bind to enzyme before GSH does. It might appear that the observations from affinity chromatography suggest a different order of binding since the enzyme attaches to adsorbent, having active arms resembling IV, in the absence of maleylacetone. This is not really a contradiction. Free enzyme might be able to bind GSH in the absence of maleylacetone but may result in a nonproductive complex. There are a few cases of ordered sequential mechanisms where high concentrations of the second substrate appear to inhibit the reaction because binding of the second substrate first leads to an inactive species incapable of further reaction unless it first dissociates (Dalziel, 1957).

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Conformational Changes in Subfractions of Calf Thymus Histone H1†

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ABSTRACT: This paper presents the first study of conformational changes in the subfractions of calf thymus H1. H1 was fractionated by the method of Kincade and Cole (Kincade, J. M., and Cole, R. D. (1966), *J. Biol. Chem. 241,* 5790) using a very shallow Gdn-HCl gradient. A possible new H1 subfraction, about 5–8% of the H1, has been found and characterized by amino acid analysis and electrophoresis. The effects of salt concentration and pH on the conformation of each of the four major subfractions have been studied by measuring the fluorescence anisotropy of the tyrosine emission and the circular dichroism (CD) of the peptide bond. Upon the addition of salt to aqueous solutions at neutral pH, all four subfractions show an instantaneous change in fluorescence anisotropy, fluorescence intensity, tyrosine absorbance, and CD. The folding associated with this instantaneous change is highly cooperative, and involves the region of the molecule containing the lone tyrosine, which becomes buried in the folded form. The folding of subfraction 3a is more sensitive to salt than the other major subfractions. Upon folding, approximately 13% of the residues of subfractions 1b and 2 form α and β structure; 3a and 3b have approximately 16% of the residues in α and β structures. There is no evidence for interactions between the subfractions. In salt-free solutions, each of the four major subfractions shows very little change in conformation in going from low to neutral pH, but each shows a very sharp transition near pH 9. This transition gives rise to a marked increase in fluorescence anisotropy and fluorescence intensity, and involves the formation of both α and β structure in a manner similar to that of the salt-induced state.

Histones have been known since 1884 (Kossel, 1884), but it was only the discovery of the subunit structure of chromatin (Hewish and Burgoyne, 1973; Woodcock, 1973; Olins and Olins, 1973, 1974; Sahasrabuddhe and Van Holde, 1974; Kornberg, 1974; Noll, 1974) that established at least some of the functions of histones H2a, H2b, H3, and H4. Compared to our knowledge of the non-H1, or inner histones,[1] our understanding of H1 is meager indeed. It is known that H1 is heterogeneous (Kincade and Cole, 1966a,b; Bustin and Cole, 1968, 1969; Kincade, 1969; Panyim and Chalkley, 1969a; Fambrough and Bonner, 1969; Langan et al., 1971; Seale and Aronson, 1973; Stout and Phillips, 1973; Sherod et al., 1974; Ruderman et al., 1974; Ruderman and Gross, 1974; Gurley et al., 1975; Spiker, 1976), in contrast to the evolutionary conservation of the inner histones, especially H3 and H4 (DeLange et al., 1969; DeLange and Smith, 1971; Panyim et al., 1971). Evidence has been presented that H1 is, in some fashion, involved in chromatin condensation and mitosis (Littau et al., 1965; Mirsky et al., 1968; Bradbury et al., 1973a), with the phosphorylation of H1 perhaps serving as a trigger for mitosis (Lake and Salzman, 1972; Lake, 1973;

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[1] In this paper, histones H2a, H2b, H3, and H4 will be called the *inner histones* (Isenberg, 1976), since they are inside of the nucleosomes.

Bradbury et al., 1973b, 1974a,b; Gurley et al., 1974, 1975; Balhorn et al., 1975). However, despite all this, it must be acknowledged that we are only in a primitive position in understanding the function of H1.

The work of the past few years on the structural and cross-complexing properties of the inner histones (Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1972, 1973, 1974a–e; Smerdon and Isenberg, 1974; Van Holde and Isenberg, 1975; Isenberg, 1976) played an essential part in developing current ideas of chromatin structure, simultaneously, of course, pinpointing one important function of these histones. It appears possible, and perhaps likely, that a structural study of H1 will be important in understanding the cross-complexing and functioning of H1. To this end, we have studied the physical properties of H1, which we report here.

H1 is commonly prepared by the use of solvents that denature proteins. As will be seen below, H1, so obtained, is strung out in a more or less unfolded, and denatured, condition. We have no way of knowing what the native state is, but we can be reasonably sure that the *unfolded* form is *not* it. Furthermore, at the present time, it is not possible to give a functional criterion for the native state of H1. In contrast to studies on enzymes, for example, we have no ready test. Nevertheless, we know that when enzymes are renatured from an unfolded state they undergo a folding to a compact form, usually in a highly cooperative manner (Baldwin, 1975). For this reason, in the absence of a better criterion, we might, at least tentatively, adopt an indirect criterion for a presumptive native state of H1. However, since there is really no functional reason to call any particular state native, we shall, in this paper, use the terminology folded and unfolded, rather than native and denatured. Nevertheless, the implication is clear that the folded state could reasonably be either the native state or close to it.

In the following paper of this issue (Smerdon and Isenberg, 1976) we will show that the various subfractions of calf thymus H1 differ dramatically from each other in binding to certain nonhistone chromosomal proteins. These findings demand that a careful investigation be made to see if distinct structural differences in the subfractions can be found. This is one of the goals of the present paper.

It will be shown below that each subfraction of H1 has a highly cooperative transition from an unfolded to a folded form. As already indicated, it is much more reasonable, when studying interactions of H1, to study them in the folded state. This was essential, in fact, in studying the inner histones (Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1972, 1974a–e) and helped elucidate certain binding and structural characteristics of these histones. Similar work on H1 may lay the structural framework for understanding the functions of the H1 subfractions. This is another aim of the present paper.

To date, there are only a few papers in the literature devoted to physical studies of the conformation of H1 (Boublik et al., 1970; Vladimirov et al., 1970; Bradbury et al., 1967, 1972, 1975) and these are limited to studies on unfractionated H1. We have asked: Do the subfractions have the same or different structural features? To this end, we have fractionated H1 using the method of Kincade and Cole (1966a) but applying a very shallow Gdn-HCl gradient to achieve high resolution. In the process, we have discovered what we believe is a new subfraction comprising about 5–8% of the total calf thymus H1.

