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This work represents the first study of the conformational changes in histone H1 subfractions as well as the interactions of these subfractions with two nonhistone chromosomal (NHC) proteins. Calf thymus H1 was fractionated by the method of Kincade and Cole (1966a) using a very shallow guanidinium chloride gradient. A possible new H1 subfraction, about 5-8% of the whole 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, the circular dichroism (CD) of the peptide bond, and the tyrosine absorbtion. 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, with no further time dependence. The folding associated with this instantaneous change is highly cooperative, and involves the region of the molecule containing the lone tyrosine (at, or near, residue 72), which becomes buried in the folded form. The folding of subfraction 3a is approximately twice as sensitive to salt as 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, as measured by fluorescence anisotropy and sedimentation equilibrium.

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.

The interactions of the NHC proteins HMG1 and HMG2 with the four major H1 subfractions were studied using fluorescence anisotropy of the tryptophan emission, sedimentation equilibrium, sedimentation velocity, and CD. 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.

The formation of the two strong complexes involves little or no change in α-helical or β-sheet content. Also, the H1(2)·HMG1 complex has an overall tertiary structure that is more globular than that of the H1(2) molecule alone.

HMG2 interacts with 3a and 3b but shows no detectible complexing with 1a and 2.

Physical Properties of Calf Thymus H1 Histones: Conformational Changes and Interactions With Two Nonhistone Chromosomal Proteins

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PHYSICAL PROPERTIES OF CALF THYMUS H1 HISTONES: CONFORMATIONAL CHANGES AND INTERACTIONS WITH TWO NONHISTONE CHROMOSOMAL PROTEINS

I. INTRODUCTION

Classification of Chromosomal Proteins

One of the most intriguing endeavors in molecular biology today is the search for an understanding of the organization and functioning of DNA in the eukaryotic nucleus. DNA, the fundamental genetic material, does not exist as merely a free polynucleotide in the cell. Rather, it is associated with protein and RNA to form a very specifically controlled complex called chromatin. Studies on the composition of chromatin show a DNA:protein:RNA ratio of approximately 1.0:2.5:0.1 (dePomerai, *et al.*, 1974).

The properties of the chromatin complex are quite different from those of free DNA. For instance, only a small portion of the DNA in chromatin is transcribed in any given cell (McCarthy, *et al.*, 1973). This transcribable DNA differs in different cell types of the same organism (Felsenfeld, *et al.*, 1975) and, thus, gives rise to differentiation. Therefore, the presence of protein and RNA greatly influences the state of the DNA component.

A meaningful question that one can ask is: "What roles do these different components play in the structure and function of chromatin?" One approach toward answering this question has been to isolate the individual chromosomal components and study their properties free of any other components. One may then look for clues that can help decipher their functional roles in a more complicated structure

such as chromatin.

Of concern to this work is the protein component of chromatin. Although, as one can imagine, chromatin is comprised of hundreds of different proteins, it has proven fruitful in the past to divide them into two general groups. These are the histones and nonhistone chromosomal (NHC) proteins.

Each group is present in approximately equal amounts in chromatin (Garrard, *et al*., 1974), however, the number of unique proteins in the two groups is quite different. Whereas there may be hundreds of NHC proteins (Garrard, *et al*., 1974; Peterson and McConkey, 1976), the number of histones is relatively small.

There are, in general, only five major histone fractions (Johns, 1971; Hnilica, 1972). Two are arginine-rich (H3 and H4), two slightly lysine-rich (H2a and H2b), and one very lysine-rich (H1) in amino acid composition. Table I lists some of the chemical characteristics of these different histone fractions.

Since 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), it has become apparent that four of these histones, namely H2a, H2b, H3 and H4, are involved in performing the same functions. Results suggest that two each of these four histones exist "inside" the subunit particle (Van Holde, *et al*., 1975; Thomas and Kornberg, 1975; Pardon, *et al*., 1975; Shaw, *et al*., 1976a) and, thus, have become classified as the inner histones (Isenberg, 1976). The

TABLE I

Characterization of the Histones[a]

Class	Fraction	Lys/Arg	Total Residues	Molecular Weight	Basic/Acidic
Outer Histone	H1 (I, f1, KAP)	$\sim$ 22.0	$\sim$ 215	$\sim$ 21,500	$\sim$ 7.7
Inner Histone	H2a (IIb1, f2a2, LAK)	1.17	129	14,004	2.9
" "	H2b (IIb2, f2b, KAS)	2.50	125	13,774	3.1
" "	H3 (III, f3, ARE)	0.72	135	15,324	2.8
" "	H4 (IV, f2a1, GRK	0.79	102	11,282	3.6

[a] Data taken from Elgin and Weintraub (1975), and Van Holde and Isenberg (1975).

remaining histone fraction, H1, is not found in the subunit particle (Van Holde, _et al_., 1975; Thomas and Kornberg, 1975; Shaw, _et al_., 1976a). For the sake of distinguishing this fraction from the others, I will refer to the H1 histones as the _outer histone_ class (Isenberg, 1976).

There are a number of distinctions that can be made between the inner and outer histone classes. For instance, each of the inner histones shows a remarkable stability against evolutionary changes in amino acid sequence (Delange and Smith, 1975; Elgin and Weintraub 1975). The conservatism found in H3 and H4 represents the lowest mutation rate yet observed (Delange and Smith, 1975). On the other hand, the outer histone class is much more divergent in amino acid sequence (Rall and Cole, 1971; Jones, _et al_., 1974; Arutyunyan, _et al_., 1975; Dixon, _et al_., 1975; Elgin and Weintraub, 1975). Just enough of its characteristics have survived in such widely disparate systems as the pea plant and higher mammals to enable recognition of a lysine-rich histone in all these systems (Fambrough, _et al_., 1968; Fambrough and Bonner, 1969; Bustin and Cole, 1969; Kincade, 1969; Rall and Cole, 1971; Panyim, _et al_., 1971).

Furthermore, in almost all cases, each inner histone fraction is comprised of just one parent molecule (Delange and Smith, 1975; Elgin and Weintraub, 1975), although microheterogeneity can be introduced by post-synthetic modifications, such as phosphorylation, acetylation, and methylation (Dixon, _et al_., 1975; Elgin and Weintraub, 1975). The H1 class, however, generally consists of

more than one parent molecule (Kincade and Cole, 1966a,b; Bustin and Cole, 1968, 1969; Kincade, 1969; Panyim and Chalkley, 1969a; Fambrough and Bonner, 1969; 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). Within a given organism, these different gene products (or subfractions) vary as much as 10% to 20% by amino acid substitution (Bustin and Cole, 1968; Kincade, 1969; Rall and Cole, 1971; Delange and Smith, 1975). Further heterogeneity can be introduced by the modifications mentioned above (Dixon, et al., 1975; Elgin and Weintraub, 1975).

From amino acid sequences of the inner histones (Delange and Smith, 1975; Elgin and Weinbraub, 1975), it has become apparent that each of these molecules have asymmetrical amino acid distributions. A predominance of the basic residues occur in the N-terminal half as well as in a short region at the C-terminal end. The region in between has a composition and distribution much like what is found for globular proteins (Delange and Smith, 1975; Isenberg, 1976; Van Holde and Isenberg, 1975). This pattern is reversed in the outer histone class. Although the complete sequence of an H1 molecule has not yet been published, large portions of a number of different H1 subfractions have been sequenced (Rall and Cole, 1971; Jones, et al., 1974; Arutyunyan, et al., 1975; Dixon, 1975; Dixon, et al., 1975; Delange and Smith, 1975; Elgin and Weintraub, 1975) and it is evident that the C-terminal half of H1 is the dominant basic region. H1 also has a small basic region at the

N-terminal end. Between these two highly basic regions is a non-basic region (approximately residues 40 to 110) analogous to the globular protein-like C-terminal regions of the inner histones. More distinctions between these two groups of histones will be presented in the sections to follow.

The group of NHC proteins is considerably more complex than the histones. The number of distinct NHC polypeptides has been estimated to be as high as 450 (Peterson and McConkey, 1976). However, it appears that approximately 15-20 of these proteins comprise 50% to 70% of the NHC pool (Elgin and Bonner, 1972).

Many of the physicochemical properties of the NHC protein group differ markedly from those of the histones. The ratio of acidic to basic residues of total NHC protein fractions are generally in the range of 1.2 to 1.6 (calculated without considering amides) (Benjamin and Gellhorn, 1968; Marushige, et al., 1968; MacGillivray, et al., 1972). The molecular weights of the individual peptide chains are estimated to range from about 10,000 daltons to several hundred thousand daltons with isoelectric points from less than 3.7 to 9.0 (Elgin and Bonner, 1972; MacGillivray and Rickwood, 1974).

A number of different subclasses of NHC proteins have been isolated and are being further separated and characterized at the present time (MacGillivray, et al., 1972; Elgin and Bonner, 1972; Wu, et al., 1973; Smith and Stocken, 1973; Goodwin and Johns, 1973; Goodwin, et al., 1973; Patel and Holoubek, 1974; Wakabayashi, et al., 1974). One such subclass, of particular interest to this

work, is the HMG (high mobility group) class of NHC proteins being characterized by Johns and coworkers (Goodwin and Johns, 1973; Goodwin, _et al_., 1973; Johns, _et al_., 1975). This subclass is obtained by extracting chromatin with 0.35 M NaCl followed by treatment of this extract with 2% (w/v) trichloroacetic acid (TCA). The precipitate brought down by the 2% TCA contains most of the low mobility material observed in polyacrylamide gel patterns of the original 0.35 M NaCl extract. The 2% TCA supernatant contains the HMG proteins.

Separation of the HMG proteins (of which there are approximately 17 in all) on carboxymethylcellulose results in the purification of two of the major HMG components. These two proteins are called HMG1 and HMG2 and comprise from 10% to 20% of the total HMG pool. Their amino acid compositions are very similar and quite unusual. Both HMG1 and HMG2 contain 20-25% basic residues and 25-30% acidic residues. Preliminary results suggest that the basic and acidic residues are asymetrically distributed (Johns, _et al_., 1975). Both proteins have molecular weights of about 26,000 daltons (Shooter, _et al_., 1974; Goodwin, _et al_., 1975) and both contain cysteine and tryptophan (Baker, _et al_., 1976).

It is evident that the protein component of chromatin is comprised of a whole spectrum of different protein classes. The histone group, although containing only a small number of different protein fractions, can be divided into a class of proteins that are quite stable to evolutionary changes (inner histones) and a

class that appears to be both species and tissue specific (outer histones). The NHC protein group contains both acidic and basic proteins and is comprised of some proteins present in relatively large amounts as well as other proteins that are present in only minute quantities. From this it is understandable why the properties of chromatin are so much more complex than those of free DNA.

Biological Implications for Chromosomal Proteins

The observations of a number of laboratories over the last few years have greatly changed our basic concept of chromatin substructure (Hewish and Burgoyne, 1973; Woodcock, 1973; Olins and Olins, 1973, 1974; Sahasrabuddhe and Van Holde, 1974; Kornberg, 1974; Noll, 1974; Oudet, *et al.*, 1975; Van Holde, *et al.*, 1975; Pardon, *et al.*, 1975). What was thought to be a smooth, linear, regular (Pardon and Wilkins, 1972) or irregular (Bram and Ris, 1971) supercoil of the DNA double helix is now envisaged as fairly regularly spaced particles (like beads on a string) along the chromosome fiber where the DNA in each particle folds, in some fashion, around the inner histones. These four histones exhibit very specific interactions with one another *in vitro* (D'Anna and Isenberg, 1973, 1974b,d,e) and, from a number of studies (Kornberg and Thomas, 1974; Weintraub and Van Lente, 1974; Thomas and Kornberg, 1975; Martinson and McCarthy, 1975; Van Lente, *et al.*, 1975; Weintraub, *et al.*, 1975), it appears that these same histone:histone interactions are responsible for maintaining the subunit particle *in vivo*. Thus, one of the primary functions of the inner histones, implied by these

studies, is the packaging of the DNA duplex in the eukaryotic nucleus.

The location of H1 along the chromatin fiber is not yet known. It appears that H1 is not intimately associated with the subunit particle and that multimers of particles do contain H1 (Van Holde, et al., 1975; Thomas and Kornberg, 1975; Shaw, et al., 1976a). This has led to the proposal that H1 lies between particles, and is associated with the DNA in the spacer regions (Shaw, et al., 1976b). Recent results by Varshavsky, et al., (1976), who have analysed electrophoretically isolated monomers and dimers of subunit particles, suggest that the spacer regions can contain two, one, or no H1 molecules.

