AFFINITY CHROMATOGRAPHY IN THE STUDY OF MACROMOLECULAR INTERACTIONS

I. BINDING OF DNA TO IMMOBILIZED HISTONES

II. INTERACTION OF TWO NONHISTONE CHROMOSOMAL PROTEINS WITH H1 HISTONE AND ITS SUBFRACTIONS IMMOBILIZED ON AGAROSE

A Thesis

Submitted to

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Sai-Hung Yu

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ABSTRACT

Part I

The six major histone fractions of chicken erythrocytes have been individually immobilized on agarose. The binding of native and denatured DNA to these immobilized histones was studied. It was found that the binding efficiency of various immobilized histone fractions for native DNA decreases in the order of: H5, H1, H2B=H3, H4, H2A; which is in good agreement with previous results obtained using free solution techniques. Denatured DNA bound to the histone-agarose anomalously, giving a tight complex which was not dissociated by 2 M NaCl.

Part II

The interaction of calf thymus H1 histones with two calf thymus nonhistone proteins, HMG1 and HMG2, (High Mobility Group proteins, Walker et al., Eur. J.

Biochem. 62, 461, 1976) has been studied using columns of H1 immobilized on agarose. HMG2 does not interact with immobilized H1, but HMG1 binds to H1 columns at low ionic strength and can be eluted with NaCl in the range of 0.05 to 0.15 M. Three chromatographic subfractions of H1 have also been immobilized and tested for their ability to bind HMG1. Based on the NaCl concentration required to elute HMG1 from the H1 subfraction columns, it is found that affinity of the H1 subfractions for HMG1 is 2>1>3.

PART I

BINDING OF DNA TO IMMOBILIZED HISTONES

INTRODUCTION

The term "Affinity Chromatography", first introduced by Cuatrecasas, Wilchek and Anfinsen /1/ in 1968, refers to the chromatographic technique for the separation and purification of enzymes or other macromolecules, based on their biospecific affinity for a given ligand immobilized (covalently attached) to an insoluble support such as agarose, glass, or polyacrylamide. The potential of this technique in the quantitative purification of enzymes and other highly specific macromolecules has been greatly exploited in recent years /2,3,4 and references therein/. Its analytical potential has also been realized in the studies of enzyme mechanisms /5,6/, probing of enzyme active sites /7/, and in studying the interactions of actin and myosin /8/, DNA and histones /9,10/, as well as DNA and nonhistone chromosomal proteins /11/.

Under favorable conditions, the analytical application of affinity chromatography on reversible

interaction systems can often provide considerable operational advantages over free solution techniques. By immobilizing one of the interacting species, various parameters affecting the interaction can be easily determined, and nonspecific aggregation is less likely to occur. The technique also offers the advantage of being preparative and also possessing a reusable ligand.

Interest in the interaction between histones and DNA is not new. They are the two major components of all eucaryotic chromosomes. The early report of Huang and Bonner /12/ and the later ones to follow /13-15/ on the strong inhibitory effect of histones on DNA template activity have stirred up great interest in their interaction for the past ten years. Many studies were concentrated on the ability of various histone fractions to stabilize the DNA helix at high temperature (see review by Hnilica /16/ and references therein), while others were to measure the binding constant /17/, to show the nonspecificity of the binding/18/ and to show the structure of histones when complexed to DNA /19,20/. The general agreement

is that histones bind to DNA primarily through electrostatic linkages between the amino groups of the histone and the phosphate groups of the DNA helix. Although different histone fractions may show a slight preference for a given G-C content, there is probably no specific sequence recognition involved.

Application of affinity chromatography to DNA-histone interaction had been reported by Ayad, et al. /9,10/. These investigators studied the binding of DNA to calf thymus histones immobilized on kieselguhr as well as on cyanogen-bromide-activated agarose. They showed that DNA bound to the immobilized histones and could be eluted with a salt gradient, and that differences exist in the salt concentration required to elute the bound DNA from the three immobilized histone fractions they studied. Although their results did not show any strong evidence that the immobilied histones were as native as their unimmobilized counterparts, the idea of using a similar system to study the interaction of histones to various chromosomal constituents is sound and should be advantageous over some other in vitro techniques in many respects.

By immobilizing the histones on agarose through a short spacer arm which would allow more structural freedom for the histone molecules, one can investigate the affinity of various nonhistone chromosomal proteins to histones, the interaction between different histone fractions, and between various forms of DNA to histones.

The major objective of this first study is to characterize the histone-agarose affinity chromatography system in terms of its DNA binding behavior. The results thus obtained are to be compared with those obtained using unimmobilized techniques. Such characterization is necessary for further application of this technique in studies of histone and nonhistone chromosomal protein interaction which is to be reported in Part II of this Thesis, and for other potential applications.

In this study, six major fractions of histone of the chicken erythrocytes (H1, H2A, H2B, H3, H4 and H5)* were each immobilized on agarose through a short spacer

---

* Histone nomenclature used in accordance to CIBA Foundation Symposium on the Structure and Function of Chromatin, 1975.

arm made up of a glycyl derivative. Histone-agarose were packed into columns and the binding of native and heat denatured calf thymus DNA to each fraction was investigated. In contrast to previous histone-agarose-DNA binding study, but compatible with results obtained from some other nonimmobilized techniques /21,22/, it was observed that the binding efficiency of native DNA to histone-agarose is primarily predicted by the net positive charge of the corresponding histone fraction. As for heat denatured DNA, some unexplainable results were obtained.

EXPERIMENTAL PROCEDURE

Isolation of Nuclei from Chicken Erythrocyte

All procedures were performed at 4°C unless mentioned otherwise. Approximately 350 ml of fresh chicken blood was collected from ten mature egg-laying hens, to which was added 0.1 volume of 10% Na_3 citrate (pH 7.4) to prevent clotting. The blood was filtered through four layers of cheescloth to remove debris before centrifuging at 1,000 x g for 10 min. to sediment the red blood cells. The blood cells were washed three times with a buffer consisting of 0.14 M NaCl, 0.01 M Na_3 citrate pH 7.4, and were lysed by suspending for two hours with occasional stirring, in an equal packed-cell-volume of the nuclei buffer (0.05 M Na acetate, 0.1 M NaCl, 5 mM $NaHSO_3$, 1 mM $CaCl_2$ 0.5% Triton X-100 pH5.0) containing 0.6% saponin. Nuclei from the lysed cells were sedimented at 1,000 x g for 1½ hours and then washed repeatedly until no red color was present. Fifteen minutes of centrifugation time was sufficient to sediment the nuclei during the washing procedure. The white colored nuclear pellet,

consisted of intact nuclei (as shown by phase contrast microscopy) was well suspended in the nuclei buffer containing 60% glycerol, and stored at -20°C until use.

Isolation and Purification of Histones

Histones H1 and H5 were extracted from the nuclear pellet by blending in 20 pellet volumes of 5% PCA (perchloric acid). Extraction was repeated twice and extracts were pooled. The remaining histones in the chromatin pellet were extracted three times with 20 pellet volumes each of 0.25 N HCl. Both the PCA and HCl extracts were dialysed extensively in H_2O followed by lyophilization.

Histone fractionation was done according to van der Westhuyzen et al. /23/ with some modifications.

Each of the extracts was chromatographed on a 2.5 x 160 cm column of Bio-Gel P-60 (Bio-Rad Laboratories) equilibrated in 0.02 M HCl, 0.05 M NaCl, 0.02% NaN_3 pH 1.7. Lyophilized PCA extract (30 mg) was dissolved in 2 ml column buffer containing 8 M urea, incubated at 30°C for 10 min. and cooled down to room temperature before loading on the column. Chromatography was performed at room temperature with a constant flow rate

of 6 ml/hr. Conditions for the HCl extract were identical except that 70 mg of histones were used.

Appropriate fractions containing the H2B-H3 mixture were pooled, dialysed in water and concentrated by lyophilization. The mixture was chromatographed on a 2.5 x 60 cm column of Sephadex G-100 (Pharmacia) equilibrated and run in 50 mM sodium acetate, 5 mM $NaHSO_3$ pH5.1, using 40 mg of the lyophilized sample. The flow rate was set at 7.5 ml/hr.

Appropriate fractions from each peak representing a purified histone fraction were pooled, extensively dialysed in distilled water and lyophilized. Each lyophilized histone sample was made 10 mg/ml in H_2O according to dried weight and stored at -20°C.

Coupling of Histones to Agarose

Histones were coupled to beaded agarose (Bio-Gel A-15m, Bio-Rad Laboratories) according to the procedure of Loeffler and Pierce /25/. Briefly, the agarose was activated by cyanogen bromide as described by Cuatrecasas and Anfinsen /26/. Approximately 100 g of CNBr activated wet gel (excess water removed by suction

filtration) was washed in 0.1 M sodium bicarbonate pH 9.0 and coupled to 25 mmole of glycine methyl ester in the same buffer by gentle stirring at 4°C overnight. The agarose-ester derivative was washed with 1 M HCl and H_2O followed by absolute methanol. It was subsequently converted to glycyl hydrazide derivative by adding 95 ml of abs. methanol containing 5 g of hydrazine hydrate, and stirring at room temperature for 6 hrs.

After washing with water, 2.5 g of wet hydrazide gel was added to 5 ml of 0.9 N HCl and cooled to 0°C on ice. 0.5 ml of 1 mM $NaNO_2$ was added and the gel suspension incubated for 20 min. on ice. The resulting glycyl azide derivative was washed with water and suction-filtered before coupling to histones.

Lyophilized histone (10 mg) was dissolved in 2 ml of 20 mM sodium borate and added to the gel. The total volume was adjusted to 5 ml with the borate buffer and the pH adjusted to 8.0. The mixture was gently stirred for 20 hrs. at 4°C.

The coupled histone-agarose mixture was washed thoroughly with a cold solution of 0.1 M NH_4Cl, 0.1 M NH_4OH, 0.1 M $CaCl_2$ pH 9.0 (initial wash saved for protein

determination) followed by a cold solution of 0.1 M $CaCl_2$, 1 mM HCl. Coupling of histones to the gel was quantitative as determined by Lowry protein determination on washes. The washed histone-agarose was suspended in a phosphate buffer (10 mM PO_4, 5 mM $NaHSO_3$ pH 6.7) and packed into columns made up of Pasteur pipettes. Each column had a gel bed volume of about 1 ml (0.65 g wet gel) and contained approximately 2.5 mg of histones. The columns were equilibrated in the same phosphate buffer and stored at 4°C. Reproducible results in DNA binding ability of these columns were obtained for up to six months.

A blank column made up of derivatized agarose for control experiments was prepared with identical procedures but without histones.

DNA Preparation

Two mg of calf thymus DNA (Sigma) was dissolved in 20 ml of phosphate buffer (10 mM PO_4, 5 mM $NaHSO_3$ pH 6.7), sheared in a Branson sonifier at 40 watts for three 2 min. intervals. The DNA solution was submerged in ice water during sonication to prevent overheating. After shearing, the DNA solution was centrifuged to

sediment insoluble materials in a clinical centrifuge. The supernatant was adjusted to 1 A_{260} (Absorbance unit at 260 nm) by adding phosphate buffer. After dialysing in 500 ml of the same buffer overnight, it was used as native DNA in the binding experiments.

Denatured DNA was prepared by heating the native DNA solution in boiling water for 15 min. followed by chilling quickly in a bath of ice water.

Affinity Chromatography

Chromatography was performed at room temperature. DNA (25 ug in 0.5 ml) was applied to each histone column. The column was washed with 3.5 ml of buffer, followed by a discontinous salt gradient of 0.05M to 1.0 M NaCl (total volume 10 ml). The gradient was effected by adding 0.5 ml increments of phosphate buffer each containing an increasing concentration of NaCl while collecting 0.5 ml fractions. The increase in the salt concentration was 0.05 M per increment. NaCl concentration in the eluant was determined by refractive index, according to a standard curve. Binding of DNA to the column was monitored by absorbance at 260 nm.

