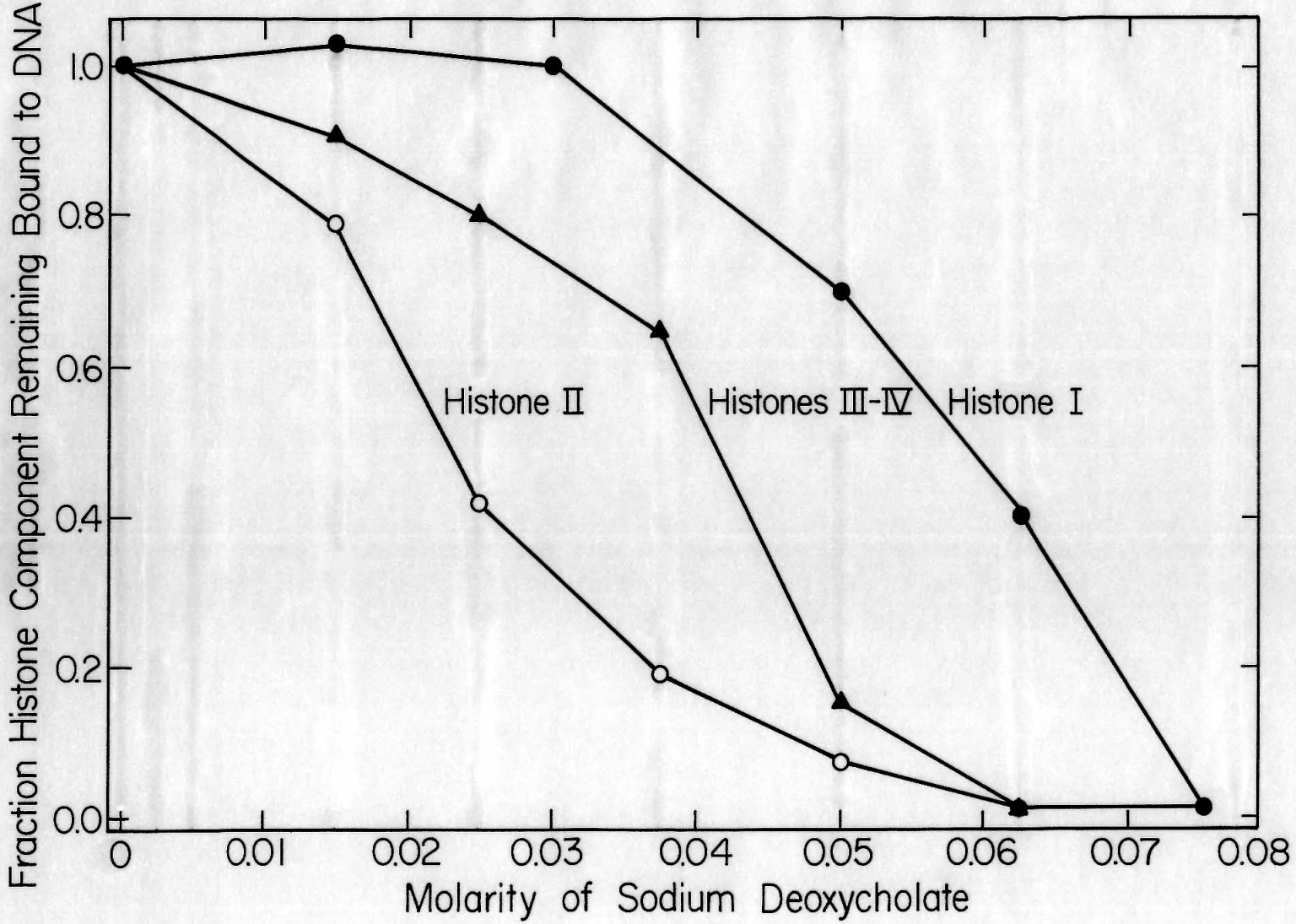
all of the micelles by the methods employed in this study. Consequently, we can account for the apparent increase in the weight fraction of nonhistone protein in chromatin samples extracted with 0.025 and 0.0375 M DOC. It is also obvious that we should anticipate an artifactually high yield of apparent DNA-bound nonhistone protein in samples of chromatin treated with higher concentrations of DOC.

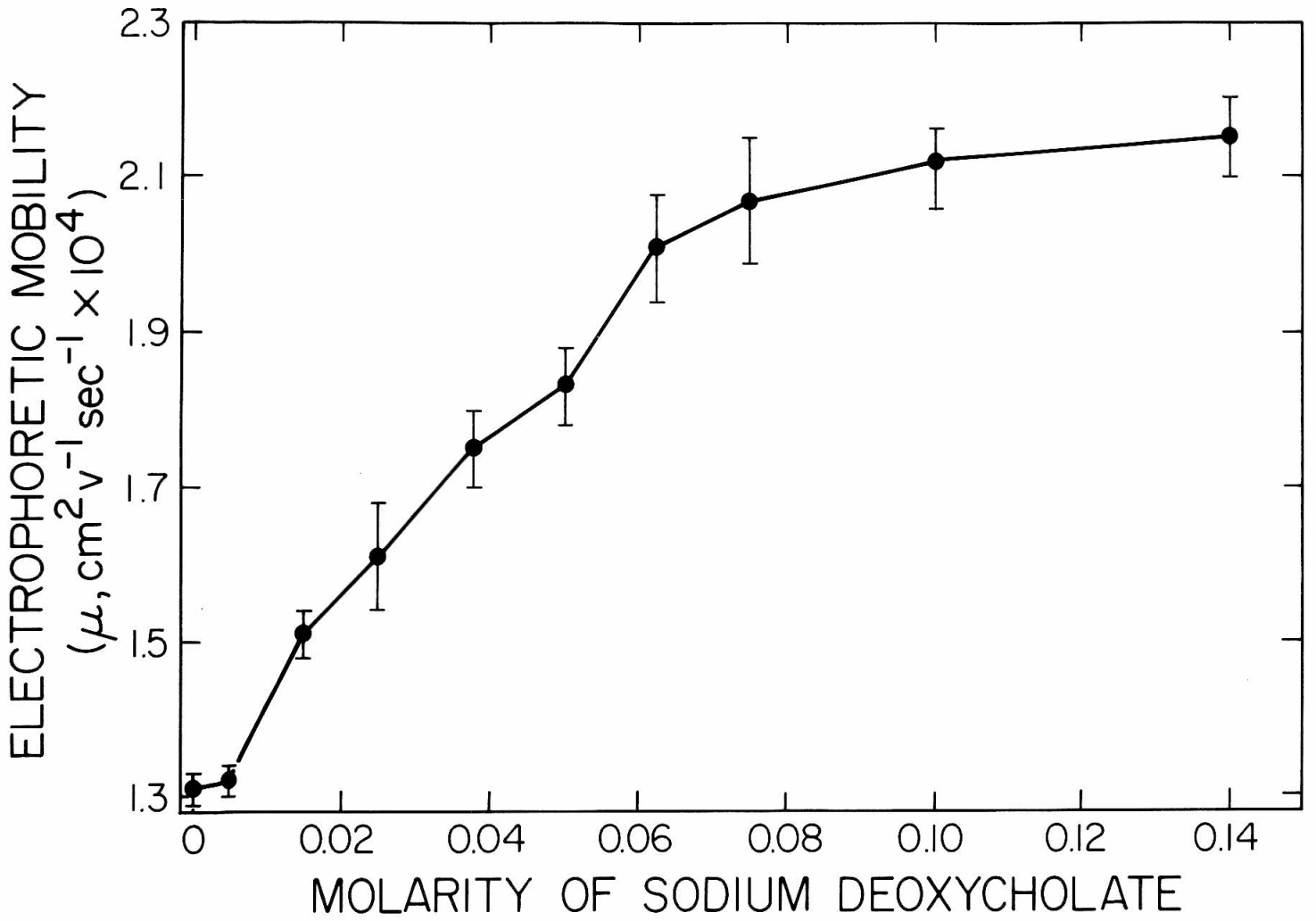
Figure 2. The fraction of individual histone components remaining bound to the DNA after extraction of chromatin with increasing concentrations of DOC. Histone I = lysine-rich = f1; histone II = slightly lysine-rich = f2b and f2a2; histone III = arginine-rich = f3; histone IV = arginine-rich = f2a1.


Fraction Histone Component Remaining Bound to DNA
1.0
0.8
0.6
0.4
0.2
0.0
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
Molarity of Sodium Deoxycholate
Histone II
Histones III-IV
Histone I

while leaving histone I bound to the DNA; whereas, all other reported dissociation agents selectively remove histone I at low concentrations, while leaving histone II, III, and IV bound to the DNA. It should also be noted that the concentration of dissociating agent required for the extraction of a given weight fraction of total histone is approximately an order of magnitude less for DOC than for other dissociating agents.

Free-zone electrophoresis of partially dehistonized samples has been considered as a method which provides an independent indication of the histone coverage of the samples (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Levinson, Smart, & Bonner, 1969). The relationship of electrophoretic mobility of the partially dehistonized samples to molarity of DOC used for dissociation of chromatin is presented in Figure 3. These data show that extraction of chromatin with 0.0375 M DOC removes approximately 67 per cent of total histone. Such partially dehistonized chromatin exhibits an electrophoretic mobility of 1.75×10^4 $cm^2 sec^{-1} volt^{-1}$, which is intermediate (51 per cent) between that of fully covered chromatin ($1.31 \times 10^4 cm^2 sec^{-1} volt^{-1}$) and pure DNA ($2.17 \times 10^4 cm^2 sec^{-1} volt^{-1}$). These data may be compared with those of Ohlenbusch, Olivera, Tuan, and Davidson (1967), who studied NaCl extraction of calf thymus chromatin, and of Levinson, Smart, and Bonner (1969),

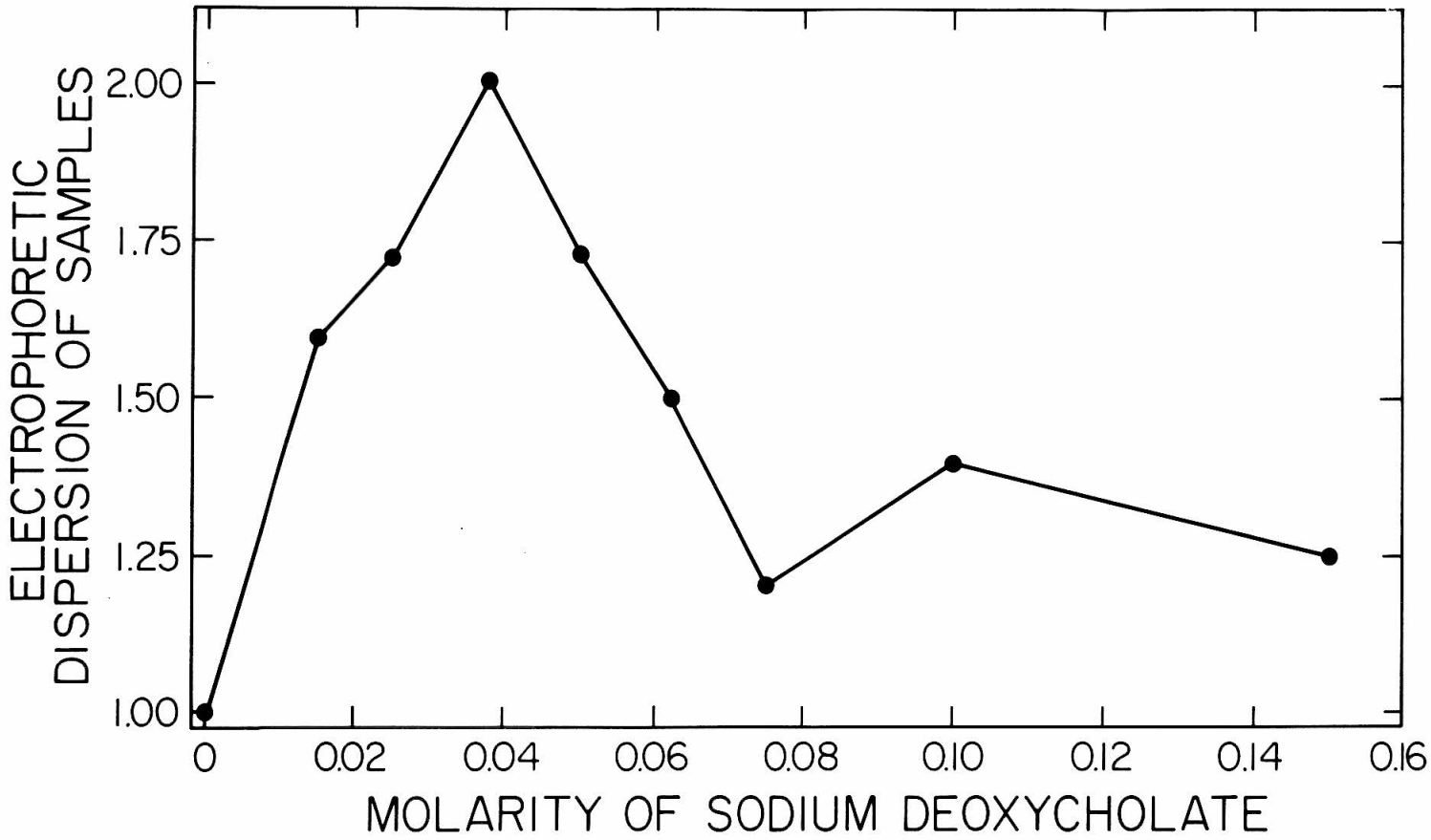
Figure 3. The free-zone electrophoretic mobility of chromatin samples partially dehistonized by increasing concentrations of DOC. Each point represents the average value from four experiments. Free-zone electrophoresis was done in 0.01 M NaCl, 0.001 M Tris, pH 7.5


ELECTROPHORETIC MOBILITY
(μ, $cm^2 v^{-1} sec^{-1} \times 10^4$)
2.3
2.1
1.9
1.7
1.5
1.3
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
MOLARITY OF SODIUM DEOXYCHOLATE

who studied guanidine hydrochloride extraction of pea bud chromatin. Both found an increase in electrophoretic mobility of approximately 50 per cent after removal of approximately 30 per cent of total histone by extraction with 0.6 M NaCl or 0.5 M GuCl. In both cases, the large change in electrophoretic mobility was consistent with the fact that histone I, the most positively charged histone, was selectively removed. Our data are consistent with the fact that histone II, the least positively charged histone, as well as a small amount of histones III and IV are removed by 0.0375 M DOC. In fact, consideration of the net positive charge density of the histones (calculated from amino acid composition data of Fambrough, 1967) removed by this concentration of DOC shows that 52 per cent of the net positive charges of the total histones have been removed. This number is in very good agreement with the 51 per cent increase in electrophoretic mobility of this sample. These data also indicate that the negatively charged DOC molecule does not remain bound to the partially dehistonized samples to any significant extent.

Figure 4 shows the electrophoretic dispersion (defined as the band width of the migrating $A_{260m\mu}$ peak at one half peak height; native chromatin is assigned the value of 1.0) of chromatin samples which have been extracted with increasing concentrations of DOC. Although the partially de-

Figure 4. The free-zone electrophoretic dispersion (the band width of the $A_{260m\mu}$ peak at one half peak height) of chromatin samples partially dehistonized by increasing concentrations of DOC. The electrophoretic dispersion of native chromatin is assigned the value of 1.0. Free-zone electrophoretic dispersion of native chromatin is assigned the value of 1.0. Free-zone electrophoresis was done in 0.01 M NaCl, 0.001 M Tris, pH 7.5.


ELECTROPHORETIC
DISPERSION OF SAMPLES
2.00
1.75
1.50
1.25
1.00
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
MOLARITY OF SODIUM DEOXYCHOLATE

histonized samples all migrate as single $A_{260m\mu}$ peaks, it is clear that partial removal of the histones from chromatin creates a set of molecules of much higher diversity of charge distribution than that of either unextracted chromatin or pure DNA.

The thermal denaturation behavior of chromatin from which discrete histone fractions have been removed provides yet another independent indication of the amount of histone coverage of each sample. It is also of interest because it may provide some insight into the distribution of histones along the DNA chain. Figure 5 shows the melting profiles of chromatin samples which have been partially dehistonized by various concentrations of DOC. Under the conditions employed in this study, the T_m (midpoint of thermal transition) of native chromatin is in the temperature range, 73 to 76° C., while that of DNA is 43 to 45° C. The values of T_m, hyperchromicity, and dispersion (defined as that temperature span required to raise the $A_{260m\mu}$ from 0.333 to 0.667 of the final hyperchromicity) for partially dehistonized samples are presented in Table 1. As mentioned previously, extraction of chromatin with 0.0375 M DOC removes about 67 per cent of total histone, which corresponds to approximately 52 per cent removal of the net positive charges contained in the histones. Inspection of Table 1 reveals that treatment with 0.0375 M DOC reduces the T_m of the

Figure 5. Melting profiles of chromatin samples partially dehistonized by increasing concentrations of DOC. Melting was done in 2.5×10^{-4} M EDTA, pH 8. No correction for thermal expansion.

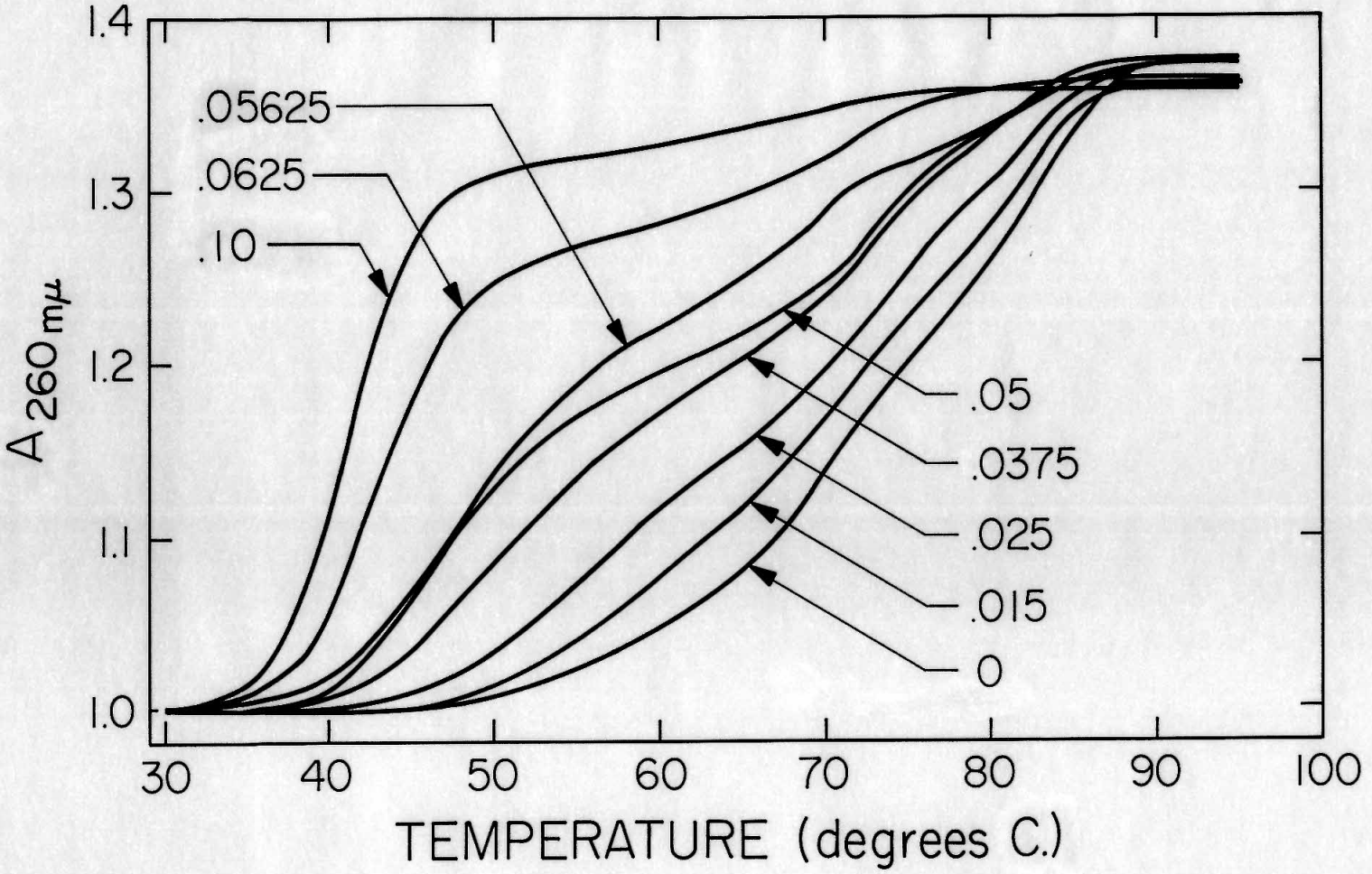

1.4
1.3
1.2
1.1
1.0
A 260 mμ
.05625
.0625
.10
.05
.0375
.025
.015
0
30
40
50
60
70
80
90
100
TEMPERATURE (degrees C.)

Table 1

Thermal Denaturation Measurements

Description	T_m [1]	h_t [2]	$\sigma_{2/3}$ [3]
Control	73.9 ± 0.5	33.9 ± 0.8	10.0 ± 0.2
0.005 M DOC	73.4 ± 0.6	34.1 ± 0.6	10.2 ± 0.2
0.015 M DOC	71.5 ± 0.6	35.1 ± 0.7	10.9 ± 0.3
0.025 M DOC	68.3 ± 0.7	35.1 ± 0.5	12.9 ± 0.3
0.0375 M DOC	64.1 ± 0.9	35.8 ± 0.6	15.6 ± 0.5
0.04375 M DOC*	60.5 ± 1.0	35.9 ± 0.5	16.5 ± 0.9
0.05 M DOC	58.5 ± 1.6	36.6 ± 0.7	17.2 ± 0.5
0.05625 M DOC*	52.5 ± 1.4	35.0 ± 0.5	15.4 ± 1.5
0.0625 M DOC	47.8 ± 1.0	36.4 ± 0.8	11.1 ± 1.5
0.075 M DOC	44.6 ± 0.5	36.3 ± 0.5	5.3 ± 0.3
0.10 M DOC	44.0 ± 0.6	36.3 ± 0.8	4.6 ± 0.3
0.15 M DOC	44.0 ± 0.7	36.0 ± 0.7	4.5 ± 0.4

1. $T_m \equiv$ midpoint of thermal transition at which $h = 0.5\ h_t$
2. $h_t \equiv$ hyperchromicity
3. $2/3 \equiv$ the dispersion, defined as that temperature span required to raise h from $0.333\ h_t$ to $0.667\ h_t$

* Data from three independent experiments; all other points from greater than 7 independent experiments

nucleoprotein complex from 73.9 to 64.1° C. This corresponds to a 33 per cent decrease in T_m (where 73.9 = 0% and 44.0 = 100%). It therefore appears that decreases in the T_m of partially dehistonized samples lag considerably behind decreases in the weight fraction of histones complexed with the DNA, and also behind decreases in the net positive charges contributed by the histones.

A general observation from Figure 5 is that the melting profiles of partially extracted chromatin samples are considerably broadened, but not cleanly divided into a DNA-like region and a native chromatin region. The general broadening of the melting profiles can be more clearly seen by inspection of the values for $\sigma_{2/3}$ given in Table 1. Olivera (in Bonner et al., 1968a) reported that the average length of a cooperative melting unit in DNA was of the order of 200 base pairs. He subsequently suggested that if extraction with a given salt concentration exposed tracts 200 base pairs long completely devoid of histone, leaving other tracts completely histone covered, one would expect a biphasic melting curve, with one component melting as DNA and the other as native chromatin. Following this argument, if the average molecular weight of histone molecules is taken to be 15,000, such a DNA segment of fully complexed chromatin would contain about 12 histone molecules. That the melting profiles of partially dehistonized chromatin

samples show considerable broadening, but are not cleanly divided into DNA-like and native chromatin regions, has been observed with chromatin dissociation by increasing concentrations of sodium chloride (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Smart & Bonner, 1969) and with increasing concentrations of guanidinium hydrochloride (Levinson, Smart, & Bonner, 1969).