A number of H1 subfractions have been partially sequenced (Rall and Cole, 1971; Bustin, 1972; Jones et al., 1974; Arutyunyan et al., 1975; Dixon, 1975; Dixon et al., 1975). There are three regions: a relatively short basic one at the N-terminal end, a much longer basic region at the C terminus, and, between these, a nonbasic region containing one tyrosine at, or near, position 72. Bradbury et al. (1975) have reported that it is the central region that undergoes most of the salt-induced conformational change. The tyrosine at position 72, the only tyrosine in H1, is in this region and acts as an excellent intrinsic fluorescence probe for following conformational changes.

In this paper we study the physical properties of the individual major subfractions, concentrating on the folding that occurs either upon the addition of salt at neutral pH, or upon raising the pH to higher values. In the following paper of this issue (Smerdon and Isenberg, 1976), we shall show that there are striking binding differences between the various H1 subfractions. We believe that this is the first physical study of the subfractions of H1, and the first time it has been reported that the H1 subfractions have markedly different binding properties.

Materials and Methods

Preparation of Unfractionated H1. Calf thymus glands were frozen quickly in CO_2 within 5 min of the animal's death, and transported to the laboratory frozen. Chromatin was extracted at 4 °C by method B of Busch (1968), modified in either of two ways to minimize proteolysis. In the first modification, the extraction buffer contained 0.05 M $NaHSO_3$ at pH 5.0 (Panyim et al., 1968; Bartley and Chalkley, 1970). In the second modification, the extraction buffer was 0.14 M NaCl, 0.05 M acetate, pH 5.0, containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF)[2] (Ballal et al., 1975). In using the latter procedure, a stock solution of 0.1 M PMSF in 2-propanol was diluted 1000-fold into fresh homogenizing buffer solution prior to each wash, as PMSF is known to eventually hydrolyze in aqueous solution (Gold, 1967).

Crude H1 was prepared by the first method of Johns (1964), yielding an acetone dried powder. At this stage, the preparation contains an appreciable amount of H2B, nonhistone chromosomal (NHC) proteins and some unknown higher molecular weight contaminants. To separate out the H2b and the higher molecular weight contaminants, 100 mg of the dry powder was dissolved in 2 ml of 0.01 N HCl, and run through a 4 cm × 2 m Sephadex G-100 column, equilibrated with 0.01 N HCl. The fractions across the H1 peak were electrophoresed as described below. The last eluting one-third of the H1 peak contained an appreciable amount of two nonhistone proteins, which were identified as the NHC proteins, HMG1 and HMG2 (Goodwin and Johns, 1973). (HMG1 and HMG2 comprised approximately 3–6%, by weight, of each crude H1 preparation.) The middle and front sections of the H1 peak showed only H1; no contaminants were seen by gel electrophoresis. Purified H1 fractions were pooled, lyophilyzed, and stored at −20 °C.

Separation of H1 Subfractions. H1 subfractions were prepared by the method of Kincade and Cole (1966a). Purified whole H1 (150–200 mg) was dissolved in 7% Gdn-HCl (10 ml), 0.1 M NaH_2PO_4, pH 6.8. (The practical grade Gdn-HCl, purchased from Sigma, was purified according to the method of Bonner et al., 1968.) The sample was applied to a 5 × 45 cm Bio-Rex 70 column, equilibrated with 7% Gdn-HCl, 0.1 M Na_2PO_4, pH 6.8. The sample was eluted with a very shallow linear gradient of Gdn-HCl, in which the concentration varied from 9.6 to 11.2% during an 8-day period. The flow rate was

[2] Abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; NHC, nonhistone chromosomal; CD, circular dichroism

50 ml/h. It, and the gradient, were controlled by an ISCO Dialagrad gradient former, Model 382. Fifteen-milliliter fractions were collected. To 0.2 ml of each fraction was added 1 ml of 1.32 M Cl_3CCOOH. This was shaken vigorously and, after 13 min, assayed turbidometrically at 400 nm. Gdn-HCl concentrations were determined on fractions devoid of protein by use of a Zeiss refractometer at 25 °C, according to Bonner et al. (1968). Separate fractions were pooled and concentrated in an Amicon ultrafiltration cell, dialyzed for 24 h against 100 × volume of 0.01 N HCl, with three volume changes, and lyophilyzed to dryness. The lyophilyzed samples were then dissolved in 0.5 ml of 0.01 N HCl, run through a 1.2 × 26 cm Sephadex G-25 column, to assure desalting of the material, and lyophilyzed back to dryness. Samples were stored dry at −20 °C until used.

Electrophoresis. Purity from other protein contaminants, and degradation products, was determined by electrophoresis on 15% acetic acid–urea gels (Panyim and Chalkley, 1969b) and 15% sodium dodecyl sulfate gels (Laemmli, 1970). For the former, 5-mm diameter gels were loaded with 25 and 50 μg of protein, and for the latter, with 25 μg. All purified whole H1 samples, and purified subfractions, showed only one band on both gel systems.

Determinations of the purity of any given subfraction from contamination by the other subfractions were made by running approximately 1 μg of sample on a vertical 1.5-mm thick slab, using a Bio-Rad, Model 200, vertical slab gel electrophoresis unit. Both sodium dodecyl sulfate and acetic acid–urea systems were used. For the sodium dodecyl sulfate slab gels, the three modifications of Thomas and Kornberg (1975) were employed.

The photograph of the acetic acid–urea gel slab presented in this paper was taken using the method of Oliver and Chalkley (1971).

Analytical Techniques. Amino acid analyses were carried out in the standard fashion on a Beckman/Spinco 120B modified automatic amino acid analyzer (Spackman et al., 1958). Hydrolysis was carried out in constant boiling HCl at 110 °C for 22 h in evacuated sealed tubes.

Concentrations of the H1 subfractions in H_2O were determined spectrophotometrically using an extinction coefficient of 1345 cm^{-1} M^{-1} at 275 nm. This value was obtained by assuming the extinction at 275 nm to be the sum of the extinction coefficients of one *N*-acetyl ethyl ester of tyosine (1340 cm^{-1} M^{-1}), as given by Herskovitz and Sorenson (1968), and one phenylalanine in aqueous solution. (The validity of this procedure is verified by the excellent stoichiometry obtained in the complexes of the H1 subfractions (Smerdon and Isenberg, 1976).) Spectra were measured on a Cary 14 spectrophotometer.

Digestion of H1 with alkaline phosphatase (*Escherichia coli*, BAPC, Worthington) was performed by the method of Sherod et al. (1970).