DNA binding studies for the six affinity columns,

each made up of an individual histone fraction, were performed simultaneously under identical conditions. The salt gradient as monitored by refractive index was roughly linear, the slope of which was similar and reproducible for all columns.

DNA & Protein Determination

DNA concentration was determined by assuming 1 A_{260} of the native DNA is equal to 50 ug/ml. Protein concentration was mostly determined by dried weight of the lyophilized samples which had been extensively dialysed in distilled water. A modified procedure of Lowry et al./27/ was used in monitoring the histone-agarose coupling procedure.

Electrophoresis

Purity of histone was determined by polyacrylamide gel electrophoresis in the acid-urea gel system described by Panyim and Chalkley /24/. Electrophoresis was performed on a 0.15 x 12 x 16 cm slab gel at 30 mA for 3 hrs. at room temperature.

RESULT

Purification of Histones

Chromatography of the 5% PCA extract on a Bio-Gel P-60 column resolved the mixture into two peaks, corresponding to histone H1 and H5 (Fig.1). Due to the high ratio of H5 to H1 in the mixture and the closeness of the two peaks, H1 samples were quite often contaminated with H5. Although not practiced in this work, further purification could be achieved by chromatography on cationic exchange resins such as Bio-Rex 70 /28/.

Chromatography of the 0.25 N HCl extract on the same column resolved the mixture into four protein peaks (Fig.2). The first peak appeared at the void volume which consisted of small quantity of residual H1 and some nonhistone proteineous contaminants (as shown by gel electrophoresis) was discarded. Peak 2 and peak 4 consisted of, respectively, histone H2A and H4 in good purity. The third peak consisted of the H2B and H3 mixture, which was resolved by chromatography on the Sephadex G-100 column (Fig.3) with H3 being eluted first.

Figure 1

Chromatographic profile of the 5% PCA extract on Bio-Gel P-60 column. Size of the column was 2.5 x 160 cm, flow rate was adjusted to 6 ml/hr. and 6 ml fractions were collected. 30 mg of histones was used in this run.

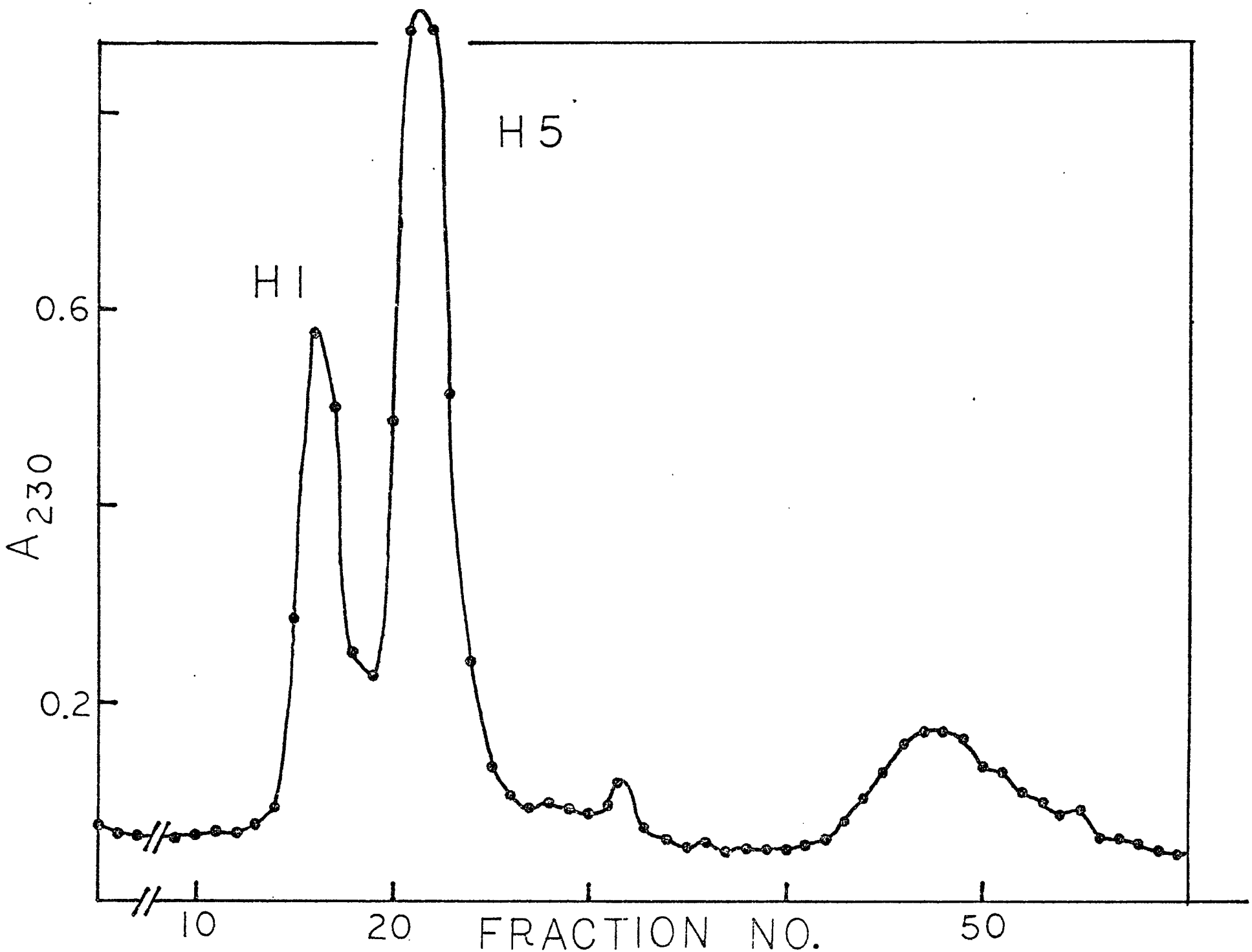


Fig 1

Figure 2

Chromatographic profile of the 0.25 N HCl extract on Bio-Gel P-60 column. 70 mg of histones was applied to the 2.5 x 160 cm column. Flow rate was adjusted to 6 ml/hr. and 6 ml fractions were collected. Peak X consisted of small amount of residual H1 histone and some nonhistone proteinaceous contaminants.

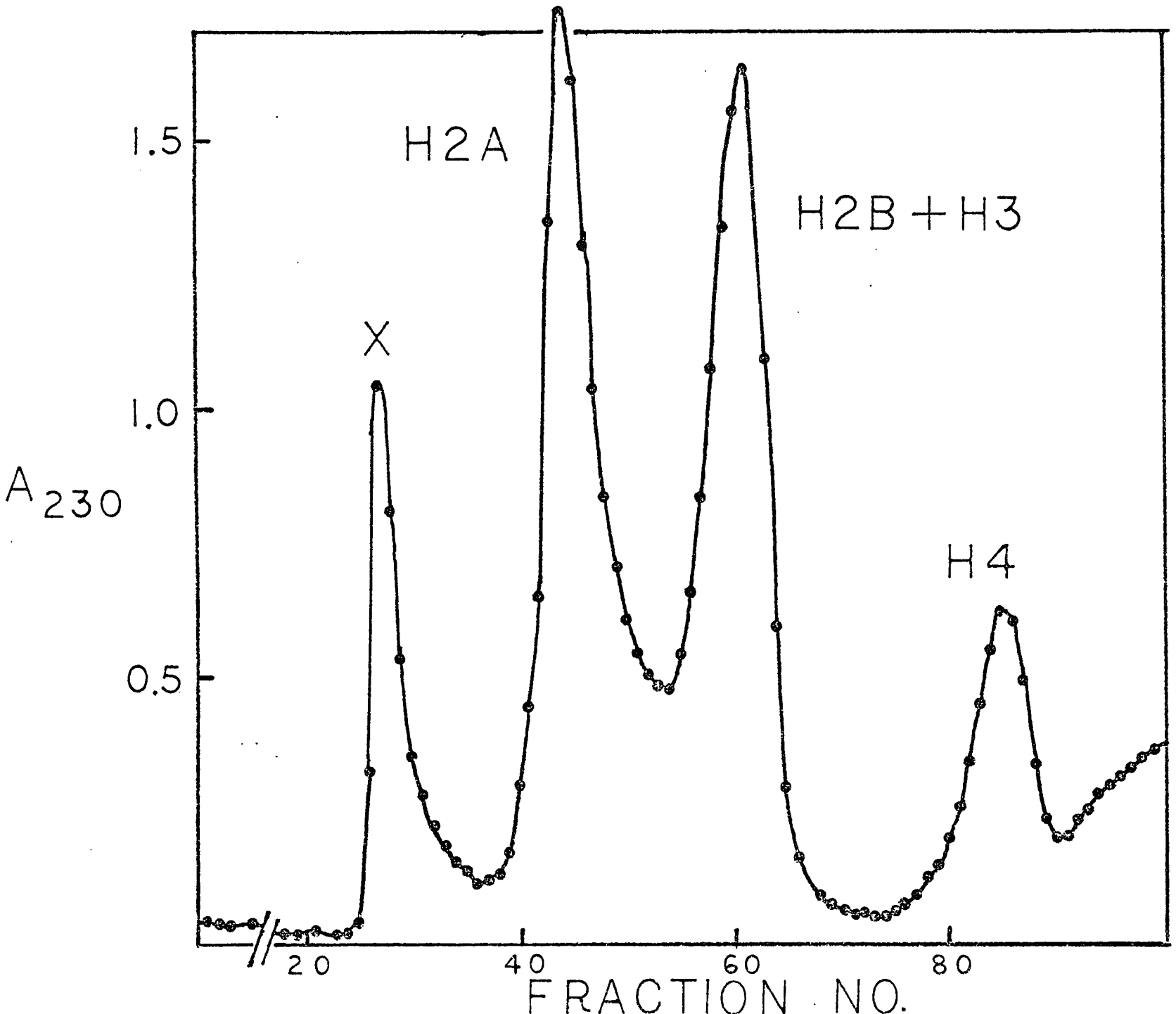


fig 2
pg 15

Figure 3

Chromatographic profile of the H2B-H3 mixture on a Sephadex G-100 column. 40 mg of histones was applied to the 2.5 x 60 cm column. The flow rate was adjusted to 7.5 ml/hr. and 6 ml fractions were collected.