DNA in chromatin is hyperchromic with respect to pure DNA (Doty, Marmur, Eigner, & Schildkraut, 1960; Tuan & Bonner, 1969). Data for the values of the hyperchromicity of chromatin samples which have had increasing amounts of total histone removed by extraction with DOC are presented in Table 1. The anticipated increase in hyperchromicity upon melting of the partially dehistonized samples is observed. The increase in the hyperchromicity, however, is rather gradual over the intermediate ranges of histone removal. This same gradual increase in hyperchromicity upon melting is observed in chromatin samples that have been partially dehistonized with increasing concentrations of sodium chloride (Tuan & Bonner, 1969; Smart & Bonner, 1969) and guanidinium hydrochloride (Levinson, Smart, & Bonner, 1969). Tuan and Bonner (1969), who have studied calf thymus, found a similar rather gradual decrease in the molar extinction coefficients of the DNA contained in samples of chromatin which had been increasingly dehistonized by

extraction with increasing concentrations of NaCl.

DISCUSSION

We have found that increasing concentrations of sodium deoxycholate (DOC) dissociate the various histone fractions in an order which is different from the order observed with other previously reported dissociating agents. DOC most readily dissociates histone II (slightly lysine-rich), while histones III and IV (arginine-rich) are dissociated by slightly higher concentrations of DOC. Histone I (lysine-rich) is the last histone fraction to be extracted by this agent. All other reported methods of histone dissociation (Ohlenbusch, Olivera, Tuan, Davidson, 1967; Murray, 1966; Levinson, Smart, & Bonner, 1969) result in the selective removal of histone I at the lowest concentrations of dissociating agent. Extraction of chromatin with 0.05 M DOC yields a nucleoprotein sample which is almost completely devoid of histones II, III, and IV, but which retains practically all of histone I. This agent thus provides a method for the interpretation of the roles of the various histone fractions in the structure and function of chromatin.

Our studies show that sodium deoxycholate is about ten times as effective as sodium perchlorate in the dissociation of total histone from chromatin. Sodium perchlorate, in

turn, is about twice as effective as sodium chloride or guanidinium hydrochloride, and requires about one half the molar concentrations of the latter to cause an equivalent amount of histone dissociation. These facts, together with the evidence presented in the second section of this paper, suggest that the effectiveness of a given salt in dissociating histones is more dependent upon differences in the binding of the anion to specific sites of the protein, than upon differences in the binding of the cation to the phosphate residues of DNA.

Ilyin and Georgiev (1969) have found that when 0.6 M NaCl extracted chromatin of calf thymus or of Ehrlich ascites carcinoma cells is treated with formaldehyde to prevent dissociation of the complex in high salt concentrations, and then centrifuged to equilibrium in a CsCl density gradient containing 2 M urea, it yields a wide plateau with two partially resolved peaks of $A_{260m\mu}$ material. The total protein to DNA ratio in the denser peak is approximately 0.95, while that in the less dense peak is about 1.3. Since 0.6 M NaCl removes histone I, they explain the presence of two peaks by suggesting that the remaining histone fractions (II, III, IV) are unequally distributed in the partially dehistonized complex. Ohlenbusch, Olivera, Tuan, and Davidson (1967) subjected a sample of calf thymus chromatin which had been extracted with 0.6 M NaCl to free-zone elec-

trophoresis. They reported that although the electrophoretic band was broader than that of pure DNA or native chromatin, it was not bimodal. As has been mentioned earlier, extraction of chromatin with 0.05 M DOC yields a nucleoprotein sample which is almost completely devoid of histones II, III, and IV, but which still has practically all of histone I still attached. Free-zone electrophoresis of this sample also yields a broader electrophoretic band; however, again there is no indication of any bimodal characteristics. It, therefore, appears that the 25 to 30 per cent difference in the distribution of total protein in the 0.6 M NaCl extracted chromatins observed by Ilyin and Goergiev (1969) is not completely due to differences in distribution of histones II, III, and IV. Moreover, since free-zone electrophoresis, which separates mainly on the basis of charge differences in chromatin molecules, shows no bimodal nature, we must look to other sources for the cause of the bimodal distribution of formaldehyde-fixed, 0.6 M NaCl extracted samples in a CsCl density gradients.

Thermal denaturation and electrophoretic mobility data both eliminate the possibility that a large number of molecules of a single histone fraction sit side by side over long stretches of DNA. They, however, do little more to precisely define the situation. One possible arrangement of the various histone fractions along the DNA chain would

be the situation in which individual molecules of the seven major species of histones succeed one another down the chain in a regular, repeating fashion, producing a repeating and completely invariant sequence of histone molecules. Since partially dehistonized samples yield more broadly migrating $A_{260_{m\mu}}$ peaks upon free-zone electrophoresis than does pure DNA or native chromatin, the DNA segments (molecular weight approximately 3×10^6) of chromatin must be complexed with varying amounts of the different histone fractions. Consequently, partial removal of histones generates a set of nucleoprotein molecules of higher diversity in charge distribution than would be expected if the histones were distributed along the DNA chain in an invariant, repeating sequence. We conclude that the various histone fractions are somewhat heterogeneously distributed along the DNA.

DNA contained in native chromatin has undergone a conformational change which is expressed as an increased absorptivity at $260_{m\mu}$ and a reduced hyperchromicity upon melting (Doty, Marmur, Eigner, Schildkraut, 1960; Tuan & Bonner, 1969). Although the dissociation of a small amount of RNA of unknown secondary structure in pea bud chromatin makes the interpretation of melting profiles more difficult, we observe a gradual increase in hyperchromicity upon melting as increasing amounts of total histone are removed by extraction of chromatin with increasing concentrations

of DOC. The same gradual increase in hyperchromicity is seen with samples which have had increasing amounts of total histone removed by NaCl (Tuan & Bonner, 1969) and GuCl (Levinson, Smart, & Bonner, 1969). If this increase in hyperchromicity upon melting represents a loss of the conformational change characteristic of DNA contained in native chromatin, then the change would not appear to be dependent upon the presence of any one particular histone fraction.

Chapter 1

Part II

A POSSIBLE MECHANISM OF REMOVAL

INTRODUCTION

Deoxycholic acid and cholic acid have been used extensively in the isolation of many subcellular components. Practically all of the procedures employed have taken advantage of their detergent-like properties to solubilize various components of the cellular homogenate. Some examples include gentle lysis of bacterial membranes (Razin & Argaman, 1963; Godsen & Sinsheimer, 1967), isolation of specific membrane fractions from several organisms (Burkhard & Kropf, 1964; Lenaz et al., 1968), isolation of polysome and other ribosome fractions (Monroy, Maggio, & Rinaldi, 1965), and purification of nucleic acids (Colter, Casper, & Ellem, 1962). Despite the widespread use of deoxycholic acid and cholic acid, relatively little has been done towards furthering the understanding of the mechanism by which these agents act.

We have reported that increasing concentrations of the sodium salt of deoxycholic acid dissociate the various histone fractions in an order which is very different from that reported with other dissociating agents, and at concentrations which are an order of magnitude less than those reported with other agents. The various histone fractions differ markedly in the ratios of lysine to arginine, net

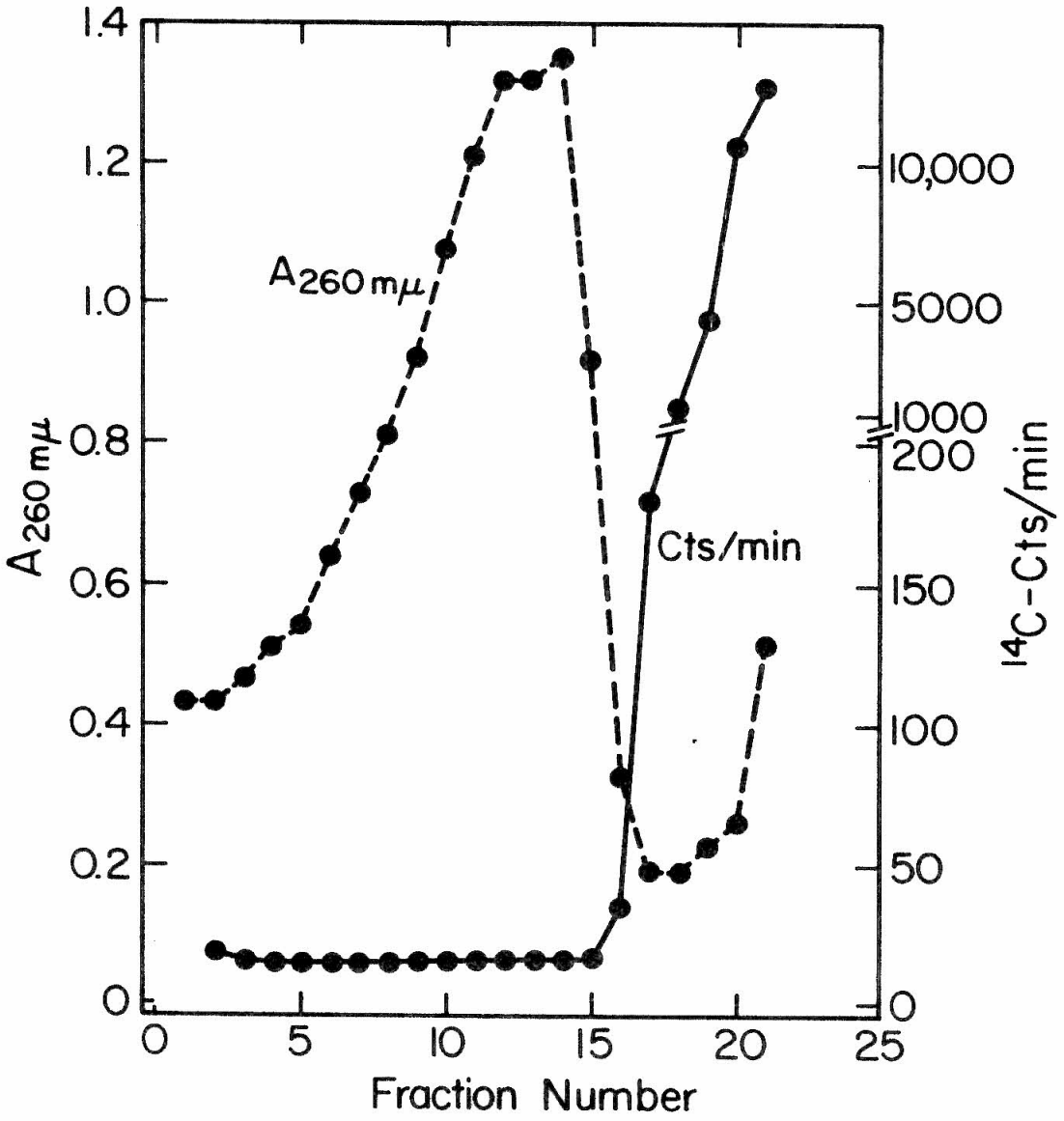
positive charge per amino acid, mole per cent hydrophobic amino acid content, and α-helical content (Fambrough, 1968; Tuan & Bonner, 1969). In an attempt to gain some insight into the mechanism by which sodium deoxycholate dissociates the various histone fractions from chromatin, we have studied the reversibility of the binding of DOC to chromatin and the effects of increasing concentrations of a few cholanic acids and their conjugated derivatives on histone dissociation patterns.

RESULTS

Because irreversible binding of the detergent-like DOC anion to partially dehistonized nucleoprotein would significantly alter biophysical and biological properties of the nucleoprotein complex, we have investigated binding of C^{14}-labelled DOC to chromatin.

The reversibility of binding of DOC to native chromatin was measured by incubating chromatin with varying concentrations of C^{14}-DOC for 30 minutes at 0^{o} C., and then subjecting the chromatin-DOC mixture to centrifugation through a sucrose gradient. Sheared chromatin (Virtis "45," 30 volts, 90 seconds) has a $s_{20,w}$ 25 to 30 S; consequently, it is readily separated from the small DOC molecules and

Figure 6. Separation of chromatin and ^{14}C-DOC by sedimentation through sucrose. 0.29 ml of native chromatin (34.3 $A_{260m\mu}$) was incubated at 0° C. for 30 minutes with 0.01 ml of ^{14}C-24-DOC (approximately 65,000 cts/min under the conditions employed). The ^{14}C-DOC-chromatin solution was then layered over a linear 5 to 20 per cent sucrose gradient (4.83 ml) and centrifuged at 65,000 rpm for 3 hours at 4° in a Spinco SW65 rotor. Fractions were collected dropwise, analyzed for $A_{260m\mu}$, and then plated on stainless steel planchets for counting in a Nuclear Chicago D181 planchet counter. Approximate concentration of DOC in the incubation mixture was 10^{-4} M (specific activity = 3.6 mc/mmole).


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A260 mμ
A260 mμ
Cts/min
10,000
5000
1000
200
150
100
50
0
14C-Cts/min
0
5
10
15
20
25
Fraction Number

micelles. The results obtained from such an experiment are shown in Figure 6. It is clear that no C^{14}-DOC molecules sediment with the $A_{260_{m\mu}}$ peak. If each histone molecule in this sample had irreversible bound one DOC molecule, then $A_{260_{m\mu}}$ of chromatin would have approximately 3000 cts/min of C^{14}-DOC bound to it. The same result, indicating no irreversible binding of DOC to native or partially dehistonized chromatin, has been obtained for samples treated with increasing concentrations of DOC (up to 0.1 molar) and then separated from DOC by sucrose gradient centrifugation or by chromatography on Biogel P-150. Therefore, the binding of sodium deoxycholate to fully covered or partially dehistonized chromatin appears to be readily and completely reversible.

Rudman and Kendall (1957) studied the effect of pH upon the binding of deoxycholic acid by human serum albumin. They found a suppression of binding above pH 9. They suggested that this result was compatible with the existence of an electrostatic bond between the positively charged ε-amino group of lysine (pK 9.3), and the negatively charged carboxylate group of the deoxycholic acid, as the primary force responsible for the binding. If the binding of DOC to the lysine of histones were the primary force involved in the histone dissociation pattern observed with increasing

Table 2

Binding of Various Cholanic Acids by Human Serum Albumin[1]

(Rudman & Kendall, 1957)

Common Name	Structure	Moles of cholanic acid bound by one mole of albumin
Monohydroxycholanic Acids		
Lithocholic Acid	3-OH-cholanic acid 7-OH-cholanic acid	6.5 6.7
Dihydroxycholanic Acids		
Deoxycholic Acid	3,12-di-OH-cholanic acid	2.3
Hyodeoxycholic Acid	3,6-di-OH-cholanic acid	3.2
Chenodeoxycholic Acid	3,7-di-OH-cholanic acid	3.0
Trihydroxycholanic Acids		
Cholic Acid	3,7,12-tri-OH-cholanic acid	0.94
Conjugated Cholanic Acids		
Glycodeoxycholic Acid	3,12-di-OH-cholanyl glycine	2.3
Taurodeoxycholic Acid	3,12-di-OH-cholanyl taurine	2.3
Glycocholic Acid	3,7,12-tri-OH-cholanyl glycine	0.92
Taurocholic Acid	3.7.12-tri-OH-cholanyl taurine	0.54

Table 2 (continued)

Binding of Various Cholanic Acids by Human Serum Albumin

1. Ten ml. of a 1 per cent human serum albumin solution (1.4×10^{-3} mM of albumin) was equilibrated by dialysis with 50 ml of buffer containing 12.7×10^{-3} of cholanic acid. After equilibration with buffer or serum protein, the cholanic acid concentration in the outer solution was determined spectrophotometrically.

concentrations of DOC, we would expect to find that histone I (25.5 mole per cent lysine) would be removed at the lowest concentrations of dissociating agent. Histone II (16.1 mole per cent lysine) would follow at slightly higher concentrations of DOC, while histones III (8.6 mole per cent lysine) and IV (8.5 mole per cent lysine) would be the last histone fractions to be dissociated. Since the experimentally observed order of dissociation is histone II, followed closely by histones III and IV, which are in turn followed lastly by histone I, we feel that the formation of such an electrostatic bond is not the primary force responsible for the histone dissociation patterns observed when chromatin is extracted with increasing concentrations of DOC.

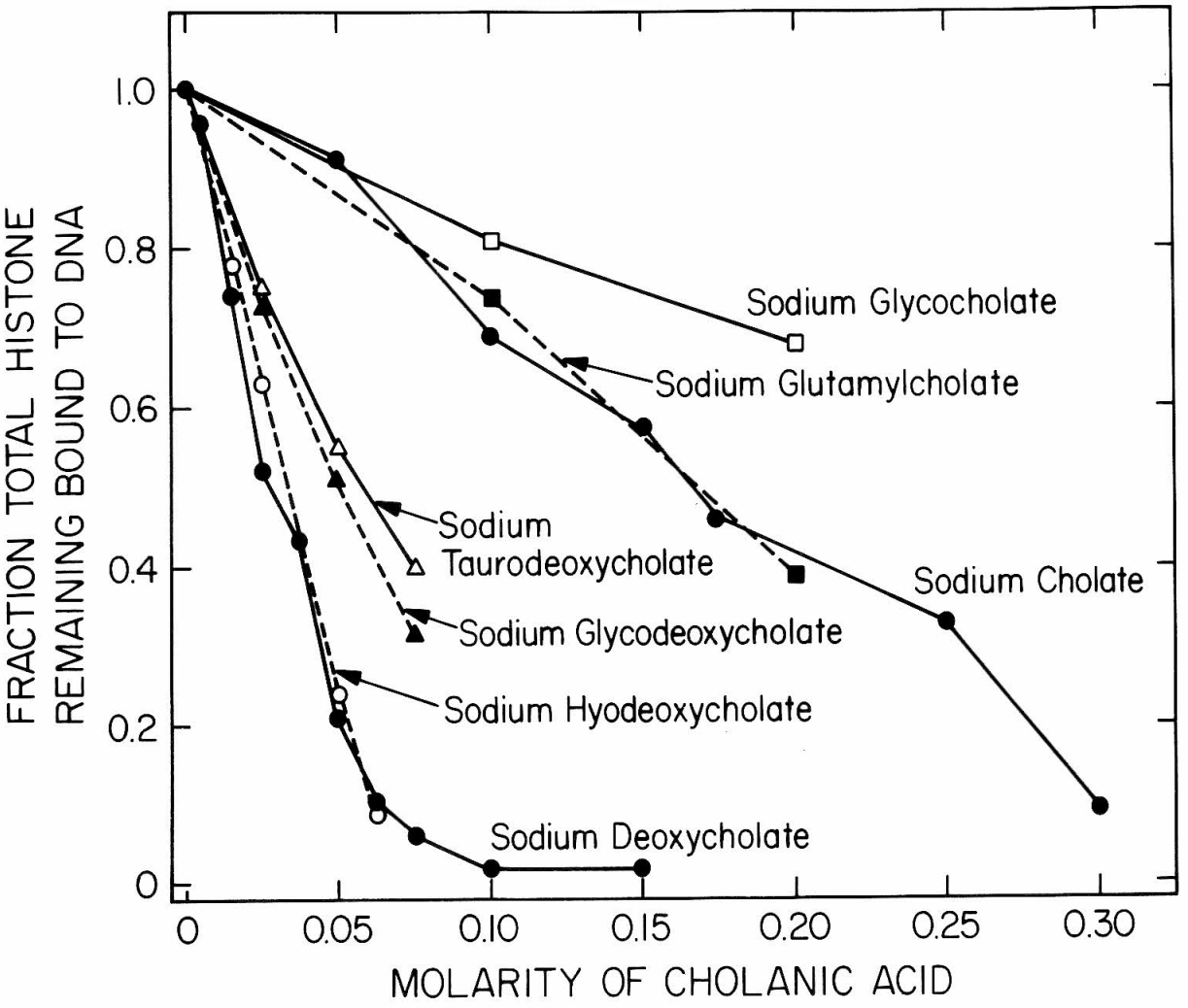
Rudman and Kendall (1957) also reported that the affinity of various cholanic acids for albumin is reduced by the introduction of polar groups into the steriod nucleus. Table 2 shows their results on the binding of various cholanic acids by human serum albumin. It is readily apparent that the extent of binding decreases in the order monohydroxy > dihydroxy > trihydroxy cholanic acid. This variation in binding among closely related cholanic acids indicates the existence of forces, other than electrostatic, between albumin and cholanic acids. The data suggested

that information of interest might be obtained from studying how changes in the structure of cholanic acids affect histone dissociation patterns obtained when chromatin is extracted with increasing concentrations of various cholanic acids.

The fraction of total histone which remains bound to DNA of chromatin after extraction with the sodium salts of several cholanic acids is shown in Figure 7. Sodium cholate is 3 to 5 times less effective as a histone dissociating agent than is sodium deoxycholate. Movement of one of the hydroxyl groups of sodium deoxycholate from the 12-position to the 6-position (sodium hyodeoxycholate) has relatively little effect on the amount of histone dissociated by a given concentration of cholanic acid. The monohydroxycholanic acid series was not sufficiently soluble in our buffer system to allow a histone dissociation study. Conjugation of the carboxyl group of deoxycholic acid or cholic acid with either glycine or taurine causes approximately a 1.5 fold reduction in the histone dissociating capacity of the cholanic acid. Conjugation of cholic acid with glutamic acid, however, restores the ability to dissociate histones to its original level (same as unsubstituted cholic acid).

Because sodium cholate occupies an intermediate position between sodium deoxycholate and sodium chloride in its

Figure 7. The weight fraction of total histone remaining bound to DNA after extraction of chromatin with increasing concentrations of the sodium salts of various cholanic acids and some of their conjugated derivatives.


FRACTION TOTAL HISTONE
REMAINING BOUND TO DNA
1.0
0.8
0.6
0.4
0.2
0
0
0.05
0.10
0.15
0.20
0.25
0.30
MOLARITY OF CHOLANIC ACID
Sodium Glycocholate
Sodium Glutamylcholate
Sodium
Taurodeoxycholate
Sodium Glycodeoxycholate
Sodium Cholate
Sodium Hyodeoxycholate
Sodium Deoxycholate

ability to dissociate histone from chromatin, its ability to selectively dissociate the various histone fractions was investigated. Figure 8 presents data on the fraction of each histone fraction remaining bound to the DNA of chromatin after extraction with increasing concentrations of sodium cholate. At a given amount of total histone removal, more histone I is removed by sodium cholate than by sodium deoxycholate. The ability to selectively leave histone I attached to the DNA while removing histone II, III, and IV is apparently diminished by the higher concentrations of dissociating agent required for a given amount of total histone removal when a third hydroxyl group is introduced into the steroid nucleus.

Thermal denaturation of partially dehistonized samples was studied in order to provide an independent indication of the degree and type of histone fraction removal. Figure 9 shows the T_m of samples which have been partially dehistonized by extraction with increasing concentrations of deoxycholic acid, cholic acid, and a few of their conjugated derivatives. In order to provide a clearer feeling of the type of histone fraction being removed at a given per cent total histone removal, the data have been replotted as fraction decrease in T_m (where 74° C. = 0 and 44° C. = 1.00) versus fraction of total histone remaining bound to the

Figure 8. The fraction of individual histone components remaining bound to DNA after extraction of chromatin with increasing concentrations of sodium cholate. Histone I = lysine-rich = f1; histone II = slightly lysine-rich = f2b and f2a2; histone III = arginine-rich = f3; histone IV = arginine-rich = f2a1.