Measurements in solution containing NaCl were performed by dissolving the lyophilyzed protein in 0.01 M cacodylate buffer, pH 7.0, before adding NaCl. (This concentration of cacodylate induced no observable change in the conformation of subfractions 1a, 2, and 3b, and only a small change in 3a. 0.001 M cacodylate buffer, pH 7.0, showed no effect on the conformation of 3a, and gave the same results as the measurements in 0.01 M cacodylate.) Samples were titrated with 5 M NaCl, 0.01 M cacodylate buffer, pH 7.0, with rapid stirring, to the desired NaCl concentration. Measurements in phosphate were performed in a similar fashion. All data were corrected for dilution.

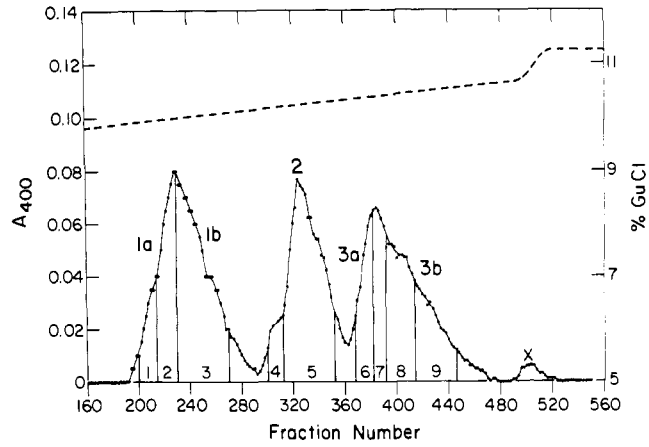

FIGURE 1: Chromatographic profile of calf thymus H1. 150 mg were loaded on a Bio-Rex 70 column (5 × 45 cm) and eluted with a linear gradient of Gdn-HCl (9.6–11.2%) containing 0.1 M sodium phosphate buffer, pH 6.8, as described under Materials and Methods. Sections 1–9 are defined in the text. The Gdn-HCl concentration is given by the dashed line.

pH titrations, measured on a Corning Model 112 pH meter, were performed by dissolving the lyophilyzed protein in 0.01 N HCl and titrating to the desired pH with NaOH.

Fluorescence anisotropy, $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, and fluorescence intensity, were measured on a computer interfaced polarization spectrometer (Ayres et al., 1974). All measurements were made at 20 °C. Samples were excited at 279 nm and the emission was measured at 325 nm.

Circular dichroism (CD) measurements were made on a Jasco Model J-10 CD recorder at 20 °C. Data reported as $\Delta\epsilon$ in units of cm^{-1} l. (mole of residue^{-1}). CD spectra were analyzed using the method of Baker and Isenberg (1976). The reference spectra used for α-helix and β-sheet determination were the CD spectra for poly(L-lysine) (Greenfield and Fasman, 1969). The reference spectra for the random state was the CD spectrum obtained for the individual H1 subfractions in 0.01 N HCl. These choices gave good sum tests and good wavelength invariances (Baker and Isenberg, 1976).

Sedimentation measurements were performed at 20 °C according to the method of Yphantis (1964) using a Beckman Model E ultracentrifuge with interference optics. A value of $\bar{v} = 0.766$, calculated from the amino acid composition (Cohn and Edsall, 1943), was used.

Results

Isolation and Characterization of H1 Subfractions. Figure 1 shows a typical elution profile from the preparative Bio-Rex 70 column. Three major peaks may be seen, as reported previously (Kincade and Cole, 1966a; Bustin and Cole, 1968; Kincade, 1969). The use of a very shallow gradient resulted in excellent resolution. (The small peak marked X may be the subfraction reported by Panyim and Chalkley, 1969a.)

We have divided the chromatogram of Figure 1 into nine sections based on electrophoretic results discussed below. Aliquots from each section were electrophoresed on sodium dodecyl sulfate slab gels (Figure 2a) and acetic acid–urea slab gels (Figure 2b). There are two bands in each of the three major peaks. (These results were found in preparations using either $NaHSO_3$ or PMSF.)

An aliquot from section 3 runs as a single band on the slab gels (Figure 2). In fact, the approximate limits of section 3 were determined in this fashion. Section 1 clearly contains 2 bands and section 2 is a transition region in which more of the second

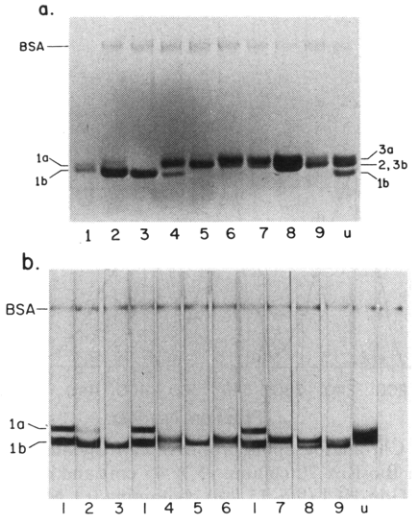

FIGURE 2: Sodium dodecyl sulfate slab gel (a) and acetic acid–urea slab gel (b) of aliquots from sections 1–9 (Figure 1) and unfractionated H1 (denoted "u"). Bovine serum albumin (BSA) marker is included.

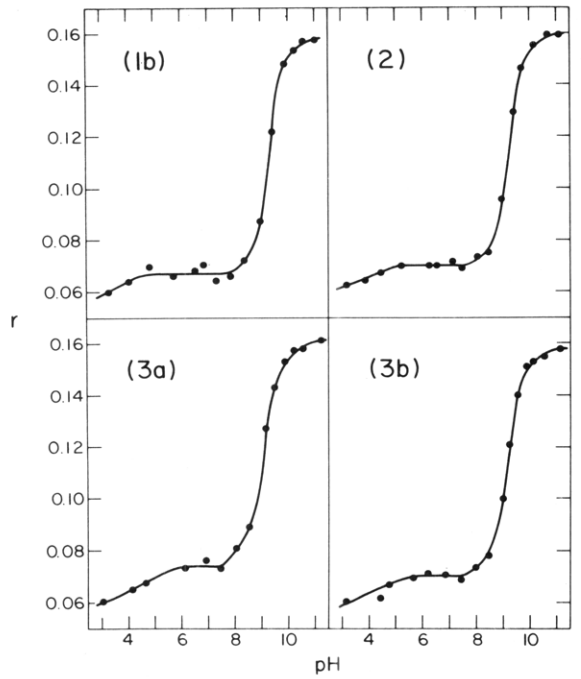

FIGURE 3: Fluorescence anisotropy of 2.0×10^{-5} M solutions of the major H1 subfractions in water as a function of pH. Solutions were titrated with NaOH, and measured at 20 °C.

band is seen as one goes from section 3 toward section 1.