fig. 3
17.16

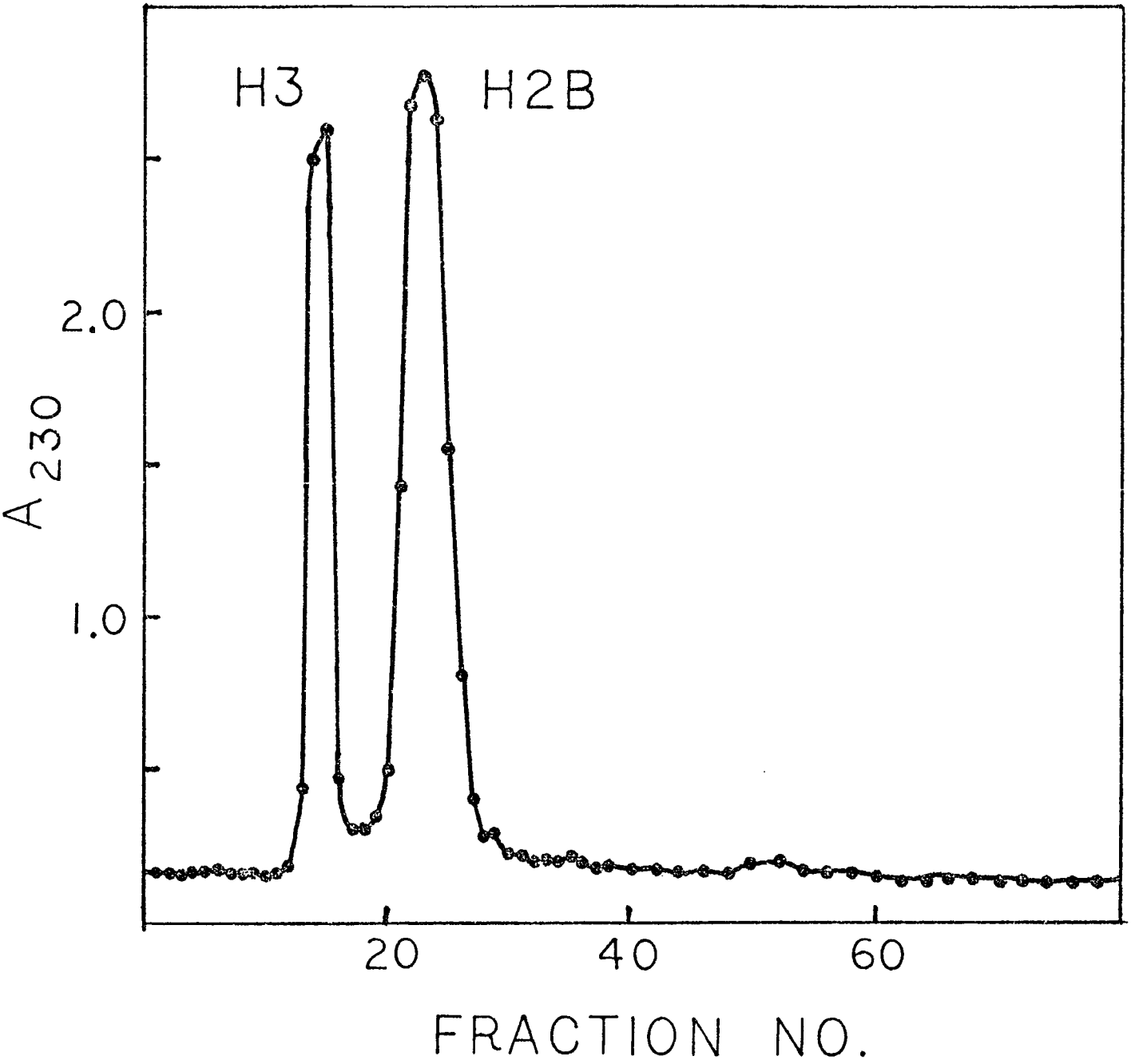


Figure 4

Electrophoretic patterns of purified histone samples.

1. 5% PCA extract (H1 + H5),

2. 0.25 N HCl extract (H2A, H2B, H3 & H4),

3. H1, 4. H5 5. H3,

6. H2A, 7. H2B, 8. H4.

Electrophoresis was performed in the acid-urea gel system described by Panyim and Chalkley /24/.

In patterns 1 & 2, 0.4 cm (diameter) x 7 cm (length) tube gels were used at 2 mA per gel for 2.5 hrs.

The purified histones were electrophoresed in a slab gel (0.15 x 12 x 16 cm) at 30 mA for 3 hrs.

Fig. 4
Pg. 17

Electrophoretic patterns of water dialysed and lyophilized histone samples are shown on Figure 4.

Binding of Native DNA to Histone-agarose Columns

Native calf thymus DNA bind to all histone-agarose columns and was recovered as a single peak in the salt gradient (Fig.5 & 6). In all cases, a small peak representing up to 5% of the DNA sample's uv. absorbance appeared in the first few fractions during the buffer wash. This material had an absorption profile roughly comparable to that of DNA and was thought to be oligonucleotides and other impurities which would not bind to the histone-agarose. Recovery of DNA from the histone column ranged from 90 to 100 %.

The chromatograms showed significant differences in the NaCl concentration required to elute the bound DNA from the columns which were made up of various histone fractions. This observation is graphically represented by superimposing the six chromatograms in Figures 5 & 6 and replotting by using the salt concentration of effluents in the abscissa (Fig.7).

The molarity of NaCl corresponding to the highest point of the DNA peak for each histone column was

Figure 5

Affinity chromatograms showing binding of native calf thymus DNA to the immobilized histone fractions H2A, H2B, H3 and H4.

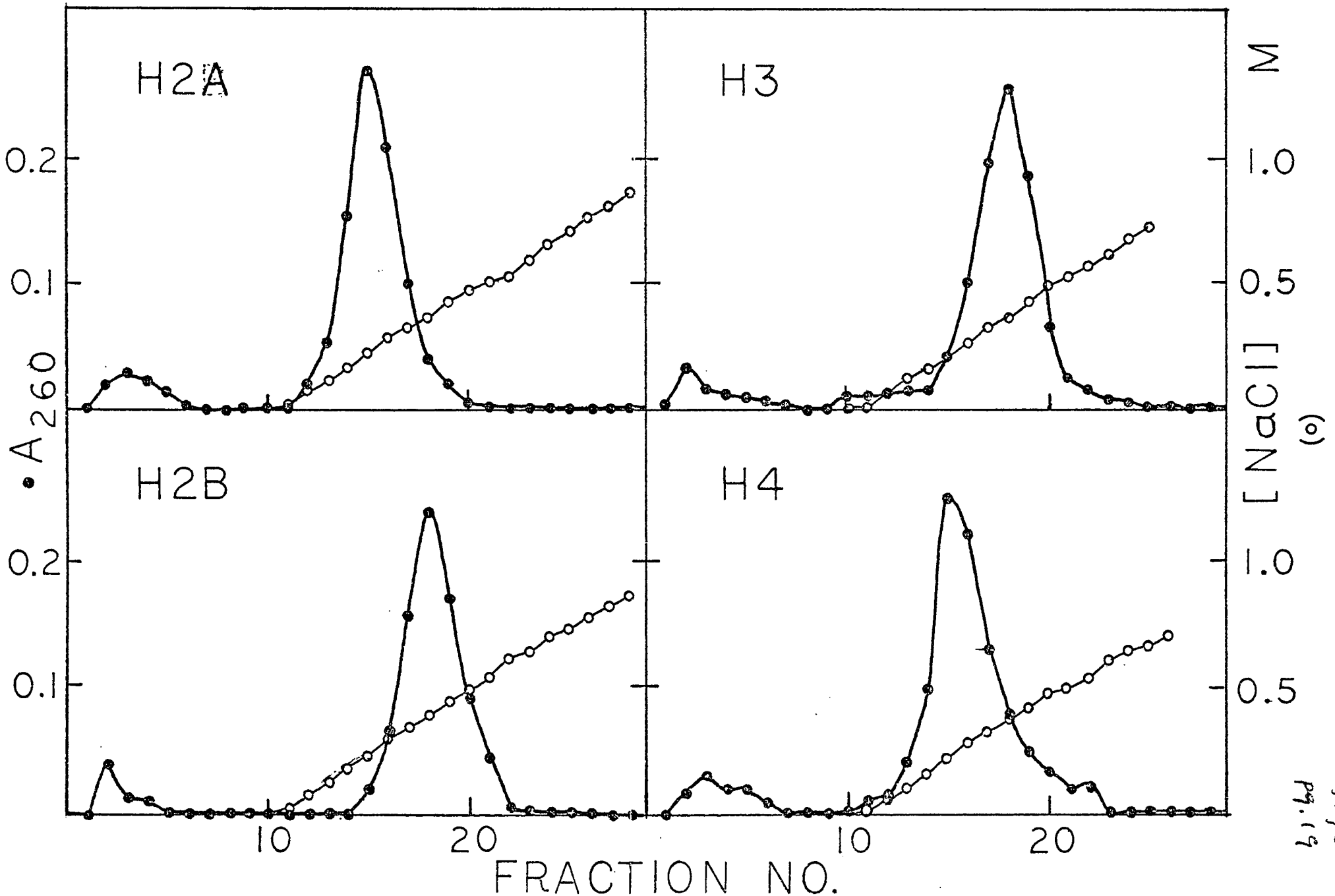


Fig 5
Pg. 19

Figure 6

Affinity chromatograms showing binding of native calf thymus DNA to immobilized histone fractions H1 and H5.

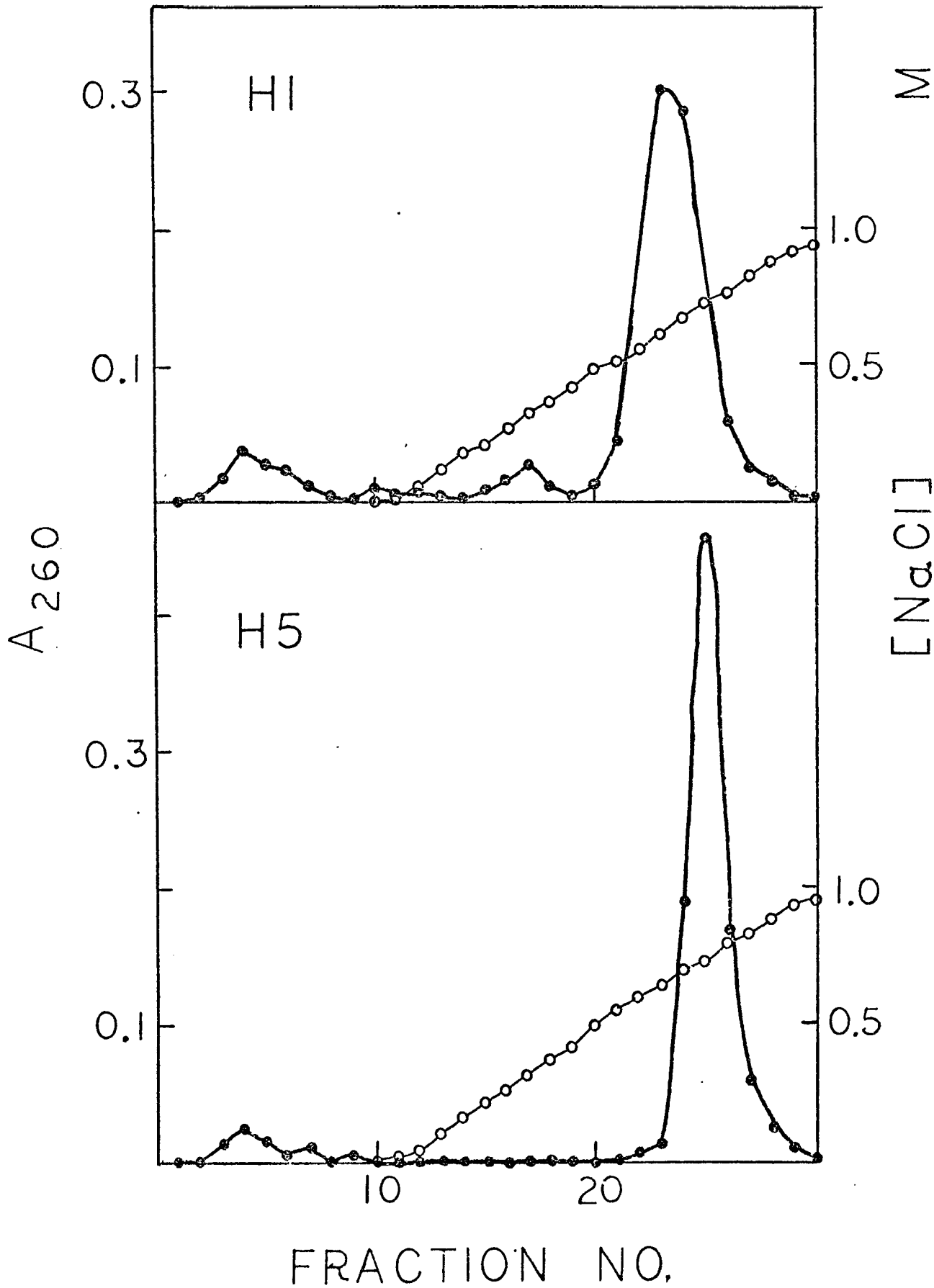

H1
H5
0.3
0.1
0.3
0.1
1.0
0.5
1.0
0.5
M
[NaCl]
A260
10
20
FRACTION NO.