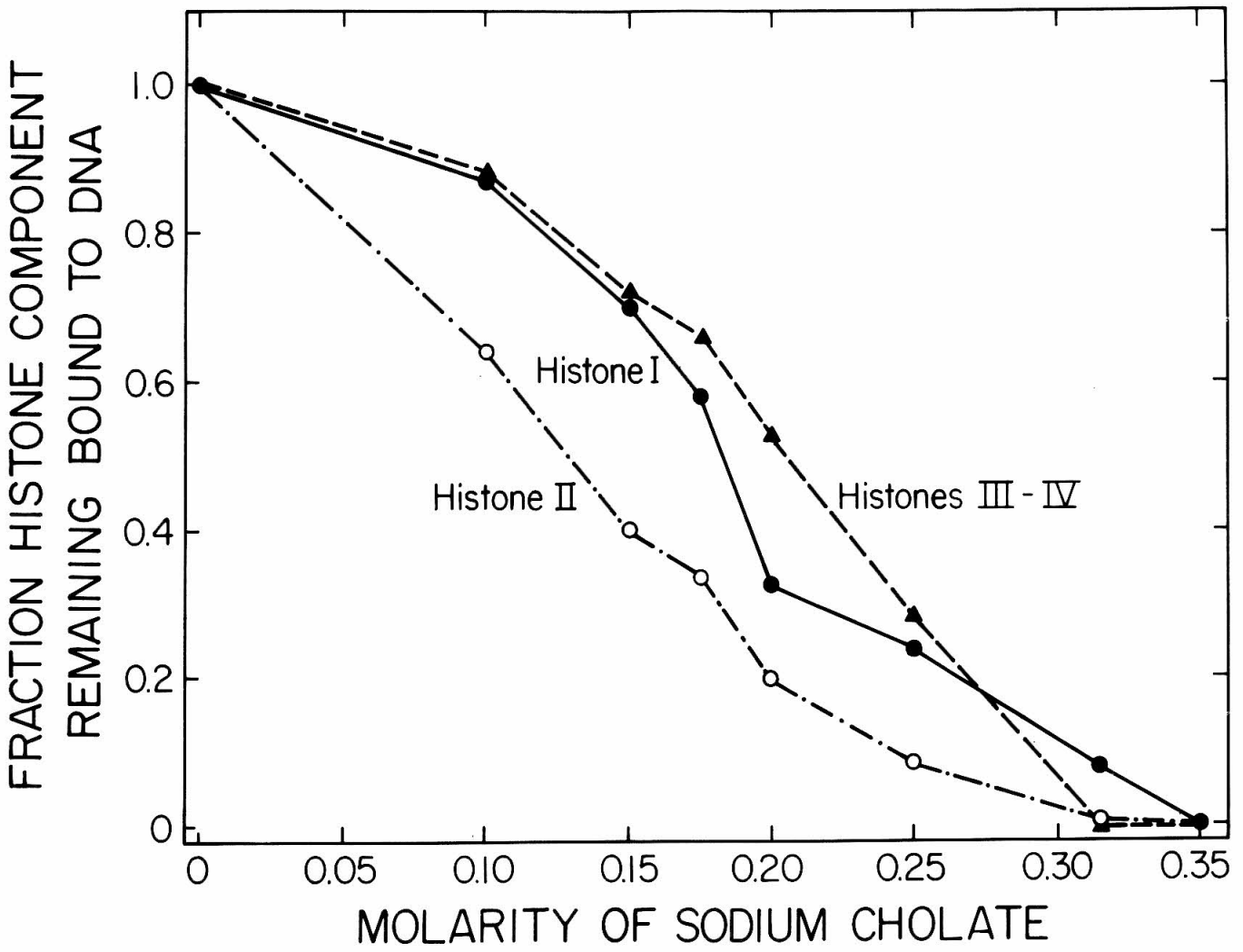
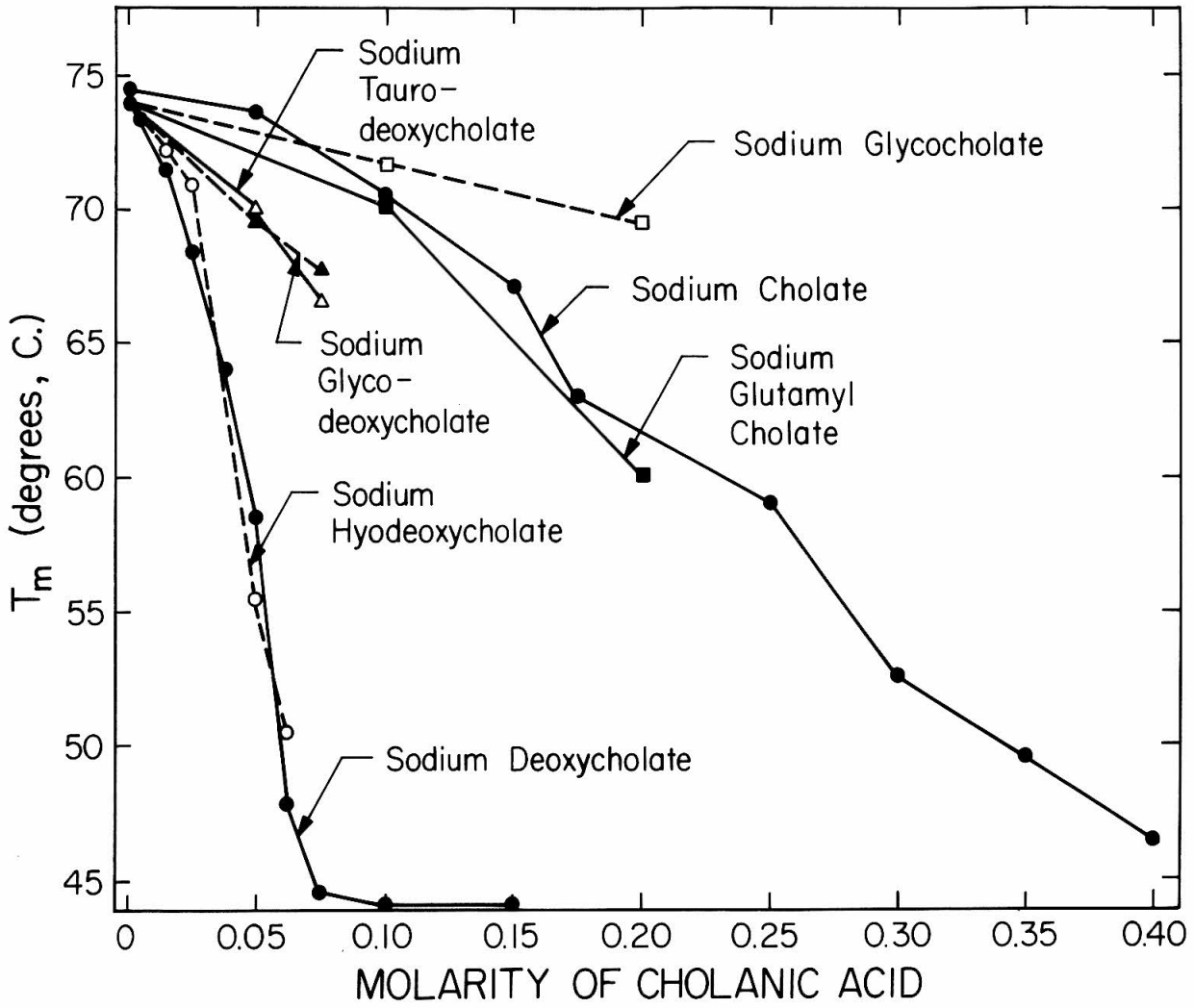

FRACTION HISTONE COMPONENT
REMAINING BOUND TO DNA
1.0
0.8
0.6
0.4
0.2
0
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
MOLARITY OF SODIUM CHOLATE
Histone I
Histone II
Histones III - IV

Figure 9. The T_m of chromatin samples partially dehistonized by increasing concentrations of the sodium salts of various cholanic acids and some of their conjugated derivatives. Melting was done in 2.5×10^{-4} M EDTA, pH 8. No correction for thermal expansion.

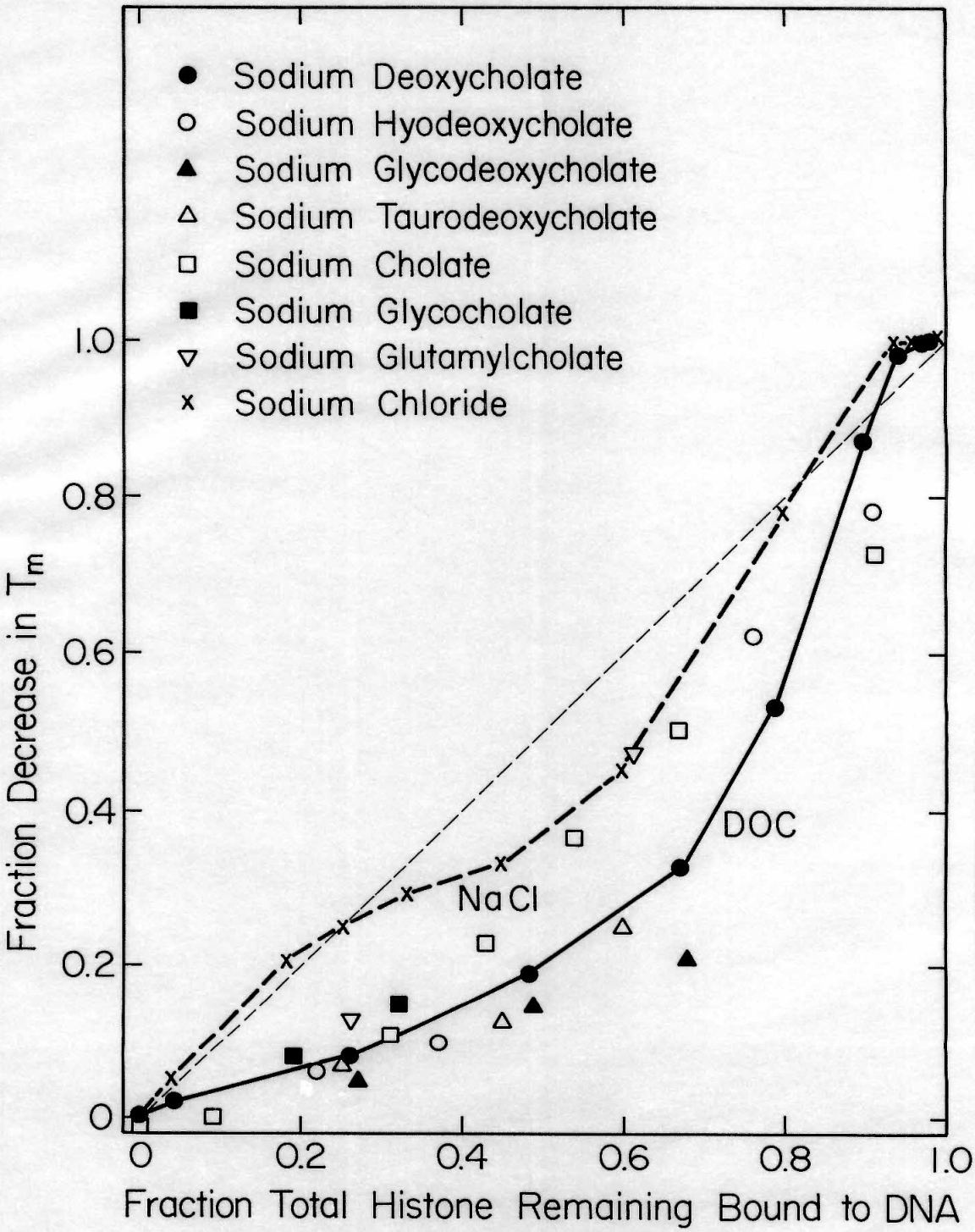

Sodium
Tauro-
deoxycholate
Sodium Glycocholate
Sodium Cholate
Sodium
Glutamyl
Cholate
Sodium
Glyco-
deoxycholate
Sodium
Hyodeoxycholate
Sodium Deoxycholate
T_m (degrees, C.)
75
70
65
60
55
50
45
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
MOLARITY OF CHOLANIC ACID

DNA (Figure 10).

The stabilization of the DNA double helix against thermal denaturation by increases in the ionic strength of the melting medium has been well documented, and for a given DNA increases by approximately 18° C./log [Na] (Dove & Davidson, 1962). Therefore, a decrease in the T_m's of partially dehistonized samples of chromatin would be expected to show some dependence on the number of histone positive charges still attached to the DNA, and, consequently, some dependence on the arginine plus lysine content of the histone fractions still attached to the DNA. Comparison of sodium chloride data (Smart & Bonner, 1969) and sodium deoxycholate data yields the expected differences in T_m changes. Thus, removal of 20 per cent of total histone by NaCl (only histone I removed) produces a decrease in the T_m of the partially dehistonized sample which is approximately 2 to 3 times as great as that observed when 20 per cent of total histone is removed by DOC (only histone II removed). These decreases are in good agreement with the differences in the net positive charge per amino acid of the two histone fractions (from amino acid composition data of Fambrough, 1967); histone I has 0.183 net positive charges per amino acid, while histone II has only 0.084.

Figure 10 also shows that decreases in the T_m's pro-

Figure 10. The fraction decrease in the T_m (where 74° C. = 0 and 44° C. = 1.00) versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of sodium chloride or the sodium salts of various cholanic acids and some of their conjugated derivatives. Melting was done in 2.5×10^{-4} M EDTA, pH 8. No correction for thermal expansion.


Sodium Deoxycholate
Sodium Hyodeoxycholate
Sodium Glycodeoxycholate
Sodium Taurodeoxycholate
Sodium Cholate
Sodium Glycocholate
Sodium Glutamylcholate
Sodium Chloride
DOC
NaCl
Fraction Decrease in T_m
Fraction Total Histone Remaining Bound to DNA
0
0.2
0.4
0.6
0.8
1.0

duced by extraction of chromatin with increasing concentrations of various deoxycholic acid derivatives as a function of total histone remaining bound to the DNA closely parallel those for deoxycholic acid. On the other hand, decreases in T_m produced by extraction of chromatin with increasing concentrations of cholic acid and its derivatives are slightly greater per weight fraction histone removed. This observation is in accord with the relatively early removal of histone I by sodium cholate (see Figure 2 and 8). It also appears that the sodium salts of the various derivatives of deoxycholic acid and cholic acid selectively dissociate histone fractions in a pattern similar to that of the parent compounds.

DISCUSSION

The first conclusion of this section is that the binding of sodium deoxycholate to chromatin components appears to be readily and completely reversible. Secondly, we conclude that the formation of an electrostatic bond between the positively charged ε-amino group of lysine and the negatively charged carboxylate group of deoxycholic acid (suggested by Rudman and Kendall (1957) for the binding of deoxycholic acid to human serum albumin) is not the primary

force responsible for the histone dissociation pattern observed when chromatin is extracted with increasing concentrations of DOC.

The lowering of the chemical potential of the aqueous chromatin-cholanic acid solution, which occurs when the cholanic acid complexes with histones and other chromatin components, certainly involves changes in the activity coefficients of histones and DNA. These changes, however, are very complex and the actual effect on the activity coefficients of the DNA and histones is difficult to predict. We feel, on the other hand, that the complexing of the relatively hydrophobic DOC anion with hydrophobic regions of the positively charged histones, certainly lowers the activity coefficient of the cholanic acid.

Consideration of the amino acid composition data of Tambrough (1967) shows that the various histone fractions contain the hydrophobic amino acid residues of valine, isoleucine, leucine, tyrosine, and phenylalanine in the following amounts: histone I, 12.1 mole per cent; histone II, 23.4 mole per cent; histones III and IV, 26.0 mole per cent. Although these data do not consider sequential arrangement of these residues in the histones, they are consistent with the hypothesis that DOC should form more stable hydrophobic complexes with histones II, III, and IV

than with histone I. With no correction for amide groups, the (lysine + arginine) - (glutamic acid + aspartic acid) content for the various histone fractions is: histone I, 18.3 mole per cent; histone II, 8.4 mole per cent; histones III and IV, 9.1 mole per cent. The mole per cent of net positive charges per histone fraction, therefore, is an inverse function of the order of removal with increasing concentrations of DOC.

We suggest that the driving force for the interaction between the cholanic acid anion and histones is the lowering of the activity coefficient of the cholanic acid anion which occurs when it is partially removed from aqueous solution by interaction with hydrophobic regions of the histones. When a cholanic acid anion interacts with a histone molecule, it effectively lowers the net positive charge of the histone molecule, thereby lowering the ionic strength of dissociating agent required to remove the histone molecule. This binding also probably cancels some of the histone-histone and histone-DNA hydrophobic interactions. It is possible that these interactions may account for the fact that ionic dissociating agents, such as NaCl, $NaClO_4$, and GuCl selectively remove histone I, the histone fraction with the highest net positive charge density per amino acid, at lower concentrations of dissociating agent than those required to

remove histones II, III, and IV.

We propose that cholanic acid anions preferentially interact with those histone fractions which contain the highest mole per cent hydrophobic amino acids, namely histones II, III, and IV. This interaction will lower the net positive charge of these histone fractions and reduce histone-histone and histone-DNA hydrophobic interactions. With these assumptions we expect sodium deoxycholate to remove histones at much lower concentrations of dissociating agent than do ionic dissociating agents - such is the experimental observation. We also expect DOC to selectively remove histones II, III, and IV at lower concentrations of dissociating agent than those required for removal of histone I - this is the experimentally observed pattern of histone dissociation. Since the introduction of a hydroxyl group into the steroid nucleus effectively lowers the activity coefficient of the cholanic acid in aqueous solution, we expect sodium cholate to be less effective in dissociation of histones from chromatin, as is experimentally observed. Conjugation of the cholanic acid with a compound which is relatively less hydrophobic will result in a lowering of the activity coefficient of the conjugated derivative in aqueous solution. Consequently, conjugation of deoxycholic acid or cholic acid with taurine or glycine

should, and does, make the product less effective in the dissociation of histones than the original cholanic acid. The importance of the negatively charged carboxylate group of the cholanic acid in the original binding of the cholanic acid anion to histones, and/or the subsequent lowering of the net positive charge of the histone to which it has bound, and/or its contribution to the ionic strength of the dissociating medium, is demonstrated by the effectiveness of sodium glutamylcholate as a histone dissociating agent. It appears that the presence of the additional negatively charged carboxyl group compensates for the expected lowering of the activity coefficient (due to the presence of the hydrophilic carboxyl group) of sodium glutamylcholate in aqueous solution.

Since addition of a third hydroxyl group to the steroid nucleus lowers the activity coefficient of the cholanic acid in aqueous solution, less cholate anion is expected to be bound to the histones than deoxycholate anion at a given molarity of cholanic acid. Consequently, a given molarity of cholic acid would not lower the net positive charge of the various histone fractions as much as the same concentration of deoxycholic acid. Dissociation of histones by sodium cholate, therefore, would be expected to be more dependent upon the ionic strength of the dissociating me-

dium, and consequently, would be expected to yield a dissociation pattern of the various histone fractions which would be intermediate between sodium deoxycholate and ionic dissociating agents, such as sodium chloride. The data show that although sodium cholate is about 7 to 10 times as effective as sodium chloride in the dissociation of total histone, it is only 2 to 3 times as effective in the dissociation of histone I.

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STUDIES ON THE ROLE OF HISTONES IN THE STRUCTURE OF CHROMATIN

INTRODUCTION

The DNA contained in the nucleus of higher organisms is complexed with proteins and usually a small amount of RNA. This nucleoprotein complex, as found in interphase cells, is referred to as chromatin. The major portion of the proteins contained in chromatin are histones, a family of seven major species all of which possess a 20 to 30 mole per cent arginine plus lysine content. The complexing of histones to DNA results in neutralization of a large portion of the negative phosphate groups of the DNA, and a few concommitant structural changes in the DNA. Although the structure of chromatin is complex, and probably concerns several levels or organization, recent studies indicate that its primary organization is at the level of supercoiling of the individual chromatin fibers (Griffith & Bonner, 1969; Pardon, Wilkins, & Richards, 1967; Moundrianakis, 1969). The model which appears to best account for experimental observations is that of a DNA double helix (width approximately 30 Å with associated protein) which is supercoiled to yield a molecule with a width of 80 Å and a pitch of 110 Å. Calculations show that the resulting structure produces about a 40 per cent shortening of the DNA molecule in chromatin, and tilts the plane of the bases in the double helix to an angle of approximately 35° with respect to the

molecular axis of the chromatin molecule. It is also observed experimentally that the DNA contained in chromatin has undergone a conformational change which is expressed as an increased absorptivity and, also, a decreased hyperchromicity upon melting (Tuan & Bonner, 1969).

The dissociation of chromatin with increasing concentrations of NaCl selectively removes histone I (lysine-rich) at lowest concentrations of dissociating agent. Higher concentrations of NaCl remove histone II (slightly lysine-rich) and histones III and IV (arginine-rich) (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Fambrough & Bonner, 1968; Georgiev, Ananieva, & Kozlov, 1966; Tuan & Bonner, 1969; Smart & Bonner, 1969). Smart, Hadler, and Bonner (1969) have reported that increasing concentrations of sodium deoxycholate (DOC) dissociate histone II at lowest concentrations of dissociating agent, while slightly higher concentrations remove histones III and IV. Histone I is the last histone fraction to be dissociated by increasing concentrations of DOC. We have compared the effects of selective removal of histones from chromatin by increasing concentrations of sodium deoxycholate with those caused by increasing concentrations of sodium chloride. Properties studied include thermal denaturation, sedimentation velocity, flow dichroism, relaxation times of molecules oriented in a flow field, and the irreversible disruption of a 130S,

cross-linked component of sheared chromatin. The data indicate that as evidenced by selective removal of histone studies, none of the structural or chemical parameters with which these properties are correlated show a dependence on the presence of one particular histone fraction.

METHODS AND MATERIALS

Preparation of Chromatin. Pea bud and calf thymus chromatins were prepared by the methods previously described (Smart, Hadler, & Bonner, 1969). The chromatin had a final concentration of 20 to 40 $A_{260m\mu}$, a ratio of $A_{230m\mu}/A_{260m\mu}$ of approximately 0.75, and a ratio $A_{320m\mu}/A_{260m\mu}$ of less than 0.034. The pea bud DNA contained in chromatin is complexed with histone protein, nonhistone protein, and RNA in the mass ratios DNA, 1.0; histone, 1.05; nonhistone, 0.50; RNA, 0.12. Calf thymus DNA is complexed in the mass ratios DNA, 1.00; histone, 1.00; nonhistone, 0.30.

Isolation of Chromatins of Differing Sedimentation Coefficients. Chalkley and Jensen (1968) reported that preparations of calf thymus chromatin (referred to as nucleohistone in their studies) contain a continuous distribution of chromatin molecules which range in sedimentation coefficient from 25 to about 200 S in 0.01 M Tris, pH 8. Two fractions with $s_{20,w}$ of 30 and 130 S were isolated by them

as previously described (Chalkley & Jensen, 1968).

Histone Dissociation Studies. To assure that the chromatin was not subjected to a sodium deoxycholate or sodium chloride concentration higher than the desired final one, partially dehistonized samples were prepared as described previously (Smart, Hadler, & Bonner, 1969).

Thermal Denaturation and Ultraviolet Absorption. The partially dehistonized samples were dialyzed exhaustively against 2.5×10^{-4} M EDTA, pH 8, diluted to approximately 1 $A_{260 m\mu}$ with dialysate, and melted in a Gilford Model 2000 multiple sample absorbance recording apparatus adapted for the recording of melting profiles. The rate of temperature increase was 0.5 to 1.0 degree/minute. Ultraviolet absorption spectra were determined with a Cary recording spectrophotometer, Model 11.

Sedimentation Analysis. Sedimentation velocity was studied using band-sedimentation techniques either on preformed sucrose gradients in a Spinco Model L ultracentrifuge or on self-generating density gradients in a Spinco Model E analytical ultracentrifuge (Vinograd, 1963). In the analytical centrifuge D_2O was used to form the gradients. The partial specific volume of the partially dehistonized chromatins was determined as a weight average of the partial specific volumes of DNA (0.555 cc/gm) (Brunner & Vinograd, 1965) and of histones (0.745 cc/gm) (Brutlag, Schlehuber,

& Bonner, 1969).

Flow Dischroism Measurements. Flow dichroism was performed as described by Callis and Davidson (1969a) in 0.0025 M Tris, pH 8, using their apparatus. In their apparatus, the flow is down a long, narrow channel with an unpolarized light beam along the direction of flow. The apparatus has the following advantages: dilute macromolecule solutions can be used (0.2 $A_{260m\mu}$), high shear gradients are easily obtained (up to 21,000 sec^{-1}), and only small volumes of solution are needed.

Relaxation Times of Molecules Oriented in a Flow Field. The relaxation of the flow dichroism signal of native and dehistonized chromatin was performed as described by Callis and Davidson (1969b) in 0.0025 M Tris, pH 8, using their apparatus. Under the conditions employed in this study, the actual time required for stoppage of flow is 3 to 5 x 10^{-3} sec.

Precipitation of Chromatin. In order to obtain a standard and reproducible measure of precipitation, we have defined precipitated chromatin as that material sedimented from solution (0.15 M NaCl - 0.0025 M Tris, pH 8) in 10 minutes at 10,000 x g. The assay was performed by slowly adding 1/3 volume of 0.6 M NaCl - 0.0025 M Tris, pH 8, to 1 volume of chromatin solution (approximately 10 $A_{260m\mu}$) containing 0.0025 M Tris, pH 8, with vigorous stirring on a

Vortex mixer. After 10 minutes on ice, the solution was centrifuged in a Servall SS-34 rotor at 10,000 rpm for 10 minutes. The supernatant was removed by pipetting, analyzed for $A_{260_{m\mu}}$ material, and then dialyzed extensively against 0.0025 M Tris, pH 8. The pellet was removed, resuspended by Teflon homogenization, and dialyzed extensively against 0.0025 M Tris, pH 8. Dialysis of supernatants and pellets was carried out in a glass stoppered, graduated cylinder mounted on a slowly rotating carrier. A small air bubble in the dialysis tubes resulted in constant stirring. All operations were carried out at 0 to 4° C.