The first peak therefore contains two bands of histone H1, which we designate as 1a and 1b. Kincade and Cole (1966a) found that degradation products appear as fractions eluting either at the front edge of peak 1, or earlier. For reasons to be discussed later, however, it appears unlikely that 1a is a degradation product. 1a comprises 5–8% of whole H1.

Section 5 comprises almost all of peak 2. It shows only a single band on both sodium dodecyl sulfate and acetic acid–urea gels. However, there is a shoulder (section 4) on the front end of the second peak. This shoulder yields the band of section 5 plus a minor component. This band may be a new H1 subfraction comprising less than 1% of the whole H1, but, since it coelectrophoreses with 1b, it may merely be some cross contamination from peak 1. (We have no evidence to discriminate between these two possibilities.)

TABLE I: Amino Acid Composition of Calf Thymus H1 Subfractions.[a]

	H1 Subfraction				
Amino Acid	1a + 1b	1b	2	3a	3b
Lysine	27.5	27.8	28.7	28.9	29.0
Histidine	0	0	0	0	0
Arginine	1.9	2.0	1.5	1.4	2.1
Aspartic acid	2.1	2.0	1.9	2.1	2.0
Threonine	5.8	5.9	5.6	5.5	5.0
Serine	7.1	5.6	6.3	6.0	5.8
Glutamic acid	3.4	3.5	3.4	3.7	3.4
Proline	9.0	9.1	9.7	9.3	9.8
Glycine	6.4	6.5	7.5	7.3	6.9
Alanine	23.7	26.2	24.4	25.0	26.1
Half-cystine	0	0	0	0	0
Valine	6.6	5.3	5.2	4.6	4.0
Methionine	0	0	0	0	0
Isoleucine	1.0	1.1	1.0	1.0	0.9
Leucine	4.5	4.2	4.1	4.3	4.1
Tyrosine	0.4	0.4	0.4	0.4	0.4
Phenylalanine	0.6	0.5	0.5	0.5	0.5
Calculated $\bar{v}$		0.766	0.766	0.765	0.764

[a] Data is presented as mole percent of total amino acid content.

Kincade and Cole (1966a) found two subfractions in peak 3. As Figure 2 shows, we verify their finding. These authors also observed only one subfraction in peak 2. With the possible exception of the minor component in section 4, we also agree with this conclusion. However, we believe our report of component 1a is new.

The mobility of the subfractions on sodium dodecyl sulfate gels follows the pattern: 1b > 1a > 2 ~ 3b > 3a. Therefore, if the separation is due only to the differences in molecular weights between the subfractions, 3a is the largest subfraction and is approximately 1500 daltons larger than the smallest subfraction 1b. However, these numbers may be erroneous, since it has been shown that histones migrate anomalously on sodium dodecyl sulfate gels (Panyim and Chalkley, 1971; Hayashi et al., 1974).

We note that subfraction 1a is the slowest moving component on acetic acid–urea gels, although it is the second fastest component on sodium dodecyl sulfate gels. This suggests that the overall charge characteristics of 1a differs from those of the other subfractions.

Table I shows the amino acid compositions of the four major subfractions, 1b, 2, 3a, and 3b, and a mixture of 1a and 1b, approximately 1:1. (It should also be noted that, as judged by gel scans, 3a and 3b are only 85–90% pure and cross-contaminate each other.) Our values are consistent with, and support, the conclusion of Kincade and Cole (1966b) that 3a has 3 arginines and 3b has 4 arginines.

The (presumptive) new subfraction, 1a, shows the highest amounts of serine and valine and the lowest amount of alanine. As we will discuss later, we believe that these data support the proposal that 1a is a new subfraction, and not a degradation product.

To determine if the resolution seen in Figure 2a was due partly to differential phosphorylation, we digested the H1 with alkaline phosphatase (Sherod et al., 1970). At enzyme to H1 ratios of 1:7, no change in the electrophoretic pattern of Figure 2a was observed, even after 20-h digestion.

Physical Changes During pH Titrations. Each of the four major H1 subfractions shows very little change in conformation in going from pH 3 to 7 (Figures 3, 4, and 5). Each sub-

TABLE II: pH Induced Physical Changes.

A. Fluorescence Anistropies and Intensities

Subfraction	r(pH 7) − r(pH 3)	r(pH 11.2) − r(pH 3)	I(pH 7)/I(pH 3)	I(pH 10.2)/I(pH 3)
1b	0.009	0.100	1.12	2.70
2	0.009	0.099	1.20	3.09
3a	0.014	0.101	1.30	3.35
3b	0.011	0.099	1.20	3.24

B. CD Analyses[a]

	pH 7				pH 10.2				
Subfraction	% α	% β	% R	Sum Test[b]	% α	% β	% R	Sum Test[b]	% α + % β
1b	1.1	−0.1	96.3	97.3	9.1	4.3	80.6	94.0	13.4
2	1.0	1.3	99.2	101.5	8.1	8.1	85.6	101.8	16.2
3a	−0.1	2.9	102.6	105.4	7.9	8.3	87.1	103.3	16.2
3b	1.3	0.1	97.8	99.2	8.4	7.4	83.3	99.1	15.8

[a] Results reported as percent α helix (% α), percent β sheet (% β), and percent random coil (% R) using poly(L-lysine) as reference spectra for α helix and β sheet and spectra of individual H1 subfractions at pH 3 for random coil. [b] See Baker and Isenberg (1976).

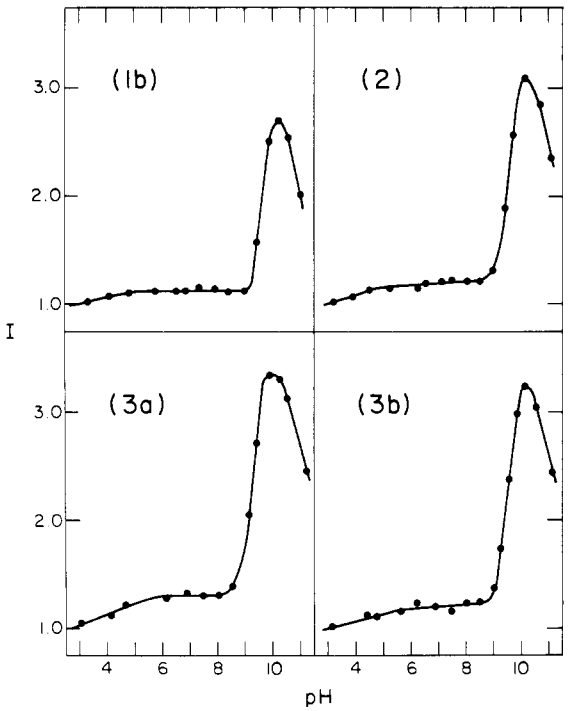

FIGURE 4: Fluorescence intensity, relative to that at pH 3, of the H1 subfractions as a function of pH. Solutions were the same as those for Figure 3.