Figure 7

Binding efficiency of native DNA to various immobilized histone fractions. Replotted from Figures 5 & 6 using NaCl concentration of the effluent in the abscissa.

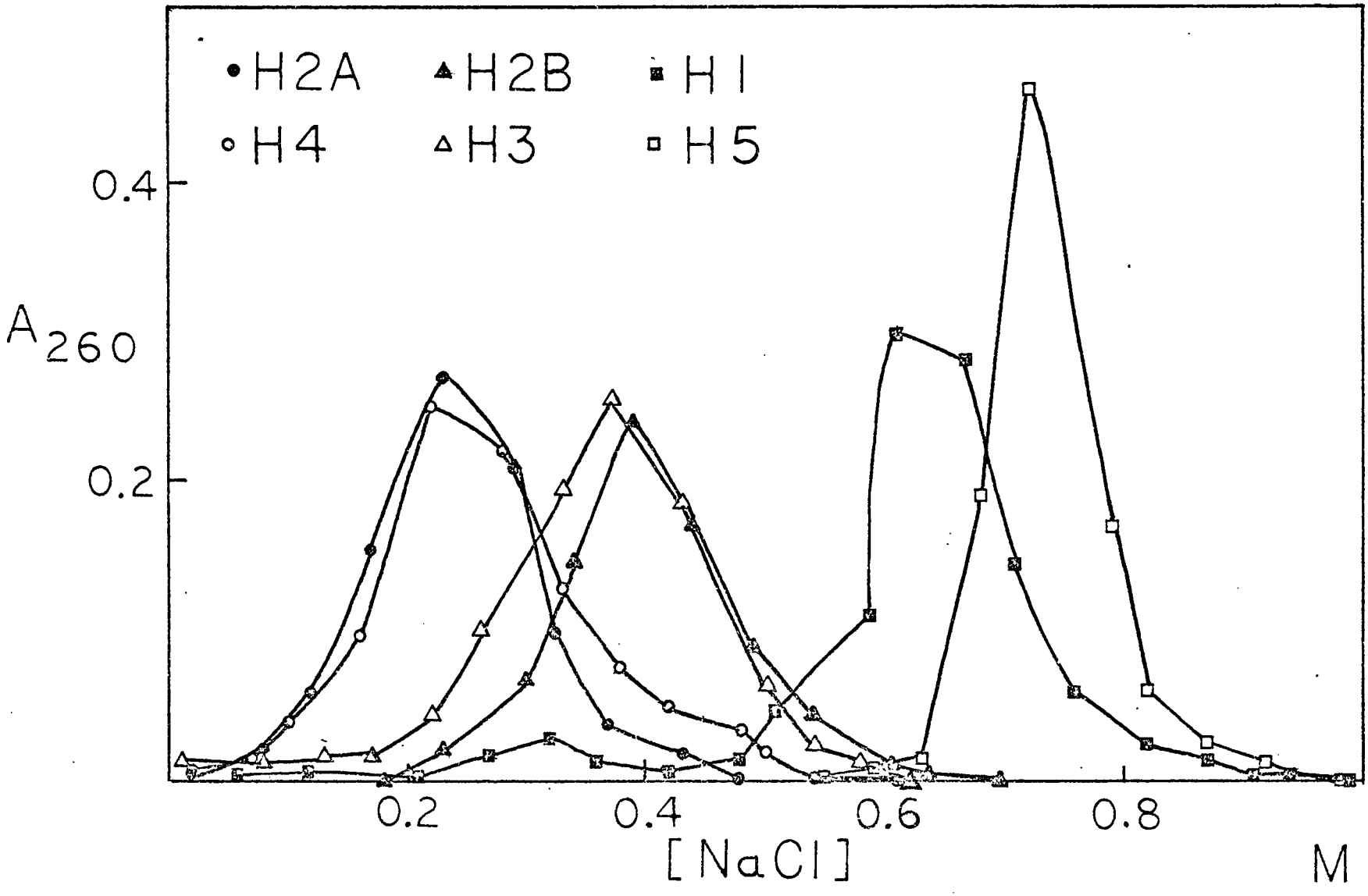


fig 7
pg. 21

tabulated as follows:

Table 1. Molarity of NaCl Required to Elute the Bound DNA

Histone	Molarity of NaCl at Peak *
H5	0.74 ± 0.02
H1	0.60 ± 0.02
H2B	0.39 ± 0.01
H3	0.38 ± 0.01
H4	0.23 ± 0.02
H2A	0.20 ± 0.03

* Average value of two experiments performed between a time interval of four months on the same column, where ± symbol is used to indicate maximum deviation from the mean.

The difference in the salt concentration required to elute bound DNA from various columns was taken to be an indication for an increasing efficiency (or strength) of binding of native calf thymus DNA for the six immobilized histone fractions in the order of:

H2A and H4, H2B and H3, H1, H5.

Reproducibility of the above data was good and further strengthened by another set of similar experiments not included in this Thesis, whereby bacteriophage PM2 form II DNA (kindly provided by Mr. Paul P. L. Lau and Dr. Horace B. Gray, Jr.) was used on the same columns. The order of the phage DNA being eluted from the six histone columns was identical to that of calf thymus DNA, although for each given column, the phage DNA seemed to bind slightly tighter and required about 0.05 M to 0.1 M more of NaCl to be eluted. This variance might be due to differences in the base composition as well as molecular weight between the two DNA species.

In the control experiments, neither the native DNA nor the heat denatured DNA bind to control agarose columns prepared identically, but without histones. DNAs were totally recovered from the column by washing with two bed volumes (2 ml) of the starting buffer.

Binding of Heat Denatured DNA to Histone-agarose

In contrast to native DNA, heat denatured DNA binds to the histone columns anomalously. For all the histone columns, only 30% of the applied DNA was recovered, as

a single peak, in the salt gradient (0 M to 1.0 M NaCl). For any given histone fraction, the postion of this peak in the chromatogram was two fractions behind that of its native DNA counterpart, or approximately 0.1 M more of NaCl was required to elute this fraction from the column as compared to native DNA. The remaining 65 or 70% of the applied DNA was not released from the column even by increasing the salt concentration to 2 M. Not until elution with 4 M guanidine HCl did this tightly bound DNA become dissociated from the histone-agarose. (In the guanidine fraction, DNA was assayed by uv. absorbance at 260nm on the pooled fractions after extensive dialysis in the phosphate buffer to remove the guanidine)

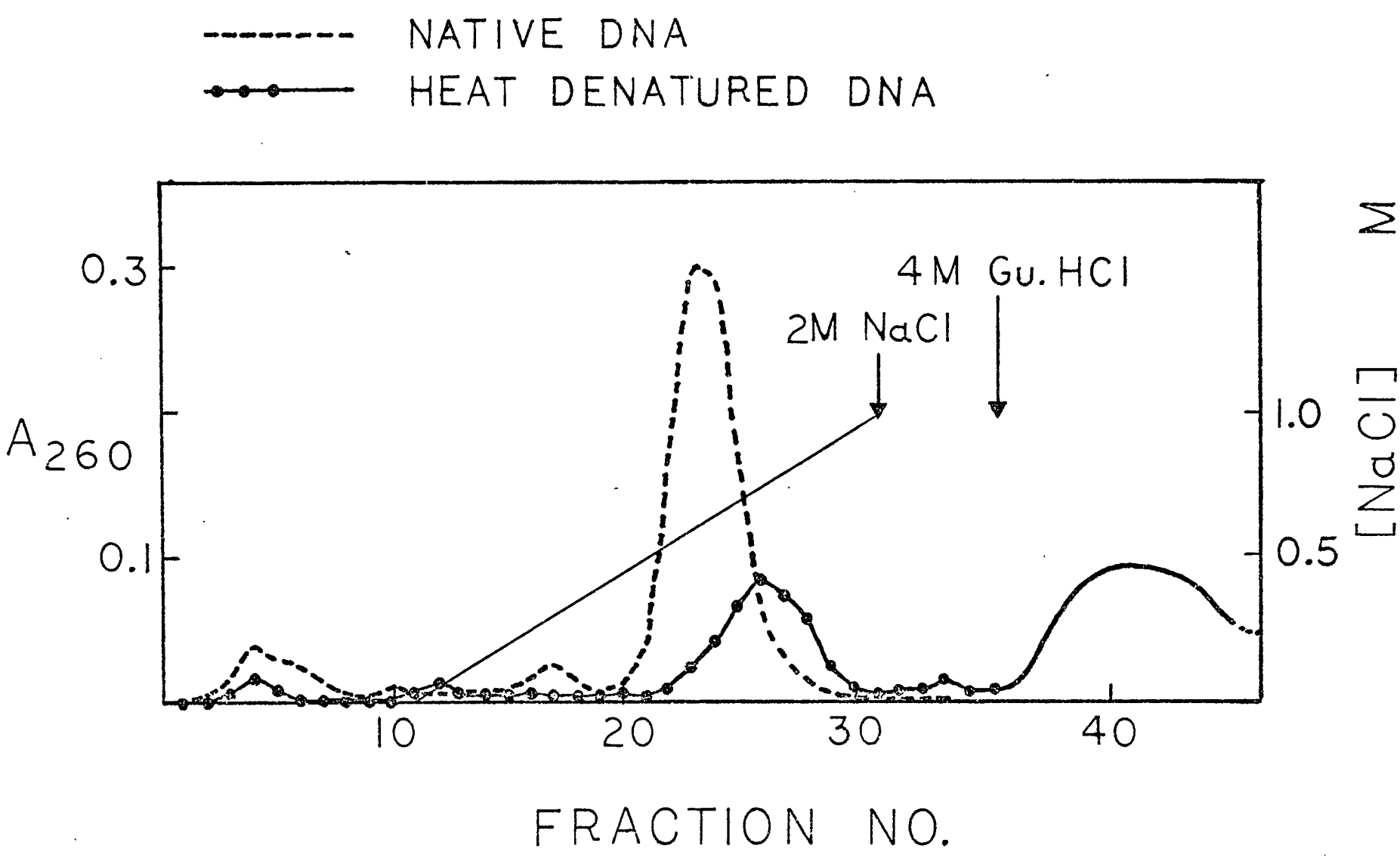
A chromatogram showing the binding of denatured DNA to histone H1 column, the scheme of which was typical and representative for other histone fractions, is shown in Figure 8.

Nature of the DNA in the salt gradient peak and the guanidine eluted fraction was examined in terms of its degree of denaturation (single-strandedness) by a simple but rather low sensitivity method of measuring

Figure 8

Affinity chromatogram showing the binding of heat denatured DNA to immobilized H1 histones. Broken line represents a superimposed chromatogram depicting the native DNA binding to the same column.
In the 4 M guanidine HCl fraction, DNA was assayed by absorbance at 260 nm after the collected fractions were pooled and extensively dialysed in the phosphate buffer.

Fig 8
Pg. 25


NATIVE DNA
HEAT DENATURED DNA
0.3
0.1
A_{260}
4M Gu. HCl
2M NaCl
1.0
0.5
[NaCl] M
10
20
30
40
FRACTION NO.

the percentage hyperchromicity of the DNA sample after "complete" denaturation at melting temperature for prolonged period. DNA from both fractions (collected from the H1 column) were adjusted to 0.2 A_{260}, heated in boiling water for 25 min., quickly chilled in ice water for 30 min., and equilibrated at room temperature for 15 min. before measuring the A_{260} for calculation of hyperchromicity. The original single stranded DNA sample used in these binding studies was used as control, and native DNA was also included for reference (Table 2).