Chemical Analysis. DNA, histone protein, nonhistone protein, and RNA analysis were performed as previously described (Smart, Hadler, & Bonner, 1969).

Disc Gel Electrophoresis of Histones. Acrylamide disc gel electrophoresis of isolated histones was performed by the method of Bonner et al. (1968a). The quantity of each electrophoretic component was determined by densitometry after Fambrough (1967).

Note: All assays were performed within one week of the preparation of chromatin.

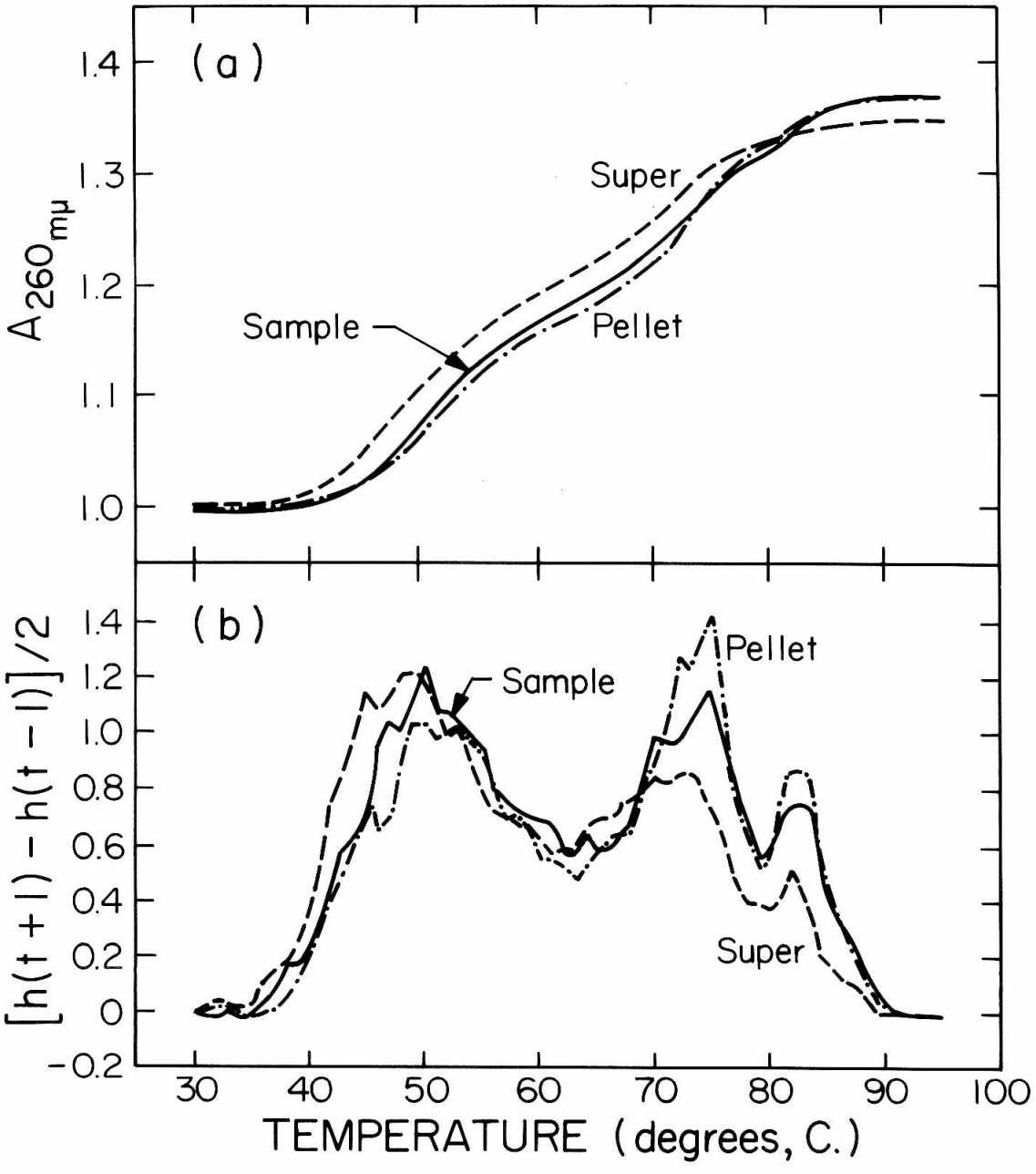
RESULTS

Samples of chromatin which have been partially dehistonized by increasing concentrations of sodium chloride (NaCl) or sodium deoxycholate (DOC) are more heterogeneous in protein distribution than native chromatin or pure DNA. This is shown by an increase in the band width of formaldehyde-fixed samples in a cesium chloride density gradient (Ilyin & Georgiev, 1969; Farber, Smart, & Bonner, 1969). These samples also have an increased heterogeneity in charge distribution over that of native chromatin or pure DNA, as shown by the increase in electrophoretic dispersion (band width) of the samples upon free-zone electrophoresis (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Smart & Bonner, 1969; Levinson, Smart, & Bonner, 1969). To provide still more evidence that a sample of partially dehistonized chromatin is composed of many discrete, slightly different types of molecules, we have subjected the samples to fractionation by precipitation in 0.15 M NaCl. Whether chromatin remains soluble or aggregates is largely dependent upon the effective charge on the chromatin molecules (Jensen & Chalkley, 1968). When the solvated, soluble form with its over-all negative charge is electrostatically neutralized in 0.15 M NaCl, solvation is reduced and the molecules interact to yield the aggregated form. Thus, we expect the

precipitability of chromatin to decrease as increasing amounts of histone are removed. If partially dehistonized chromatin samples are in fact composed of molecules which contain varying proportions of DNA to histone, we would anticipate that partially dehistonized samples should be precipitable to an extent intermediate between the observed 90 to 98 per cent characteristic of native chromatin and the 0 per cent characteristic of pure DNA. The pellet (precipitated portion) of samples with intermediate precipitation values should consist of a class of nucleoprotein molecules of a higher histone to DNA ratio than that found in the supernatant (soluble portion). Figure 1a presents the melting profiles of a chromatin sample which has been extracted with 0.04375 M DOC, and of the supernatant and pellets obtained from this sample by fractionation in 0.15 M NaCl (approximately 66 per cent of the $A_{260 m\mu}$ material of this sample was recovered in the pellet). The T_m of the original sample is 2.6° C. lower than the T_m of that portion which remained soluble. Inspection of Table 1 and Table 2 indicates that the 8.1° C. difference in the T_m's of the precipitated soluble fractions obtained by fractionation with 0.15 M NaCl, can represent a 15 per cent difference in histone content of the two fractions. In order to obtain a better understanding of the fine structure of the melting profiles, we have replotted the data as a derivative of

Figure 1a. The melting profiles of a chromatin sample which had been extracted with 0.04375 M DOC, and of the soluble (super) and precipitable (pellet) portions obtained from this sample upon fractionation in 0.15 M NaCl. Approximately 66 per cent of the $A_{260m\mu}$ material of this sample was precipitable. Melting was done in 2.5×10^{-4} M EDTA, pH 8. No correction for thermal expansion.

Figure 1b. The derivative with respect to temperature, $(h[T+1] - h[T-1])/2$, of the melting profiles presented in Figure 1a. Histone stabilized material under peaks from 70 to 75° C. and 80 to 85° C., and DNA-like material under broad peak from 40 to 55° C.


(a)
1.4
1.3
1.2
1.1
1.0
$A_{260_{m\mu}}$
Super
Sample
Pellet
(b)
$[h(t+1) - h(t-1)]/2$
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
Sample
Pellet
Super
30
40
50
60
70
80
90
100
TEMPERATURE (degrees, C.)

$A_{260_{m\mu}}$ with respect to temperature in Figure 1b. It is clear that the original sample contains more histone-stabilized material (peaks 70 to 75° C. and 80 to 85° C.) and less DNA-like material (broad peak 40 to 55° C.) than does the 0.15 M NaCl soluble portion of the sample, and, conversely, less histone-stabilized and more DNA-like material than the portion which precipitated in 0.15 M NaCl. Thus, partial removal of histones does not result in a uniform alteration of chromatin.

The value obtained by measurement of any biophysical or biochemical property of a partially dehistonized chromatin sample, therefore, can only be considered an average value made up of the contributions of all the individual, discretely different nucleoprotein molecules in the sample.

A comparison of dissociation of total histone from chromatin by increasing concentrations of sodium chloride (appendix of this paper) and sodium deoxycholate (Smart, Hadler, & Bonner, 1969) is presented in Table 1. NaCl selectively and completely dissociates histone I at concentrations less than 0.6 molar, while higher concentrations remove histones II, III, and IV. In contrast, histone II is selectively removed by low concentrations of DOC. Slightly higher concentrations of DOC remove histones III and IV. Histone I is the last histone fraction to be extracted by this agent.

Table 1

Dissociation of Total Histone Protein from Chromatin by NaCl and DOC

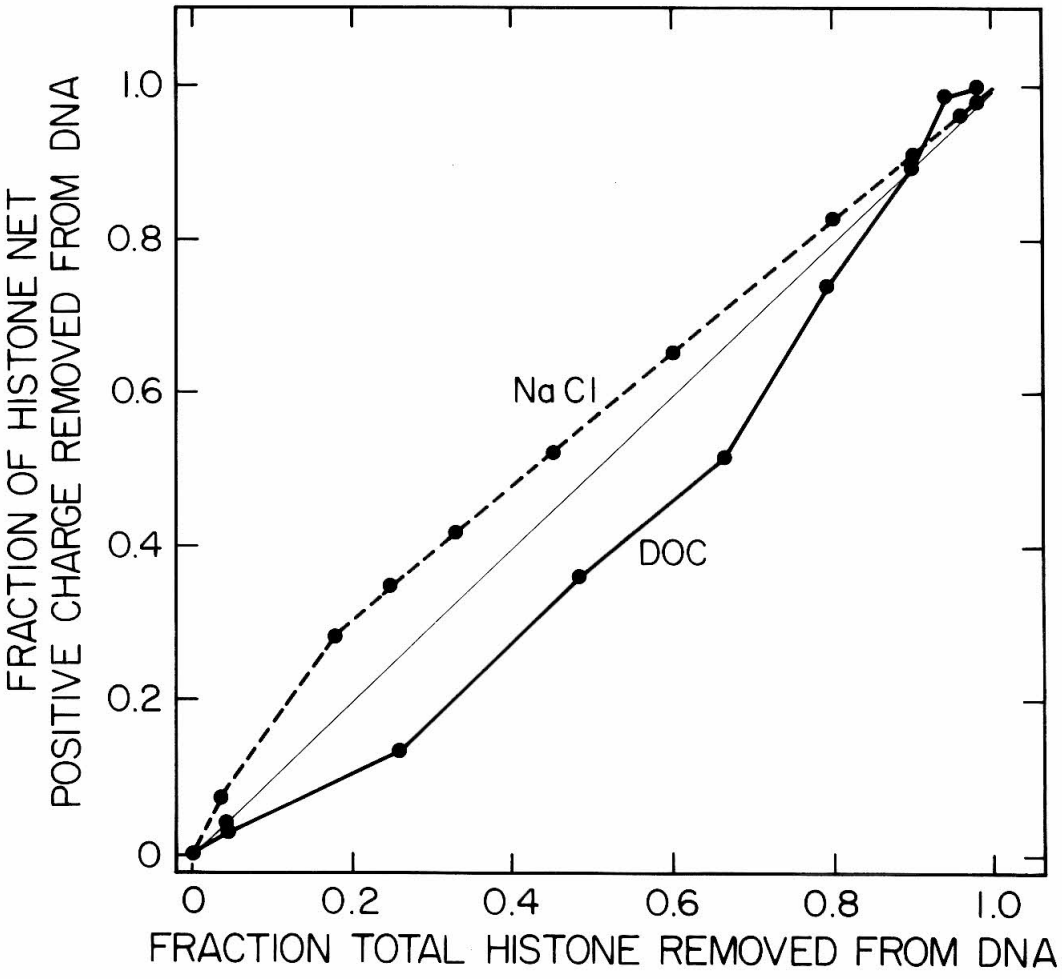
Description	Fraction Total Histone Removed from DNA[1]	Description	Fraction Total Histone Removed from DNA[2]
Control	0.00	Control	.000
0.15 M NaCl	0.01	0.0050 M DOC	.043 ± .014
0.30 M NaCl	0.04	0.0150 M DOC	.260 ± .040
0.50 M NaCl	0.18	0.0250 M DOC	.485 ± .027
0.60 M NaCl	0.25	0.0375 M DOC	.668 ± .041
0.75 M NaCl	0.33	0.04375 M DOC	.752
1.00 M NaCl	0.45	0.0500 M DOC	.792 ± .060
1.25 M NaCl	0.60	0.05625 M DOC	.862
1.50 M NaCl	0.80	0.0625 M DOC	.895 ± .035
2.00 M NaCl	0.90	0.0750 M DOC	.940 ± .035
2.50 M NaCl	0.96	0.1000 M DOC	.978 ± .011
3.00 M NaCl	0.99	0.1500 M DOC	.974 ± .009

1. Data from appendix of this paper.

2. Data from Smart, Hadler, and Bonner (1969).

Consideration of the amino acid composition data of Fambrough (1967) reveals that the (arginine + lysine) - (glutamic acid + aspartic acid) content of the histone fractions (no correction for amide group losses) is: histone I, 18.3 mole per cent; histone II, 8.4 mole per cent; histones III and IV, 9.1 mole per cent. The weight fraction of the various histone components in the chromatin used for these studies is histone I, 0.14; histone II, 0.60; histones III and IV, 0.26. The combination of these data with those for fraction of each histone component removed from chromatin by increasing concentrations of NaCl (Ohlenbusch, Olivera, Tuan & Davidson, 1967; Fambrough & Bonner, 1968) Tuan & Bonner, 1969; appendix of this paper) and DOC (Smart, Hadler, & Bonner, 1969) makes it possible to calculate the fraction of histone net positive charge removed from the chromatin by the two methods of dissociation. Figure 2 shows the calculated fraction of histone net positive charge removed from the DNA of chromatin versus the fraction of total histone removed from the DNA. The removal of a given weight fraction of histone by NaCl generates a set of nucleoprotein molecules which, on the whole, have a much higher over-all negative charge than does the set of nucleoprotein molecules generated by the removal of an equivalent weight fraction of histone by DOC. For

Figure 2. The calculated fraction of histone net positive charge removed from DNA versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. Amino acid composition of individual histones from Fambrough (1967); data on total histone and individual histones removed by DOC from Smart, Hadler, and Bonner (1969); data on total histone and individual histones removed by NaCl from appendix of this paper and Fambrough and Bonner (1968).


FRACTION OF HISTONE NET
POSITIVE CHARGE REMOVED FROM DNA
1.0
0.8
0.6
0.4
0.2
0
Na Cl
DOC
0
0.2
0.4
0.6
0.8
1.0
FRACTION TOTAL HISTONE REMOVED FROM DNA

example, dissociation of 26 per cent of total histone by DOC removes only 13 per cent of the histone net positive charge, while dissociation of 26 per cent of total histone by NaCl removes about 35 per cent of the histone net positive charge.

T_m values for chromatin samples partially dehistonized by increasing concentrations of NaCl (appendix of this paper) and DOC (Smart, Hadler, & Bonner, 1969) are presented in Table 2. The melting profiles of the partially dehistonized samples are considerably broadened, but not cleanly divided into a DNA-like and native chromatin region. Figure 3 plots the data presented in Table 2 as fraction decrease in T_m (where 73.8° C. = 0 and 43.0° C. = 1.00) versus fraction total histone removed from DNA. The removal of a given weight fraction of total histone by NaCl generates a nucleoprotein sample which has a lower T_m than does the sample generated by the removal of an equivalent weight fraction of total histone by DOC. Figure 4 shows the fraction decrease in T_m versus the fraction of histone net positive charge removed from the DNA. When plotted in this manner, the two methods of histone dissociation yield essentially identical curves. These data are consistent with the interpretation that the different histone fractions do not vary greatly in their ability to stabilize the DNA

Table 2

T_m of Chromatin Partially Dehistonized by NaCl and DOC

Description	T_m	Description	T_m
Control	73.8 ± 0.5	Control	73.9 ± 0.5
0.15 M NaCl	73.2 ± 0.8	0.0050 M DOC	73.4 ± 0.6
0.30 M NaCl	72.3 ± 0.8	0.0150 M DOC	71.5 ± 0.6
0.50 M NaCl	67.6 ± 0.2	0.0250 M DOC	68.3 ± 0.7
0.60 M NaCl	66.2 ± 0.2	0.0375 M DOC	64.1 ± 0.9
0.75 M NaCl	64.6 ± 0.4	0.04375 M DOC	60.5 ± 1.0
1.00 M NaCl	63.6 ± 0.7	0.0500 M DOC	58.0 ± 1.6
1.25 M NaCl	59.9 ± 0.8	0.05625 M DOC	52.5 ± 1.4
1.50 M NaCl	50.2 ± 1.1	0.0625 M DOC	47.8 ± 1.0
2.00 M NaCl	46.2 ± 0.8	0.0750 M DOC	44.6 ± 0.5
2.50 M NaCl	43.0 ± 1.5	0.1000 M DOC	44.0 ± 0.6
3.00 M NaCl	43.0 ± 1.0	0.1500 M DOC	44.0 ± 0.7

Figure 3. The fraction decrease in T_m (where 73.8° C. = 0 and 43.0° C. = 1.00) versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. Melting was done in 2.5×10^{-4} EDTA, pH 8. No correction for thermal expansion.

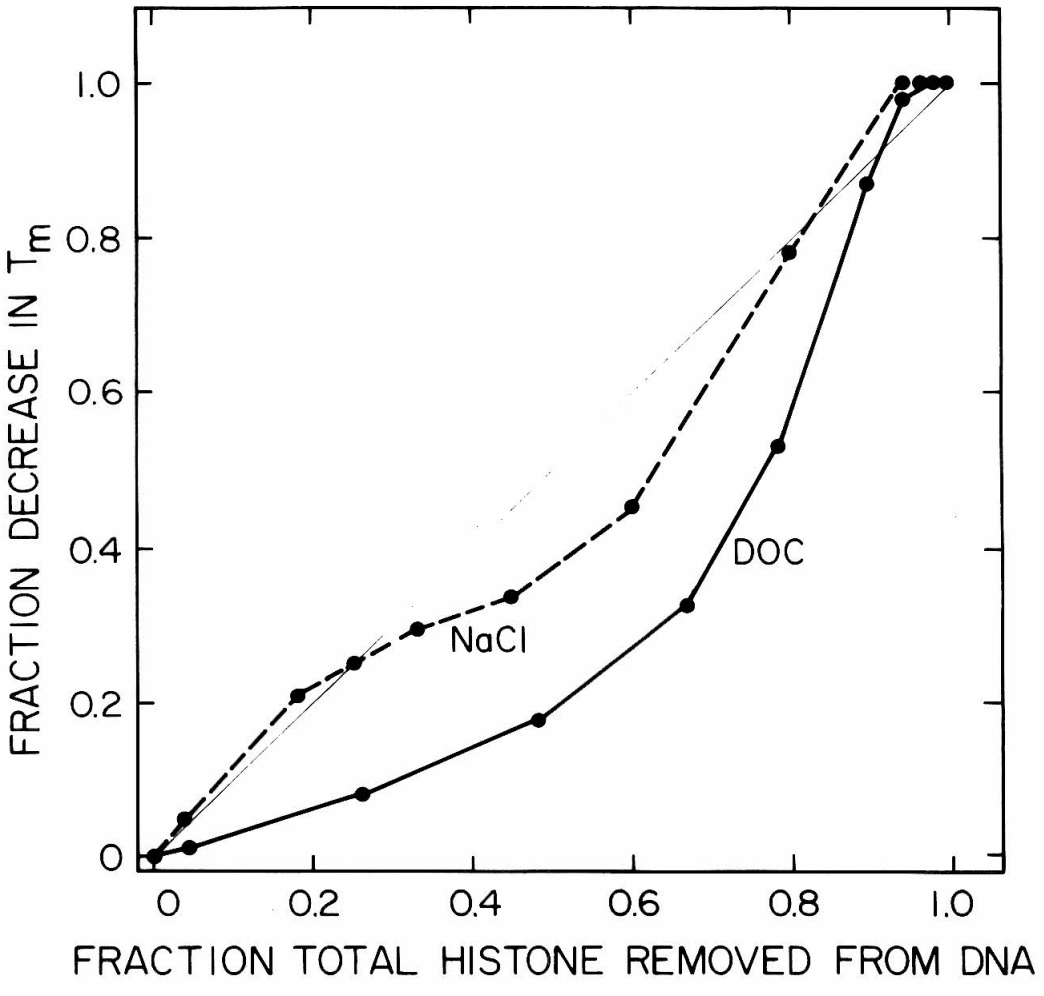
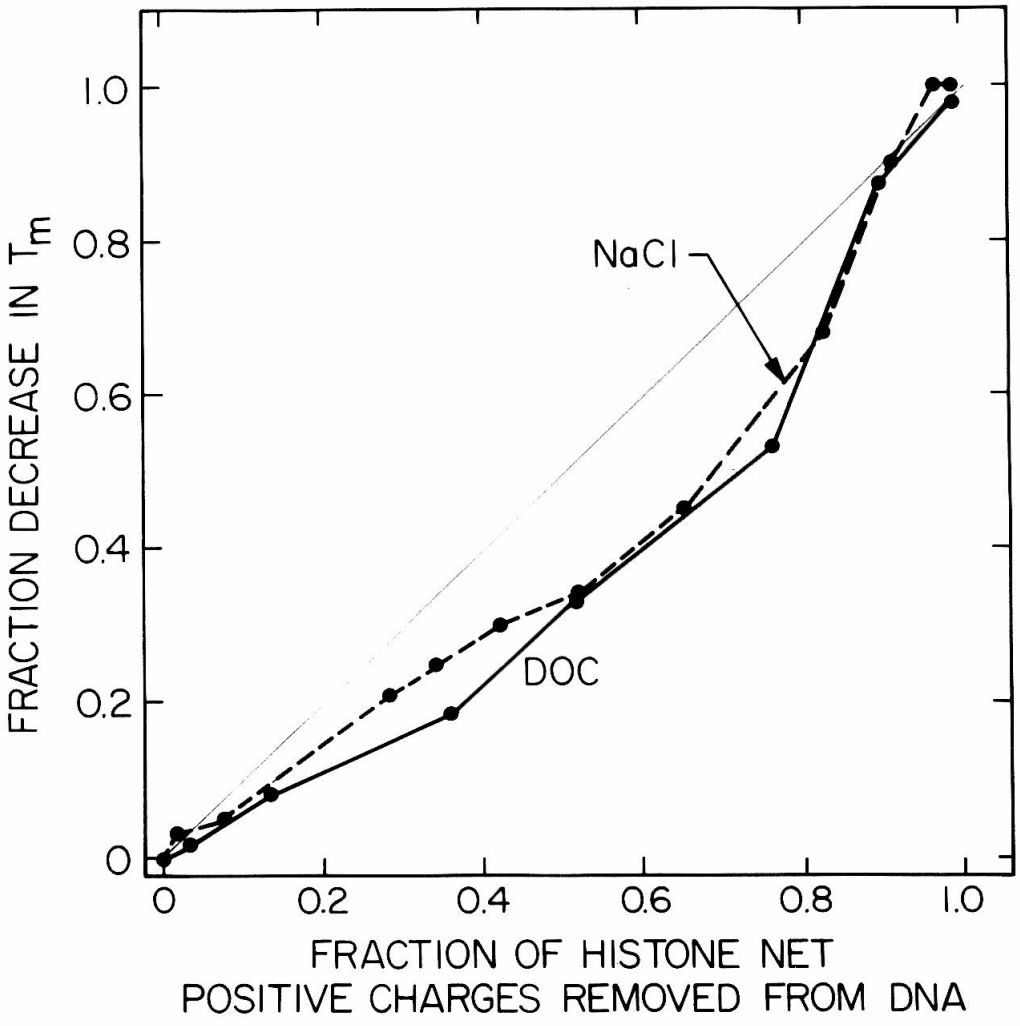

1.0
0.8
0.6
0.4
0.2
0
FRACTION DECREASE IN T_m
NaCl
DOC
0
0.2
0.4
0.6
0.8
1.0
FRACTION TOTAL HISTONE REMOVED FROM DNA

Figure 4. The fraction decrease in T_m (where 73.8° C. = 0 and 43.0° C. = 1.00) versus the fraction of histone net positive charges removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. See Figure 2 and text for calculation of histone net positive charges removed from DNA. The melting was done in 2.5 x 10^{-4} M EDTA, pH 8. No correction for thermal expansion.