Further implications have come from the work of Vogel and Singer (1975a,b, 1976). These authors have found that H1 histones bind preferentially to simian virus 40 supercoiled DNA, while the inner histones show no specificity for this DNA type over relaxed closed circular, or linear, DNA. This data implies that H1 histones interact preferentially with twisted double-helices, i.e., with four-stranded DNA structures. Such twisted configurations, stabilized by H1 histones, interacting between subunit particles, may be involved in the next level of folding of the chromatin fiber. However, it must be acknowledged that our understanding of the location of H1 in chromatin is still at a very primitive stage.

Although the location and function of the outer histone class remains unknown, a number of observations have been made that imply very important functional roles for these histones. One of the

earliest observations was that removal of H1 from either interphase chromatin (Littau, *et al.*, 1965) or metaphase chromatin (Mirsky, *et al.*, 1968), by extraction in 0.1 M citric acid-0.125 M NaCl, resulted in much more disperse (less dense) structures as viewed with the electron microscope. When histone depleted chromatin was re-combined with the total lysine-rich histone pool, the dense structures re-appeared, while re-combination with the total arginine-rich histone pool showed only disperse chromatin (Mirsky, *et al.*, 1968). Further studies by Bradbury, *et al.*, (1973a) showed that H1-depleted chromatin in going from 0 to 0.2 M NaCl does not undergo the contraction observed for whole chromatin. These observations imply that H1 is, in some way, involved in chromatin condensation.

More implications have come from studies on the phosphorylation of the H1 histones. The presence of phosphoserine and phosphothreonine in histones was reported as early as 1966 by Ord and Stocken (1966) and by Kleinsmith, *et al.*, (1966). Since that time, the phosphorylation of H1 has been a phenomenon of great interest (see reviews by: Delange and Smith, 1971; Elgin, *et al.*, 1971; Allfrey, 1971; Hnilica, 1972; Dixon, *et al.*, 1975; Ord and Stocken, 1975; Elgin and Weintraub, 1975). Studies of the last few years have shown that H1 phosphorylation changes during the cell cycle (Lake and Salzman, 1972; Lake, 1973; Bradbury, *et al.*, 1973b, 1974a,b; Gurley, *et al.*, 1974, 1975; Balhorn, *et al.*, 1975). Gurley, *et al.*, (1975), using synchronized Chinese hamster cells (line CHO), found that three distinct H1 phosphorylation events occur in sequence during the cycle of these cultured cells. The

first event takes place in G1 phase approximately 2 hours prior to entry into S phase. The second event commences simultaneously with initiation of DNA synthesis, i.e., at the beginning of S phase. The third event occurs when the cells enter mitosis. The mitotic phosphorylation event, which has been observed by others (Lake and Salzman, 1972; Lake, 1973; Bradbury, et al., 1973b, 1974a,b; Balhorn, 1975), is associated with a much higher H1 phosphate incorporation than the other two events and it appears that at least four different sites on the H1 molecules are being phosphorylated during this event. These observations prompted Gurley, et al., (1975) to propose a model whereby the G1 phosphorylation event is involved with chromatin structural changes necessary for cell proliferation; the S phase event is involved with DNA replication, and the mitotic event is involved in chromosome condensation.

Other studies by Bradbury, et al., (1973b, 1974a,b) have correlated the phosphorylation of H1 histones with the condensation of chromatin into chromosomes in the naturally synchronous system, Physarum polycephalum (a true slime mold). These authors were also able to advance the onset of mitosis in this system by adding an extract of a heterologous H1 specific phosphokinase at times prior to those where mitosis naturally occurred (Bradbury, et al., 1974b). This led to the proposal that H1 phosphokinase triggers an increase in the H1 phosphate content which is the initiation step in mitosis.

At this time it is not known if each of the H1 subfractions are phosphorylated in an identical fashion in vivo. It is noteworthy, however, that Langan, et al., (1971), have demonstrated that certain

H1 subfractions from calf thymus, rabbit thymus, and rat thymus are not phosphorylated *in vitro* by a partially purified H1 specific kinase from calf liver (Langan, 1968). Upon examining the subfractions of rabbit thymus H1, these authors found that, in those subfractions that were not phosphorylated, the phosphorylation site for this enzyme, a serine residue, was replaced by an alanine residue. Thus, by virtue of a single amino acid replacement in these H1 subfractions, there occurred the presence or absence of a major site of enzymatic phosphorylation.

Another area of study that has led to implications of possible roles for the outer histones is in development. Studies on the synthesis of histones and histone mRNA during sea urchin embryogenesis (Seale and Aronson, 1973; Ruderman and Gross, 1974; Ruderman, *et al*., 1974; Berrill and Karp, 1976) have shown that cells from stages of early development (the morula stage) produce H1 that is distinctly different from the H1 produced during gastrulation. Since these two forms of H1 appear to have different RNA messengers (Ruderman, *et al*., 1974) and are resolved on SDS electrophoresis gels, it seems likely that the two H1 forms differ in primary structure. These observations have aroused much interest since it is known that differentiation occurs during the transition of sea urchin embryos from morula to gastrula (Berril and Karp, 1976; Timourian and Watchmaker, 1975). Therefore, this correlation of a new form of H1 with the onset of gastrulation implies that the H1 histones (or more specifically, the formation of H1 subfractions) may be involved in cellular differentiation.

Thus, although the precise locations within chromatin, or functions, of the outer histone class are unknown, it appears likely that these histones play very important roles in the structure and function of chromatin.

In contrast to the histones, the NHC proteins are associated with a number of different enzymatic activities (see Table 4 of Elgin and Weintraub, 1975). Nucleic acid polymerases, nucleases, and enzymes of histone metabolism appear to be integral components of chromatin. For example, it is estimated that approximately 10% to 50% of the total cellular RNA polymerase is chromatin bound _in vivo_ (Liao, _et al_., 1968; Cox, 1973).

In addition, nuclear proteins have been isolated that can bind to and affect the conformation of DNA. These include proteins that stablize single stranded DNA (Hotta and Stern, 1971a,b; Herrick and Alberts, 1973) and proteins that unwind double-stranded superhelical DNA (Wang, 1971; Champoux and Dulbecco, 1972; Baase and Wang, 1974; Keller, 1975; Vosberg, _et al_., 1975; Pulleyblank and Morgan, 1975).

Although these numerous enzymatic activities appear to be associated with the NHC proteins, it is not known which proteins are responsible for the various activities. Indeed, very little is known about the functions of any of the individual NHC proteins.

HMG1 and HMG2 represent two of the first proteins from the NHC pool that have been isolated in large quantities. Therefore, some preliminary studies pertaining to possible functional roles have been done on HMG1 and HMG2. One such study (Johns, _et al_., 1975)

indicates that these two NHC proteins appear to show no tissue or species specificity. Comparative polyacrylamide gel electrophoresis of NHC proteins from thymus, liver, kidney, and spleen of calves , rats, and rabbits, as well as from chicken erythrocytes, show that HMG1 and HMG2 are present in each of these systems in approximately the same amount. It is noted that in calf thymus both proteins are present in about 10^5 to 10^6 copies per nucleus (Johns, et al., 1975; Walker, et al., 1975). These observations, along with the fact that HMG1 and HMG2 have highly unusual amino acid compositions, imply that these two proteins have structural roles in chromatin rather than being specific gene activators.

HMG1 and HMG2 have been shown to bind DNA (Shooter, et al., 1974; Goodwin, et al., 1975). However, in studies using HMG1-DNA complexes, it was found that this binding has little effect on the ability of the DNA to act as template for the in vitro synthesis of RNA using an E. coli RNA polymerase (Johns, et al., 1975). Even at protein to DNA ratios of 5:1 the template activity of the DNA is only slightly decreased. This is in marked contrast to the inhibitory effect of histones on DNA dependent RNA synthesis (Allfrey, 1971; Hnilica, 1972).

The effect of HMG1 on the template activity of native chromatin using E. coli RNA polymerase has also been studied (Johns, et al., 1975). Addition of HMG1 to either calf thymus chromatin or chicken erythrocyte chromatin resulted in a stimulation of template activity in both cases by about 50%. However, care must be taken in interpreting these observations since, as pointed out by these authors,

the HMG1 binding may merely make the chromatin more soluble and, thus, easier for transcription to occur.

It has become evident from these studies, and studies involving other NHC proteins, that a wealth of information about chromatin structure and function is to be gained from an understanding of the various roles of these proteins. Since major research efforts in this area are just now starting to develop, it seems likely that great strides will be made toward unraveling the NHC protein puzzle in the near future.

Conformational Studies on Chromosomal Proteins

Conformational studies on pure histones have not been done until the last decade. However, during this time period there have been numerous physical studies, especially on the inner histones (see reviews by: Isenberg, 1976; Van Holde and Isenberg, 1975). Since pure histones have been obtainable only through the use of denaturing solvents, the general trend for doing physical studies has been to follow the changes that occur when these proteins are subjected to more physiological conditions.

One of the earliest observations, which has been extensively investigated, was that the addition of salt to aqueous solutions of the inner histones results in aggregation (Cruft, *et al.*, 1954, 1957, 1958; Luarence, 1966; Davison and Shooter, 1956; Mauritzen and Stedman, 1959; Phillips, 1965, 1967; Johns, 1968, 1971; Fambrough and Bonner, 1968; Edwards and Shooter, 1969; Boublik, *et al.*, 1970a; Diggle and Peacocke, 1971; Bradbury, *et al.*, 1973c; Bradbury and

Rattle, 1972; Bradbury, et al., 1975a; Lewis, et al., 1975; Li, et al., 1972; Wickett, et al., 1972; D'Anna and Isenberg, 1974c; Sperling and Bustin, 1974, 1975; Smerdon and Isenberg, 1973, 1974). This property of the inner histones has led to some anomolous reports on their structures. However, the studies by Li, et al., (1972) on very dilute solutions of histone H4, as well as subsequent dilute solution studies on each of the inner histones (Wickett, et al., 1972; D'Anna and Isenberg, 1972, 1974a,c; Smerdon and Isenberg, 1973, 1974), have shown that the complex conformational changes, that occur almost instantaneously at higher histone concentrations, can be broken down into rather simple events. Initially, upon salt addition, each of the inner histones undergo a rapid, cooperative folding where part of the molecule forms α-helix. This folding bears all of the earmarks of the renaturation of denatured globular proteins (Isenberg, 1976) and involves the region of each inner histone molecule that is analogous, in primary structure, to globular proteins. Also, during this initial change, histone H4 (and possibly other inner histones) forms dimers (Li, et al., 1972; D'Anna and Isenberg, 1973; Smerdon and Isenberg, 1974).

At sufficiently high salt concentrations (Li, et al., 1972; Wickett, et al., 1972; D'Anna and Isenberg, 1974c), the fast refolding of histones H3 and H4 is followed by a much slower conformational change involving β-sheet formation. This change, which has a time constant dependent on the histone concentration and type of salt used (Wickett, et al., 1972), has been shown to be associated

with the self-aggregation of these histones (Smerdon and Isenberg, 1973; D'Anna and Isenberg, 1974c). Histones H2a and H2b also aggregate in salt solutions, but higher protein concentrations are required (Boublik, et al., 1970b; Diggle and Peacocke, 1971; D'Anna and Isenberg, 1972, 1974a; Bradbury, et al., 1975a; Sperling and Bustin, 1975).

Since all indications suggest that the aggregated state of these histones is not the native state, it was imperative to understand the conditions required for these different events to occur as well as the properties associated with both the aggregated and non-aggregated (but folded) states. This gave, then, criteria for the different states and allowed for a constant check on whether or not one was dealing with histones in the aggregated form. This basic understanding of the refolding properties of the inner histones laid the structural framework for studies on the interactions between these proteins.

D'Anna and Isenberg (1973, 1974b,d,e), using the dilute solution techniques employed by Li, et al., (1972), studied the interactions between pairs of inner histones in salt solutions. These studies were done by adding salt to mixtures of inner histones in aqueous solutions and monitoring the changes that occurred in the fluorescence and CD properties. This procedure allowed for interactions to take place between histones that were in the folded, but not aggregated, forms. These authors showed that some of the inner histones formed very strong 1:1 complexes while others interacted only weakly. The cross-complexing pattern that evolved from these

studies (D'Anna and Isenberg, 1974d) has played an essential part in developing current ideas of chromatin structure and simultaneously helped pinpoint one important function of these histones. This interaction pattern also helped emphasize the importance of protein-protein interactions to chromatin structure.