Table 2. Single-strandedness of DNA

DNA Sample	% H*	%H - Control
Control (denatured DNA)	6.6	0
Native DNA	30.0	24.0
Salt Grad. Peak	13.1	6.5
Guanidine Peak	9.2	2.6

* % Hyperchromicity is defined as

$$\frac{A_{260}(\text{after heating}) - A_{260}(\text{before heating})}{A_{260}(\text{before heating})} \times 100\%$$

The result showed that when subject to prolonged heating, DNA eluted in the salt gradient (Peak 1) had twice as much % H as DNA from the guanidine wash (Peak 2). This was taken as an indication that Peak 1 DNA was somewhat less denatured, or had more double stranded regions than that of Peak 2 DNA.

In conclusion, the results showed that heat denatured DNA was consistently resolved into two fractions by the histone-agarose columns. The smaller fraction (app. 30% of the applied DNA) was eluted in the salt gradient and seemed to bind to the column slightly tighter than native DNA. The larger fraction (70% of the applied DNA) binds very tightly to the column, but could be eluted with 4 M guanidine HCl. Apparently, more than ionic binding was involved since 2 M NaCl failed to release the bound DNA. The result also suggested that DNA of the salt gradient fraction was more "native" than the guanidine fraction.

It is to be reemphasized here that denatured DNA did not bind to the blank column at all.

DISCUSSION

The observed binding efficiency of native DNA to various histone fractions in the order of

$$H5 > H1 \gg H2B \simeq H3 > H4 \geq H2A$$

is strikingly similar to the results of Johns and Forrester /22/, who studied the ability of different histone fractions to precipitate DNA in low salt (0.14 M NaCl), and showed a "precipitation efficiency" in the order of

$$H1 \gg H2B \geq H3 \geq H4 > H2A$$

The studies of Anseven and Brown /21/ based on thermal denaturation data on the DNA-histone complexes also showed a complexing efficiency of a similar order:

$$H1 \gg H2B > H3 \geq H4 \simeq H2A \ .$$

A numerical comparation of the above data is given in Table 3.

This order of binding efficiency seems to be at least partially explainable by the net positive charge on each histone fraction, with the reasonable assumption that the DNA-histone interaction is predominantly

Table 3. Binding Efficiency of Native DNA to Various Histone Fractions.

Histone Fraction	Net (+) Charge (Lys/Arg)	(a) Molarity of NaCl required to elute the bound DNA	(b) mg Histone required to ppt. 50% 0.5mg DNA	(c) % free DNA in nucleohistone complexes
H5	70 (2.2)	0.74 ± 0.02	N.A.	N.A.
H1	54 (20)	0.60 ± 0.02	0.20	0
H2B	22 (2.5)	0.39 ± 0.01	0.44	46 ± 1
H3	22 (0.72)	0.38 ± 0.01	0.48	51 ± 7
H4	19 (0.79)	0.23 ± 0.02	0.55	53 ± 11
H2A	20 (1.25)	0.20 ± 0.03	0.67	56 ± 9

(a) see Table 2 and Result section for details.
(b) estimated from data of Johns & Forrester /22/. complex formed at 0.14 M NaCl.
(c) published data of Anseven & Brown /21/, determined from plots of derivative of hyperchromicity vs. temperature for reconstituted nucleohistone complexes; ± indicates deviation from the mean of three experiments.

electrostatic, and that relatively few basic residues are buried in the coiled hydrophobic region of the histone molecule, enabling a majority of the basic groups to be exposed and thus be available for DNA binding. Other modes of interactions could, of course, be involved but would be difficult or impossible to detect with the present system.

Ayad and Parker /10/ have coupled calf thymus histones directly to cyanogen bromide activated agarose and observed the binding efficiency of calf thymus DNA to the three immobilized histone fractions to be

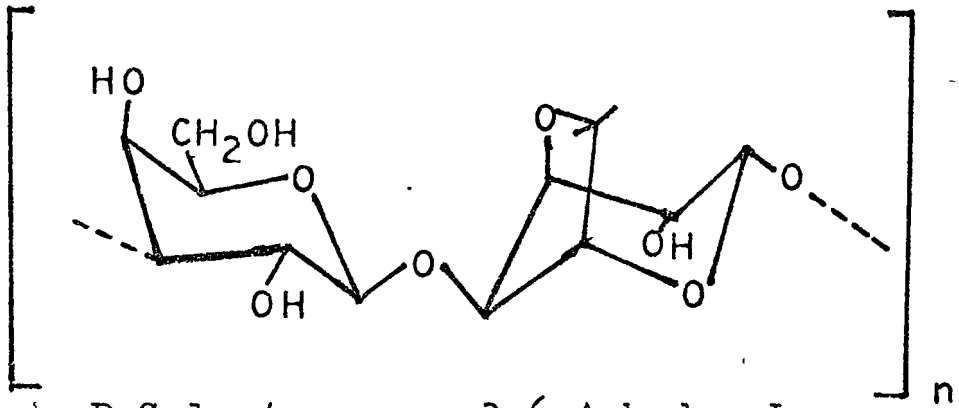
$$H3 > H2B > H1 ,$$

which appears to be just opposite to what has been discussed here. This discrepancy quite possibly has arisen from the difference in the coupling procedures. It is estimated from their cyanogen bromide activation procedure that about one in every three hydroxyl groups in the gel matrix was activated. If histones are directly coupled to these highly populated cyanogen groups, a large number of lysine residues per histone molecule can become covalently bound. In such case, the capability for DNA binding will be inversely proport-

ional to the lysine/arginine ratio of that histone fraction. The lys/arg ratios of H1, H2B and H3 (see Table 3) seem to support this view.

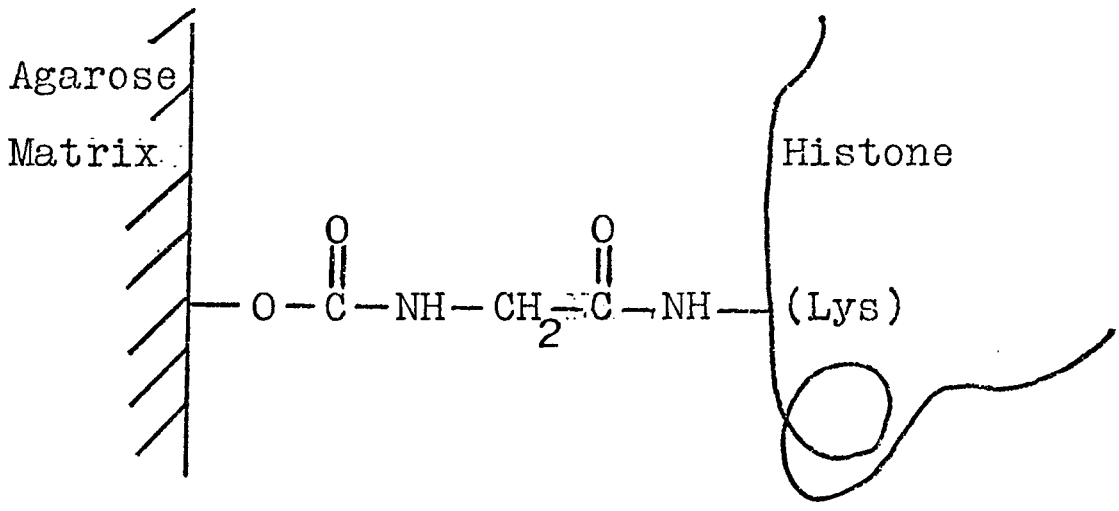
The coupling procedure used in this work had been originally employed by Loeffler and Pierce /26/ to immobilize trypsin on agarose with considerable success. Protein molecules are coupled through their ϵ-amino groups to the gel through glycyl spacer arm (Fig.9). Based on their calculation on the spacer arm density in the gel matrix /26/ and the quantity of histone used in the coupling mixture, the maximum number of points of attachment on each histone molecule will be on the average of seven for H1 and H5, and four for the other fractions, assuming that the histone molecules are fully extended at the time of coupling. This presumed low number of attaching points probably would not have any large effect on the net charge of the coupled histone molecules. Since the histone is coupled through its lysine residues, the relatively lysine hydrophobic region of the protein chain will not be as physically restricted and might be available for protein to protein interactions.

FIGURE 9



D-Galactose 3,6-Anhydro-L-galactose

A. The Structure of the Repeating Unit of Agarose.
(Reproduced from page 18 ref. 4).



B. Coupling of Histone to Agarose Through a Glycyl Spacer Arm.
(Reproduced from Loeffler & Pierce /25/)

In another study, Ayad and Wilkinson /9/ have examined the binding of calf thymus DNA to calf thymus histones absorbed on kieselguhr (diatomaceous earth) and showed that the binding efficiency of the various histone-kieselguhr for DNA was in the order of

$$H3 > (H2A+H4) \simeq H1 > H2B \ .$$

However, it is difficult to compare this data to the previous ones due to the undefined nature of protein absorption (or immobilization in this instance) on the kieselguhr.

Binding behavior of the heat denatured DNA on the histone-agarose columns is highly reproducible but is difficult to interpret. An early study using the method of equilibrium dialysis by Akimrimisi et al./17/ has demonstrated that histone had higher affinity for native rather than for denatured DNA, whereas the reult of this work clearly indicates that heat denatured DNA, or at least a large portion of it, forms a very tight complex with the histone-agarose which is not dissociated even in 2 M NaCl. The complex formation is at least partially histone mediated since the DNA does not bind to derivatized control agarose at all.

However, a small portion of the applied DNA does not bind as tightly and can be eluted in the salt gradient at a NaCl concentration not much higher than that required for native DNA. Although the results have suggested that the salt eluted fraction may differ from the tightly bound fraction in having more double stranded regions, it seem unlikely that it would account for the observed difference in binding.

That denatured DNA can form a tight complex with other immobilized basic proteins may have already been observed in the early work of Mandell and Hershey /29/. These investigators employed a column of methylated serum albumin (a highly positively charged protein resulting from extensive methylation of its carboxyl groups) adsorbed on kieselguhr and observed that heat denatured bacteriophage T2 or T4 DNA was totally retained on the column even at 4 M NaCl, whereas the native DNA was eluted at low salt. Later works of Roger et al. /30,31/ using partially, and completely (heat) denatured pneumonococcal DNA on a similar type of column have also demonstrated that 35 and 45%, respectively, of the applied DNA had remained bound

on the column when the NaCl concentration was increased to 1.5 M. No explanation for the "unelutable" DNA was given in these studies.

It is of interest to mention at this point some preliminary results on the binding of supercoiled bacteriophage PM2 (Form I) DNA to the same histone-agarose columns, which had not been included in this Thesis. The binding behavior of this DNA is very similar to that of denatured DNA except that the salt eluted fraction is generally so small that it is often barely detectable. The page DNA is only partially dissociated from the histone-agarose by 4 M guanidine HCl, but complete elution from the histone column is attained with 1% SDS (sodium dodecyl sulfate). As mentioned earlier in the Result section, the nonsupercoiled form (Form II) of the same DNA binds very similarly to calf thymus DNA. Different from the denatured DNA situation, however, it is well documented in the recent literature that H1 histones have a much higher affinity for supercoiled SV40 DNA than for its nonsupercoiled form /32-34/, and that giant aggregates can result from such DNA-histone complexes /35/.

Even not knowing the nature of these tight histone-agarose-DNA complexes at this time, it is still of great interest to see that DNAs varying in their secondary or tertiary structure can show such a profound difference in their binding behavior to the histone-agarose. It is also of interest to point out that the similarity in the binding behavior between denatured DNA and the supercoiled DNA may very well be reflecting the transient but frequent occurrence of single stranded regions in the latter molecules.

In conclusion, the results of this work show that even histones covalently bound to the agarose still exhibit DNA binding ability very compatible with that of histones in solution , and therefore strongly suggests the validity for analytical application of the present technique to other interacting systems such as histone-histone interactions, and histone to non-histone chromosomal proteins interactions.

The binding behavior of denatured DNA to the histone agarose seems anomalous and difficult to interpret. On the other hand, it should be clear that such anomalous behavior can most probably be exploitted in the frac-

tionation of different forms of DNA on histone affinity columns.