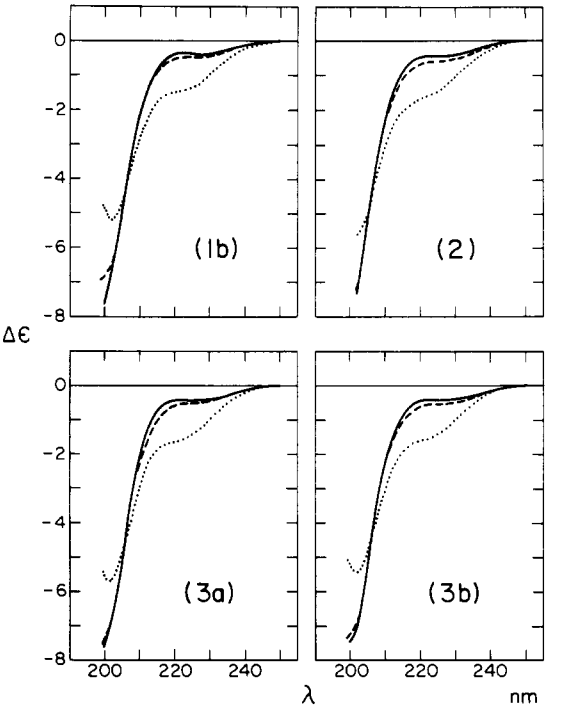

FIGURE 5: CD spectra of 0.5×10^{-5} M solutions of the H1 subfractions in water at pH 3.0 (—), pH 7.0 (- - -), and pH 10.2 (···). Solutions were titrated with NaOH and measured at 20 °C in a 2-mm path length cell.

fraction has a typical random-coil spectrum. As the pH is raised to about 9.1, there is a sharp transition, which is also found for whole H1. There is a small rise in anisotropy in going from pH 3 to 6, but this is not the result of a major folding of the protein, since the CD spectrum hardly changes from pH 3 to 7 (Figure 5). We estimate that only 1–3% of the residues form α and/or β structure (Table II) during this pH change. Because this change in CD is so small, we ascribe no significance to the differences in the CD results found for the various subfractions. However, the fluorescence parameters are different; subfraction 3a shows a larger change in fluorescence anisotropy and intensity than the others (Table II).

Our results are in disagreement with those of Bradbury et al. (1975), who find that the pH-induced conformational change in whole H1 occurs in going from pH 3 to 6. We do not find this and do not understand the source of this disagreement.

The initial increase in fluorescence intensity at pH 9, followed by a decrease in intensity above pH 10.2 (Figure 4), can be easily explained, since two competing processes occur in this pH region. First, the partial titration of the lysine residues in each subfraction molecule results in a conformational change to a folded form. In this form, the mobility of the tyrosine decreases and the anisotropy rises. Also, in the folded form, the tyrosine is in a less quenching environment than the neutral pH conformation, and the intensity goes up. Second, as the pH is raised to still higher values, deprotonation of the tyrosine increases. Tyrosinate has a much lower quantum yield than tyrosine (Truong et al., 1967; Eisinger et al., 1969) and the

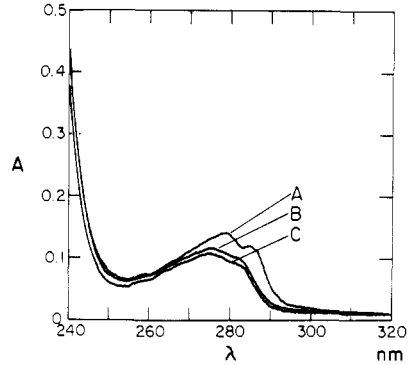

FIGURE 6: Absorbance, A, of 6.9×10^{-5} M solutions of subfraction 2 in H_2O (C), in 0.002 M sodium phosphate, pH 7.0 (B), and in 0.04 M sodium phosphate, pH 7.0 (A).

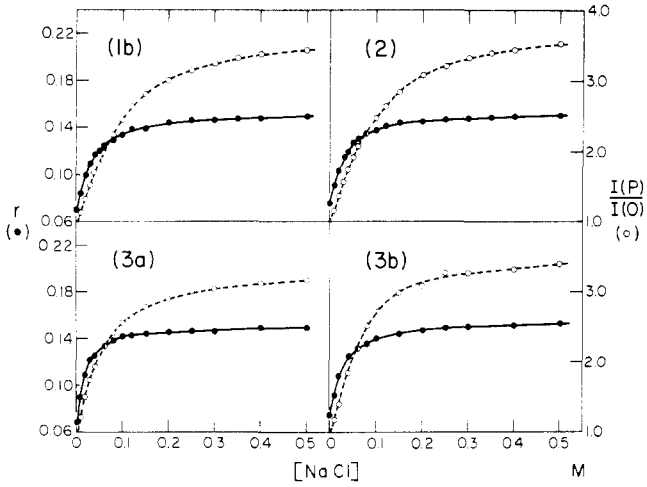

FIGURE 7: Fluorescence anisotropy and relative intensity, at 20 °C, of 2.0×10^{-5} M solutions of the H1 subfractions in 0.01 M cacodylate buffer, pH 7.0, as functions of NaCl concentration. $I(P)$ is the intensity for salt concentration P.

intensity decreases. These two competing events lead to first a rise, and then a fall, in intensity as the pH is raised.

That the pH 9.1 transition is indeed a molecular folding is supported by the CD data (Figure 5). Appreciable amounts of both α-helix and β-sheet form (Table II).

In going from pH 7 to 10.2, the α-helical content jumps from essentially 0 to 8–9% and the β-sheet content goes to 4–8%. The differences in α-helical or β-sheet content for the various subfractions are probably within experimental error; no significance can be attached to the differences. The same may be noted for the anisotropy values of the folded form: all of the subfractions show essentially the same anisotropy.