In contrast to the inner histones, our knowledge of the conformational properties of the outer histone class is meager indeed. To date, there are relatively few reports in the literature devoted to physical studies on H1 (Boublik, et al., 1970a; Vladimirov, et al., 1970; Bradbury, et al., 1967, 1972, 1975b,c; Chapman, et al., 1976) and these are limited to studies on unfractionated H1 (which in some cases was partially contaminated). However, even though these properties are an average of the properties of the individual H1 subfractions, a number of conclusions can be made. As was the case for the inner histones, pure H1 is usually obtained through the use of harsh solvents. Therefore, the same format used for physical studies on the inner histones was applied to H1. One of the first observations was that H1 differed from the other histones in that addition of salt to aqueous solutions of H1 did not yield aggregation (Teller, et al., 1965; Haydon and Peacocke, 1968; Edwards and Shooter, 1969). This is yet another distinction between the inner and outer histone classes.

The recent reports by Bradbury and coworkers (Bradbury, et al., 1975b,c; Chapman, et al., 1976) show that addition of NaCl to H1 in $^{2}H_2O$ at low pH gives rise to changes in the NMR spectra. A major change is due to ring-current shifts of the methyl resonances

of valine, leucine, and isoleucine indicating that several groups of protons of the apolar residues are in close proximity to an aromatic sidechain in the salt induced state of these molecules. These authors state that ring-current shifts of this type are an indicator of precise and repeatable molecular structure, and suggest that at least part of the H1 molecules have defined and specific folding analogous to that of globular proteins (Bradbury, et al., 1975b). It is further noted by these authors that calf thymus H1 contains only two aromatic residues (a tyrosine at, or near, residue 72 and a phenylalanine at, or near, residue 106) and that approximately 85% of the apolar residues are in the region from 40 to 115. Therefore, these results imply that it is the non-basic, central region of the H1 molecules that undergoes most of the structure formation induced by salt.

Changes in NMR spectra were also observed when the pH was raised from 3 to 6 (Bradbury, et al., 1975b). The chemical shifts observed during this titration were almost coincident with the titration of glutamic acid residues of which there are approximately five in the central region of the H1 molecules. These authors suggest that this data implies that the glutamic acid residues in the central region of the H1 molecules are involved in salt links in the folded form.

Further studies were done on the two fragments of the H1 molecules that are produced by restricted chymotrypsin digestion (Bradbury, et al., 1975c). When salt was added to aqueous solutions, at pH 3, of the N-terminal fragments (residues 1 to 106), changes were

observed in the NMR spectra. However, these changes were not identical to those observed for the intact molecules. This fragment, which contains most of the non-basic region, was estimated, from CD results, to form approximately 22% α-helix and 6% β-sheet in 1.5 M NaCl, pH 3. On the other hand, the C-terminal fragment showed very little change in the NMR spectra upon salt addition and formed only 6% α-helix and no β-sheet. These authors suggest that this implies that the N-terminal region of the H1 molecules is associated with the structure formation. They also suggest that this structure requires more of the residues than are present in the chymotrypsin produced N-terminal fragment since the NMR spectra differed from that of the intact molecules. It is further noted by these authors that this fragment appears to aggregate at NaCl concentrations above 0.75 M and that there was no evidence for structure formation upon titration of the glutamic acid residues. These observations are also contrary to what was observed for the intact molecules (Bradbury, _et al_., 1975b).

Recently, Bradbury and coworkers have reported studies on calf thymus H1 cleaved with thrombin (Chapman, _et al_., 1976). It was found that digestion with thrombin results in cleavage of the H1 molecules at approximately residue 122 or about 15 residues further from the N-terminus than the cleavage point of chymotrypsin. Again, upon isolation of the fragments, NMR and CD spectra were taken. The N-terminal half in this case showed an NMR spectrum in 1 M NaCl, pH 3, that was identical to what was observed for the intact molecules under these same solution conditions. CD spectra of the N-terminal half showed that approximately 20% α-helix and

and 16% β-sheet was formed in 1 M NaCl. The C-terminal fragment showed little or no structure formation under these conditions. The close correlation between the NMR spectrum of the N-terminal fragment with unfractionated H1 led these authors to propose that the region of the H1 molecules containing residues 1 to approximately 122 was involved in the formation of a globular structure whereas the C-terminal half is much more elongated. (It is noted that the CD results reported for this N-terminal fragment are significantly different from those reported by these authors for intact H1 molecules. Bradbury, et al., (1975b) reports 15% of the H1 molecule forms α-helix in 1 M NaCl, pH 3, with no β-sheet formation.)

Thus, the studies on H1 to date suggest a more globular structure in the N-terminal half with the C-terminal region being more like an extended "tail". Bradbury, et al., (1975c) and Chapman, et al., (1976) have reported that the C-terminal fragments bind DNA as tightly as the intact H1 molecules and that the N-terminal region does not bind DNA. These authors have suggested that H1 molecules in chromatin may bind DNA along the more extended C-terminal and that the more globular N-terminal region may then be available for interactions with either subunit particles or NHC proteins.

The physical properties of the individual NHC proteins have not been studied with the exception of HMG1 and HMG2. Recently, Baker, et al., (1976) reported on the effects of salt and pH on the refolding of HMG1 and HMG2. It was found that over a wide pH range (pH 4 to pH 10) both proteins have a folded structure, with appro-

ximately 45% of their residues in an α-helical conformation. In high and low pH solutions, both molecules unfold and form more random structures. The folded structures of these two proteins are insensitive to salt addition, even up to 2 M NaCl, suggesting that charge-charge interactions play little or no role in stabilizing their tertiary structures. From the fluorescence properties of the tryptophan residues in HMG1 and HMG2 it appears that these residues are buried in the folded form. Also, it was found that both HMG1 and HMG2 have sedimentation coefficients of approximately 2.4S (Carl Baker, personal communication). This gives a frictional ratio (f/f_0) of about 1.4 for these proteins, indicating that both HMG1 and HMG2 are rather globular in shape (see Sober (1968) for a comparison of f/f_0 for globular and elongated proteins).

The only major difference between HMG1 and HMG2, observed by these authors, was that HMG1 aggregates at about pH 5.7 whereas HMG2 aggregates at about pH 9.0. Thus, it appears that HMG1 is an acidic protein at neutral pH and HMG2 is a basic protein. This difference is possibly due to different levels of amidation (Johns, et al., 1975).

These physical studies on HMG1 and HMG2 represent the first of such studies on individual NHC proteins. Undoubtedly, physical studies will extend to more of the NHC proteins as they become isolated. As knowledge of the structures and interactions of these very important components of chromatin accumulates, it seems likely that more meaning will be brought to, what has been up until now, a total mystery.

II. MATERIALS AND METHODS

Preparation of Unfractionated H1

Calf thymus glands were frozen quickly in CO_2 within 5 minutes of the animal's death, and transported to the laboratory frozen. Chromatin was extracted at $4^{o}C$ 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 phenylmethylsulfonyl fluoride (PMSF) (Ballal, et al., 1975). In using the latter procedure, a stock solution of 0.1 M PMSF in isopropanol was diluted 1000 fold into a fresh homogenizing buffer solution prior to each wash, as PMSF is known to eventually hydrolyse 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 histone 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 x 2 m Sephadex G-100 column, equilibrated with 0.01 N HCl. Fractions were assayed by measuring the absorbance at 230 nm. (It is noted that a good criterion for purity of the

H1 fractions is the A_{230}/A_{275} ratio, which is greater than 25 for pure H1.) The fractions across the H1 peak were electrophoresed as described below. The last eluting 1/3 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% to 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, lyophilysed, and stored at -20°C.

Separation of H1 Subfractions

H1 subfractions were prepared by the method of Kincade and Cole (1966a). 150-200 mg of purified whole H1 was dissolved in 10 ml of 7% guanidinium chloride (GuCl), 0.1 M NaH_2PO_4 pH 6.8. (The Practical Grade GuCl, purchased from Sigma, was purified according to the method of Bonner, et al., 1968.) The sample was applied to a 5 cm x 45 cm Bio-Rex 70 column (200-400 mesh, sodium form, prepared according to Bonner, et al., 1968), equilibrated with 7% GuCl, 0.1 M NaH_2PO_4 pH 6.8. The sample was eluted with a very shallow linear gradient of GuCl (see text) in which the concentration was varied over an 8-day period. The flow rate was 50 ml/hr. It, and the gradient, were controlled by an ISCO Dialagrad gradient former, Model 382. 15 ml fractions were collected. To 0.2 ml of each fraction was added 1 ml of 1.32 M TCA. This was shaken vigorously

and, after 13 minutes, assayed turbidometrically at 400 nm. GuCl 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 (using an Amicon DIAFLO um2 membrane), dialysed for 24 hours against 100 x volume of 0.01 N HCl, with three volume changes, and lyophilysed to dryness. The lyophilysed samples were then dissolved in 0.5 ml of 0.01 N HCl, run through a 1.2 cm x 26 cm Sephadex G-25 column, to assure desalting of the material, and lyophilysed back to dryness. Samples were stored dry at -20°C until used.

Preparation of HMG1 and HMG2

Calf thymus HMG1 and HMG2 were kindly provided by Drs. G. H. Goodwin and E. W. Johns. Their method of preparation is described in Goodwin and Johns (1973) where HMG1 and HMG2 are fractions A and C, respectively.

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% SDS gels (Laemmli, 1970). For the former, 5 mm diameter gels were loaded with 25 μg and 50 μg of protein, and for the latter, with 25 μg. All purified whole H1 samples, and purified H1 subfractions showed only one band on both

gel systems. However, both HMG1 and HMG2 showed other minor bands under these conditions. When lower amounts of each were used (5 - 10 μg) so that a good estimation of purity could be made, it was estimated that approximately 1% - 2% of the HMG1 and HMG2 was contamination.

Determinations of the purity of any given H1 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 SDS and acetic acid-urea systems were used. For the SDS slab gels, the three modifications of Thomas and Kornberg (1975) were employed.

The photograph of the acetic acid-urea gel slab presented in this work 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 analyser (Spackman, *et al*., 1958). Hydrolysis was carried out in constant boiling HCl at 110°C for 22 hours in evacuated sealed tubes.

Protein concentrations were determined spectrophotometrically on a Cary 14 spectrophotometer. Concentrations of the H1 subfractions (and unfractionated H1) in H_2O were determined using an extinction coefficient of 1345 $cm^{-1} l\ M^{-1}$ 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 tyrosine (1340 $cm^{-1} l\ M^{-1}$),

as given by Herskovitz and Sorenson (1968), and one phenylalanine in aqueous solution. The extinction coefficients used for HMG1 and HMG2 were 2.1 x 10^{4} $cm^{-1} l M^{-1}$ and 2.0 x 10^{4} $cm^{-1} l M^{-1}$ at 280 nm, respectively (Baker, et al., 1976).

Digestion of H1 with alkaline phosphatase (Escherichia coli, BAPC, Worthington) was performed by the method of Sherod, et al., (1970). Digestions were terminated by the addition of the SDS sample solution (2.0% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue, 0.127 M Tris, pH 6.8) to an equal volume aliquot of the digestion solution, followed by boiling for 2 minutes. Aliquots were taken at ½ hr., 1 hr., 1½ hrs., 2 hrs., 3 hrs., 5 hrs., 7 hrs., and 20 hrs. after starting the incubation.

Measurements on H1 subfractions in solution containing NaCl were performed by dissolving the lyophilysed 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. Measurements on subfraction 3a were repeated in 0.001 M cacodylate buffer at pH 7.0. This concentration of cacodylate 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 except that the lyophilysed histones were first dissolved in H_2O; phosphate, added subsequently, was used as its own buffer at pH 7.0. All data were corrected for dilution.

pH titrations were performed by dissolving the lyophilysed protein in 0.01 N HCl and titrating to the desired pH with NaOH. pH was measured on a Corning Digital Model 112 pH meter equipped with a Corning semimicro combination electrode.

For studies on H1·HMG interactions, stock solutions (4-6 x 10^{-5} M) of reduced HMG1 and HMG2 were prepared as described in Baker, et al., (1976) and stored at $4^{o}C$ or $-20^{o}C$ in 0.01 N HCl - 1 mM dithiothreitol. Aliquots of protein stock solutions were electrophoresed on acetic acid-urea gels during, and at the end of, the time period in which they were used, which was no longer than two weeks. Neither oxidation nor degradation was observed. In addition, no observable changes in tryptophan spectral properties occurred during the storage period. Stock solutions (about 10^{-4} M) of the H1 subfractions (and unfractionated H1) in H_2O were made the same day used.