PART II

INTERACTION OF TWO NONHISTONE CHROMOSOMAL PROTEINS WITH H1 HISTONE AND ITS SUBFRACTIONS IMMOBILIZED ON AGAROSE

INTRODUCTION

Goodwin et al /36/ reported the isolation of a group of nonhistone proteins from a 0.35 M NaCl extract of calf thymus chromatin by a simple 2% trichloroacetic acid precipitation procedure. This moderately heterogeneous group of proteins was designated High Mobility Group (HMG) protein due to the high electrophoretic mobility in low pH gels. Two of these proteins, HMG1 and HMG2, are thought to have structural roles in the chromatin due to their presence in relatively large quqntity (10^5-10^6 molecules each, per nucleus) /37/. Several chemical and physical studies /37-41/ have been published on HMG proteins and it has been shown that HMG1 can form a complex with H1 histone /41/, can bind to DNA at ionic strength up to 0.4 M NaCl /40/, and can stimulate chromatin template activity /37/.

Current models on the subunit structure of chromatin /42-44/ generally assume that the smaller and less lysine rich histones H2A, H2B, H3 and H4 are directly involved in forming the core of chromatin subunits

(also known as nu bodies /45/ or nucleosomes /46/). The H1 histones are thought to organize these subunits into higher ordered structures. Assuming that HMG proteins do participate in the superstructure of chromatin, it would be likely that they do so by interacting with H1 histones. This study is an attempt to investigate such interaction using affinity chromatography on immobilized H1 columns.

EXPERIMENTAL PROCEDURE

Total HMG Proteins

All preparative procedures were performed at 4°C unless mentioned otherwise. HMG proteins were isolated from the calf thymus according to the methods of Goodwin et al./47/. In summary, 300 g of calf thymus tissue (Pel-Freez Biologicals, Rogers, Ark.) was homogenized in a blendor and washed repeatedly in a solution containing 7 mM NaCl, 25 mM EDTA, pH 7.5, then extracted three times with 250 ml each of a 0.35 M NaCl pH 7.0 solution. The extract was made 2% in trichloroacetic acid (TCA) by adding 0.02 volumes of 100% (w/v) TCA to precipitate the LMG (Low Mobility Group) proteins. HMG proteins in the supernatant were precipitated by adding 15 ml of conc. NH_4OH per liter of supernatant, followed by rapid addition of 3 volumes cold acetone. The HMG protein precipitate was washed twice with 0.1 M HCl/acetone (1:6 v/v) and twice with acetone, then vacuum dried and stored at -20°C until use.

H1 Histones

H1 histones were extracted with 5% perchloric acid from calf thymus chromatin which had been pre-extracted with 0.35 M NaCl for nonhistone proteins. The extract was extensively dialysed in distilled water before lyophilization and storage at -20°C. Fractionation of H1 into subfraction 1, 2 and 3 was done according to the method of Kinkade and Cole /28/ by gradient elution chromatography on a BioRex 70 column at room temperature, using a linear gradient of guanidine chloride (8.5 to 14%) conatining 0.1 M sodium phosphate buffered at pH 6.8. Appropriate fractions were collected, dialysed in distilled water and then lyophilized before coupling to agarose.

HMG1 and HMG2 Proteins

A mixture of HMG1 and HMG2 was prepared by adding 10 mg of vacuum dried total HMG proteins to 10 ml of chilled phosphate buffer (10 mM Na phosphate, 5 mM $NaHSO_3$, pH 7.0). Insoluble material which consisted of the bulk of HMG proteins other than HMG1 and (to a lesser extent) HMG2 were centrifuged out, leaving in the supernatant a mixture of HMG1 and HMG2 proteins.

Affinity Chromatography

Electrophoretically pure H1 histone and its three chromatographic subfractions were each coupled to agarose (Biogel A-15m, BioRad Laboratories) according to the method of Loeffler and Pierce /25, also see Part I for summarized procedure/. This method allowed histone molecules to be quantitatively coupled to the agarose through a glycyl spacer arm using the glycyl azide derivative of the agarose as the activated species. Histone-agarose (1 ml, containing 2.5 mg of coupled histone) was packed into columns made from pasteur pipettes. Histone-agarose columns were stored at 4°C in the phosphate buffer and were reusable for up to six months in terms of their ability to bind DNA. Such DNA binding experiments were done using the procedures described in Part I. NaCl concentration required to elute calf thymus DNA from the H1 column was 0.6 M.

Chromatography of HMG proteins was performed at room temperature. A mixture of HMG1 and HMG2 (3 ml of a 0.35 mg/ml solution in phosphate buffer) was applied to the column, which was then washed with 2 ml of buffer,

followed by a discontinous gradient of sodium chloride (0.0 M to 0.5 M in buffer). The gradient was effected by layering 0.5 ml increments of buffer with increasing ionic strengths on the gel column while collecting 0.5 ml fractions. Increase in salt concentration was 0.025 M per increment. Elution of HMG proteins from the columns was monitored by absorbance at 280 nm and polyacrylamide gel electrophoresis. The NaCl gradient was measured by refractive index, using known NaCl standards in buffer.

Protein Determination

Protein concentration was either determined by dried weight of lyophilized samples which had been extensively dialysed in distilled water, or by a modified method of Lowry et al /27/.

Electrophoresis

Histone and HMG proteins were electrophoresed in 1.5 mm slab gels using the acid-urea system according to Panyim and Chalkley /24/ with the exception that a 20% acrylamide gel was used instead of 15%. LMG proteins and the 0.35 M NaCl extracted nonhistone proteins were electrophoresed in an 8.75% acrylamide gel system

in the presence of SDS according to Laemmli /48/.

RESULT

Isolation of HMG1 and HMG2

The 0.35 M NaCl extract consisted of a highly heterogeneous group of nonhistone proteins which was qualitatively similar to the total nonhistone proteins of the chromatin preparation (Fig1A, patterns 1 & 4).

× Most of the proteins in the 0.35 M NaCl extract ~~was~~ were precipitated by 2% TCA, leaving in the supernatant the HMG proteins. The HMG proteins migrated electrophoretically in the SDS gel as a single band, corresponding to a molecular weight of roughly 24,500 daltons (Fig. 10A, patterns 2 & 3), as estimated by using bovine serum albumin and rabbit muscle dehydrogenase as molecular markers. The total nonhistone proteins were resolved into nine observable bands by electrophoresis in a 20% acrylamide acid-urea gel (Fig.10B, pattern 1). The banding pattern matches reasonably well with that reported by Goodwin et al./36,37/, who used a slightly different gel system /49/.

FIGURE 10

(A) Polyacrlamide gel electrophoresis pattern of HMG & other nonhistone proteins during extraction.
1. 0.35 M NaCl extract of calf thymus chromatin;
2. LMG proteins; 3. HMG proteins; 4. Chromatin pellet after extraction with 0.35 M NaCl.
Electrophoresis was performed on a 16x12x0.15 cm gel slab for 3 hrs. at 32 mA. 50 ug of protein was applied per gel slot. The gel consisted of a 3.3% acrylamide stacking gel, 8.75% separating gel, and 0.1% SDS in Tris buffer as described by Laemmli /48/.

(B) Polyacrylamide gel electrophoresis pattern of total HMG and HMG1 & 2 mixture in acid-urea gels.
1. Total HMG protein (8 ug);
2. HMG1 & 2 mixture (4 ug).
Proteins were dissolved in the same buffer consisting of 0.9 N acetic acid, 4 M urea, 2% 2-mercaptoethanol and 25% glycerol, heated in boiling water for 2 min., and layered on the gel. Electrophoresis was performed in a 16x12x0.075 cm gel slab, for 3.5 hrs. at 16 mA. The gel conatined 20% acrylamide and was otherwise described by Panyim and Chalkley /24/.

(A)

1 2 3 4

66,500

33,500

(B)

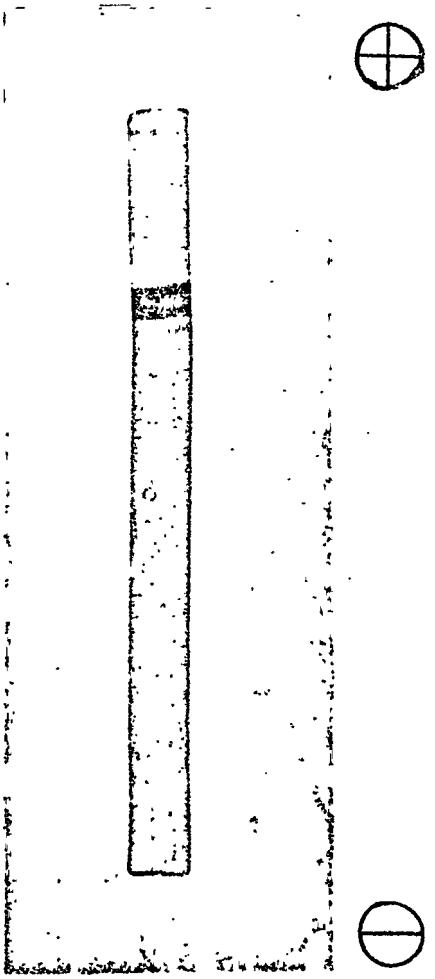
1 2

Fig. 10
pg. 46

FIGURE 10

(C) Polyacrylamide gel electrophoresis pattern of purified calf thymus H1 histone.
Electrophoresis was performed on a 0.4 x 8 cm cylindrical gel, at 2 mA per gel for 2.5 hrs. 4 ug of histone was used. Procedures were otherwise identical to (B).

fig. 10
pg. 47.



Although the total HMG proteins in vacuum dried form was soluble in dilute acid and in a concentrated salt solution, it was found that all the proteins except HMG1 and HMG2, were insoluble in the chilled phosphate buffer used here, and could be sedimented in a clinical centrifuge. This finding is contrary to previous reports /36,37/ that total HMG proteins are completely soluble in water. It did, however, enable the isolation of a moderately pure mixture of HMG1 and HMG2 proteins for these studies (see Fig.10B, pattern 2).

The chromatographic separation of H1 subfractions 1(a,b), 2 and 3(a,b) is shown in Figure 11 and is similar to previous reports in the literature /28,50/.

Binding of HMG1 and HMG2 to Histone-agarose Columns

The HMG mixture was resolved into two peaks after application to the H1-agarose column (Fig.12A). The runoff peak was shown to be HMG2 by gel electrophoresis while the second peak, which was eluted with a dilute NaCl gradient, was shown to be HMG1 (Fig.13). Other experiments in which 10 mM 2-mercaptoethanol was included in the buffer gave the same result.

Similar results were obtained using the columns

FIGURE 11

Chromatographic profile for H1 histone subfractions of the calf thymus.

Chromatography was performed according to Kinkade & Cole /28/ on a 1.4 x 17 cm column of BioRex 70, eluted with a linear gradient of guanidine chloride (8.5 to 14%, 250 ml total volume) at a flow rate of 1 ml/hr. One ml fractions were collected. Peak 1(a,b), 2 and 3(a,b) represent the three H1 components used in this study. H1 subfraction nomenclature corresponds to that used by Smerdon and Isenberg /51/.