Conformational Changes at Neutral pH, Induced by Salt Addition. Upon the addition of salt to each of the four major H1 subfractions, there is an instantaneous change in fluorescence anisotropy, fluorescence intensity, tyrosine absorbance, and CD. However, there is no evidence for the slow change that has been observed in studies of the inner histones, H3 and H4 (Li et al., 1972; Wickett et al., 1972; Smerdon and Isenberg, 1973, 1974; D'Anna and Isenberg, 1974; Isenberg, 1976).

There is a striking red shift plus an increase in tyrosine absorbance upon the addition of salt (Figure 6). Accompanying this shift there is an enhancement of the resolution of the fine structure. These changes, particularly the increased resolution, are typical of what is observed when tyrosine, phenol, or, for that matter, a number of different chromophores, move from

TABLE III: NaCl Induced Physical Changes.

A. Fluorescence Anisotropies and Intensities[a]

Subfraction	r_w	$r(\infty)$	$r(\infty) - r(0)$	$I(\infty)/I(0)$
1b	0.067	0.153	0.083	3.9
2	0.070	0.154	0.080	4.0
3a	0.074	0.156	0.083	3.4
3b	0.070	0.156	0.080	3.8

B. CD Analyses[b]

Subfraction	% $\alpha(\infty)$	% $\beta(\infty)$	% R(∞)	Sum Test[c]	% $\alpha(\infty)$ + % $\beta(\infty)$
1b	7.2	6.1	92.8	106.1	13.3
2	4.8	8.5	94.8	108.1	13.3
3a	7.2	8.8	87.3	103.3	16.0
3b	6.5	10.1	93.7	110.3	16.6

[a] $r(P)$ and $I(P)$ are anisotropy and intensity at NaCl concentration P. r_w is anisotropy in water. [b] Results reported as percent α helix, β sheet, and random coil at ∞ salt concentration (see Appendix). Reference spectra are same as those used for Table II. [c] See Baker and Isenberg (1976).

a more polar to a less polar environment (Beaven, 1961; Jaffe and Orchin, 1962; Wetlaufer, 1962, Herskovitz and Sorenson, 1968).

Both the fluorescence anisotropy and the intensity are increasing functions of the NaCl concentrations (Figure 7). As was indicated in discussing the high pH change, this indicates that the tyrosine-containing region of each subfraction undergoes a salt-induced conformational change, which decreases both the mobility of the tyrosine and the quenching of its fluorescence. All of the spectral changes show that the tyrosine is buried in the folded form. Table III (section A) lists the magnitude of the change in spectral parameters for each of the four major subfractions. As can be seen, each subfraction has a low anisotropy in water, where the tyrosine is relatively free to rotate, and a much higher anisotropy in the folded form, the same for each subfraction.

The addition of salt gives rise to marked changes in the CD spectra (Figure 8). We took spectra at different salt concentrations and extrapolated to infinite salt concentration in the manner shown in the Appendix. At infinite salt concentrations the equilibrium has presumably been shifted completely to the folded form and one can thereby determine the amount of α and β structure in the folded form for each subfraction molecule. These results are shown in Table III (section B). Each subfraction shows both α and β structure in the folded form; subfractions 1b and 2 show approximately 13% of their residues in α helices or β sheet, and subfractions 3a and 3b show approximately 16% of their residues in these forms. However, since the error in each measurement is somewhat amplified by extrapolation, the differences in α and β content between the various subfractions may not be significant.

Assuming a two-state model (Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1972), the fraction of molecules in the salt-induced state was calculated from CD data, and compared to the fraction calculated from fluorescence data. Figure 9 shows that, in every case, the functional dependence of this fraction on salt concentration is essentially the same when measured either by fluorescence or by CD. Since these two techniques are sensitive to entirely different properties of the molecule, these data indicate that the folding of the H1 subfraction molecules is highly cooperative (Holcomb and Van Holde, 1962; Ginsburg and Carrol, 1965; Anfinsen et al., 1972; D'Anna and Isenberg, 1974; Van Holde and Isenberg, 1975;

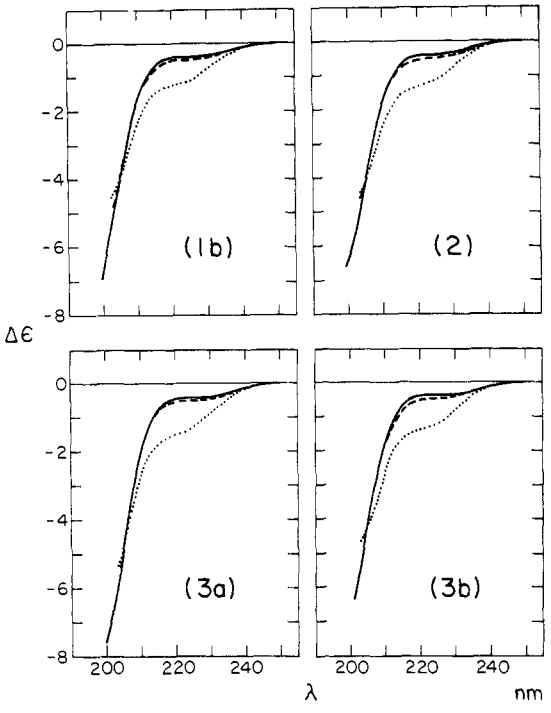

FIGURE 8: CD spectra of 0.5×10^{-5} M solutions of the H1 subfractions in water at pH 3.0 (—), in 0.01 M cacodylate, pH 7.0 (- - -), and in 0.4 M NaCl, 0.01 M cacodylate, pH 7.0 (···). Solutions were measured at 20 °C in a 2-mm path length cell.

Isenberg, 1976). A cooperative model, as defined here, is one in which there is an equilibrium between the random state of each subfraction and the folded state; as the salt concentration is raised, the equilibrium shifts to the folded form.

The inverse plots (Figure 10) are linear, showing that the data may be interpreted by a two-state model (Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1972). Furthermore, within experimental error, $K_{CD} = K_r$, indicating that both techniques are measuring different aspects of one overall conformational change, as demanded by a cooperative model.[3] As noted earlier, subfractions 3a and 3b cross-contaminate each other to approximately 10–15%. Nevertheless, even with this contamination, it is not difficult to show that if most of the intensity change results from folding, and not from the direct interaction of salt with the tyrosine in the denatured state, then a linear relationship will still hold for a two-state model (Evett and Isenberg, 1969; Wickett et al., 1972).