FRACTION DECREASE IN T_m
1.0
0.8
0.6
0.4
0.2
0
NaCl
DOC
0
0.2
0.4
0.6
0.8
1.0
FRACTION OF HISTONE NET
POSITIVE CHARGES REMOVED FROM DNA

double helix against thermal denaturation provided that corrections are made for the net positive charge contained in each histone fraction.

Table 3 presents the $s_{20,w}$ values for samples of chromatin which have been partially dehistonized by increasing concentrations of NaCl and DOC. The value of 31 S for sheared chromatin is in good agreement with the value of 30 S obtained by Chalkley and Jensen(1968) for sheared calf thymus chromatin. The value of 15 to 16 S for samples completely dehistonized by DOC or NaCl is also in agreement with the value of 14 S which they obtained for DNA purified from the chromatin. As noted above, chromatin aggregates in 0.05 to 0.30 M NaCl (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Chalkley & Jensen, 1968). When such aggregated chromatin is redissolved and dialyzed against 0.0025 M Tris, pH 8, it exhibits a $s_{20,w}$ slightly greater than that of native chromatin (Richards, Smart, & Bonner, 1969). It has also been shown that samples of chromatin which have been partially dehistonized by treatment with sodium chloride concentrations up to 1.0 M are still capable of partial precipitation in 0.15 M NaCl (Smart & Bonner, 1969). Inspection of partially dehistonized chromatin obtained in the sucrose cushions of the 50,000 rpm, 10 hour centrifugation after extraction of chromatin with 0.15 to 0.75 M NaCl

Table 3

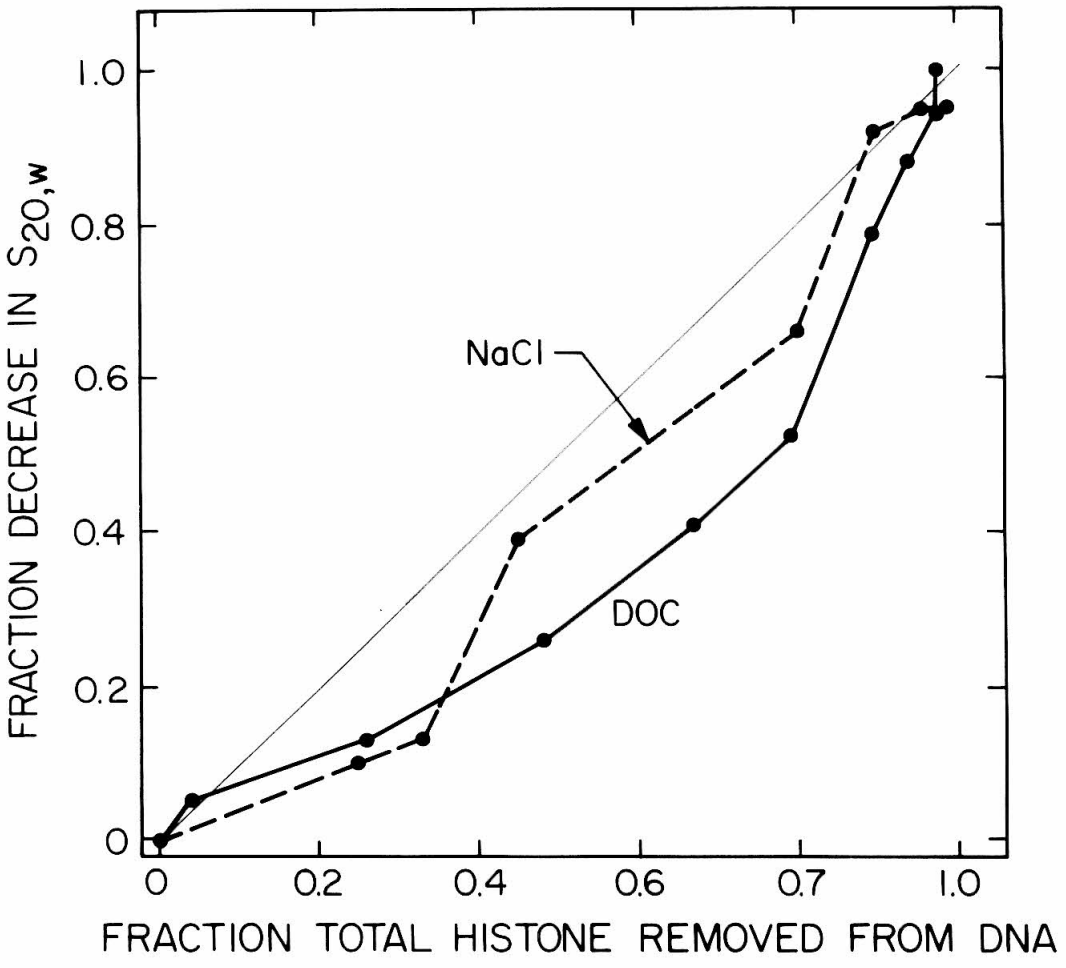
$S_{20,w}$ of Chromatin Partially Dehistonized by NaCl and DOC

Description	$s_{20,w}$	Description	$s_{20,w}$
Control	31.0 ± 0.6	Control	31.0 ± 0.6
0.15 M NaCl	35.4 ± 2.5	0.0050 M DOC	30.1 ± 0.6
0.30 M NaCl	33.5 ± 1.5	0.0150 M DOC	28.9 ± 0.2
0.60 M NaCl	29.5 ± 0.3	0.0250 M DOC	26.9 ± 0.3
0.75 M NaCl	28.9 ± 0.8	0.0375 M DOC	24.6 ± 0.6
1.00 M NaCl	24.8 ± 0.6	0.0500 M DOC	22.7 ± 0.4
1.50 M NaCl	20.6 ± 0.4	0.0625 M DOC	18.6 ± 0.2
2.00 M NaCl	16.5 ± 0.3	0.0750 M DOC	17.1 ± 0.4
2.50 M NaCl	16.0 ± 0.4	0.1000 M DOC	16.3 ± 0.5
3.00 M NaCl	16.1 ± 0.2	0.1500 M DOC	15.3 ± 0.3

reveals a hard-packed, slightly whitish pellet characteristic of partially precipitated chromatin. Therefore, we might expect an erroneously high $s_{20,w}$ value for samples of chromatin extracted with concentrations of NaCl within this range. Figure 5 shows the fraction decrease in $s_{20,w}$ (where 31 S = 0 and 15.3 S = 1.00) versus the fraction of total histone removed from the DNA by increasing concentrations of DOC or NaCl. Correction for the fraction of histone net positive charge removed from the DNA yields essentially superimposible curves above 40 per cent histone removal. Below this amount of histone removal, samples partially dehistonized by treatment with NaCl have a much lower fraction decrease in $s_{20,w}$ than samples with an equivalent amount of total histone removed by extraction with DOC. As mentioned previously, this deviation may be due to partial precipitation of these samples during the isolation procedure.

Native DNA exhibits a strong flow dichroism. This is the expectation for the β-form of the Watson-Crick structure in which the planes of the bases are perpendicular to the helix axis. The absorptivity of histone at $259_{m\mu}$ is only 1.7 per cent of that of DNA (Ohba, 1966), and the contribution of the histones themselves can therefore be neglected in measurement of flow dichroism. The value of the flow

Figure 5. The fraction decrease in $s_{20,w}$ (where 31.0 S = 0 and 15.3 S = 1.00) versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. Band sedimentation was performed in a Spinco Model E analytical ultracentrifuge with self-forming gradients of 90 per cent D_2O - 0.005 M NaCl - 0.01 M Tris, pH 8.


FRACTION DECREASE IN $S_{20,w}$
1.0
0.8
0.6
0.4
0.2
0
NaCl
DOC
0
0.2
0.4
0.6
0.7
1.0
FRACTION TOTAL HISTONE REMOVED FROM DNA

dichroism, therefore, predominantly reflects the orderliness of the planar purine and pyrimidine bases perpendicular to the flow lines. Table 4 presents the flow dichroism, $(A_{260m\mu}\text{flow} - A_{260m\mu}\text{stationary})/A_{260m\mu}\text{stationary}$, values for chromatin samples, which have been partially dehistonized by increasing concentrations of NaCl or DOC, subjected to shear forces of $\langle G \rangle$ = 21,250, 8,500, and 4,250 sec^{-1}. The value obtained for completely dehistonized chromatin is in good agreement with that expected for pure DNA of molecular weight 3×10^6 (Callis & Davidson, 1969). The data for $\langle G \rangle$ = 21,250 sec^{-1} are plotted in Figure 6 as fraction increase in flow dichroism (where 0.0185 = 0 and 0.0600 = 1.00) versus fraction of total histone removed from the DNA. Increases in flow dichroism of samples partially dehistonized by NaCl or DOC lag considerably behind increases in the fraction of total histone removed from the DNA.

When the helix axis of pure DNA is oriented parallel to the flow lines in the present apparatus, the bases of the β-form of the Watson-Crick structure are perpendicular to the direction of propagation of the light beam. The value of the flow dichroism is, therefore, dependent primarily on the orientation of the DNA molecule. Studies by Pardon, Wilkins, & Richards (1967), Griffith and Bonner (1969), and Moundrianakis (1969) indicate that although chromatin is complex, and probably capable of assuming

Table 4

Flow Dichroism ($\Delta A_{260m\mu}/A_{260m\mu}$) of Chromatin Partially Dehistonized by NaCl

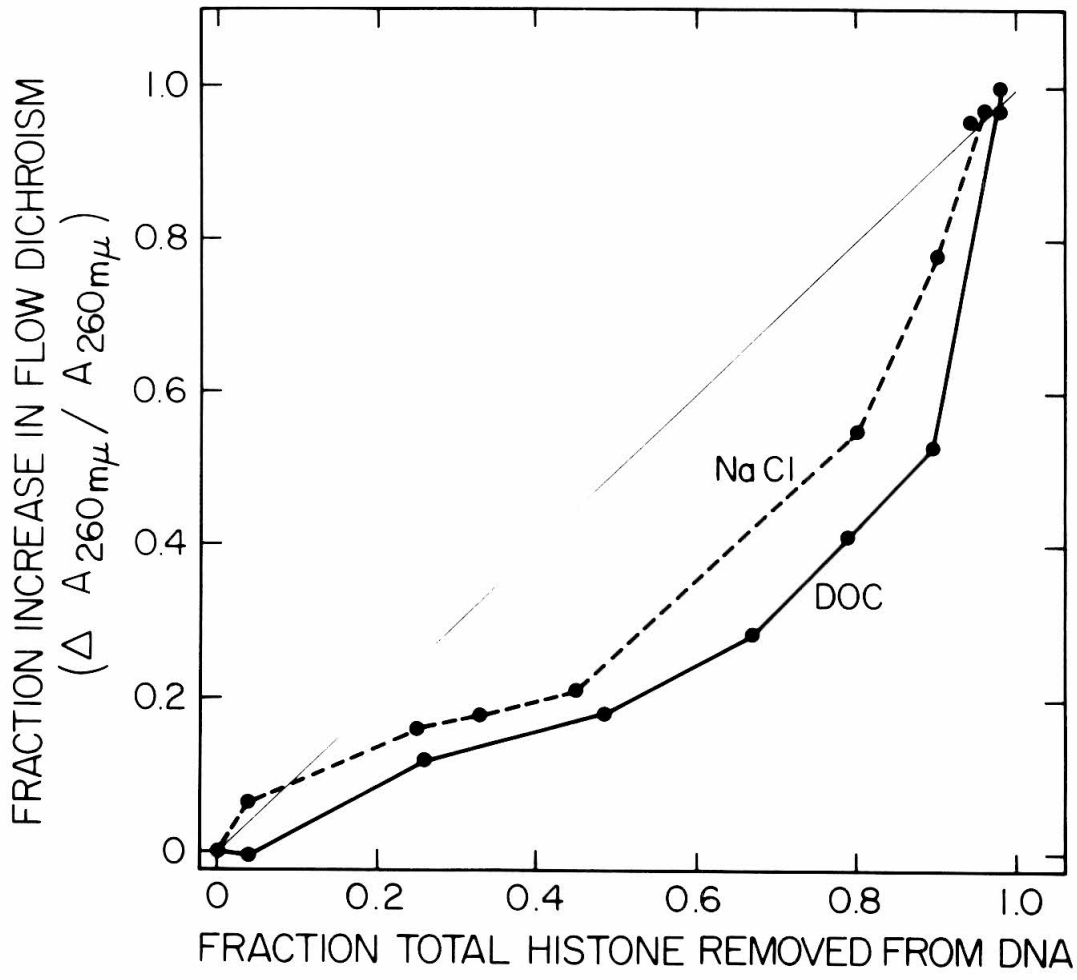
Description	$A_{260m\mu}/A_{260m\mu}$ $\langle G \rangle$ = 21,250	$\langle G \rangle$ = 8,500	$\langle G \rangle$ = 4,250
Control	0.0185	0.0118	0.0079
0.15 M NaCl	0.0204	0.0134	0.0094
0.30 M NaCl	0.0208	0.0141	0.0099
0.60 M NaCl	0.0247	0.0173	0.0115
0.75 M NaCl	0.0256	0.0178	0.0122
1.00 M NaCl	0.0267	0.0185	0.0132
1.50 M NaCl	0.0410	0.0244	0.0171
2.00 M NaCl	0.0549	0.0323	0.0238
2.50 M NaCl	0.0583	0.0386	0.0283
3.00 M NaCl	0.0585	0.0390	0.0286

Table 4 (continued)

Flow Dichroism ($\Delta A_{260m\mu}/A_{260m\mu}$) of Chromatin Partially Dehistonized by DOC

Description	$A_{260m\mu}/A_{260m\mu}$ <G>= 21,250	<G>= 8,500	<G>= 4,250
Control	0.0180	0.0118	0.0075
0.0050 M DOC	0.0177	0.0116	0.0071
0.0150 M DOC	0.0230	0.0124	0.0099
0.0250 M DOC	0.0257	0.0161	0.0115
0.0375 M DOC	0.0300	0.0174	0.0123
0.0500 M DOC	0.0353	0.0216	0.0145
0.0625 M DOC	0.0402	0.0247	0.0167
0.0750 M DOC	0.0582	0.0358	0.0243
0.1000 M DOC	0.0600	0.0398	0.0269
0.1500 M DOC	0.0600	0.0375	0.0270

Figure 6. The fraction increase in flow dichroism ($A_{260_{m\mu}}$flow - $A_{260_{m\mu}}$stationary / $A_{260_{m\mu}}$stationary) (where 0.0185 = 0 and 0.0600 = 1.00) versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. Flow dichroism was measured at $\langle G \rangle$ = 21,250 sec^{-1} in 0.0025 M Tris, pH 8, and at a DNA concentration of $A_{260_{m\mu}}$ = 0.2.


FRACTION INCREASE IN FLOW DICHROISM
(Δ A 260mμ / A 260mμ)
1.0
0.8
0.6
0.4
0.2
0
Na Cl
DOC
0
0.2
0.4
0.6
0.8
1.0
FRACTION TOTAL HISTONE REMOVED FROM DNA

several levels of organization, its primary organization is that of supercoiled chromatin fibers. The decrease in the flow dichroism of native chromatin as compared to DNA isolated from it, reflects not only the probable decreased orientability of the whole chromatin molecule (shorter contour length), but also the decreased number of base pairs perpendicular to the molecular axis of an oriented molecule.

Figure 6 shows that removal of a given weight fraction of total histone by NaCl results in a slightly greater increase in the flow dichroism than that observed when an equivalent weight fraction of histone is removed by DOC. Comparison of the fraction increase in flow dichroism versus fraction histone net positive charge removed from the DNA shows that removal of a given amount of histone net positive charge by NaCl yields a smaller increase in flow dichroism than the removal of the same amount of net positive charge by DOC.

Callis and Davidson (1969) have shown from relaxation of flow oriented molecules that the flexibility of native DNA is not due to local denaturation, but rather to noncovalent interactions which determine the flexibility and configuration of typical random-coil polymers. Extrapolation of their data shows that the expected relaxation time for pure DNA of molecular weight 3×10^6 is approximately 1.3 msec. This value is well below the 3 to 5 msec required

for complete flow stoppage in the apparatus, and as expected no measurable relaxation time for completely dehistonized samples of chromatin is observed, i.e., the molecules relax in less than 3 to 5 msec. Studies on the relaxation of electrical birefrignence of DNA by Ohlenbusch (1966) indicate that the effective rigid rod length of native calf thymus DNA is near 4000 Å. Griffith and Bonner (1969) have reported that the predominant structure of sheared chromatin, as evidenced by electron microscopic examination of the molecules, is a supercoiled nucleoprotein molecule which is 80 Å wide and has a repeat distance of 110 Å. If this molecule behaved as one rigid rod, then its calculated length would be approximately 9500 Å. The expected relaxation time for this molecule would be approximately 15 msec. However, the experimental observation is that chromatin samples oriented in a flow field have a relaxation time of less than 3 to 5 msec. Therefore, the chromatin molecules do not behave as completely rigid rods, and, consequently, they may be behaving as random-coil polymers. It should, however, be noted that the calculated relaxation time for a rigid rod of 6000 Å would be approximately 5 msec. Therefore, almost any type of flexibility in the proposed rigid rod of 9500 Å would probably reduce the relaxation time to below the measurable limits.

Chalkley and Jensen (1968) reported that sheared calf thymus chromatin is heterogeneous with respect to molecular size and can be fractionated by sedimentation through a sucrose gradient into chromatin molecules of differing complexity. They studied two fractions of $s_{20,w}$ = 30 and 130. They found that the large molecules (130 S) are made up of smaller molecules (30 S) linked together noncovalently by protein cross-links. Exposure of the two chromatins to 0.4 M NaCl, followed by removal of the salt dialysis, causes a return to 30 S in all instances. The disruption of the complex structure of 130 S chromatin by salt above 0.4 M is irreversible. Because these data suggest that the lysine-rich histones (histone I) play a role in maintaining the structure of such complexes, we have investigated the effect of removal of small amounts of slightly lysine-rich histones (histone II) upon the 130 S complex. Figure 7 shows the sedimentation profiles of 130 S calf thymus chromatin after exposure to 0.015 M DOC, which removes approximately 26 per cent of total histone (of which more than 80 per cent is histone II). Thus, removal of relatively small amounts of histone II also irreversibly disrupts the 130 S structure. To eliminate simple hydrophobic interactions as the source of the disruption of the 130 S complex, we have checked the effect of exposure to 0.015 M DOC + 0.5% Brij (a neutral

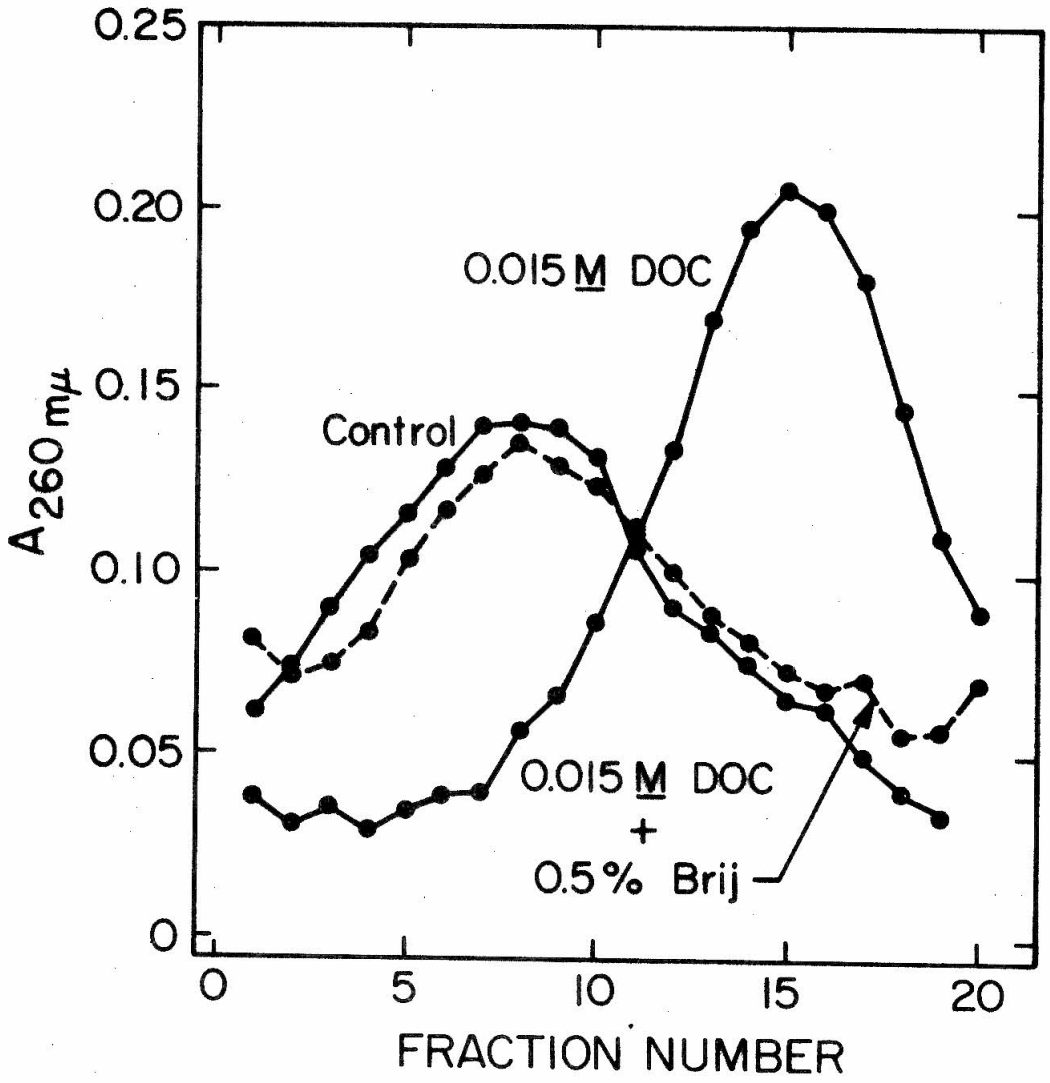

0.25
0.20
0.15
0.10
0.05
0
$A_{260\,m\mu}$
0.015 M DOC
Control
0.015 M DOC
+
0.5% Brij
0
5
10
15
20
FRACTION NUMBER

Figure 7. Sedimentation profiles of a 130 S component of chromatin after treatment with 0.015 M DOC or 0.015 M DOC - 0.5 per cent Brij (neutral detergent). 130 S chromatin treated for 30 minutes at 0° C. with appropriate concentration of detergent(s) and then layered (0.5 ml) over a linear 5 to 20 per cent sucrose gradient (4.5 ml). After centrifugation at 38.000 rpm for 1 hour at 0° C. in a Spinco SW39 rotor, 20 drop fractions were collected and diluted with 0.2 ml of 0.0025 M Tris, pH 8, for determination of $A_{260 m\mu}$. Detergents were mixed before adding to chromatin.

detergent). Treatment with this combination of detergents, which is very effective in the lysis of bacteria (Godson & Sinsheimer, 1967), removes very little histone protein and results in no significant change in the $s_{20,w}$ of the complex.