Samples for the complex studies were prepared by adding the required volumes of stock solutions to a test tube, and then adding the appropriate volume of H_2O to obtain 1/3 of the final volume needed. 0.03 M phosphate was then added to give the final volume, which contained 0.02 M phosphate at the desired pH.

Fluorescence anisotropy, $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, and fluorescence intensity, were measured on a computer interfaced polarization spectrometer constructed in our laboratory (Ayres, et al., 1974). All measurements were made at $20^{o}C$. For studies on the H1 subfractions, samples were excited at 279 nm and the emission

was measured at 325 nm. For the studies on H1·HMG interactions, samples were excited at 295 nm and emission was measured at 340 nm. Under these latter conditions, essentially only tryptophan fluorescence is observed. The anisotropies for noninteracting mixtures were calculated using the Weber addition law (Weber, 1953; Dale and Eisenger, 1975).

Circular dichroism (CD) measurements were made on a Jasco Model J-10 CD recorder at 20^{0}. Data is reported as $\Delta\varepsilon$ in units of $cm^{-1}l$ (mole of residue)$^{-1}$ for the H1 subfractions alone and $cm^{-1}l$ (mole of protein)$^{-1}$ for the H1·HMG complexes. CD spectra for the H1 subfractions were analysed using the method of Baker and Isenberg (1976). The reference spectra used for α-helix and β-sheet were the CD spectra for poly-l-lysine (Greenfield and Fasman, 1969). The reference spectra used 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).

All sedimentation measurements were performed at 20^{0}C, on a Beckman, Model E ultracentrifuge equipped with interference, schlieren, and scanner optics. A 12 mm path length cell was used for unfractionated H1 alone and a 30 mm path length cell was used for all studies done on H1·HMG mixtures. Equilibrium experiments were performed according to the high speed method of Yphantis (1964) using interference optics. Velocity experiments on HMG1 alone and HMG1·H1(2) complexes were carried out using scanner optics

at 280 nm. Velocity experiments on H1(2) alone were carried out using schlieren optics. A partial specific volume ($\bar{v}$) of 0.766 was used for unfractionated H1. The $\bar{v}$ values used for the individual H1 subfractions are given in Table II. These values were calculated from the amino acid compositions (Cohn and Edsall, 1943). A value of 0.728 was used for the $\bar{v}$ of both HMG1 and HMG2 (Shooter, et al., 1974; Goodwin, et al., 1975). A $\bar{v}$ of 0.746 was used for the complexes and was calculated from the weight average of the partial specific volumes of the two monomers. Molecular weights of 26500 and 26000 were used for HMG1 and HMG2, respectively (Shooter, et al., 1974; Goodwin, et al., 1975). A molecular weight of 24000 was used for the H1 subfractions in the complex studies and was obtained from Figure 15. The data were analysed with a computer program written by Dr. Robert Dyson of this Department.

III. RESULTS

Part A: Isolation and Characterization of Calf Thymus H1 Subfractions

Column Chromatography

Figure 1 shows the elution profile of calf thymus H1 from the preparative Bio-Rex 70 column following the method of Kincade and Cole (1966a). The GuCl conentration was varied from 7% to 14% over an 8-day period and resulted in the partial resolution of three major H1 peaks as was reported previously (Kincade and Cole, 1966a; Bustin and Cole, 1968; Kincade, 1969). The H1 applied to the column in this experiment had not been purified on the Sephadex G-100 column (see Materials and Methods) and, therefore, contained contaminants HMG1 and HMG2. It can be seen from Figure 1 that these two NHC proteins are easily separated from the H1 subfractions by this technique. Further, as shown in the Figure, when a linear gradient in GuCl concentration is run from 7% to 8%, HMG1 and HMG2 can be resolved from each other. This provides a method of separation of these two proteins.

Figure 2 shows another elution profile of H1 from the preparative Bio-Rex 70 column. However, in this experiment, the GuCl concentration was varied from 9.6% to 11.2% over the 8-day period. In comparing Figures 1 and 2, it is obvious that the more shallow GuCl gradient resulted in much better resolution of the three major H1 peaks and also resolved a minor 4th peak (marked X). This small peak (X) may be the histidine containing subfraction reported by

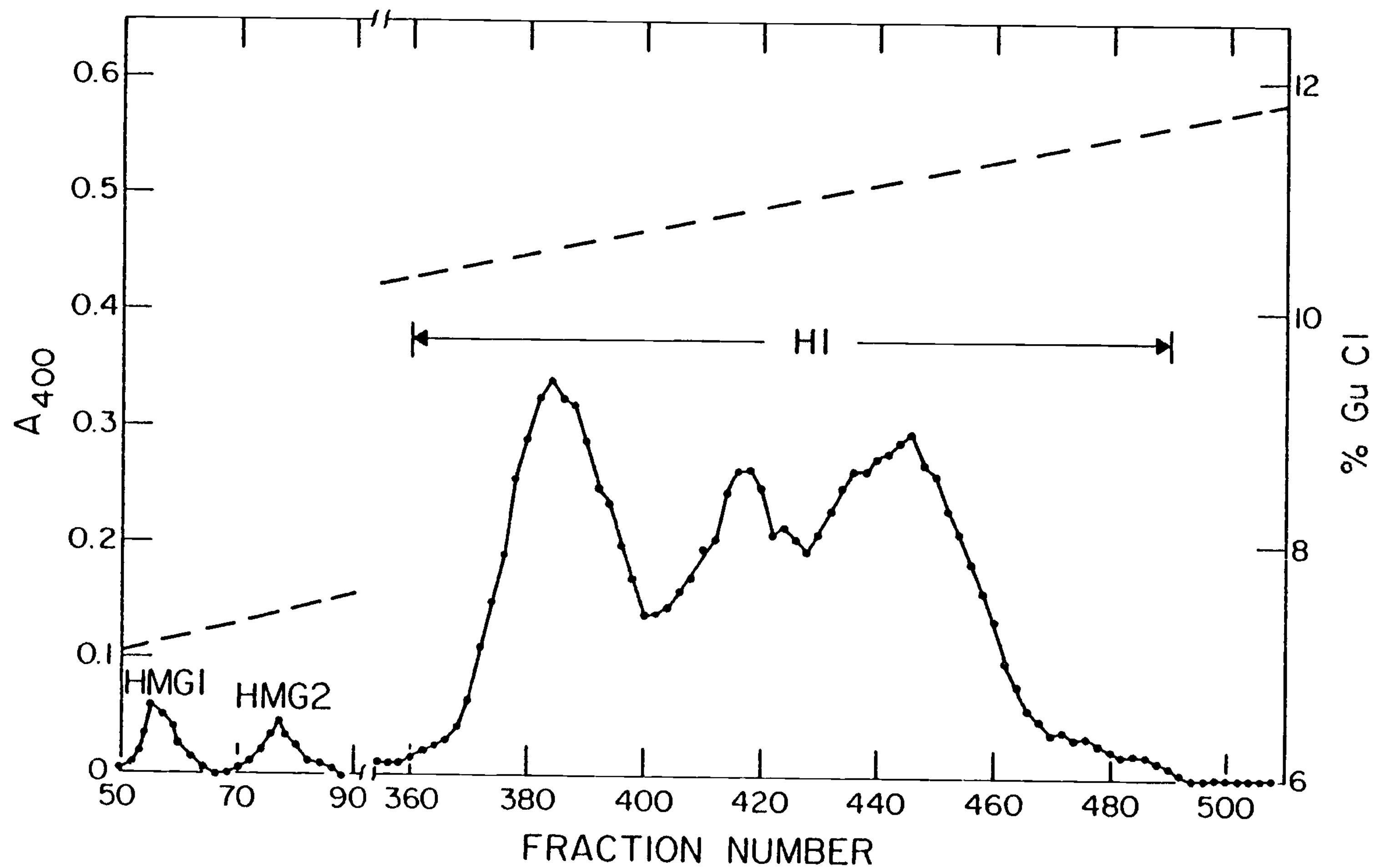

Figure 1. Chromatographic profile of calf thymus H1. Protein was eluted from the Bio-Rex 70 column with a 7% to 14% linear gradient of GuCl. Dashed line denotes GuCl concentration.

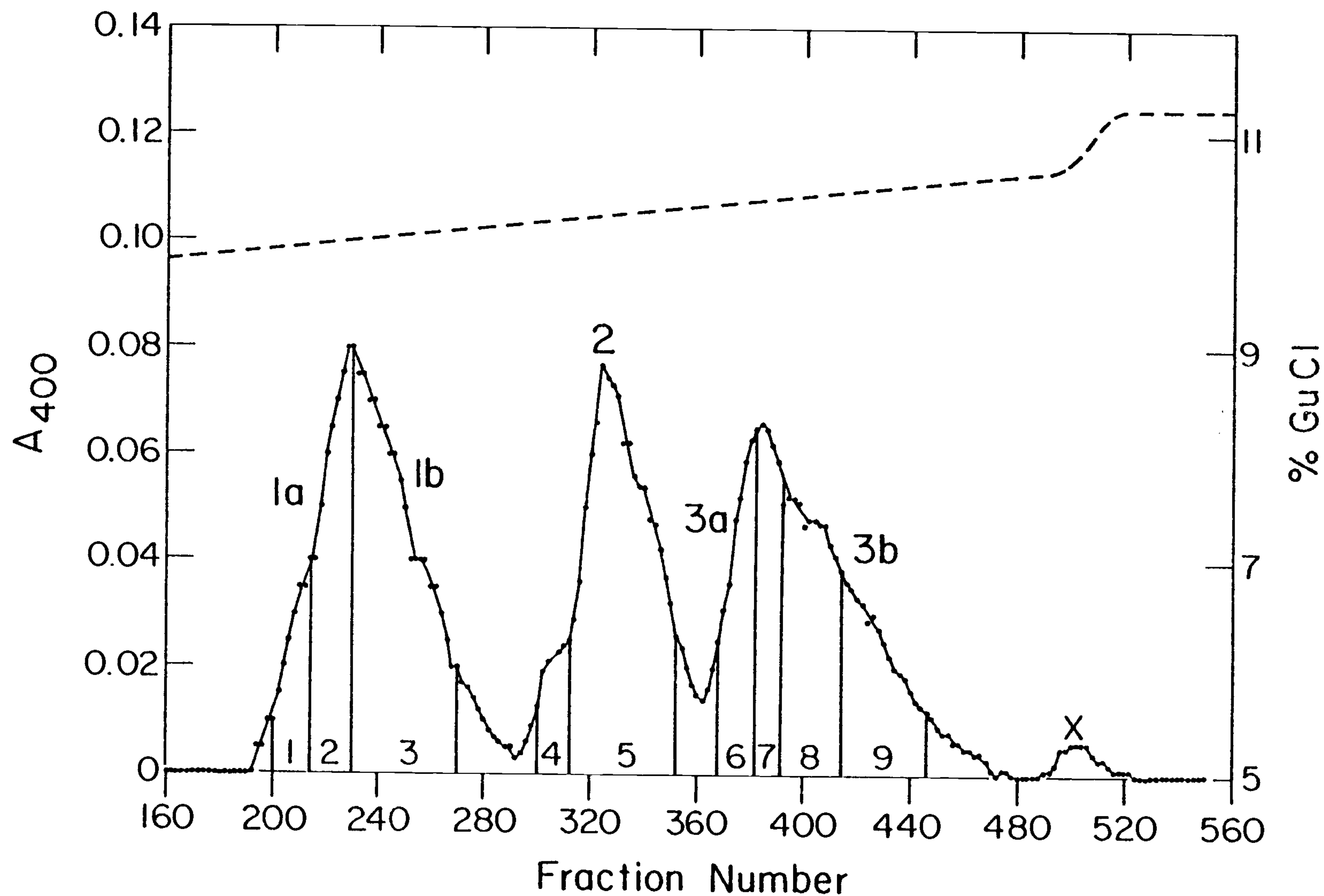

Figure 2. Chromatographic profile of calf thymus H1 using a very shallow GuCl gradient. Protein was eluted from the Bio-Rex 70 column with a 9.6% to 11.2% linear gradient of GuCl. Sections 1, 2,...,9 are defined in the text. Dashed line denotes GuCl concentration.

Panyim and Chalkley (1969a), as it is present in a very small amount and eluted at the same GuCl concentration as reported by these authors. (It is noted that the H1 used in the latter experiment had been previously run through the Sephadex G-100 column and, thus, contained no detectable contaminants.)