Even in the presence of some cross-contamination by 3b, it is clear that subfraction 3a has a significantly higher effective binding constant than the other subfractions. When the effect of contamination is estimated, the refolding of 3a is approximately twice as sensitive to the salt concentration as the other subfractions. Nevertheless, the greater sensitivity to salt does not necessarily imply that the folded form of 3a differs from the folded forms of the other subfractions. As far as our measurements go, we have not found, at least thus far, any marked differences in the physical properties of the folded forms, of the various subfractions. This makes the demonstration of dramatic differences in the binding properties of the various subfractions even more striking (Smerdon and Isenberg, 1976).

Finally, to see if there is any interaction between molecules of the same or different subfractions, the salt dependence of

[3] K_{CD} and K_r are the effective salt binding constants as defined in Li et al. (1972).

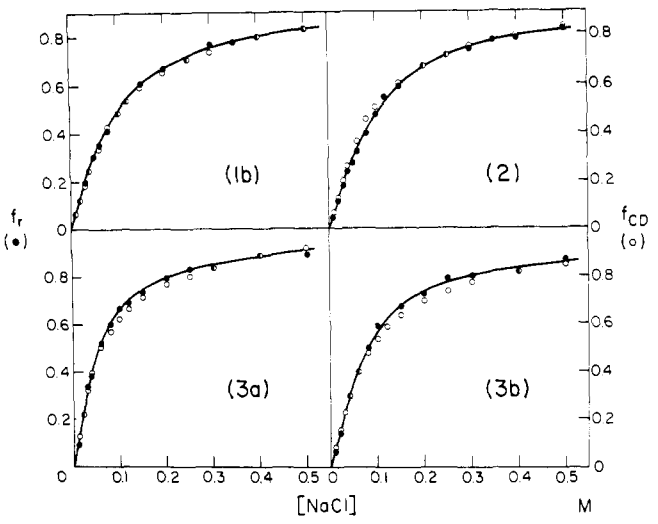

FIGURE 9: Fractional change in folding of each subfraction as measured by fluorescence (f_r) and by CD (f_{CD}) data. The curves shown are fitted to the anisotropy data.

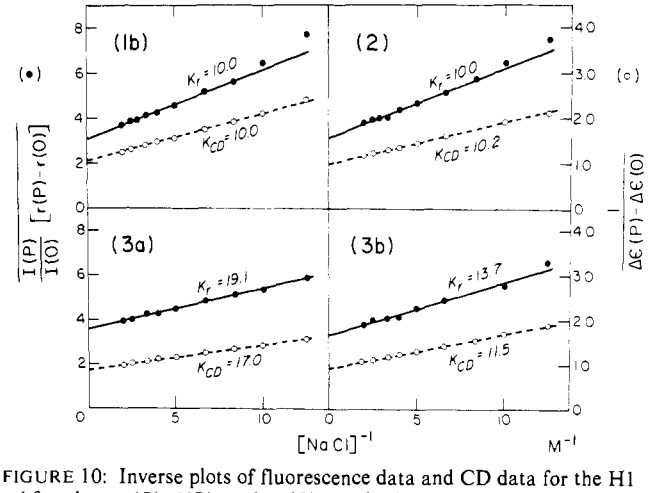

FIGURE 10: Inverse plots of fluorescence data and CD data for the H1 subfractions. $r(P)$, $I(P)$, and $\Delta\epsilon(P)$ are the fluorescence anisotropy, intensity, and CD at 220 nm for salt concentration P. K_r and K_{CD} are the effective NaCl binding constants as defined in Li et al. (1972).

the fluorescence anisotropy of whole H1 was measured at different protein concentrations, since anisotropy changes have been sensitive indicators of histone–histone interactions (Li et al., 1972; Wickett et al., 1972; D'Anna and Isenberg, 1972, 1973, 1974a–e; Smerdon and Isenberg, 1973, 1974; Van Holde and Isenberg, 1975; Isenberg, 1976). Figure 11 shows that the functional dependence of the anisotropy on phosphate concentration is invariant to changes in the protein concentration, at least over the range shown. The same result was found for NaCl titrations. These results suggest that H1 molecules do not complex with one another, regardless of whether they are of the same or of different subfractions. This conclusion was verified by sedimentation equilibrium measurements of the molecular weight of unfractionated H1 at different phosphate concentrations. We obtained 23 400, 25 100, and 26 500 for phosphate concentrations of 0.01, 0.05, and 0.20 M, respectively. These molecular weights are characteristic of monomer H1 and are in good agreement with the determinations made by others, under different solution conditions (Teller et al., 1965; Haydon and Peacocke, 1968; Edwards and Shooter, 1969). We also note that, for each of our molecular weight determinations, the ln C vs. Δr^2 plots gave good straight lines showing that only monomer was present. (The slight increase

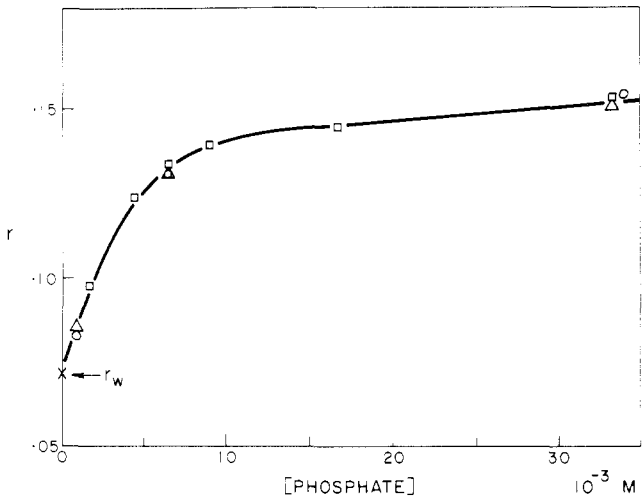

FIGURE 11: Fluorescence anisotropy, at 20 °C, of unfractionated H1 at 2.3×10^{-5} M (△), 3.7×10^{-5} M (□), and 4.7×10^{-5} M (○) as a function of phosphate concentration, at pH 7.0. r_w is the value of each in water.

in the apparent molecular weight in going from low to high phosphate concentrations is most likely due to the quenching of positive charges in the H1 molecules (Williams et al., 1958).)

Discussion

In the course of preparing subfractions for physical studies, we discovered a new fraction, which we call 1a. We must now ask if this is really a new subfraction of calf thymus histone H1, or if it is a degradation product. This is particularly important, since it is known (Kincade and Cole, 1966a) that H1 degradation products are eluted from ion-exchange resins before the H1 subfractions.