DISCUSSION

Partially dehistonized chromatin samples show an increased heterogeneity in protein distribution (increased band width of formaldehyde-fixed samples in a CsCl density gradient) (Ilyin & Georgiev, 1969; Farber, Smart, & Bonner, 1969) and charge distribution (increased band width of samples upon free-zone electrophoresis) (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Smart, Hadler, & Bonner, 1969) over that of native chromatin or pure DNA. The ability to separate a partially dehistonized sample of chromatin into two distinctly different classes of molecules by the relatively gentle method of precipitation in 0.15 M NaCl, indicates that such samples are composed of many discrete, slightly different nucleoprotein molecules. Thus, the value obtained by the measurement of any biophysical or biochemican property of a partially dehistonized chromatin sample can only be regarded as an average value which contains

the contributions of all the individual, discretely different nucleoprotein molecules in the sample. With this in mind, we have investigated the effects of selective removal of histones from chromatin by increasing concentrations of DOC (removes histone II at lowest concentrations, followed at slightly higher concentrations by histones III and IV, and lastly, at still higher concentrations, by histone I) and NaCl (removes histone I at lowest concentrations, followed at higher concentrations by histones II, III, and IV) on the following properties: thermal denaturation, sedimentation velocity, flow dichroism, relaxation times of molecules oriented in a flow field, and the irreversible disruption of a 130 S cross-linked component of chromatin. The data indicate that none of the structural or chemical parameters with which these properties are correlated show a dependence upon the presence of a particular histone fraction.

The stabilization of the DNA double helix against thermal denaturation increases by approximately 18° C./log [Na] (Dove & Davidson, 1962). Therefore, a decrease in the T_m's of partially dehistonized samples of chromatin is expected to depend somewhat on the number of histone positive charges still attached to the DNA. Figure 4 shows that when corrections are made for the net positive charge con-

tained in each histone fraction, the different histone fractions do not vary greatly in their ability to stabilize the DNA double helix. It is also apparent that the histones provide a greater stabilization of the DNA against thermal denaturation than can be accounted for simply on the basis of charge neutralization. For example, removal of 50 per cent of total histone net positive charge with DOC or NaCl causes only a 30 per cent decrease in the T_m of the samples. To produce an equivalent change in the T_m of pure DNA, about 75 per cent of the Na^+ ions required to allow an equivalent stabilization of 30° C. (44° C. in 2.5 x 10^{-4} M EDTA to 74° C. of histone stabilized DNA in chromatin) would have to be removed from the melting medium. One possible interpretation of the observation that histones provide a greater stabilization of DNA against thermal denaturation than can be accounted for simply on the basis of charge neutralization, is that histone binding shows some concentration dependence, i.e., removal of some of the histone positive charges increases the contribution of the remaining histones to the thermal stabilization of the DNA. However, it has been shown that the binding of histones to DNA at low ionic strengths is essentially irreversible (Shih & Bonner, 1969), and arguments presented by Li and Bonner (1969) suggest that the stabilization of a DNA segment by a given histone mole-

cule is not appreciably affected by the state of the DNA segments immediately adjacent to the molecule. Another possible interpretation of the increased stabilization by histones, is that the presence of a histone molecule(s) interfers with cooperative melting of adjacent, less stable DNA segments. Clarification of the interpretation of melting profiles of partially dehistonized chromatin requires more information concerning the minimum length of an independently melting segment of DNA, and more information about the details of the deposition of the individual histone molecules on the DNA chain.

Ohba (1966) observed that the sedimentation coefficients of chromatin samples were most changed by the release of the intermediately dissociable histones by NaCl. Richards, Smart, and Bonner (1969) have shown that chromatin which is caused to aggregate in 0.15 M NaCl (no removal of histones) yields a chromatin sample which exhibits an increased $s_{20,s}$ over that of untreated chromatin even after two days of exhaustive dialysis against 0.0025 M Tris, pH 8. The facts that chromatin samples with up to 40 per cent of total histone removed by NaCl are capable of partial precipitation in 0.15 M NaCl (Smart & Bonner, 1969) and that both our procedure and that of Ohba (1966) cause the samples to be exposed for rather long periods of time to NaCl con-

centrations within the range for precipitation of chromatin (0.05 to 0.30 M), require consideration of the possibility of erroneously high $s_{20,w}$ values for these samples. Chalkley and Jensen (1968) observed a decrease in the $s_{20,w}$ value of calf thymus chromatin from 30 S in 0.01 M Tris, pH 8 - 50 per cent D_2O, to 22 S in 0.6 M NaCl - 0.01 M Tris, pH 8 - 50 per cent D_2O. Their procedure, which avoided exposure of the sample to precipitating concentrations of NaCl, therefore, resulted in a 50 per cent reduction in the $s_{20,w}$ of a sample which had approximately 30 per cent of total histone removed by NaCl. Correction for the fraction of histone net positive charge removed from the DNA by increasing concentrations of DOC and NaCl yields essentially identical curves above 40 per cent histone removal. Consideration of this portion of the curves alone would suggest that the various histone fractions do not vary greatly in their effect on the structural and chemical parameters which produce the increased S value of chromatin.

Ohba (1966) observed the flow dichroism of chromatin to be approximately 20 per cent of the flow dichroism of pure DNA (at a $\langle G \rangle$ value of 2000 sec^{-1}). In a different type of apparatus and at a $\langle G \rangle$ value of 21,250 sec^{-1}, we observe the flow dichroism of chromatin to be about 30 per cent of that of DNA. At lower $\langle G \rangle$ values, the flow

dichroism of chromatin relative to DNA approaches that reported by Ohba; e.g., at $\langle G \rangle = 4,250$, the flow dichroism of chromatin is approximately 25 per cent of that of DNA. We also observe, as did Ohba (1966), that in the hydrodynamic field, the disorderliness of the base pairs of chromatin samples partially dehistonized by increasing concentrations of NaCl is largely dependent on the presence of the less dissociable histones, namely histones II, III, and IV. However, extraction of chromatin with increasing concentrations of DOC produces essentially the same effect; i.e., in the hydrodynamic field the disorderliness of the base pairs of chromatin samples partially dehistonized by increasing concentrations of DOC is largely dependent on the presence of the less dissociable histones, which in this case is histone I. Consequently, it appears that the flow dichroism of DNA contained in chromatin is very sensitive to the presence of a relatively small amount of histone protein. For example, after removal of 80 to 90 per cent of total histone, the flow dichroism is increased only about 50 per cent of the way from native chromatin to pure DNA.

As mentioned previously, the decrease in flow dichroism of native chromatin with respect to the DNA isolated from it reflects not only the probably decreased orientability due to shortening of the whole chromatin molecule, but also

the decreased number of base pairs perpendicular to the molecular axis of an oriented molecule. Ohba (1966) attempted to eliminate the effect of the increased orientability of DNA molecules over that of chromatin molecules by shearing the DNA to a size which yielded a rotatory diffusion constant which was approximately the same as that of chromatin. When he measured the difference in the flow dichroism of these two samples, which presumably reflected only the disorderliness of the base pairs of the DNA contained in chromatin, he found that the number of base pairs perpendicular to the molecular axis of the chromatin molecules was approximately 40 per cent of the base pairs of the fragmented DNA. A supercoiled structure which is 80 Å wide and has a repeat distance of 110 Å would effectively shorten the length of the molecule approximately 40 per cent and would tilt the plane of the bases to an angle of 35° with respect to the molecular axis of the chromatin molecule. Although the process which forms the condensed structure of the DNA contained in chromatin is not at all clear, it seems reasonable to assume that it is somewhat stabilized by the neutralization of the phosphate negative charges and the concomitant reduction of electrostatic repulsion of the DNA fibers which the histone positive charges provide. Consequently, it is not surprising that the frac-

tion increase of flow dichroism with respect to the weight fraction of total histone removed is greater in chromatin samples which have been extracted with NaCl than in samples extracted with DOC.

A secondary level of organization, a cross-linking of individual chromatin molecules, has been reported by Littau, Allfrey, Frenster, and Mirsky (1964), who examined the appearance of sectioned nuclei in the electron microscope. In a later report (Littau, Burdick, Allfrey, & Mirsky, 1965) they examined isolated thymus nuclei after various histone extraction procedures. After removing lysine-rich histones (histone I) with 0.10 M citric acid, they observed that the structure of the dense chromatin had been loosened. However, extraction of isolated nuclei with a graded series of HCl-ethanol mixtures showed that more than 50 per cent of total histone (mostly arginine-rich) can be extracted without any marked change in the appearance of chromatin. They concluded that lysine-rich histones combine with phosphate negative groups to cross-link DNA double helices and that arginine-rich histones combine with phosphate groups along the double helix. Chalkley and Jensen (1968) have described an intermolecular cross-linked structure which is present in small amounts in sheared calf thymus chromatin, and can be isolated by preparative sedimentation as a 130 S compo-

nent. Removal of histone I (lysine-rich) by extraction of the 130 S chromatin with 0.4 M NaCl irreversibly disrupts the cross-linked structure. They concluded that the lysine-rich histones appear to play a role in maintaining the structure of such complexes. We find that the cross-linked structure of the 130 S component of calf thymus chromatin is irreversibly disrupted by extraction with 0.015 M DOC. It, therefore, seems that if histone I is responsible for intermolecular cross-linking of the 130 S structure, then the bonds provided by histone I are not sufficiently strong to maintain the structure when 26 per cent of total histone (mostly histone II and no histone I) is selectively removed from the complex.

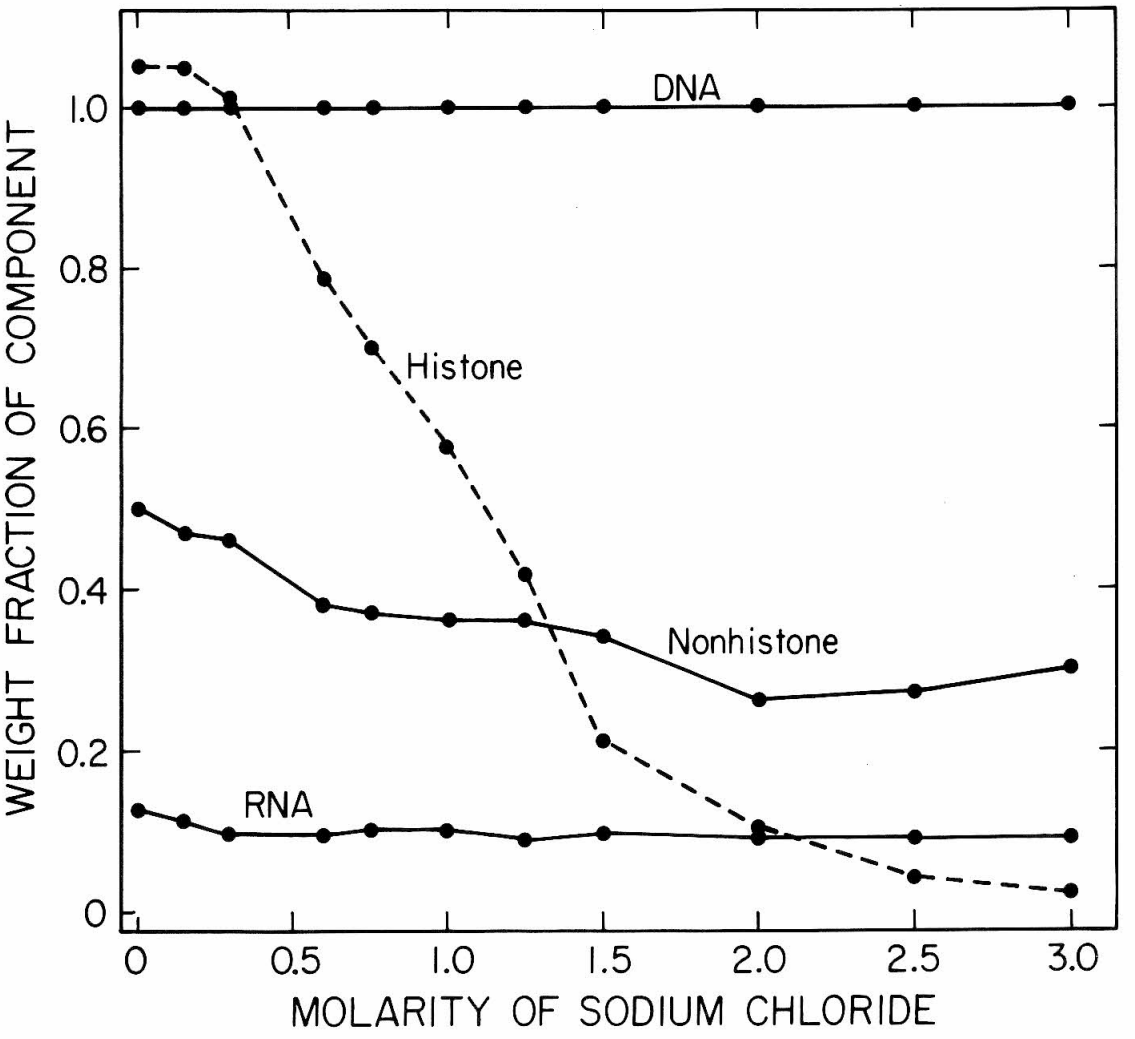
APPENDIX

The selective removal of the various histone fractions by increasing concentrations of sodium chloride has been investigated by several workers (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Ohba, 1967; Fambrough & Bonner, 1968; Tuan & Bonner, 1969). It has generally been found that histone I is removed at concentrations of NaCl between 0.3 and 0.6 M, while higher concentrations remove histones II, III, and IV at approximately the same rate. Because the

methods employed in the present study result in a relatively short exposure to the actual dissociating concentration of NaCl, we have reinvestigated the pattern of histone dissociation under our conditions of extraction with pea bud chromatin. We have also investigated the effects of increasing concentrations of NaCl upon removal of nonhistone protein and RNA from chromatin, and have determined the thermal denaturation properties of partially dehistonized samples.

The weight fractions of total histone protein, nonhistone protein, and RNA remaining bound to the DNA of pea bud chromatin as a function of molarity of NaCl used for dissociation are shown in Figure 8. The results show that under the conditions employed increasing concentrations of NaCl principally dissociate histone protein. Up to 50 per cent of nonhistone protein is dissociated by concentrations of NaCl between 2.0 to 3.0 M. As with increasing concentrations of guanidine hydrochloride (Levinson, Smart, & Bonner, 1969) and sodium deoxycholate (Smart, Hadler, & Bonner, 1969), increasing concentrations of NaCl dissociate only about 20 per cent of the RNA associated with chromatin. Our results on the fraction of each histone component dissociated from chromatin by increasing concentrations of NaCl are in complete agreement with the results of previous

Figure 8. The weight fraction of components of chromatin remaining bound to DNA after extraction of chromatin with increasing concentrations of NaCl.


DNA
Histone
Nonhistone
RNA
WEIGHT FRACTION OF COMPONENT
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1.0
1.5
2.0
2.5
3.0
MOLARITY OF SODIUM CHLORIDE

workers. The methods employed in this study, however, result in a slightly lower amount of total histone dissociation by concentrations of NaCl between 0.75 and 2.5 M than that observed by other workers. For example, Fambrough and Bonner (1968) reported that 1.0 and 1.5 M NaCl remove about 56 and 95 per cent of total histone respectively, while we observe that the same concentrations remove 45 and 80 per cent of total histone respectively. Occasional aggregation of histones at concentrations of NaCl above 1.0 M, presumably with other histone or nonhistone proteins, results in sporadically higher yields of histone in the chromatin pellet which produce no apparent effect on the thermal denaturation of the sample.

The thermal denaturation data for chromatin partially dehistonized by increasing concentrations of NaCl are presented in Table 5. Under the conditions employed in this study, the T_m of native chromatin is in the temperature range of 73 to 76° C., while that of DNA is 43 to 45° C. Increases in the T_m's of the partially dehistonized samples are considered in detail in an earlier portion of this paper.

The DNA contained in chromatin has undergone a conformational change which is expressed as an increased absorptivity at $260_{m\mu}$ (Tuan & Bonner, 1969). Consequently,

Table 5

Thermal Denaturation of Chromatin

Partially Dehistonized by NaCl

Description	T_m [1]	h_t [2]	$\sigma_{2/3}$ [3]
Control	73.8 ± 0.5	33.9 ± 0.9	10.6 ± 0.2
0.15 M NaCl	73.2 ± 0.8	32.4 ± 0.5	10.5 ± 0.4
0.3 M NaCl	72.3 ± 0.8	33.5 ± 0.6	12.1 ± 0.8
0.5 M NaCl*	67.6 ± 0.2	34.9 ± 0.1	17.3 ± 1.0
0.6 M NaCl	66.2 ± 0.2	35.3 ± 1.2	17.3 ± 0.9
0.75 M NaCl	64.6 ± 0.4	35.5 ± 0.5	18.3 ± 0.6
1.0 M NaCl	63.6 ± 0.7	35.5 ± 0.5	18.3 ± 0.4
1.25 M NaCl*	59.9 ± 0.8	36.9 ± 1.4	18.2 ± 0.8
1.5 M NaCl	50.2 ± 1.1	36.6 ± 0.7	15.0 ± 1.3
2.0 M NaCl	46.2 ± 0.8	35.7 ± 0.8	6.8 ± 0.6
2.5 M NaCl	43.0 ± 1.5	36.3 ± 1.5	5.2 ± 0.6
3.0 M NaCl	43.0 ± 1.0	36.8 ± 1.2	4.9 ± 0.6

1. $T_m \equiv$ midpoint of thermal transition at which $h = 0.5\ h_t$
2. $h_t \equiv$ hyperchromicity
3. $\sigma_{2/3} \equiv$ the dispersion, defined as that temperature span required to raise h from $0.333\ h_t$ to $0.667\ h_t$

* data from 2 independent experiments; all other points from 3 or 4 independent experiments

an increase in hyperchromicity upon melting is expected for deproteinized nucleoproteins. Table 5 shows that a rather gradual increase in hyperchromicity is seen with samples which have increasing amounts of total histone removed by NaCl.

As with all methods for the selective removal of histones, the melting curves of the partially dehistonized samples are broadened, but not divided into a DNA-like and a native chromatin region. An estimation of the cooperativity of the melting profiles can be obtained from the values for $\sigma_{2/3}$ presented in Table 5.

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STUDIES ON THE ROLE OF HISTONES IN THE BEHAVIOR OF CHROMATIN AT PHYSIOLOGICAL IONIC STRENGTHS (TEMPLATE ACTIVITY AND PRECIPITATION)

INTRODUCTION

The chromatin isolated from interphase cells of higher organisms is a chemically defined entity of DNA, histone and nonhistone protein, and a small amount of RNA. *In vivo* and *in vitro* studies have indicated that the ability of chromatin to act as a template for RNA synthesis is greatly reduced in the highly differentiated cell (Bonner & Huang, 1963; Littau, Allfrey, Frenster, & Mirsky, 1964; Paul & Gilmore, 1966; Georgiev, Ananieva, & Kozlov, 1966). Marushige and Bonner (1966) have reported that the full capacity for RNA synthesis in the presence of exogenous *E. coli* RNA polymerase can be restored to that of pure DNA by removal of histones by increasing concentrations of sodium perchlorate. It has been suggested, however, that the reduced template activity of chromatin in the low ionic strength incubation system for RNA synthesis described by Marushige and Bonner (1966) may be due to aggregation of chromatin in the incubation medium (Sonnenberg & Zubay, 1965).

Recent work has suggested that a more specific binding of *E. coli* RNA polymerase to polyoma DNA is obtained in an incubation medium of higher ionic strength (approximately 0.2 M KCl) (Pettijohn & Kamiya, 1967). This ionic strength

is within the range (0.05 to 0.3 M NaCl) which results in aggregation of chromatin (Chalkley & Jensen, 1968). We have studied the effect of selective removal of histones upon the ability of partially dehistonized chromatin to prime a high ionic strength (0.2 M KCL) DNA-dependent RNA synthesis system catalysed by E. coli RNA polymerase. We have also studied the precipitation of partially dehistonized samples in 0.15 M NaCl - 0.0025 M Tris, pH 8. The samples were dehistonized by increasing concentrations of sodium deoxycholate (DOC) (removes histone II at lowest concentrations, followed at slightly higher concentrations by histones III and IV, and lastly at still higher concentrations by histone I) (Smart, Hadler, & Bonner, 1969), and by increasing concentrations of NaCl (removes histone I at lowest concentrations, followed at higher concentrations by histones II, III, and IV) (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Fambrough & Bonner, 1968; Smart & Bonner, 1969). The data show that the template activity of chromatin samples partially dehistonized by increasing concentrations of DOC or NaCl increases from the template activity of native chromatin (approximately 25 per cent of that of pure DNA) to that of pure DNA in a fashion which shows a nearly linear relationship to the amount of total histone coverage of the template. On the other hand, the precipitability of partially

dehistonized chromatin samples in 0.15 M NaCl shows a large dependence on the presence of histone I (lysine-rich).

METHODS AND MATERIALS

Preparation of Chromatin. Pea bud chromatin was prepared by the methods previously described (Smart, Hadler, & Bonner, 1969). The chromatin had a final concentration of 20 to 40 $A_{260m\mu}$, a ratio of $A_{230m\mu}/A_{260m\mu}$ of approximately 0.75, and a ratio $A_{320m\mu}/A_{260m\mu}$ of less than 0.034. The DNA contained in pea bud chromatin is complexed with histone protein, nonhistone protein, and RNA in the mass ratios DNA, 1.00; histone, 1.05; nonhistone, 0.50; RNA, 0.12.

Histone Dissociation Studies. To assure that the chromatin was not subjected to a sodium deoxycholate or sodium chloride concentration higher than the desired final one, partially dehistonized samples were prepared as described previously (Smart, Hadler, & Bonner, 1969).

Thermal Denaturation and Ultraviolet Absorption. The partially dehistonized samples were dialyzed against 2.5×10^{-4} M EDTA, pH 8, diluted to approximately 1 $A_{260m\mu}$ with dialysate, and melted in a Gilford Model 2000 multiple sample absorbance recording apparatus adapted for the recording of melting profiles. The rate of temperature in-

crease was 0.5 to 1.0 degree/minute. Ultraviolet absorption spectra were determined with a Cary recording spectrophotometer, Model 11.

Precipitation of Chromatin. In order to obtain a standard and reproducible measure of precipitation, we have defined precipitated chromatin as that material sedimented from a 0.15 M NaCl - 0.0025 M Tris, pH 8, solution of chromatin (approximately 10 $A_{260_{m\mu}}$) in 10 minutes at 10,000 x g. The assay was performed as described by Smart and Bonner (1969).

Assay of RNA Polymerase Activity. The incubation mixture contained 10 umoles of Tris buffer, pH 8, 50 umoles of potassium chloride, 3 umoles of magnesium chloride, 3 umoles of β-mercaptoethanol, 0.05 umoles of spermidine-HCl, 0.1 umole each of GTP, CTP, UTP, and ^{14}C-ATP (1μc/μmole), and approximately 10 μg of E. coli RNA polymerase. Total volume of reaction mixture was 0.25 ml. Varying amounts of pure DNA or DNA contained in partially dehistonized chromatin samples were added to the reaction mixture as template for RNA synthesis. Incubation was carried out at 37° C. for 10 minutes. The reaction was terminated by the rapid addition of 0.5 ml of cold bovine serum albumin solution (250 μg/ml in 0.0025 M Tris, pH 8) and then 2 ml of 20 per cent trichloroacetic acid (w/w). The samples were allowed to sit

on ice for at least 30 minutes, and the acid-insoluble material then collected by filtration through presoaked (10 per cent trichloroacetic acid, w/w) membrane filters (Bac-T-Flex, type B-6, 25 mm size from Carl Scheicher & Schuell Co., Keen, N. H.). The filters were washed further with 3 portions of 10 ml of 10 per cent trichloroacetic acid (w/w). Filters, after drying, were counted with a Beckman LS-200B liquid scintillation system. The counting cocktail contained 1 kg dioxane, 4 g PPO, 0.05 g POPOP, and 120 g naphthalene. Counting efficiency is approximately 85 per cent.

Preparation of E. coli RNA Polymerase. DNA-dependent RNA polymerase was prepared by a modification of the procedure of Chamberlin and Berg (Bonner et al., 1968a). The preparation used in the assays corresponds to the f_4 fraction of Chamberlin and Berg (1962). Its specific activity ranged from 400 to 650 μμmoles incorporation of ^{14}C-ATP per ug enzyme per 10 minutes with 30 μg DNA and under our standard reaction conditions. The purified enzyme had nearly complete dependency upon added DNA. Enzyme concentration was calculated by the relation $\epsilon^{1\%}_{280_{mu}} = 6.5$ (Richardson, 1966).