Electrophoresis and Amino Acid Analysis

The chromatogram of Figure 2 was divided into the nine sections shown based on electrophoretic results discussed below. Aliquots from each section were electrophoresed on SDS slab gels (Figure 3a) and acetic acid-urea slab gels (Figure 3b). 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 3). 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 band is seen as one goes from section 3 toward section 1.

The first peak therefore contains two bands of histone H1, which are designated 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 SDS and acetic acid-urea gels. However, there is a

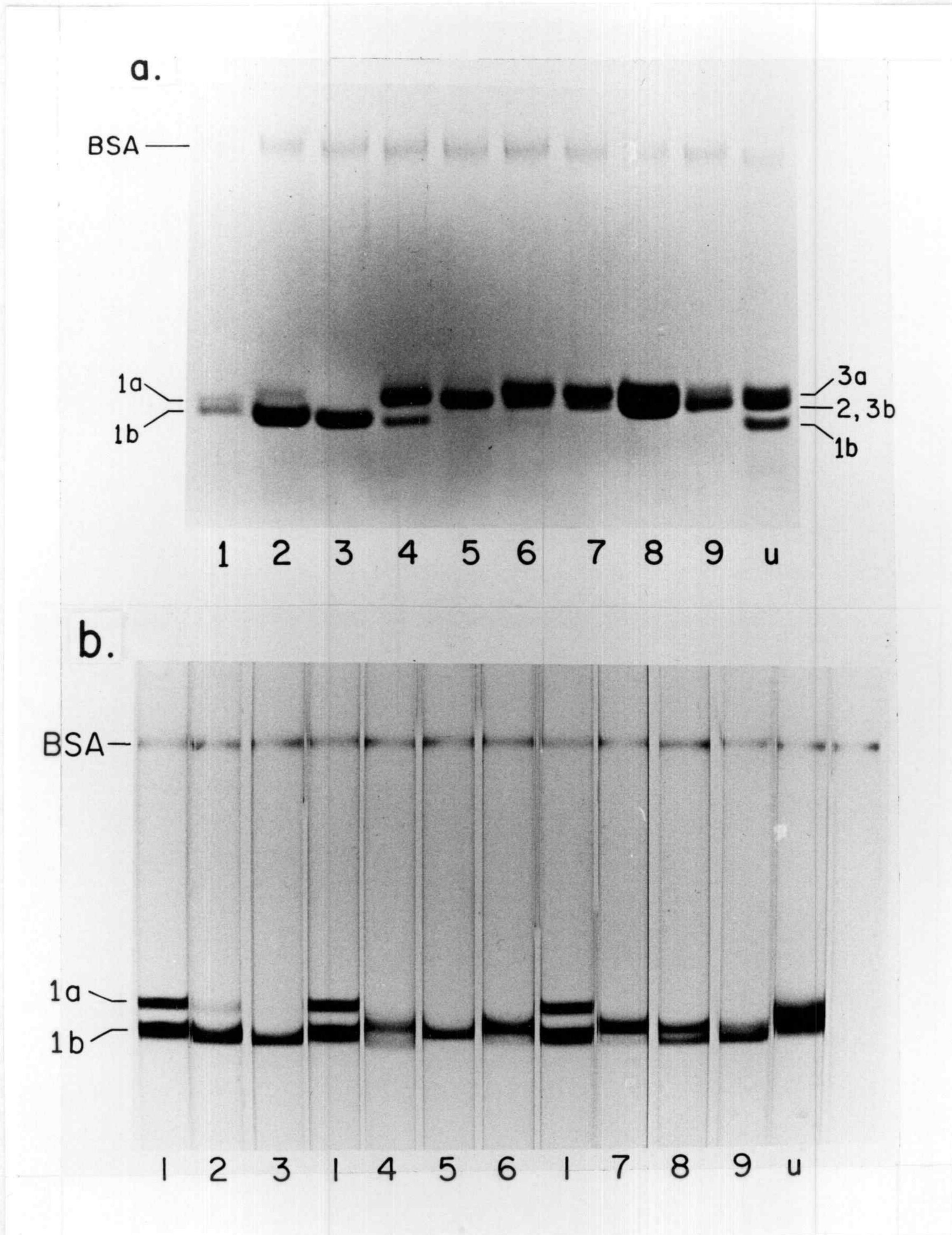

Figure 3. SDS slab gel (a) and acetic acid-urea slab gel (b) of aliquots from sections 1-9 (Figure 2) and unfractionated H1 (denoted "u"). Bovine Serum Albumin (BSA) marker is included.

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 co-electrophoreses with 1b, it may merely be some cross-contamination from peak 1. (There is no evidence to discriminate between these two possibilities.)

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

The mobility of the subfractions on SDS gels follows the pattern: $1b > 1a > 2 \sim 3b > 3a$. Therefore, if the separation is due only to the difference 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 anomolously on SDS gels (Panyim and Chalkley, 1971; Hayashi, et al., 1974).

Subfraction 1a is the slowest moving component on acetic acid-urea gels, although it is the second fastest component on SDS gels. This suggests that the overall charge characteristics of 1a differ from those of the other subfractions.

To determine if the resolution seen in Figure 3a was due partly to differential phosphorylation, unfractionated H1 was digested with

alkaline phosphatase (Sherod, et al., 1970). At enzyme to H1 ratios of 1:7, no change in the electrophoretic pattern of Figure 3a was observed, even after 20 hours digestion.

Table II 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 be noted that, as judged by gel scans, 3a and 3b are only 85% - 90% pure and cross-contaminate each other.) Kincade and Cole (1966b) found 3.5 moles of arginine/mole of protein in peak 3. They concluded that one of the subfractions of peak 3 contained 3 arginines while the other subfraction contained 4 arginines. As can be seen in Table II,my results verify their conclusion and show 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 and lysine. As will be discussed later, these data support the proposal that 1a is a new subfraction, and not a degradation product.

TABLE II

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 $\bar{v}$	--	.766	.766	.765	.764

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

Part B: Conformational Changes in the H1 Subfractions

Physical Changes During pH Titrations

Each of the four major H1 subfractions shows very little change in conformation in going from pH 3 to pH 7 (Figures 4, 5, and 6). Each subfraction has a typical random coil CD spectrum. As the pH is raised to about pH 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 pH 6, but this is not the result of a major folding of the protein, since the CD spectrum hardly changes from pH 3 to pH 7 (Figure 6). Using the method of Baker and Isenberg (1976) (see Materials and Methods), it is estimated that only 1% to 3% of the residues form α and/or β structure (Table IIIB) during this pH change. Because this change in CD is so small, there can be no significance ascribed 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 IIIA). These small changes at low pH are most likely due to the titration of the carboxyl groups of aspartic and glutamic acids which titrate in the pH range of 2 - 5.

The initial increase in fluorescence intensity at pH 9, followed by a decrease in intensity above pH 10.2 (Figure 5) can be easily explained since two competing processes occur in this pH region. First, the partial titration of the lysine residues in

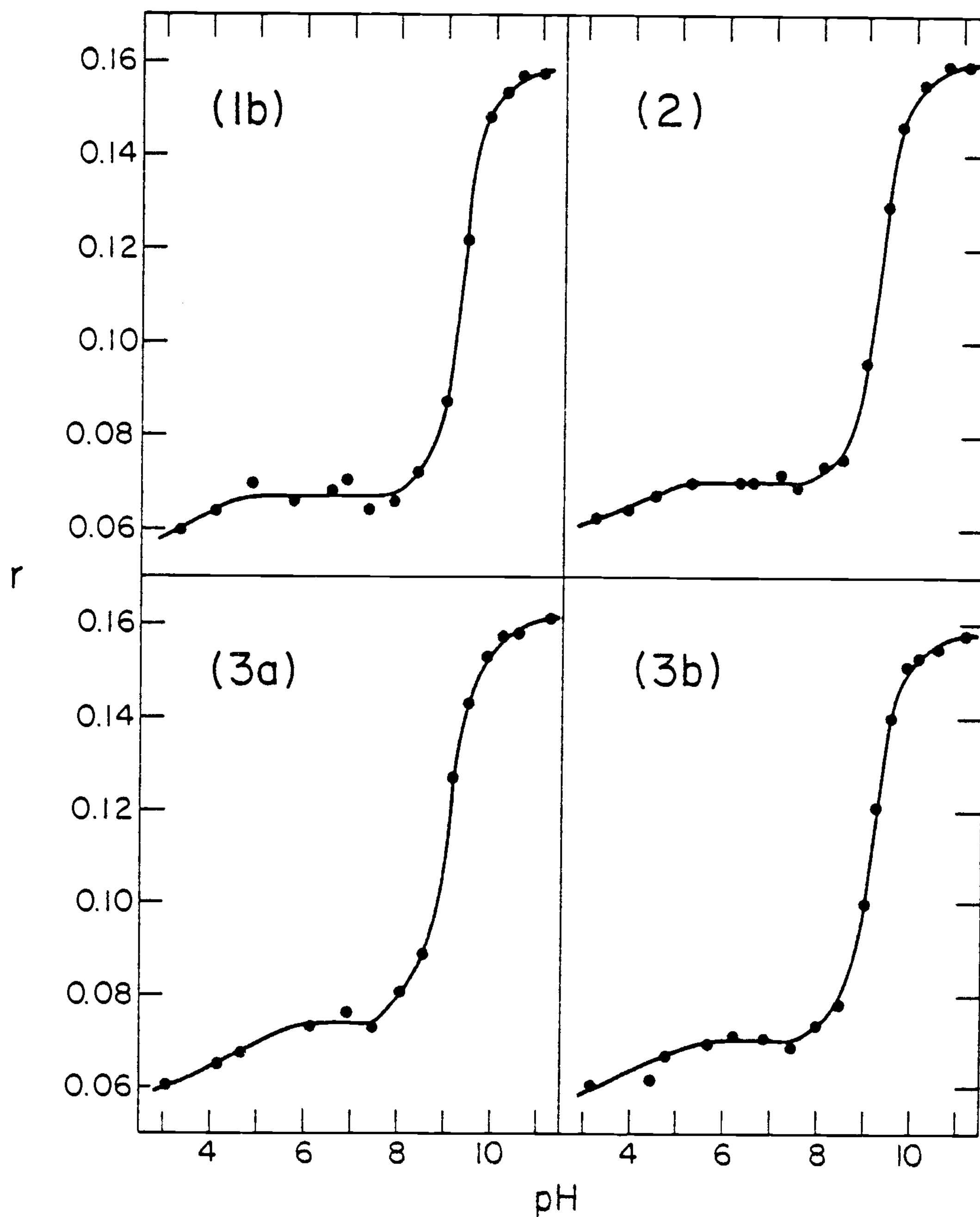

Figure 4. Fluorescence anisotropy of 2.0 x 10^{-5} M solutions of the major H1 subfractions in water as a function of pH.