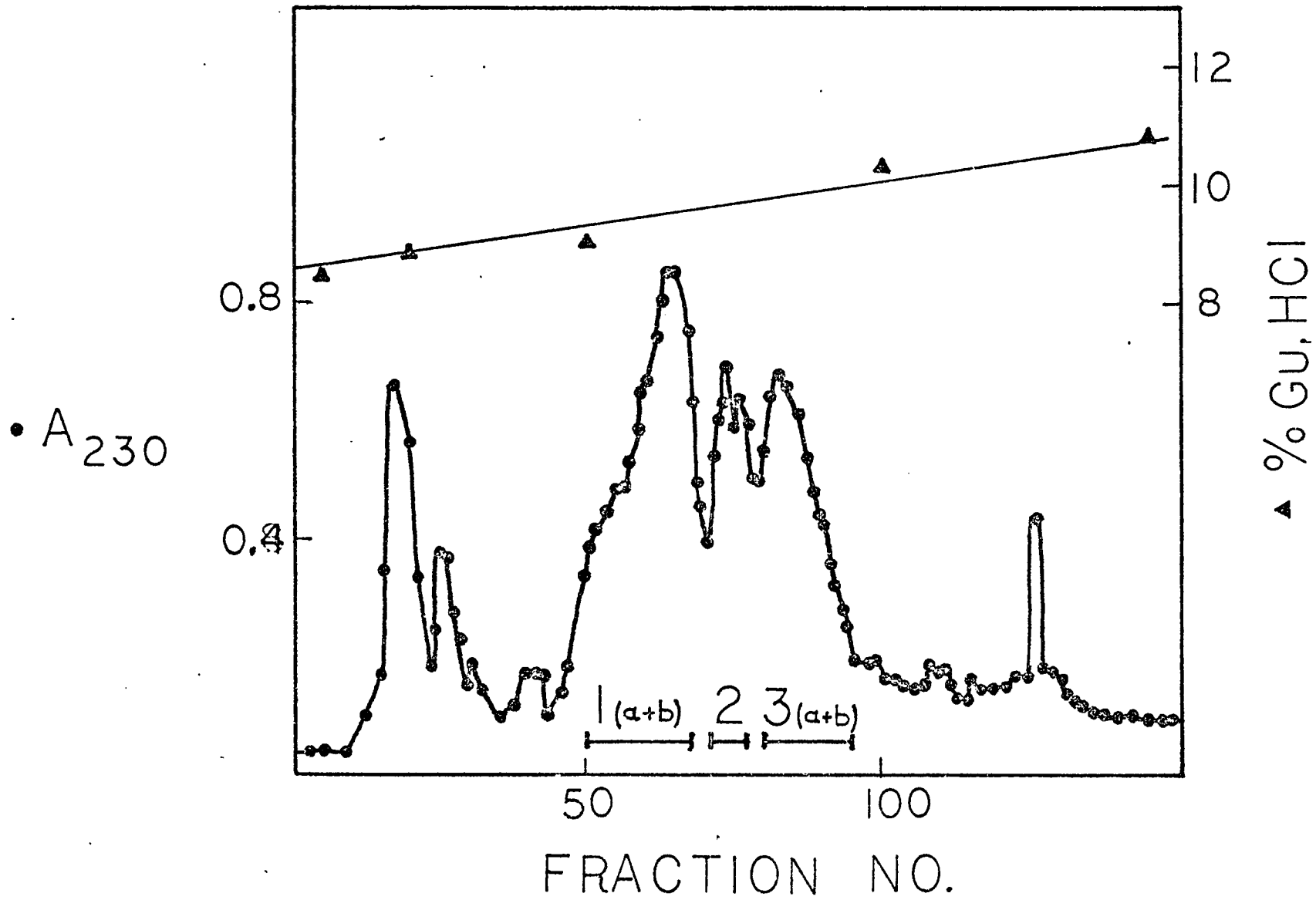

• A230
0.8
0.4
12
10
8
▲ % Gu, HCl
1(a+b)
2
3(a+b)
50
100
FRACTION NO.

FIGURE 12

Chromatographic profile for the binding of HMG1 & HMG2 proteins to H1-agarose columns.
One mg of HMG1 & 2 mixture in 10 mM phosphate buffer (containing5 mM $NaHSO_3$ pH 7.0) at 0.35 mg/ml was applied to each column, followed by a discontinous gradient of NaCl (0.00 M to 0.50 M) containing the same buffer. Each column (0.5 cm diameter) had a gel bed volume of 1 ml and contained 2.5 mg histone. Fractions of 0.5 ml were collected and read at 280 nm. Salt concentration in the effluent was determined by refractive index.

(A) H1 histone column, a mixture of all components;
(B) H1 component 1(a,b);
(C) H1 component 2;
(D) H1 component 3(a,b).

fig 12
pg. 50

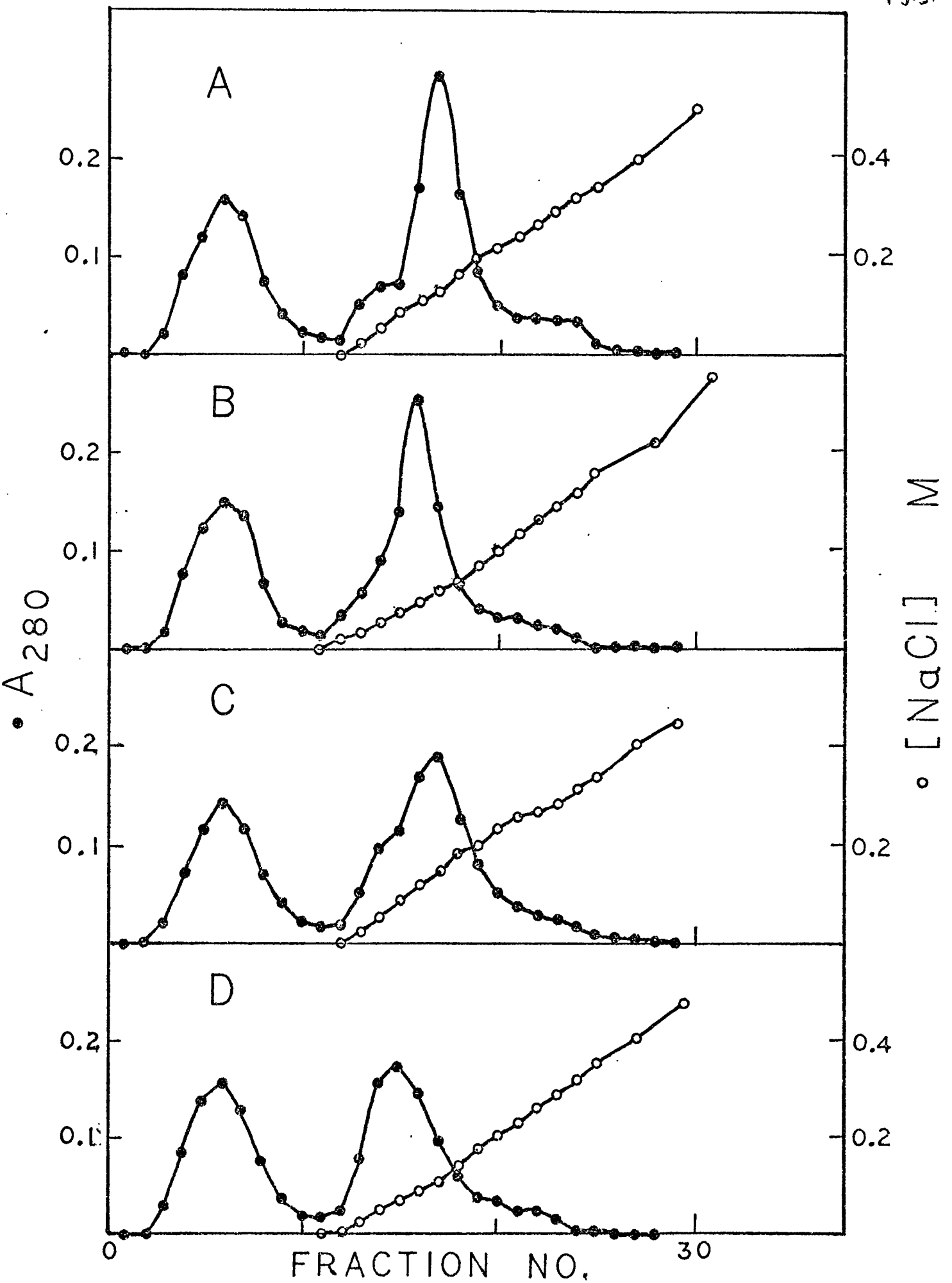
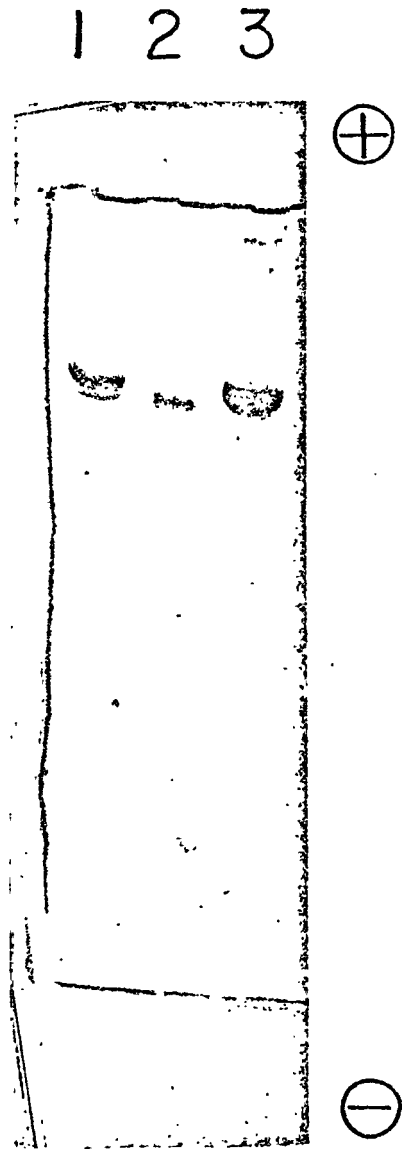


FIGURE 13

Acid-urea electrophoresis pattern showing the nature of the two protein peaks in Fig. 3A.
Procedure was identical to that decribed in Fig.1B.

1. Mixture of HMG1 & 2;
2. Peak 1 (HMG2);
3. Peak 2 (HMG1).


1 2 3
⊕
⊖

made from the three individual H1 subfractions. However, while the profile and postion of Peak 1 (HMG2) were all nearly identical in the three corresponding chromatograms, the elution postion of Peak 2 (HMG1) varied considerably (Fig.12B, C & D). By superimposing and replotting this same data using effluent NaCl concentration as the abscissa (Fig.14), it became more obvious that while Peak 1 was eluted from each column in nearly the identical position, Peak 2 (HMG1) was eluted at different ionic strengths. The NaCl concentration required to dissociate the HMG1 from the histone-agarose decreased in the order of component $2 > 1 > 3$.

In a control experiment, bovine serum albumin was coupled to agarose under the same condition as histone and its affinity for HMG1 and HMG2 proteins was examined. Both the HMG proteins were eluted from the column with the starting phosphate buffer and showed no affinity for the immobilized BSA or the agarose matrix itself.

FIGURE 14

Chromatographic profile showing preferential binding of HMG1 protein to H1 component 1, 2 and 3.
Data from Fig. 3B, C & D were combined and replotted using salt concentration of the effluent as abscissa. Part A of the plot, representing the elution profile of HMG2 (noninteracting species), is drawn by merely superimposing the profiles corresponding to the first ten fractions of Fig. 12 B, C and D.