Statistical Analysis of Enzyme Kinetic Data. The template saturation curves, rate of RNA synthesis with increasing concentrations of DNA in the presence of a constant

amount of RNA polymerase, were fitted by the least-squares method with a weighting factor of v^4 for reaction velocity. This also takes into account the fact that the principal inaccuracies are at low velocities. Data for pure DNA and chromatin samples were found to fit satisfactorily to the Michaelis-Menten equation. Fitting of the kinetic data to the equation was performed on an IBM System/360 Model time-sharing computer and by a CITRAN program translated by Brutlag (1969) from the FORTRAN program of Cleland (1967). V_{max} and K with their standard errors were obtained.

Assay for Ribonuclease Activity in Templates. The presence of ribonuclease activity in the templates used in these studies was assayed by measuring the effect of added template (10 μg DNA/0.25 ml assay solution) on acid-insoluble ^{32}P counts contained in purified soluble RNA prepared from HeLa cells by Hatlen (1969). Incubation was carried out at 37° C. for increasing lengths of time up to 4 hours. The reaction was terminated as described previously for the assay of RNA polymerase activity. Ribonuclease activity was also assayed by measuring the incorporation of ^{14}C-ATP into acid-insoluble RNA (as described previously for the assay of RNA polymerase activity) for increasing lengths of time up to 4 hours.

Chemical Analysis. DNA, histone protein, nonhistone

protein, and RNA analysis were performed as previously described (Smart, Hadler, & Bonner, 1969).

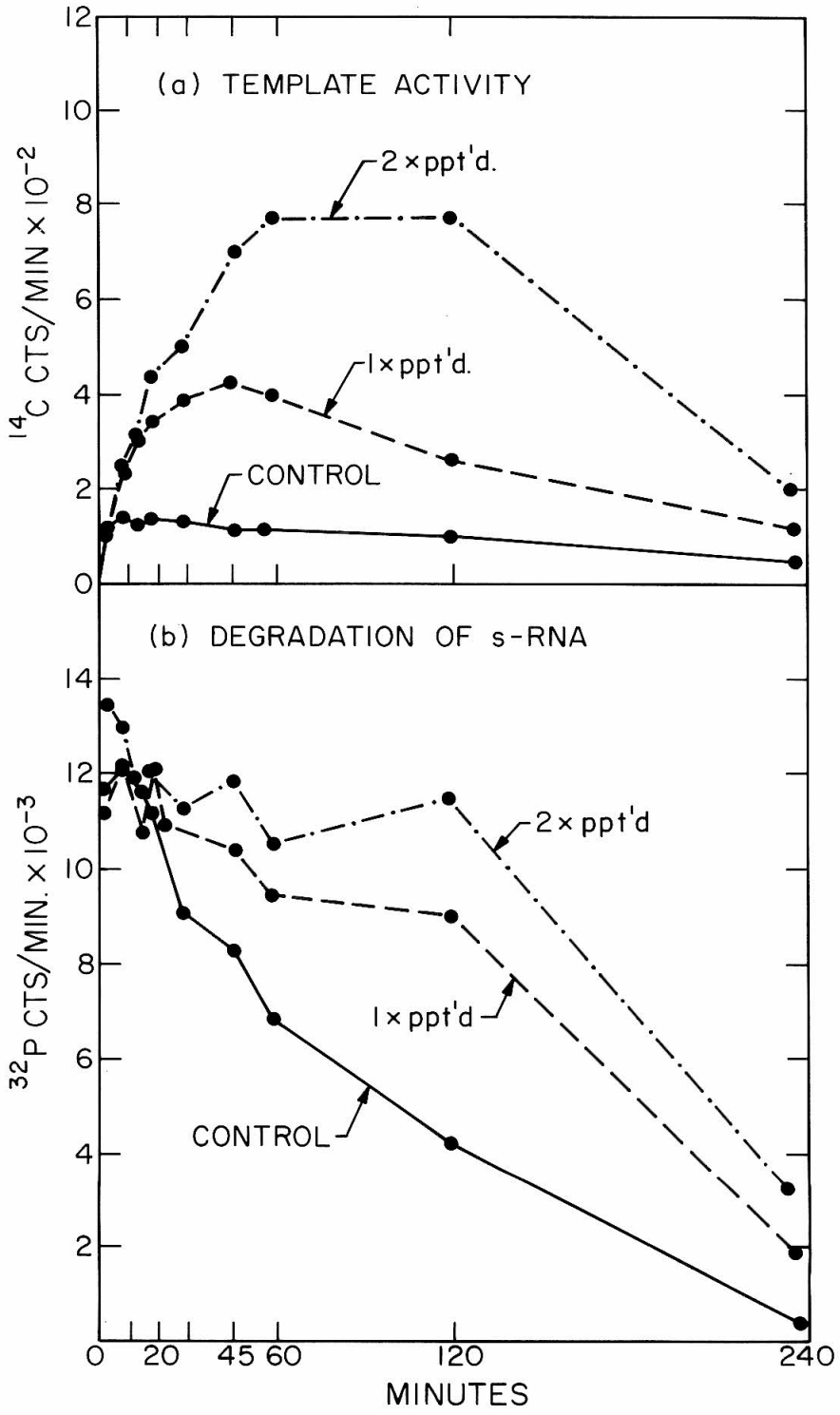
Disc Gel Electrophoresis of Histones. Acrylamide disc gel electrophoresis of isolated histones was performed by the method of Bonner et al. (1968a). The quantity of each electrophoretic component was determined by densitometry after Fambrough (1967).

RESULTS

We have observed that our preparations of pea bud chromatin are associated with varying amounts of ribonuclease activity. In an attempt to selectively dissociate ribonuclease, we have extracted chromatin with 0.15 M NaCl - 0.0025 M Tris, pH 8. Although this molarity of NaCl does not dissociate histones, it might be expected to effectively remove a protein which is less strongly bound to DNA by ionic forces. Figure 1a shows a time course of DNA-dependent incorporation of ^{14}C-ATP into acid-insoluble RNA as primed by chromatin samples (10 µg DNA/0.25 ml assay solution) which have been extracted zero, one and two times with 0.15 M NaCl. That the decreased incorporation of counts into RNA is due to ribonuclease activity is shown in Figure 1b, which presents a time course of acid-insoluble

Figure 1a. Template activity assay for ribonuclease activity associated with chromatin after zero, one, and two times extraction with 0.15 M NaCl. The chromatin samples (10 μg DNA/0.25 ml assay solution) were incubated for RNA synthesis as described in the text for increasing periods of time. 5 μg of *E. coli* RNA polymerase was added to each 0.25 ml of assay solution.

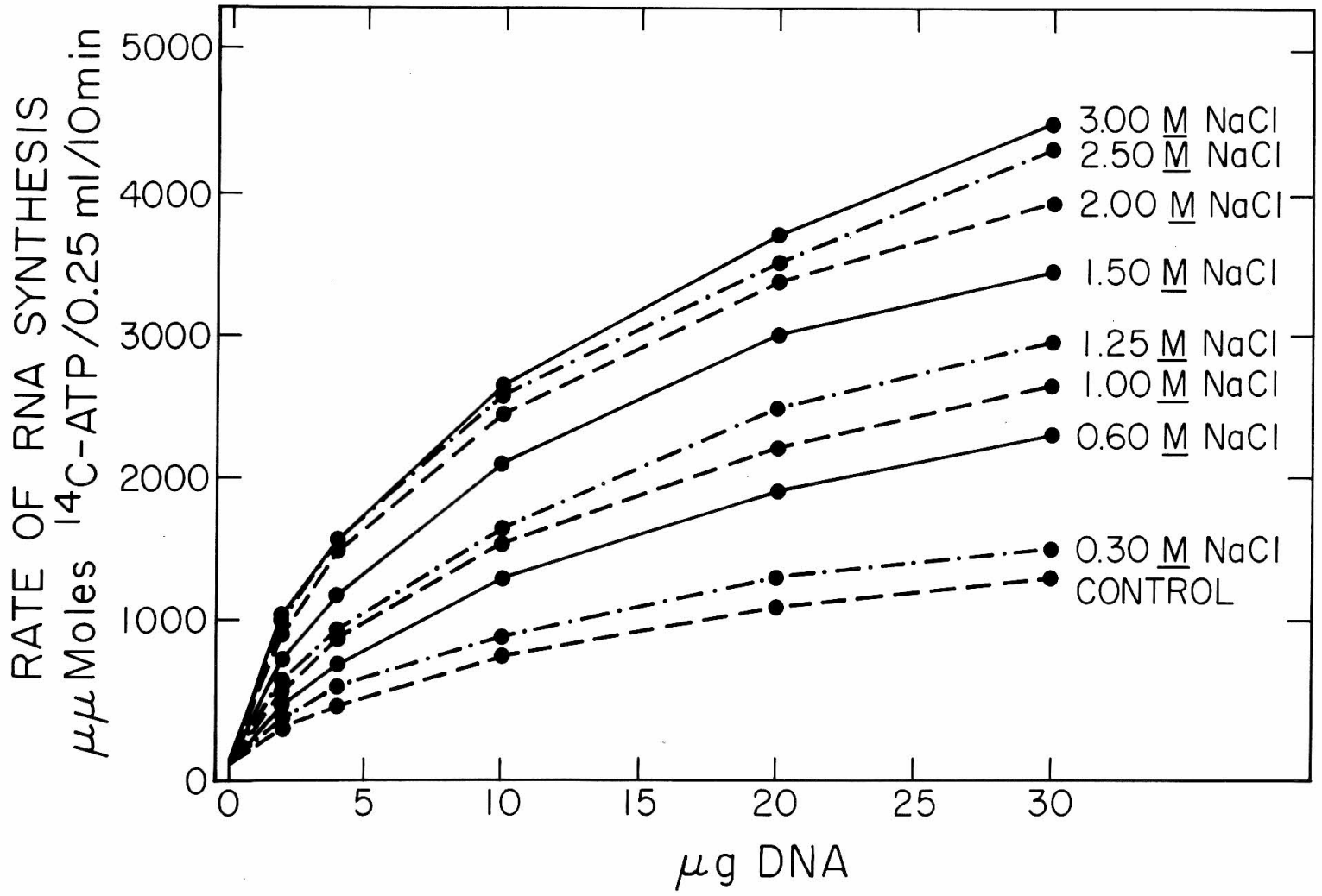
Figure 1b. Degradation of ^{32}P-labelled RNA assay for ribonuclease activity associated with chromatin after zero, one, and two times extraction with 0.15 M NaCl. The chromatin samples (10 μg DNA/0.25 ml assay solution) were incubated for increasing periods of time with purified ^{32}P-labelled sRNA from HeLa cells (Hatlen, 1969).


(a) TEMPLATE ACTIVITY
2 × ppt'd.
1 × ppt'd.
CONTROL
^{14}C CTS/MIN × 10^{-2}
(b) DEGRADATION OF s-RNA
2 × ppt'd
1 × ppt'd
CONTROL
^{32}P CTS/MIN. × 10^{-3}
0 20 45 60 120 240
MINUTES

counts contained in ^{32}P-labelled sRNA upon exposure to the same chromatin samples (10 µg DNA/0.25 ml of assay solution). Thus, in this set of experiments (which contained a rather large amount of ribonuclease) extraction of the chromatin with 0.15 M NaCl dissociated much of the chromatin-associated ribonuclease. In the preparation of chromatin samples for template activity studies we have, therefore, routinely extracted the original purified sheared chromatin three times with 0.15 M NaCl - 0.0025 M Tris, pH 8, before selectively dissociating histones with increasing concentrations of DOC or NaCl. It should be noted, however, that although this procedure removes most of the ribonuclease, it does not remove all of it. Most of our preparations of chromatin extracted three times with 0.15 M NaCl showed a slight decrease in incorporation of ^{14}C-ATP into acid insoluble RNA after incubation periods greater than 60 minutes.

Figure 2 presents saturation curves for the rate of RNA synthesis in a 0.2 M KCl, DNA-dependent RNA synthesis system as a function of template concentration. The templates are chromatin samples partially dehistonized by increasing concentrations of NaCl. Under these conditions, approximately 5000 µµmoles of ATP were incorporated into acid insoluble RNA at 37° C. for 10 minutes with 30 µg of pure DNA as the template. The template activity of native

Figure 2. Saturation curves for the rate of RNA synthesis as a function of template concentration. The templates were chromatin samples which had been partially dehistonized by increasing concentrations of NaCl. 10 μg of *E. coli* RNA polymerase was added to each 0.25 ml of assay solution. Each point represents the average of a duplicate experiment.


RATE OF RNA SYNTHESIS
μμ Moles ^{14}C-ATP/0.25 ml/10 min
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
μg DNA
3.00 M NaCl
2.50 M NaCl
2.00 M NaCl
1.50 M NaCl
1.25 M NaCl
1.00 M NaCl
0.60 M NaCl
0.30 M NaCl
CONTROL

chromatin is about 25 per cent of that of pure DNA, while that of chromatin dehistonized by dissociation in 3.0 M NaCl is approximately 90 per cent of that of pure DNA.

The curves presented in Figure 2 approximate hyperbolae and, therefore, computer fitting of the data to the Michaelis-Menton equation (after Shih, 1969)

$$v = \frac{V_{max} \cdot A}{K + A}$$

in which V_{max} is the velocity of RNA synthesis in the presence of infinite template, K is the template concentration required to half saturate the enzyme, and A is the template concentration, yields satisfactory fitting. The initial rate of RNA synthesis, v, is obtained as A approaches 0, and, consequently, is defined as V_{max}/K. In the case that K remains unchanged template activity is proportional to V_{max}.

Table 1 shows the derived rate constants and their standard errors as calculated from the template saturation curves of Figure 2. As stated previously, the chromatin used in these studies is associated with a small amount of ribonuclease activity. In a template saturation experiment this ribonuclease activity has a much greater effect on incorporation of ^{14}C-ATP into acid-insoluble RNA at high template concentrations than at lower template concentrations. In fact, in experiments which contained considerable ribonuclease activity, the saturation curves peaked

Table 1

The Rate Constants Calculated from Saturation Curves of Template Activity of Chromatin Partially Dehistonized by NaCl

Description	V_{max} (μμmoles ATP/ 10 min/0.25 ml.)	K (μg DNA/0.25 ml.)
Control	1615 ± 71	14.6 ± 1.2
0.30 M NaCl	2109 ± 117	12.6 ± 1.64
0.60 M NaCl	3599 ± 166	17.1 ± 1.7
1.00 M NaCl	3921 ± 175	14.7 ± 1.5
1.25 M NaCl	4521 ± 274	16.2 ± 2.1
1.50 M NaCl	4922 ± 151	12.8 ± 0.9
2.00 M NaCl	5238 ± 219	10.6 ± 1.1
2.50 M NaCl	5737 ± 435	11.3 ± 2.1
3.00 M NaCl	6177 ± 419	12.2 ± 2.0

at some intermediate template concentration and then sloped downwards. Independent experiments indicate that extraction with 3.0 M NaCl dissociates some (but not all) of the ribonuclease activity in highly contaminated preparations of chromatin not previously extracted with 0.15 M NaCl. The same treatment, however, does not appear to have much effect on the small amount of ribonuclease activity which remains associated with chromatin after it has been extracted three times with 0.15 M NaCl (Smart, unpublished observations). The effect of ribonuclease activity on the rate constants derived from the template saturation curves is to decrease the apparent values for K and V_{max}. It is also apparent that since digestion of RNA by ribonuclease is a substrate concentration dependent process, contamination of the partially dehistonized samples by a constant amount of ribonuclease/μg DNA will enhance the lowering of K and V_{max}, in samples which have a greater amount of RNA synthesis/μg DNA. We interpret the apparent lowering of the values of K and V_{max} as artifacts due to the presence of ribonuclease activity in the partially dehistonized chromatin samples. With these assumptions, we can say that the total removal of histones with 3.0 M NaCl yields a template which is as effective as pure DNA in priming the DNA-dependent RNA synthesis catalysed by E. coli RNA polymerase in a 0.2 M KCl

incubation system. Also, it would appear that the value of K remains essentially unchanged over the complete range of histone removal.

Figure 3 presents saturation curves for the rate of RNA synthesis in 0.2 M KCl as a function of template concentration. In this case the templates are chromatin samples partially dehistonized by increasing concentrations of sodium deoxycholate. Total removal of histone with DOC yields a template which is as effective as pure DNA in priming the DNA-dependent RNA synthesis catalysed by E. coli polymerase.

Table 2 presents the derived rate constants and their standard errors as calculated from the template saturation curves of Figure 3. Several independent experiments have indicated that extraction of chromatin with 0.025 M DOC removes (or at least inactivates) essentially all of the ribonuclease contained in chromatin samples. The essentially complete inactivation of ribonuclease is observed even in highly contaminated preparations (Smart, unpublished observations). It follows from the previous discussion that inactivation of the contaminating ribonuclease should effectively result in an apparent increase in the values of K and V_{max}.

Extraction of chromatin with 0.15 M NaCl to remove ribonuclease results in aggregation of the chromatin. The

Figure 3. Saturation curves for the rate of RNA synthesis as a function of template concentration. The templates were chromatin samples which had been partially dehistonized by increasing concentrations of DOC. 10 μg of *E. coli* RNA polymerase was added to each 0.25 ml of assay solution. Each point represents the average of a duplicate experiment.

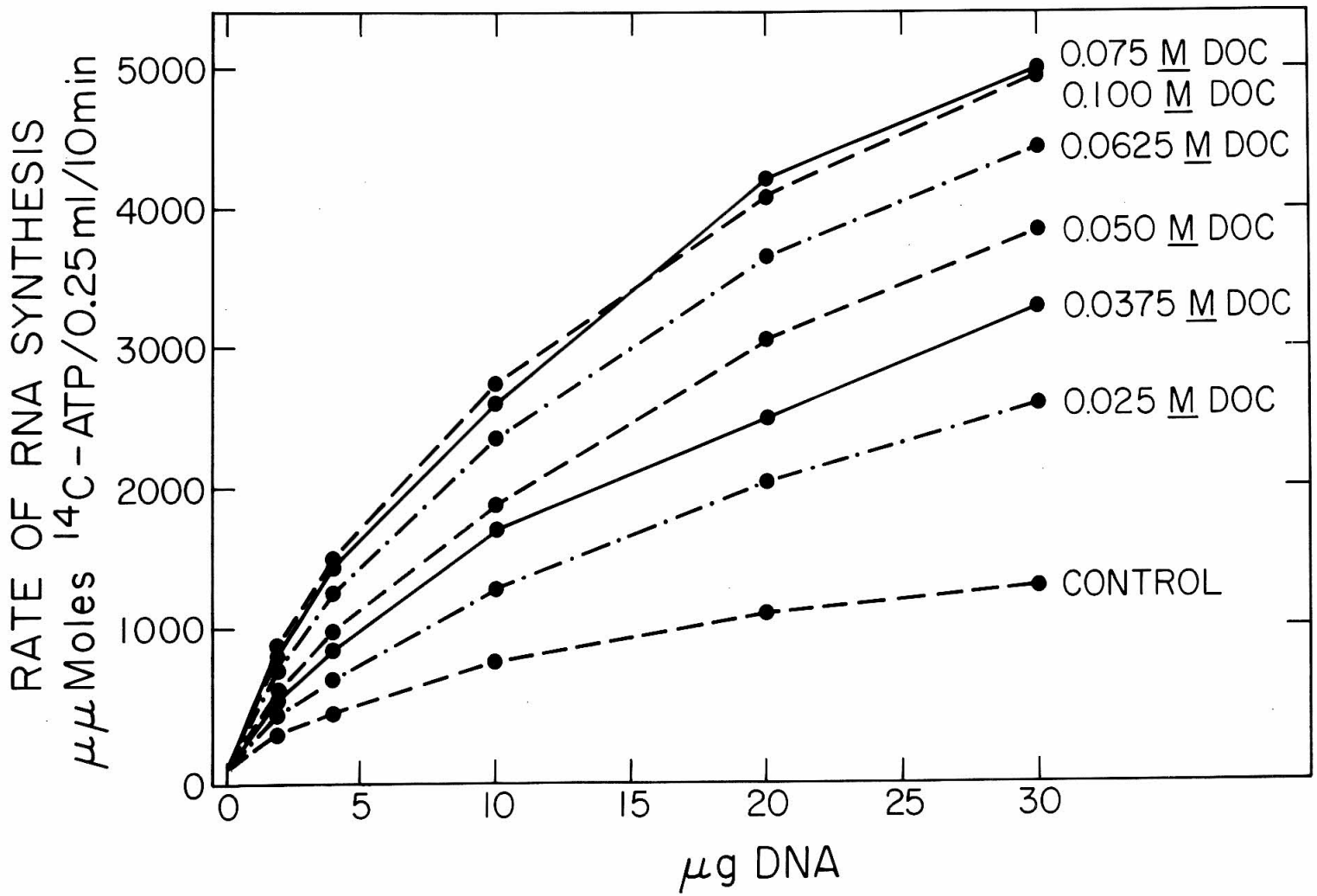

RATE OF RNA SYNTHESIS
μμMoles 14C-ATP/0.25ml/10min
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
μg DNA
0.075 M DOC
0.100 M DOC
0.0625 M DOC
0.050 M DOC
0.0375 M DOC
0.025 M DOC
CONTROL

Table 2

The Rate Constants Calculated from Saturation Curves of Template Activity of Chromatin Partially Dehistonized by DOC

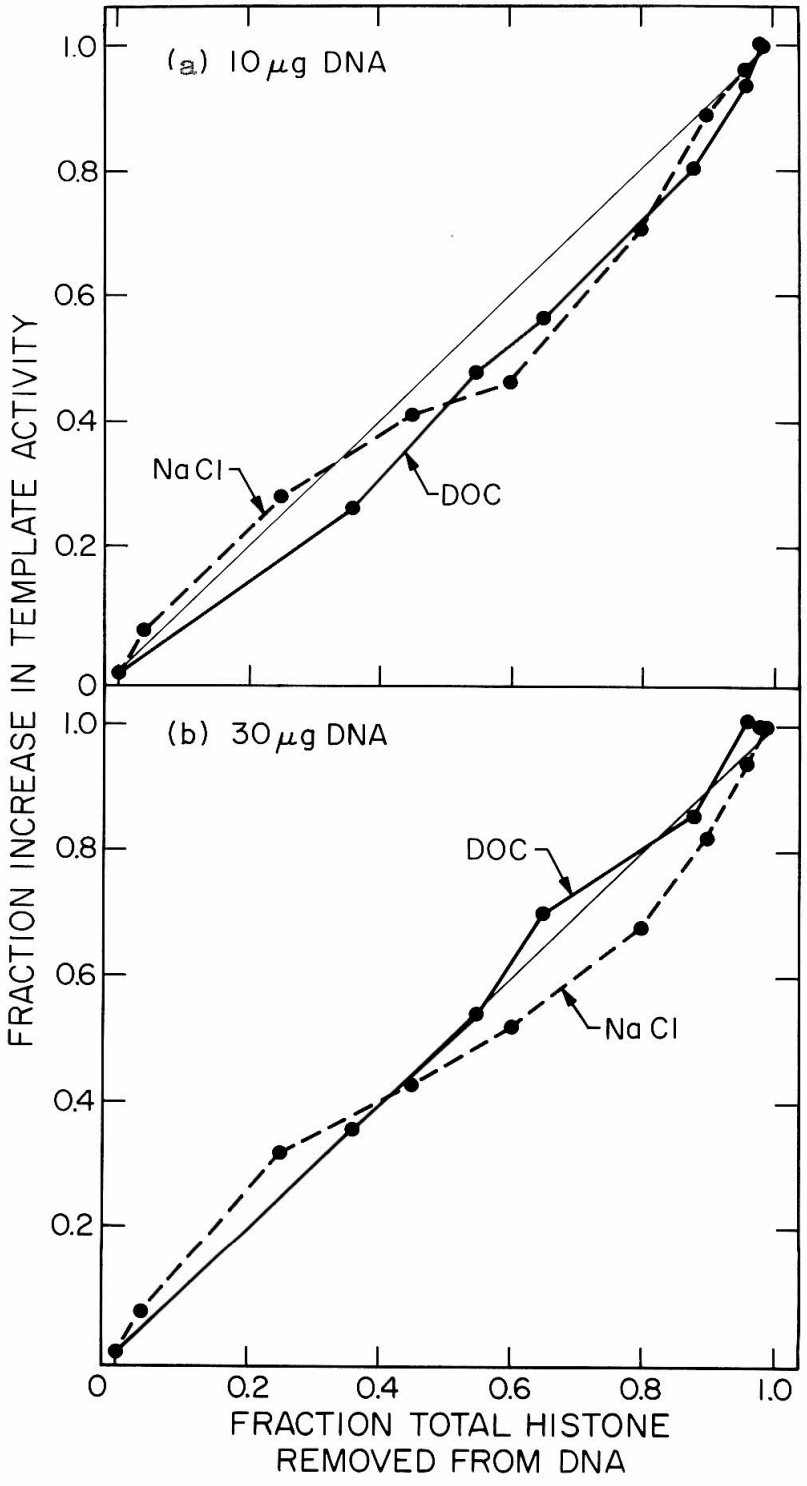
Description	V_{max} (μμmoles ATP/ 10 min/0.25 ml.)	(μg DNA/0.25 ml.)
Control	1615 ± 71	14.7 ± 1.2
0.025 M DOC	5198 ± 369	30.2 ± 3.6
0.0375 M DOC	5992 ± 600	25.7 ± 4.6
0.050 M DOC	7594 ± 514	29.2 ± 3.4
0.0625 M DOC	7600 ± 311	21.4 ± 1.7
0.075 M DOC	8526 ± 552	21.0 ± 2.6
0.100 M DOC	7912 ± 371	18.2 ± 1.7

aggregated chromatin redissolves in 0.0025 M Tris, pH 8, after resuspension in a Teflon homogenizer and extensive dialysis. However, it has been shown that the chromatin retains some of the aggregated structure even after two days of dialysis, as evidenced by an increased $s_{20,w}$ of the precipitated chromatin over that of native chromatin (Smart & Bonner, 1969; Richards, Smart, & Bonner, 1969). We have observed that samples of chromatin which have been extracted three times with 0.15 M NaCl require a slightly higher concentration of DOC to dissociate a given weight fraction of histone than do unextracted preparations of chromatin. Thus, in the saturation curves presented in Figure 3 the values for weight fraction of total histone removed from the DNA versus the concentration of DOC were as follows: 0.36 at 0.025 M DOC, 0.55 at 0.0375 M DOC, 0.63 at 0.05 M DOC, 0.88 at 0.0625 M DOC, 0.96 at 0.075 M DOC, and 0.98 at 0.100 M DOC. The values for the T_m's of these samples were in total agreement with the reduced amount of total histone removal by the dissociating agent. Values for weight fraction total histone removed from the DNA by increasing concentrations of NaCl were in agreement with those previously published by Smart & Bonner (1969).