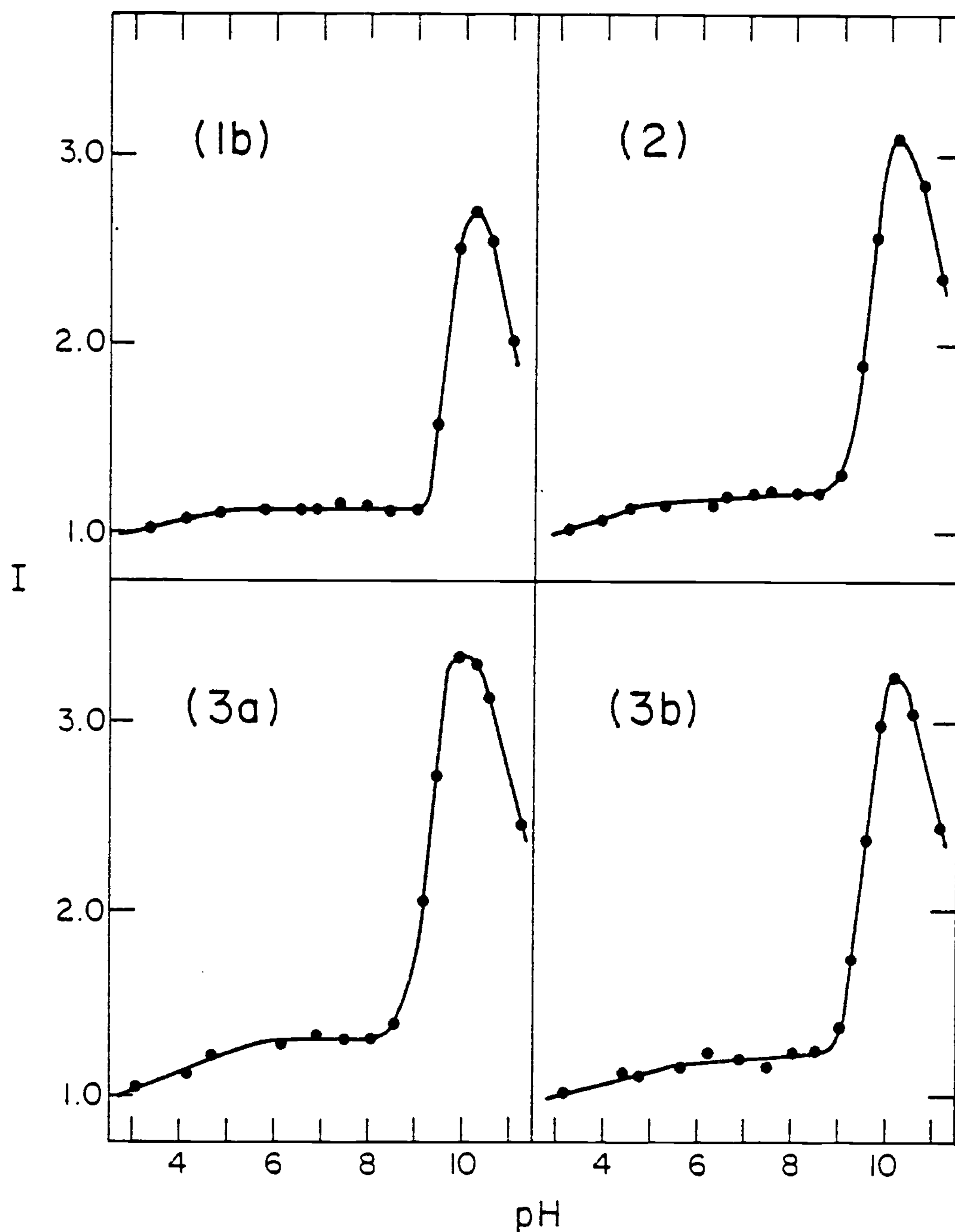

Figure 5. Fluorescence intensity, relative to that at pH 3, of 2.0×10^{-5} M solutions of the H1 subfractions as a function of pH.

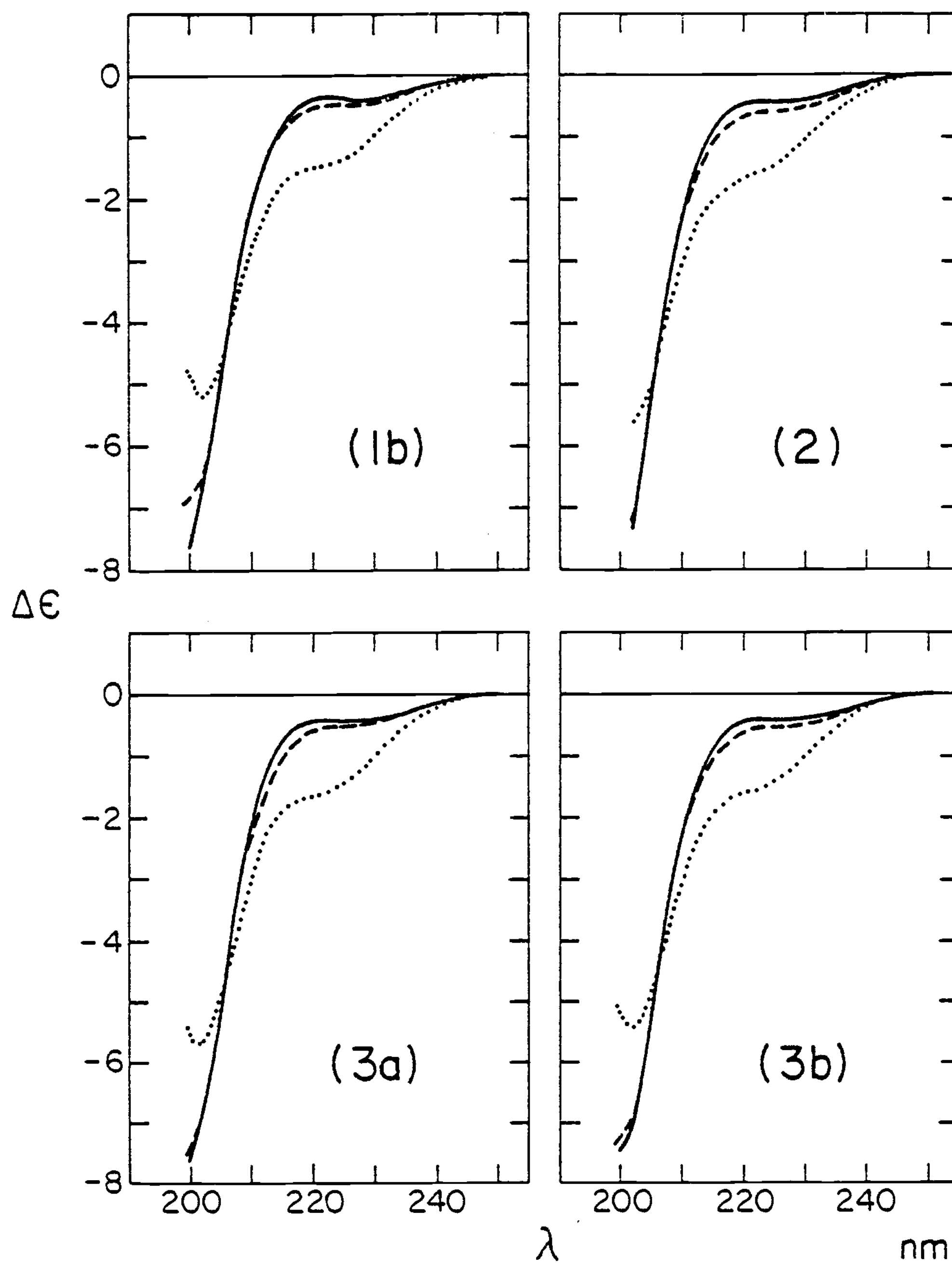

Figure 6. CD spectra of 0.5×10^{-5} M solutions of the H1 subfractions in water at pH 3.0 (———), pH 7.0 (-----), and pH 10.2 (·····). Solutions were measured in a 2 mm path length cell.

TABLE III: pH INDUCED PHYSICAL CHANGES

A. FLUORESCENCE ANISOTROPIES 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).

each subfraction molecule results in a conformational change to a folded form. In this form, the mobility of the tyrosine decreases and the anisotorpy 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 stil 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 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 6). Appreciable amounts of both α-helix and β-sheet form (Table IIIB).

In going from pH 7 to pH 10.2, the α-helical content jumps from essentially zero 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 aqueous solutions (pH 7) of 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). Figure 7 is an example of the change in fluorescence properties and the CD at 220 nm as a function of time.

There is a striking red shift plus an increase in tyrosine absorbance upon the addition of salt (Figure 8). 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 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 9). 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 IVA 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.

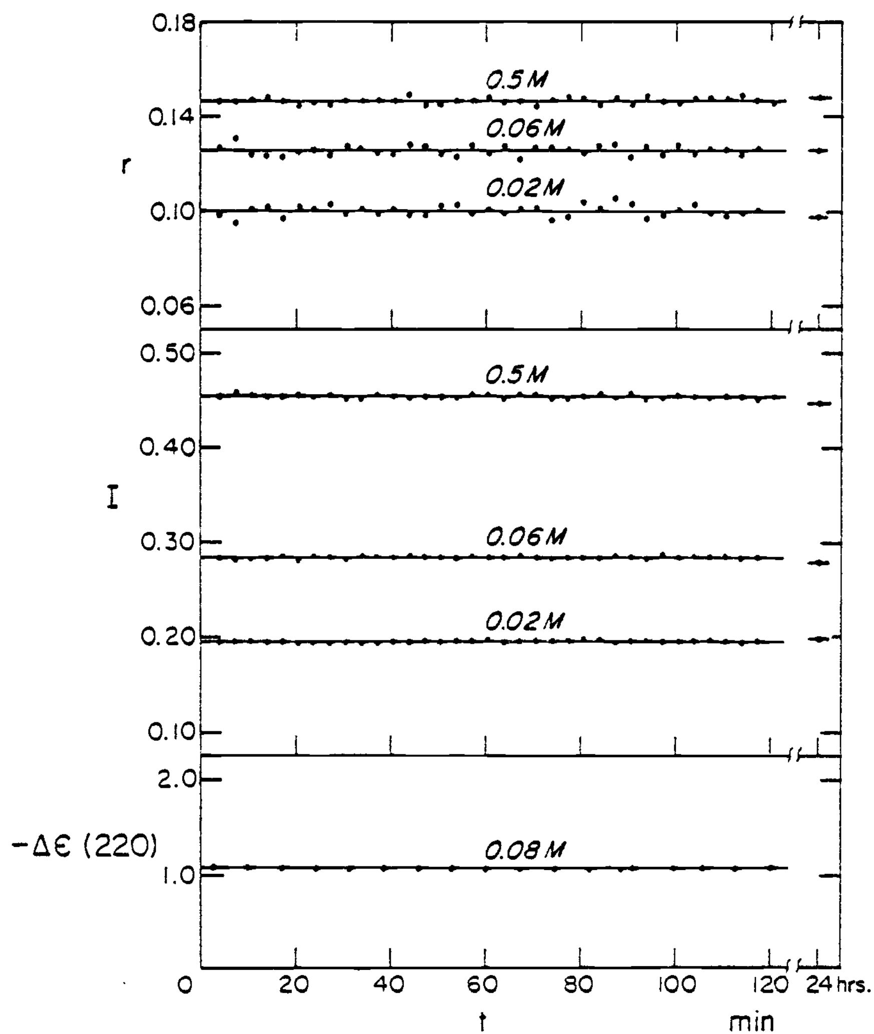

Figure 7. Fluorescence anisotropy (r), fluorescence intensity (I), and CD at 220 nm of subfraction 2 as a function of time at different NaCl concentrations. Protein concentrations were 1.25 x 10^{-5} M and 6.25 x 10^{-6} M for fluorescence measurements and CD measurements, respectively.

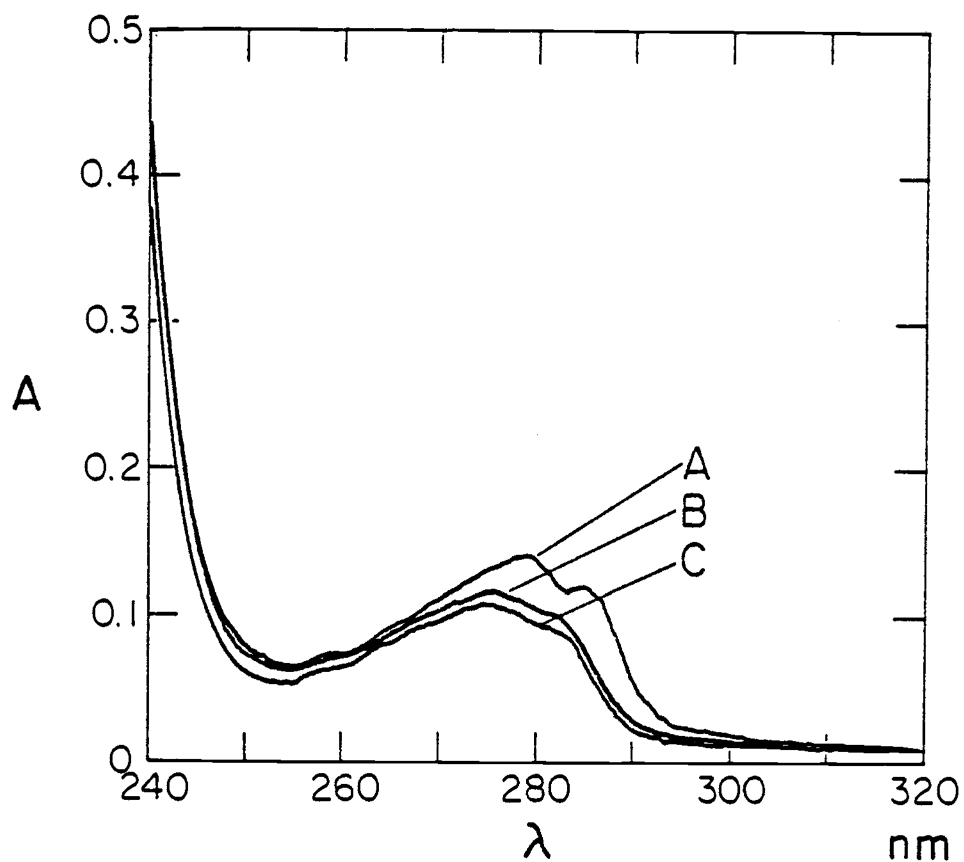

Figure 8. Absorbance, A, of 6.9 x 10^{-5} M solutions of subfraction 2 in H_2O (C), in 0.002 M sodium phosphate, pH 7.0 (B), and in 0.04 M sodium phosphate, pH 7.0 (A).