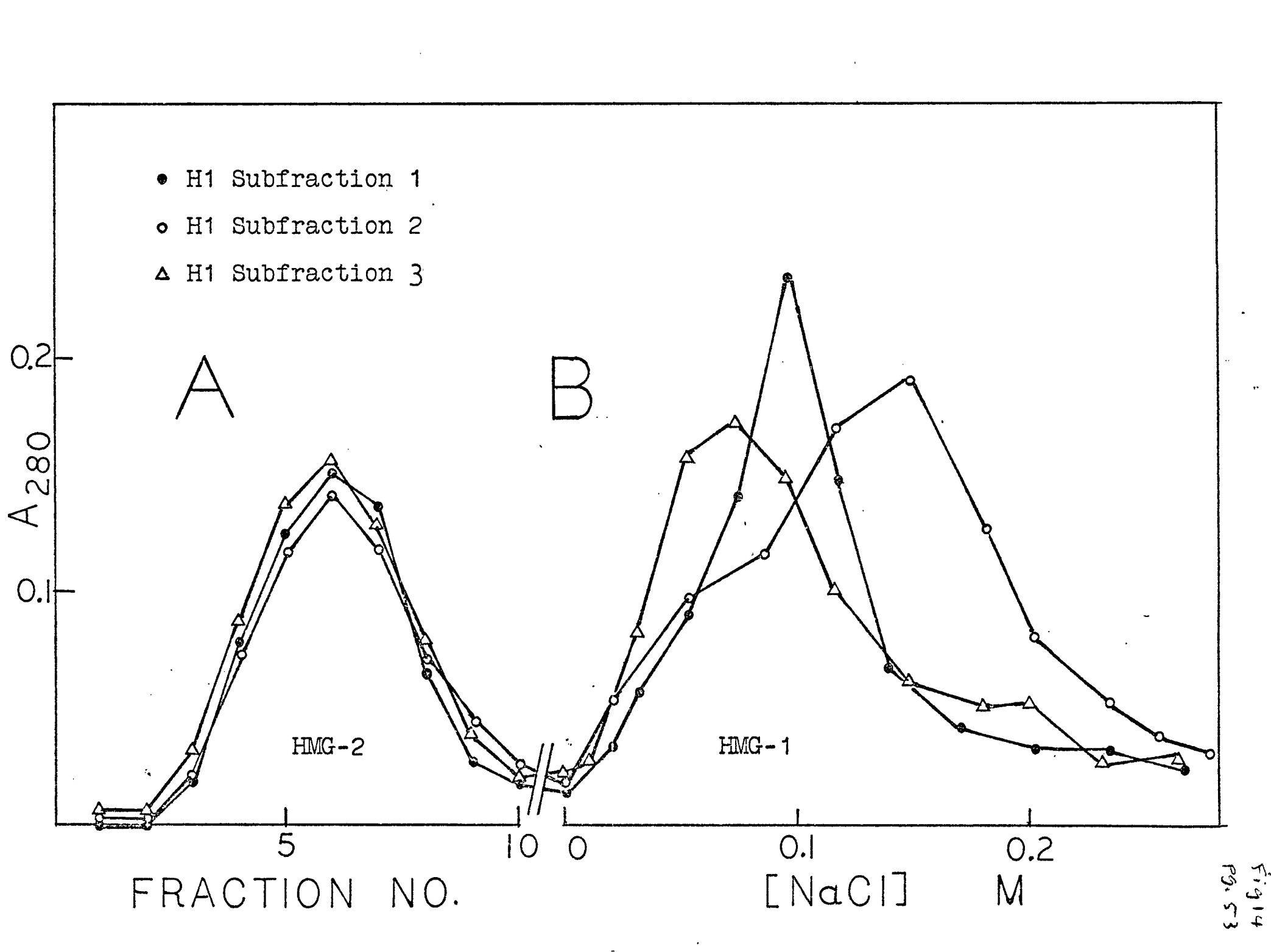

H1 Subfraction 1
H1 Subfraction 2
H1 Subfraction 3
A
B
0.2
0.1
A_{280}
HMG-2
HMG-1
5
10
0
0.1
0.2
FRACTION NO.
[NaCl] M

Fig 14
Pg. 53

DISCUSSION

Histone Affinity Columns In Nonhistone Chromosomal Protein Fractionation

It was initially anticipated that histone affinity columns might be useful for the fractionation of a more complex mixture of nonhistone proteins, such as the 0.35 M NaCl extracted proteins from the calf thymus chromatin (see Fig.10). Prior to this work, similar studies were done using chicken erythrocyte H1, H3 and H5 affinity columns, and the nonhistone proteins derived from the same source using the procedure described by Gronow and Griffiths /52/. The extracted nonhistone proteins were soluble in a solution of 10 mM Tris-HCl pH 7.4, containing 0.1% 2-mercaptoethanol and 10% glycerol, at a low protein concentration of 0.1 mg/ml. When this protein solution was applied to the histone column and eluted stepwise with buffer, followed by 0.5 M NaCl in buffer and then 6 M guanidine-HCl, three protein peaks were observed. These protein peaks were all qualitatively similar as shown by SDS gel electro-

phoresis. The result was common to all three histone columns that were examined, and seemed to suggest that nonspecific aggregation of the complex mixture of nonhistone proteins with the histone-agarose had occured.

It was this inability to show specific interactions between immobilized histones and a complex mixture of nonhistone proteins that led to this study, using the more soluble and less complex mixture of HMG proteins described by Johns et al./36,37/. Nonspecific binding of nonhistone proteins to immobilized histones has also been recently documented by McCleary et al., using immobilized beef liver histones and phosphorylated nonhistone proteins /53/. They observed some specificity of interaction, however, when the nonhistone protein was slowly annealed to the immobilized histone using a gradient dialysis procedure. Given the tendency of isolated nonhistone proteins to aggregate and become insoluble, it does not seem surprising that a rapid column procedure is inadequate to achieve specific binding. The use of slow, gradient dialysis from denaturing solvents is probably a more promising, although

slower procedure, for the fractionation of complex nonhistone chromosomal protein mixtures. The results of this work with HMG protein mixture suggest that similar mixture of more monodisperse proteins can successfully be fractionated using immobilized H1 columns. In this case the system was quite simple since HMG1 and HMG2 were the only two proteins which were recovered in a soluble form from the HMG protein mixture.

Interaction of HMG1 and HMG2 with H1 Histones

The observed differential binding of HMG1 and HMG2 to immobilized H1 columns (Fig.12) seems to suggest the nature of interaction to be primarily electrostatic. HMG1 and 2 have approximate pI's of 6.5 and 8.5 respectively /38/, corresponding to an acidic, and a basic protein in the neutral pH buffer used in the binding experiments. Based on simple electrostatic assumptions, HMG2 should not bind to H1-agarose whereas HMG1 should bind and should be dissociated by increasing the ionic strength. The results seem to agree with this prediction. Although comparative chromatography of HMG1 and 2 on anion exchange columns has not been reported, behavior of HMG1 and 2 on carboxymethyl cellulose or

Sephadex ion exchangers follows a predicatable order of binding based on ion exchange expectations. (HMG1 binds weakly to carboxymethyl ion exchangers relative to HMG2 /37,47/)

Smerdon and Isenberg /54/ have recently shown that different chromatographic subfractions of H1 (which are known to be different in amino acid sequence) bind HMG proteins differently. Using ultracentrifugation, fluorescence anisotropy and circular dichroism, they find that HMG2 interacts weakly with H1 subfractions 3a and 3b but not at all with subfraction 1a or 2; and that HMG1 interact strongly with subfractions 1b and 2, and somewhat weakly with subfractions 3a and 3b.

Affinity chromatograms obtained in this work also indicated differential binding of HMG1 and HMG2 to the H1 columns (HMG1 binds to all three chromatographic H1 subfractions but HMG2 does not bind to any), as well as a small difference in the binding affinity of HMG1 for various H1 subfractions, in the order of $2 > 1(a,b) > 3(a,b)$.

These reults differ somewhat from those of Smerdon and Isenberg in that no evidence of interaction between

HMG2 and any of the H1 subfractions was observed. However, weak interactions detected by their physical techniques may be too subtle to be distinguished by H1 column chromatography. More striking is the qualitative similarity between this observation on differential binding of HMG1 to the different H1 subfraction columns and those of Smerdon and Isenberg cited above. This similarity can not be explained on the basis of any obvious artifacts. Preparation of histone-agarose, as well as affinity chromatography for each H1 subfraction, was performed at the same time, under identical conditions. Consistency of the histone-agarose columns in terms of DNA binding activity has been clearly established in Part I of this Thesis.

The observed differential binding is probably not simply due to the different ion exchange properties of the H1 subfraction columns, since the overall net charge difference on the H1 subfractions is only 2 or 3 out of a net charge of about +57 to +60, as calculated from published amino acid compositions /50/. It is believed that a more specific type of interaction is being measured here, especially in light of the evidence

from C.D. and fluorescence measurements /51/ that salt (0.05-0.15 M NaCl) induced conformational change in H1 subfractions 3a and 3b are produced at lower salt concentrations than for subfractions 1 or 2. Ithas been proposed (T. G. Spring, personal communication) that the observation on differential elution are the result of a conformational change induced in H1 by the NaCl used to elute the column. The conformational change induced in H1 is thought to be near the center of the molecule in a relatively hydrophobic, lysine poor region of the histone molecule /51/, whereas in this study, the H1 molecule is most likely to be coupled to the agarose gel through the basic N or C terminal ends. Based on the data of Loeffler and Pierce /25/, it is calculated that the average density of reactive groups on the glycyl-agarose is approximately 1 per 1000 nm^3, which would allow an H1 molecule 72 nm long (fully extended) to be coupled at a maximum of about 7 sites. It is suggested that the hydrophobic, lysine poor region near the center of the histone is probably not covalently coupled to the agarose, but is relatively free to bind HMG1, and also to undergo a salt induced conformational

change which results in the release of HMG1. Other interpretation is possible and should be explored.

One unresolved problem with the column results is that the H1 column elution profiles of HMG1 are not all symmetrical, suggesting the possibility of microheterogeneity in HMG1. This possibility has not been explored but it is noted that isoèlectric focusing of HMG1 by Walker et al /38/ has shown four or more bands in the pI region of 6-7.

With the supporting evidence of Smerdon and Isenberg /54/ and the previous success in applying the same technique to histone-DNA interactions (see Part I), it is safe to assume that the observed HMG1-H1 binding behavior is not an artifact arising from the immobilized nature of the histone molecules. We must now ask the question: what is the physiological significance of these HMG1-H1 interactions.

The possibility of HMG1-H1 complexes existing in the chromatin has been raised by Smerdon and Isenberg /54/ who suggested that the supposedly H1 dimer observed by Thomas and Kornberg /55/ in a chromosomal protein cross-linking experiment might actually be the HMG1-H1

dimer they observed in solution. Their argument was was based on the molecular weight of the cross-linked protein product, and on other evidence that H1 does not form dimers. The results of this work, however, seem to suggest that HMG-H1 complexes observed as such, probably would not exist inside the nucleus, since the HMG1 proteins can be dissociated from the H1-agarose in subphysiological ionic strengths of about 0.05 M to 0.15 M NaCl. The HMG1-H1 dimer observed by Smerdon and Isenberg was in a buffer containing only 30 mM Na phosphate, and the effect of increasing ionic strength had not been determined. Nonetheless, it should be clear that the possibility of HMG1-H1 complex through other modes of interactions than from what is observed, inside the nucleus where other chromosomal constituents are present, has not been excluded. It has been reported that HMG1 had a high affinity for DNA /39/. On forming complex with DNA, HMG1 protein retains a somewhat acidic region of the protein chain in its native folded structure, completely free of DNA /39,56/. As part of its charge being neutralized by the bound DNA, such acidic region would interact with histones or other

nuclear components in a fashion different from that observed in the column technique where DNA is absent. It is of interest to note that DNA can complex with large excess of HMG1 protein (at protein/DNA ratio of up to 5.0) yet remain soluble /39/. This is quite contrary to H1 histone which is known to cause DNA precipitation, and its presence is a necessity for the condensation of chromatin fibers at the salt range of 0.1 to 0.4 M NaCl /57/. Given the presumption that HMG1's presence in large quantity is an indication of a structural role in the chromatin and given its ability to stimulate chromatin template activity /37/, one is tempted to suggest that HMG1 may perform a structural function in the chromatin by acting as a H1 antagonist, or that it substitutes for H1 in some template active regions inside the nucleus, allowing that part of the chromatin fiber to relax and become more accessible to various functional proteins. This possibility and others may best be explored by protein cross-linking experiments of the chromatin using reversible cross-linking reagents such as those reported by Peretz et al /58,59/,

and by locating the presence of HMG proteins in the active or inactive regions of the chromatin using the available chromatin fractionation techniques /60,61/.

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