We first note that we have seen no evidence, in our preparations, of the degradation products that were identified and shown to be such by Kincade and Cole (1966a) (see Figure 4 of Kincade and Cole, 1966a). We have seen only the peaks shown in our Figure 1. Furthermore, we obtained the same profile with the use of either $NaHSO_3$ or PMSF, two different protease inhibitors. In addition, we note that 1a is the slowest migrating band on an acetic acid–urea gel and migrates slower than 1b on a sodium dodecyl sulfate gel. One would not expect these results if 1a were a degradation product, except perhaps if there were a specific clipping of a few residues at one end. We cannot rule out this latter possibility completely; a definitive evaluation must await sequence determinations. However, we note that even now we can draw some conclusions from the amino acid composition determinations (Table I). There is, for example, significantly more serine and valine in the mixture of 1a and 1b than in 1b alone. Consequently, since we know that the molecular weights of all the subfractions are about the same, if 1a is a degradation product, it is probably not a degradation product of 1b. Furthermore, 1a has more valine than any other fraction and there is also more arginine than in fraction 2 or 3a. Thus, while we cannot, at present, rigorously eliminate the possibility that 1a is a degradation product, it appears unlikely.

Our work has shown that the single tyrosine of each H1 subfraction is buried in the folded form. The absorbance red shifts and rises, the resolution of the fine structure in the absorbance band is enhanced, and the anisotropy goes up. Our spectral studies support and verify the findings of Bustin (1971). He presented an elegant study of the reaction of tetranitromethane with the tyrosine of H1. In high salt the reaction rate was dramatically reduced, and Bustin concluded that, upon the addition of salt, the protein folded in such a way that tyrosine was protected from the solvent.

In the folded state we find about 15% of the residues are in either an α-helical or β-sheet form. There is probably additional tertiary structure, otherwise the tyrosine would not be so firmly buried. The transition to the folded state is highly cooperative. In this state at least part of the molecule has a compact, folded structure.

We have no measurements that show that the different H1 subfractions have different structural properties in the folded state. All of the physical parameters of the folded state are almost the same, and it is hard to ascribe significance to the differences that were found. 3a is much more sensitive to salt than the others are, but this describes a property of the folding and not a property of the final folded structures. In any case, subfraction 3a is clearly different from the others. It may therefore have structural features that are different, although these differences may not be discernable by the techniques we have used. In the following paper of this issue (Smerdon and Isenberg, 1976) we shall show that subfractions 3a and 3b bind the non-histone chromosomal proteins HMG1 and HMG2 in a manner that is strikingly different from the other subfractions.

Acknowledgments

The authors thank Dr. K. E. Van Holde for use of the Durrum-Jasco CD recorder and for discussions of the sedimentation results. The authors also thank Dr. R. R. Becker for discussions of the amino acid determinations and Mrs. R. B. Hopkins for her skillful technical assistance in preparing the H1 subfractions.

Appendix

Let f = fraction of molecules in the folded form; ψ_i = reference spectrum for structure i; $\Psi(P)$ = observed CD spectrum for salt concentration P; A_i = fraction of residues, in the folded form, forming structure i; a_i = fraction of residues, in the zero salt form at neutral pH, forming structure i; ξ_i = fraction of residues, calculated by method of Baker and Isenberg (1976), at salt concentration P, forming structure i. Therefore,

$$\Psi(P) = f\Psi(\infty) + (1-f)\Psi(0)$$

or

$$\xi_\alpha\psi_\alpha + \xi_\beta\psi_\beta + \xi_R\psi_R = f(A_\alpha\psi_\alpha + A_\beta\psi_\beta + A_R\psi_R) + (1-f)(a_\alpha\psi_\alpha + a_\beta\psi_\beta + a_R\psi_R)$$

Thus, by equating coefficients,

$$A_\alpha = [\xi_\alpha - (1-f)a_\alpha]/f$$

$$A_\beta = [\xi_\beta - (1-f)a_\beta]/f$$

$$A_R = [\xi_R - (1-f)a_R]/f$$

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Interactions between the Subfractions of Calf Thymus H1 and Nonhistone Chromosomal Proteins HMG1 and HMG2†

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ABSTRACT: The nonhistone chromosomal proteins, HMG1 and HMG2, interact with the various subfractions of calf thymus H1 with a high degree of specificity. Subfractions 1b and 2 interact very strongly with HMG1 to form heterodimers. In contrast, subfractions 3a and 3b interact much more weakly. HMG2 interacts with 3a and 3b but shows no detectable complexing with 1a and 2.

The nonhistone chromosomal (NHC)[1] proteins HMG1 and HMG2 (Goodwin and Johns, 1973) constitute a relatively large fraction of the NHC pool. They are present in about 10^5–10^6 copies/nucleus (Johns et al., 1975, Walker et al., 1975), but, so far, no functional role has been ascribed to them. Their amino acid compositions are unusual: about 25% of the residues are basic and 30% acidic (Goodwin and Johns, 1973). HMG1 is an acidic protein while HMG2 is basic (Baker et al., 1976), differing from each other probably in the degree of amidation of glutamic and aspartic acids. The proteins are folded over a wide pH range, approximately pH 4–10. In this range, about 40–50% of the residues are in an α-helical conformation (Baker et al., 1976). The NHC fraction called P1 by Smith and Stocken (1973) appears likely to be HMG proteins.

Smith and Stocken (1973) stated that P1 interacted with histones H1. Shooter et al. (1974) studied the interaction of HMG1 with unfractionated H1 and concluded that the interaction did not result in specific complex formation.

In this paper, we report studies of the complexing of HMG1 and HMG2 with the subfractions of H1. We find that HMG1 and HMG2 interact with the various subfractions of H1 in a remarkably specific fashion. Some subfractions interact very strongly, some not at all, and some interact with intermediate binding strength.

Histone H1 is heterogeneous both with respect to amino acid sequence (Rall and Cole, 1971; Dixon, 1975; Dixon et al., 1975; Arutyunyan et al., 1975) and the degree of its phosphorylation (Langan et al., 1971). There is evidence that the various subfractions may have distinct roles in embryogenesis (Seale and Aronson, 1973; Ruderman and Gross, 1974; Ruderman et al., 1974) and undoubtedly other distinctions will be found in future studies. However, at present, we have no knowledge re-

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received April 16, 1976.* This investigation was supported by Grant CA 10872, awarded by the National Cancer Institute, Department of Health, Education, and Welfare. Preliminary accounts of this work were given at the International Symposium on Chromatin, Glasgow, Oct 1975, and the meeting of the American Society of Biological Chemists, San Francisco, June 1976.

[1] Abbreviations used are: NHC, nonhistone chromosomal; CD, circular dichroism.