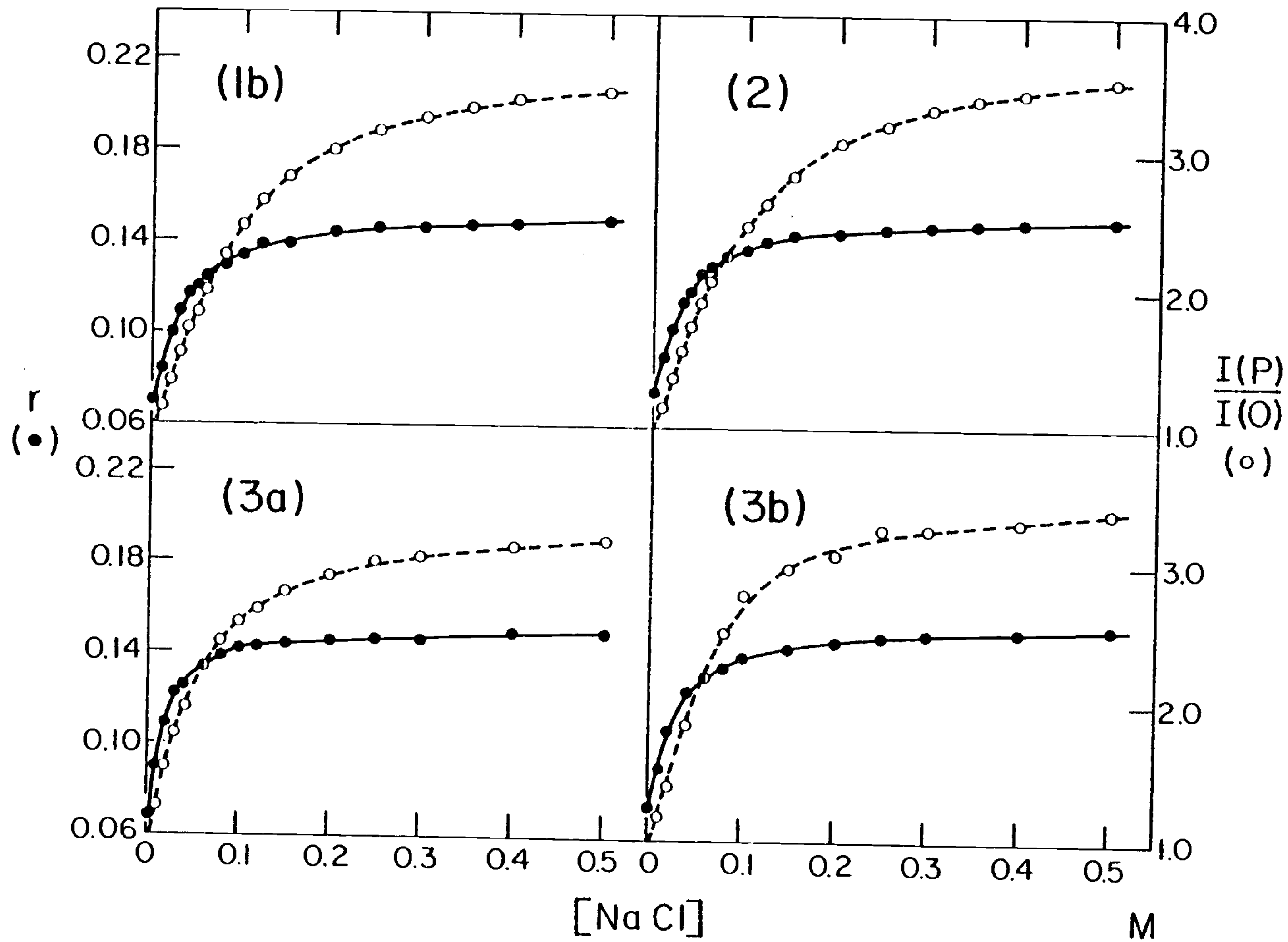

Figure 9. Fluorescence anisotropy and relative intensity of 2.0 x 10^{-5} M solutions of the H1 subfractions in 0.01 M cacodylate buffer, pH 7.0, as functions of NaCl concentration. I(P) is the intensity for salt concentration P.

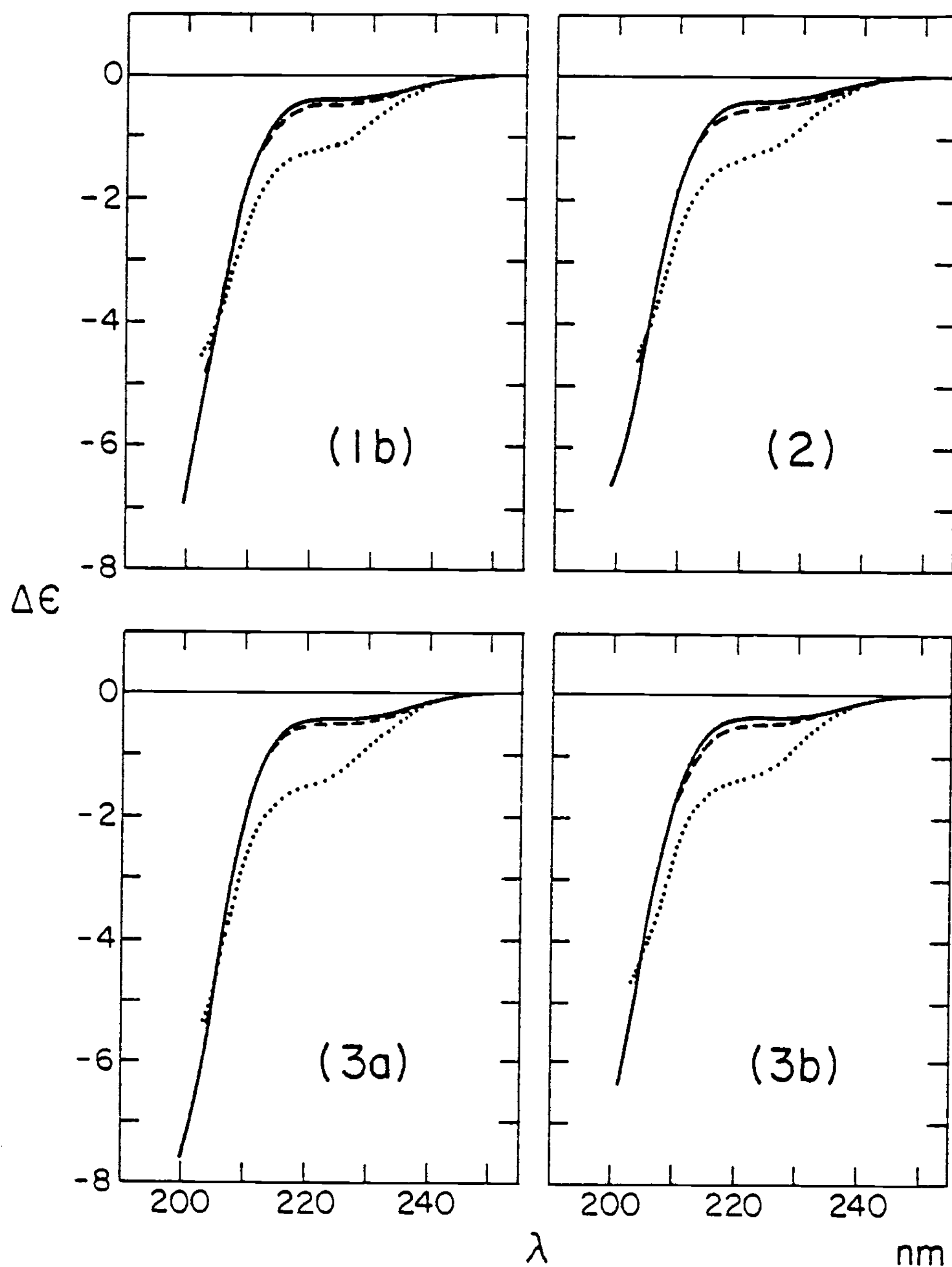

Figure 10. CD spectra of 0.5 x 10^{-5} M solutions of the H1 subfractions in water at pH 3.0 (———), in 0.01 M cacodylate, pH 7.0 (-----), and in 0.4 M NaCl, 0.01M cacodylate, pH 7.0 (·····). Solutions were measured in a 2 mm path length cell.

TABLE IV: 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	$\%\alpha(\infty)$	$\%\beta(\infty)$	$\%R(\infty)$	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

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[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 III.

[c] See Baker and Isenberg (1976).

The addition of salt gives rise to marked changes in the CD spectra (Figure 10). Spectra were taken at different salt concentrations and extrapolated to infinite salt concentration in the manner shown in Appendix I. 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 IVB where spectra at three different salt concentrations were analysed and averaged for each subfraction. 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.

Figure 11 shows the CD at 220 nm as a function of salt concentration, for each of the subfractions. 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, and compared to the fraction calculated from fluorescence data. Figure 12 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

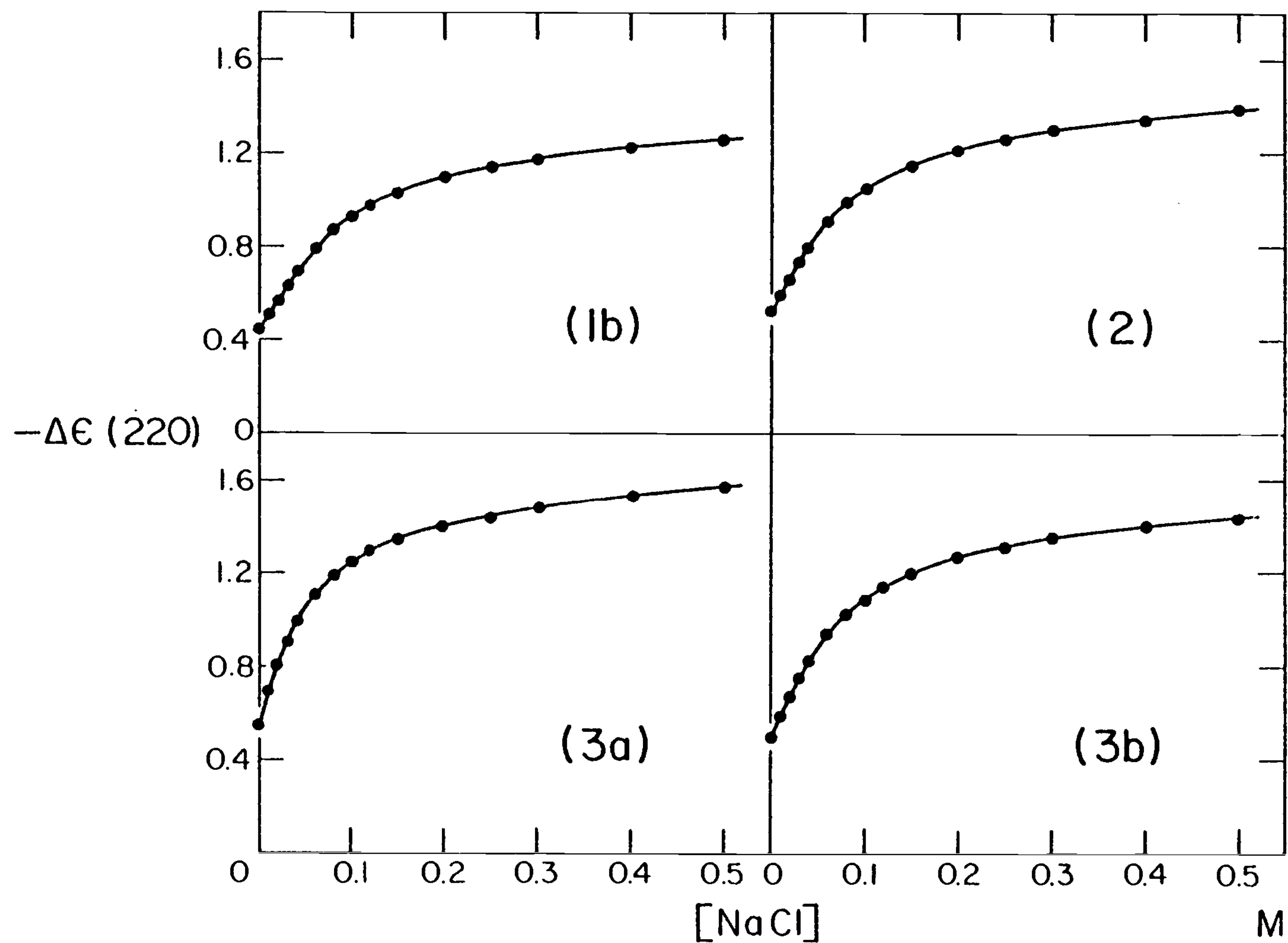

Figure 11. CD at 220 nm of 1.0×10^{-5} M solutions of the H1 subfractions as functions of NaCl concentration. Measurements were made in a 1 cm path length cell.