Figures 4a and 4b present the fraction increase in template activity (rate of RNA synthesis) versus the frac-

Figure 4a. The fraction increase in template activity (10 μg DNA/0.25 ml assay solution) versus the fraction total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl. The points are the same as those in Figures 2 and 3. The template activity of native chromatin is assigned the value of 0, while that of chromatin completely dehistonized by 3.0 M NaCl or 0.15 M DOC is assigned the value of 1.00.

Figure 4b. Same as Figure 4a except that template concentration was 30 μg/0.25 ml assay solution.


(a) 10 μg DNA
(b) 30 μg DNA
NaCl
DOC
DOC
NaCl
FRACTION INCREASE IN TEMPLATE ACTIVITY
FRACTION TOTAL HISTONE REMOVED FROM DNA
0
0.2
0.4
0.6
0.8
1.0

tion of total histone removed from the DNA for samples of chromatin partially dehistonized by increasing concentrations of NaCl and DOC at 10 and 30 μg DNA, respectively. (In each case, the template activity of chromatin at that concentration is assigned the value of zero, while that of DNA is assigned the value of 1.0.) Therefore, it appears that increases in the template activity of partially dehistonized chromatin samples in the 0.2 M KCl, DNA-dependent RNA synthesis catalysed by E. coli RNA polymerase from that observed with native chromatin to that observed with pure DNA are directly proportional to the weight fraction of total histone removed from DNA. It is also clear that the reduced template activity of chromatin is not solely dependent on the presence of any one particular histone fraction. Limited experiments have shown that these conclusions also apply to the template activities of the same templates in the low ionic strength system described by Marushige and Bonner (1966).

It has been suggested that the low template activity of chromatin in the low ionic strength incubation system for RNA synthesis described by Marushige and Bonner (1966) may be due to aggregation of the chromatin in the incubation medium (Sonnenberg & Zubay, 1965; Chalkley & Jensen, 1968). Whether or not aggregation actually occurs under these in-

cubation conditions is, however, somewhat questionable (Bonner & Huang, 1966). On the other hand, it is clear that the high ionic strength (0.2 M KCl) used in the RNA synthesis system in the present studies constitutes an aggregating condition for native chromatin. In fact, visual inspection of the incubation mixture containing chromatin, as well as some partially dehistonized samples, shows that most of the template is out of solution as a white precipitate.

Chalkley and Jensen (1968) reported that a 130 S cross-linked component of chromatin aggregates in 0.15 M NaCl to a greater extent than the predominant 30 S component of chromatin. They also observed that the template activity of the 130 S component was approximately 30 per cent less than that of the 30 S component. Since these data suggest that there may be some relationship between precipitability in 0.15 M NaCl and the template activity of native chromatin, we have investigated the precipitation (in 0.15 M NaCl) of chromatin samples partially dehistonized by increasing concentrations of NaCl or DOC. Under the conditions employed in these studies approximately 90 to 95 per cent of the $A_{260m\mu}$ material of native chromatin is precipitated in 0.15 M NaCl, while 0 per cent of the $A_{260m\mu}$ material of pure DNA is precipitated. Figure 5 presents the fraction of

$A_{260_{m\mu}}$ material soluble in 0.15 M NaCl versus the fraction of total histone removed from the DNA by increasing concentrations of DOC or NaCl. The data for the weight fraction of total histone removed from the DNA by increasing concentrations of DOC or NaCl are as reported by Smart, Hadler, and Bonner (1969) and Smart and Bonner (1969). It is apparent that dissociation of chromatin with increasing concentrations of NaCl has a much greater effect on the precipitability of the resulting partially dehistonized nucleoprotein than does dissociation with increasing concentrations of DOC (per weight fraction total histone removed from the DNA). For example, removal of 50 per cent of total histone with NaCl yields a sample which is about 65 per cent soluble in 0.15 M NaCl, while the dissociation of the same amount of total histone with DOC yields a sample which is only about 20 per cent soluble. Essentially all of the $A_{260_{m\mu}}$ material is soluble after 80 per cent of total histone has been removed by NaCl; however, only about 60 per cent of the $A_{260_{m\mu}}$ material has been solubilized by the same amount of histone removal by DOC. Figure 6 shows the fraction of $A_{260_{m\mu}}$ material soluble in 0.15 M NaCl versus the fraction of histone net positive charge removed from the DNA of chromatin samples which have been partially dehistonized by increasing concentrations of NaCl or DOC

Figure 5. The fraction of $A_{260_{m\mu}}$ material contained in chromatin which is soluble in 0.15 M NaCl - 0.0025 M Tris, pH 8, versus the fraction of total histone removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl.

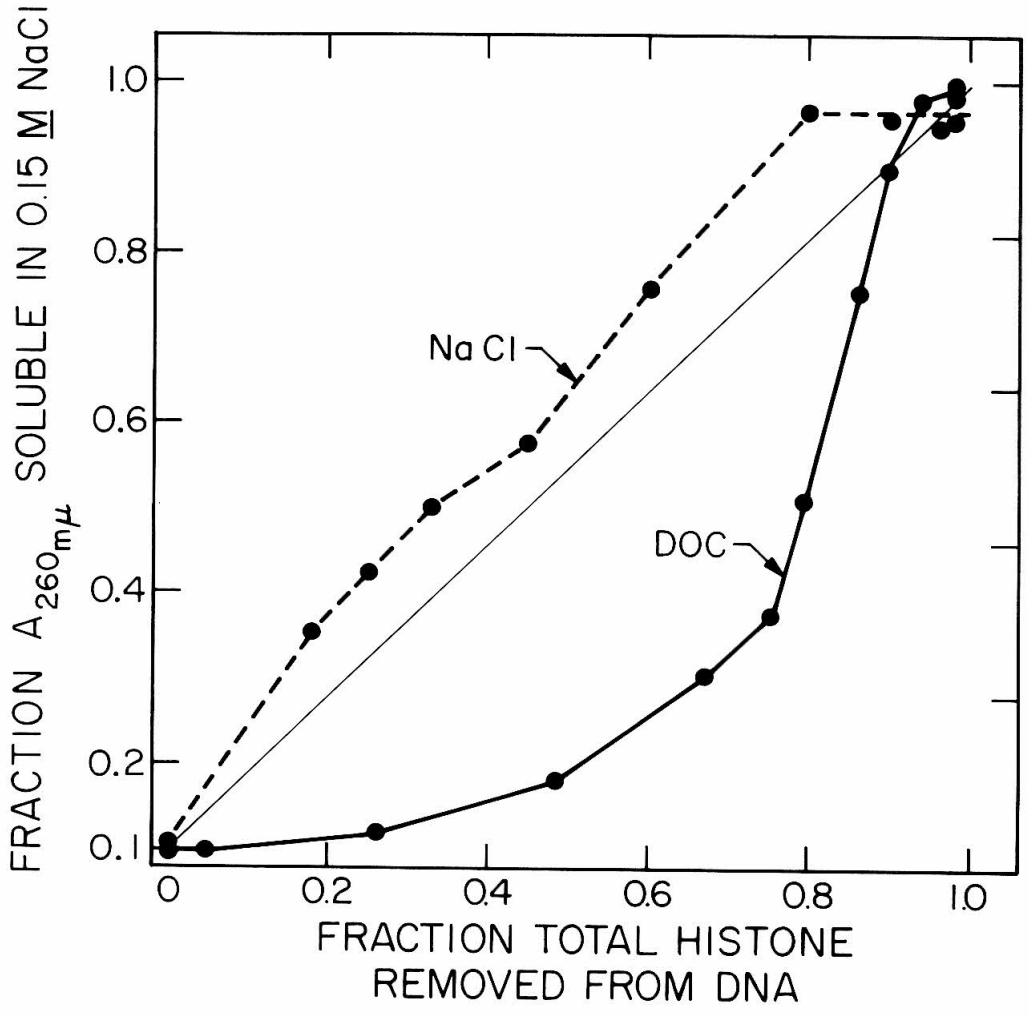
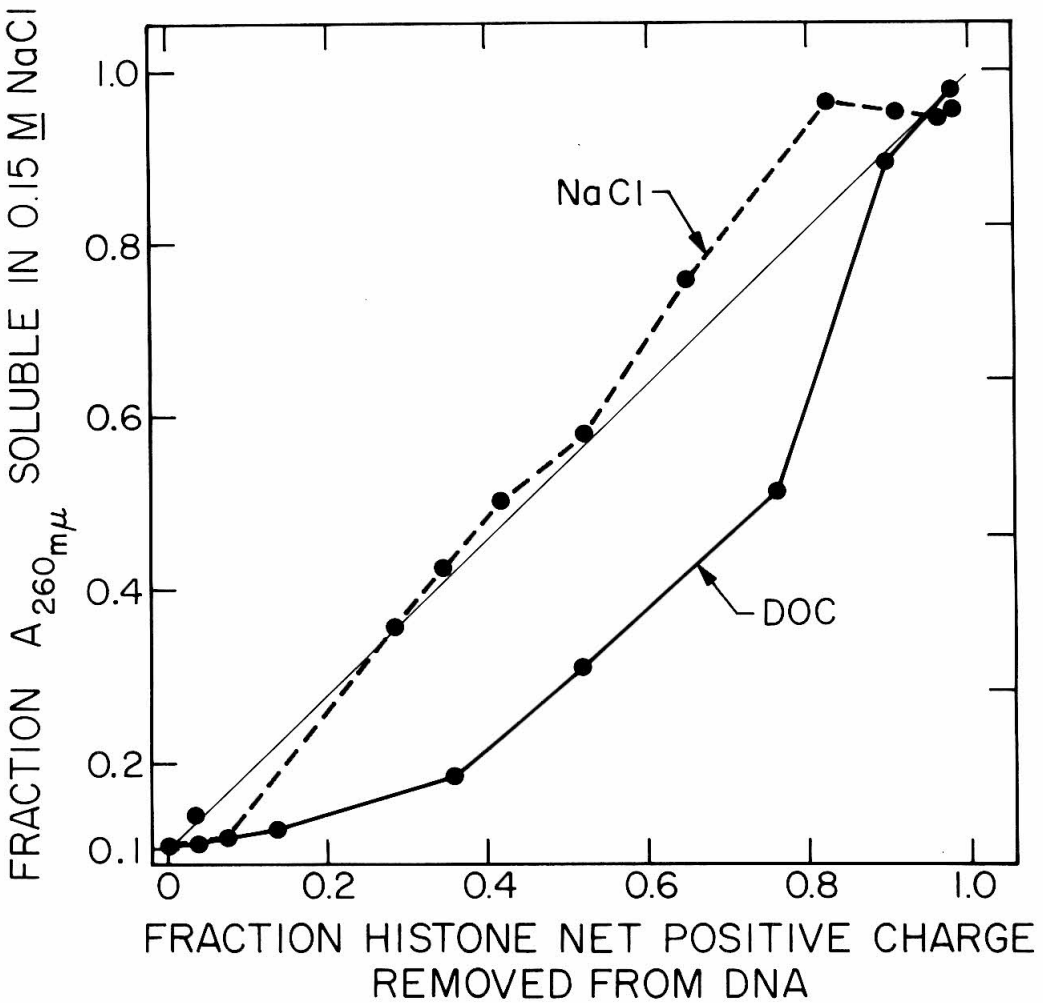

FRACTION $A_{260m\mu}$ SOLUBLE IN 0.15 M NaCl
1.0
0.8
0.6
0.4
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
Na Cl
DOC
FRACTION TOTAL HISTONE
REMOVED FROM DNA

Figure 6. The fraction of $A_{260m\mu}$ material contained in chromatin which is soluble in 0.15 M NaCl - 0.0025 M Tris, pH 8, versus the fraction of histone net positive charge removed from DNA after extraction of chromatin with increasing concentrations of DOC or NaCl.


FRACTION $A_{260m\mu}$ SOLUBLE IN 0.15 M NaCl
1.0
0.8
0.6
0.4
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
NaCl
DOC
FRACTION HISTONE NET POSITIVE CHARGE
REMOVED FROM DNA

(Smart & Bonner, 1969). Replotting the data of Figure 5 in this manner indicates that even after correction for the differences in the charge densities of the different histone fractions removed by the two methods of selective removal (Smart & Bonner, 1969), the resulting nucleoprotein samples differ quite markedly in their ability to aggregate in 0.15 M NaCl.

DISCUSSION

The priming ability (template activity) of partially dehistonized samples of chromatin in the high salt (0.2 M KCl) DNA-dependent RNA synthesis catalysed by E. coli RNA polymerase is inversely proportional to the fraction template DNA covered by histone. Thus, the template activity of chromatin samples partially dehistonized by increasing concentrations of sodium deoxycholate (DOC) or sodium chloride (NaCl) increases from the template activity of native chromatin to that of pure DNA in a fashion which shows a nearly linear relationship to the amount of total histone coverage of the template. Since the two methods employed for selective dissociation of histone from chromatin yield entirely different patterns of histone dissociation (Smart, Hadler, & Bonner, 1969; Smart & Bonner, 1969), it is clear that the lowered template activity of native

chromatin is not solely due to the presence of one particular histone fraction.

The rate constants and their standard errors for the partially dehistonized chromatin directed RNA synthesis have been obtained by computer fitting of the saturation curve data to the Michaelis-Menton equation. Within the limits of the assay (due to a slight amount of ribonuclease contamination) the template concentration required to half saturate the enzyme appears to be constant. Marushige and Bonner (1966) have interpreted the constancy of this value, K, to mean that the frequency and availablilty of sites to which RNA polymerase can bind are the same whether the DNA is supplied as chromatin or as pure DNA. If this is the situation, then it would appear that the enzyme can penetrate the aggregated template which is obtained in our RNA synthesis system when the DNA is supplied as native chromatin. Since physiological ionic strength is very close to that of our incubation system for RNA synthesis, it does not seem unreasonable to suppose that *in vivo* most of the chromatin exists in a highly aggregated state. Littau *et al.* (1964) have, in fact, observed that much of the DNA in the nucleus of a highly differentiated cell (the calf thymocyte) occurs in a condensed or compacted state, visible as dense clumps in the electron microscope.

Several workers have reported that the RNA transcribed

in vitro from chromatin by exogenous *E. coli* RNA polymerase is equivalent to the RNA transcribed *in vivo* (as evidenced by DNA-RNA hybridization competition) and that both of these RNA's represent only a limited number of the species of RNA transcribed *in vitro* from pure DNA of the same organism (Paul & Gilmour, 1966; Bonner *et al.*, 1968b; Bekhor, Kung, & Bonner, 1969). If our *in vitro* RNA synthesis system accurately represents the actual *in vivo* state, then it would appear that the limited transcription of the differentiated state is not due to physical blockage of the RNA polymerase binding sites, but rather due to interference with initiation or movement of the bound enzyme. Georgiev, Ananieva, and Kozlov (1966) combined selective removal of histones by increasing concentrations of NaCl with hybridization techniques and found that after removal of a small amount of protein from chromatin by concentrations of NaCl between 0.4 to 0.6 *M*, the number of sites transcribable by exogenous RNA polymerase increased from that of chromatin to that of pure DNA. They concluded that it was probable that the actual repressor of mRNA synthesis is contained in that protein fraction removed from the chromatin by extraction with concentrations of NaCl between 0.4 to 0.6 *M*. Thus, they supposed that either a special fraction of the lysine-rich histones (histone I), or less probably, a nonhistone protein takes the role of *in vivo* repressor in mRNA synthesis.

We have observed that partial removal of histones from chromatin by increasing concentrations of sodium deoxycholate results in increases in the template activity of the nucleoprotein which are essentially identical to those observed by removal of histones with increasing concentrations of sodium chloride when compared as a function of total histone removed from the DNA. It seems likely that the increase in template activity is due to local disruption of the structure required for repression of transcription by dissociation of the histone(s) in that immediate region. If the chance of the disruption and resulting transcription of DNA is equal to the chance of the disruption and resulting transcription of any one other sequence of DNA, then we would expect to find that the species of RNA synthesized from chromatin which had only a small amount of total histone removed from the DNA would increase from the limited number of species transcribed from chromatin to a representation of all of the species contained in the RNA transcribed from pure DNA. If the proposed "randomness of artificial derepression" is correct, then we would expect to find that the removal of a portion of histone I (the first histone fraction removed by increasing concentrations of NaCl) would yield a template from which would be transcribed (in a limited amount) all of the sequences transcribed from pure DNA (as has been observed by Georgiev, Ananieva, and

Kozlov, 1966). Likewise, we would expect the template produced by the removal of an equivalent weight fraction of histone II (the first histone fraction to be removed by increasing concentrations of DOC) to yield all of the sequences of RNA transcribed from pure DNA. Restating this, we would expect hybridization competition experiments to show that the RNA transcribed *in vitro* from chromatins which had 10 to 20 per cent of total histone removed by increasing concentrations of DOC or NaCl would compete completely with each other, and with the RNA transcribed from pure DNA.

Georgieve, Ananieva, and Kozlov (1966) reported that the increase in template activity which they observed after extraction of chromatin with 0.6 M NaCl could not be attributed to an increase in the solubility of the nucleoprotein, because its solubility in the medium used in their RNA synthesis system remained very low. Chalkley and Jensen (1968) on the other hand reported that a 130 S component of chromatin had a reduced template activity over that of the bulk of the chromatin (30 S) which could be correlated with an increased precipitability of the 130 S component in 0.15 M NaCl. We find that the removal of a given weight fraction of total histone from chromatin by increasing concentrations of NaCl generates a nucleoprotein sample which is more soluble in 0.15 M NaCl than is the sample generated by the re-

moval of an equivalent weight fraction of total histone by increasing concentrations of DOC. Even after corrections are made for the net positive charge contained in each histone fraction, the increased solubility of chromatin samples partially dehistonized by increasing concentrations of NaCl, over those partially dehistonized by increasing concentrations of DOC, still exists. Consequently, the template activity of a partially dehistonized chromatin sample in the 0.2 M KCl, DNA-dependent RNA synthesis system does not appear to be very closely related to the precipitability of the same template in 0.15 M NaCl. Both of these salt concentrations are well within the limits of precipitation of chromatin (0.005 to 0.30 M NaCl) (Chalkley & Jensen, 1968).

Chalkley and Jensen (1968) suggested that whether chromatin exists in the soluble form or in the aggregated state is largely dependent upon the effective charge on the chromatin molecules. They proposed that when the solvated, soluble form with its overall negative charge is electrostatically neutralized in 0.15 M NaCl, solvation is reduced and the molecules interact to give the aggregated form. If the various histone fractions made equal contributions to the total hydrophobicity of the chromatin molecules, we should find that, after corrections for the net positive charge contained in each fraction, the curves for the sol-

ubility (in 0.15 M NaCl) of samples of chromatin partially dehistonized by increasing concentrations of NaCl or DOC would be essentially identical. Although the poor understanding of nonhistone proteins makes the interpretation of these data more difficult, the results are consistent with the hypothesis that histone I (lysine-rich) contributes more to the total hydrophobicity of chromatin molecules than do histones II, III, and IV per weight fraction histone component. Consideration of the amino acid composition data of Fambrough (1967) shows that the various histone fractions contain the hydrophobic amino acid residues of valine, isoleucine, leucine, tyrosine, and phenylalanine in the following amounts: histone I, 12.1 mole per cent; histone II, 23.4 mole per cent; histones III and IV, 26.0 mole per cent. Consequently, the proposed increased hydrophobic contribution of histone I to chromatin molecules cannot be accounted for by known amino acid compositions of the various histone fractions.

Several isolated facts suggest that histone I is deposited on the DNA chain in such a manner that it is much more exposed to the aqueous environment than are the other histone fractions. Histone I, especially from mammalian tissues, is much more susceptible to degradation by contaminating proteolytic enzymes than are the other histone

fractions (Panyim, Jensen, & Chalkley, 1968; Fambrough, 1967). Littau et al. (1965), who examined the appearance of sectioned thymus nuclei in the electron microscope after various histone extraction procedures, concluded that histone I combines with the phosphate negative groups to cross-link DNA double helices, and that the remaining histone fractions combined with the phosphate groups along the double helix. Chalkley and Jensen (1968) observed that when precipitation of chromatin in 0.15 M NaCl is conducted in the presence of DNA or RNA of molecular weight greater than 3×10^4, the extent of chromatin aggregation is greatly reduced. They showed that this is due to an increased negative charge on the chromatin molecules following a shift of a portion of histone I to the free nucleic acid. From optical rotatory dispersion data, Tuan and Bonner (1969) have calculated that the various histone fractions complexed with DNA can contain (upper limit) the following percentages of α-helix: histone I, 15 per cent, histone II, 58 per cent, histones III and IV, 42 per cent. The amino acid compositions (Fambrough, 1967) of the various histone fractions show that histone I contains 18.3 mole per cent of net positive charge, while histone II contains 8.4 mole per cent and histones III and IV contain 9.1 mole per cent. All of the ionic dissociating agents employed for the selective

removal of histones from chromatin dissociate histone I at the lowest concentration of dissociating agent (Ohlenbusch, Olivera, Tuan, & Davidson, 1967; Fambrough & Bonner, 1968; Levinson, Smart, & Bonner, 1969). Thus, the histone fraction which should have the strongest ionic interaction with the phosphate groups of DNA (on the basis of amino acid composition) is the first to be dissociated from the DNA by several ionic methods.

We feel that all of these properties of histone I (early removal from chromatin by increasing concentrations of dissociating agents, susceptibility to attack by proteolytic enzymes, proportionately large contribution to the aggregation of partially dehistonized chromatin in 0.15 M NaCl, apparent cross-linking of DNA chains, ability to shift to free nucleic acids of moderately large size, and apparent low content of α-helix) can be accounted for by assuming that histone I is more exposed to and/or interacts more with the aqueous environment than are and/or do the other histone fractions. Consequently, if the histones are arranged along the DNA chain in a linear sequence, then (with the above assumptions) the structure of histone I in chromatin must be much more loose and open (exposed to the aqueous environment) than that of the other histone fractions. Another possible arrangement for the histones which

would account for the apparent increased hydrophobic contributions of histone I to the chromatin molecules is one in which a sheath-like structure is formed with histones II, III, and IV usually on the inside of the sheath (near or in the hydrophobic groove(s) of the DNA double helix) and histone I (when it occurs) on the outside of the sheath, exposed to the aqueous environment.

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