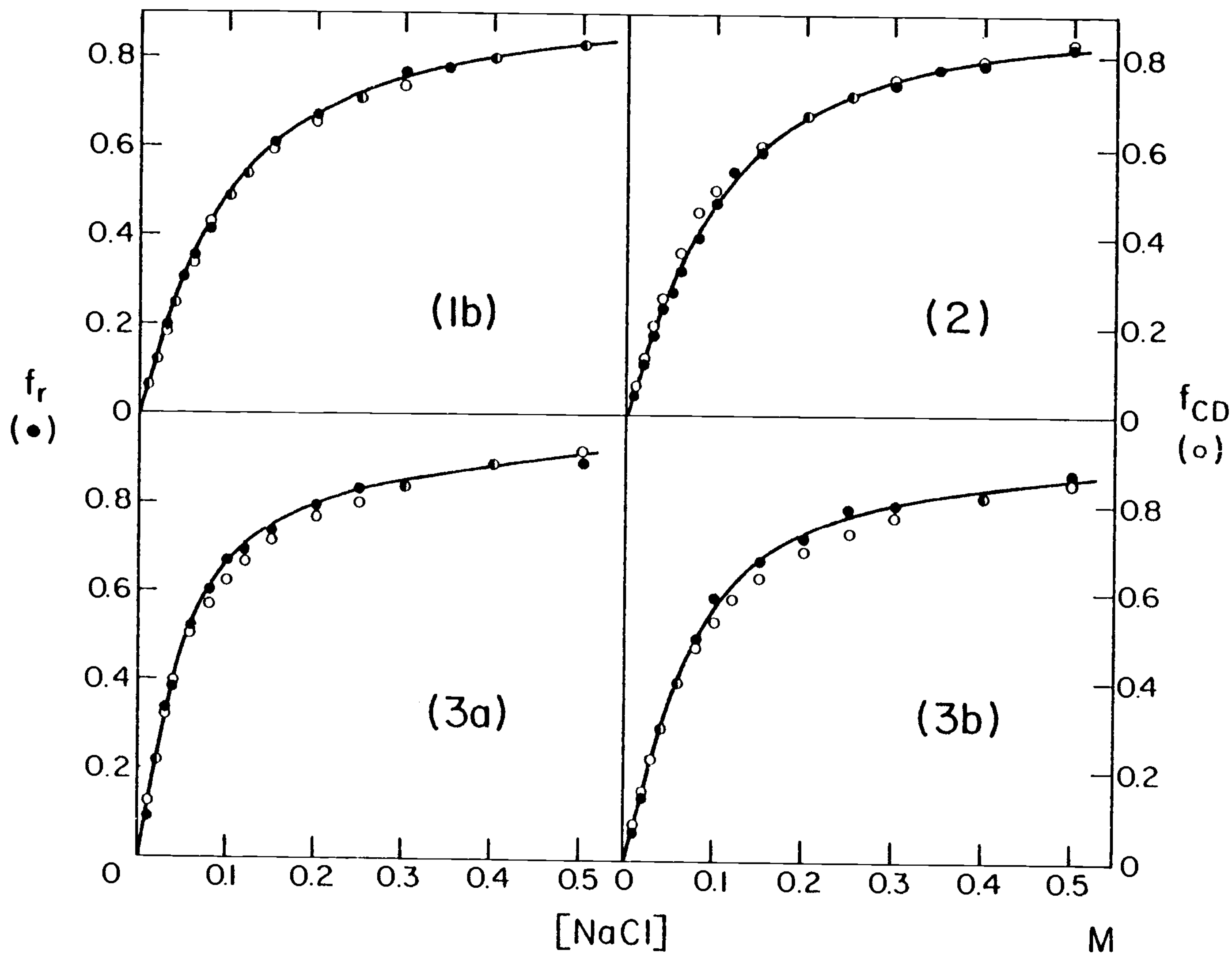

Figure 12. Fractional change in folding of each subfraction as measured by fluorescence (f_r) and by CD (f_{CD}) data. The curves shown are fitted to the anisotropy data.

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; 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 13) 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.[1] 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

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

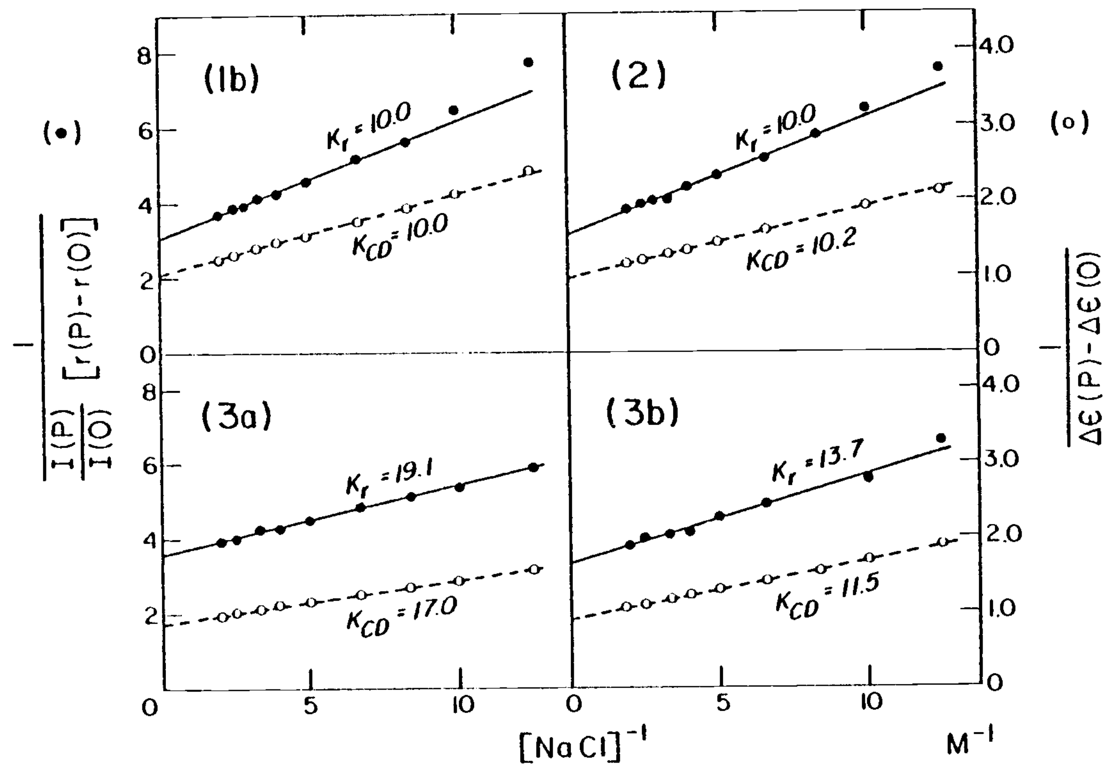

Figure 13. Inverse plots of fluorescence data and CD data for the H1 subfractions. r(P), I(P), and $\Delta\varepsilon$(P) are the fluorescence anisotropy, fluorescence intensity, and CD at 220 nm for salt concentration P. K_r and K_{CD} are the effective NaCl binding constants as defined in Li, et al., (1972).

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. Thus far, these studies have not found any marked differences in the physical properties of the folded forms of the various subfractions.

Finally, to see if there is any interaction between molecules of the same or different subfractions, the salt dependence of 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,b,c,d; Smerdon and Isenberg, 1973, 1974; Van Holde and Isenberg, 1975; Isenberg, 1976). Figure 14 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 is verified by sedimentation equilibrium measurements of the molecular weight of unfrationated H1 at different phosphate concentrations (Figure 15). 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). It is also noted that, for each molecular weight determination, the

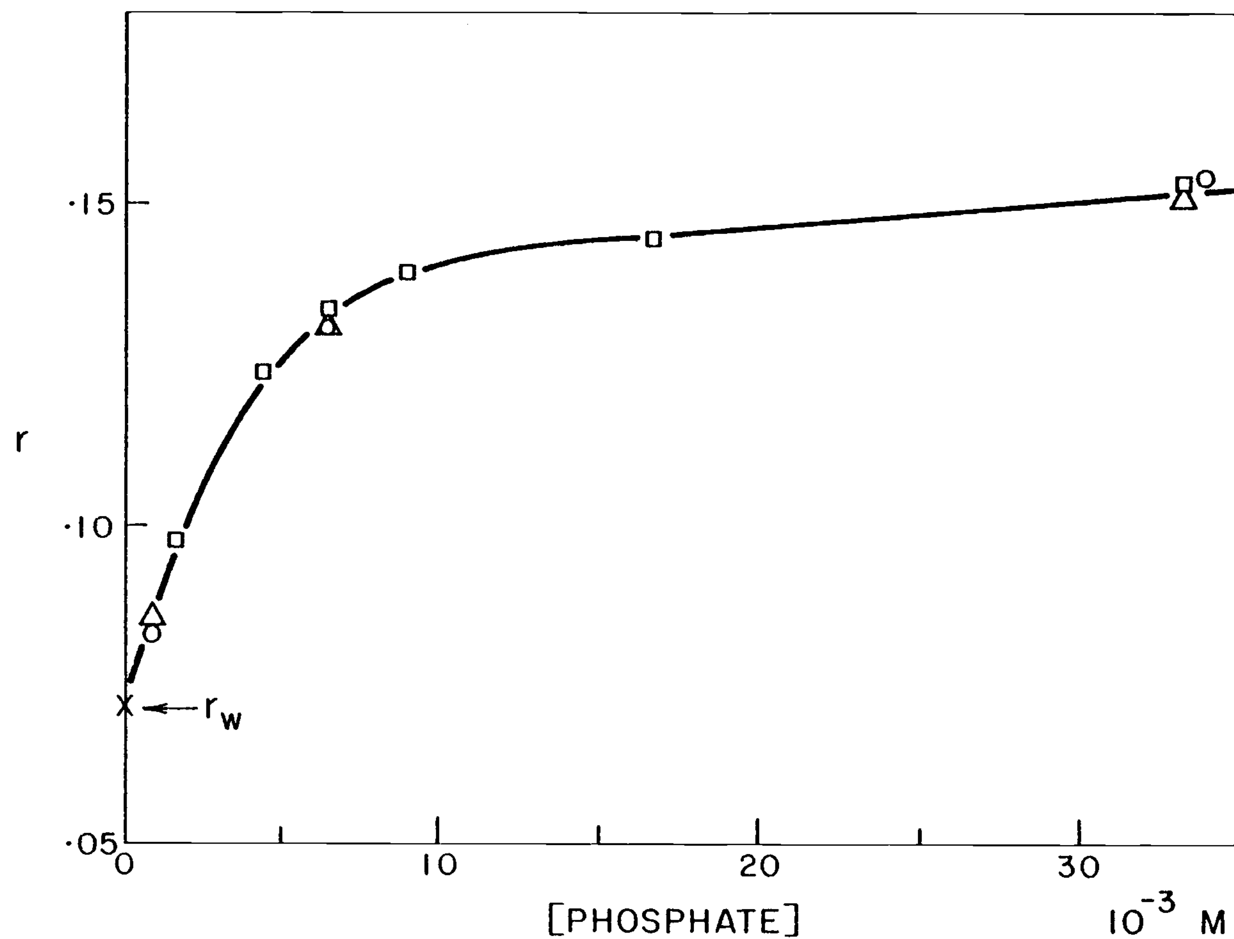

Figure 14. Fluorescence anisotropy of unfractionated H1 at 2.3 x 10^{-5} M (Δ), 3.7 x 10^{-5} M (□), and 4.7 x 10^{-5} M (O) as a function of phosphate concentration, at pH 7.0. r_w is the value of each in water.

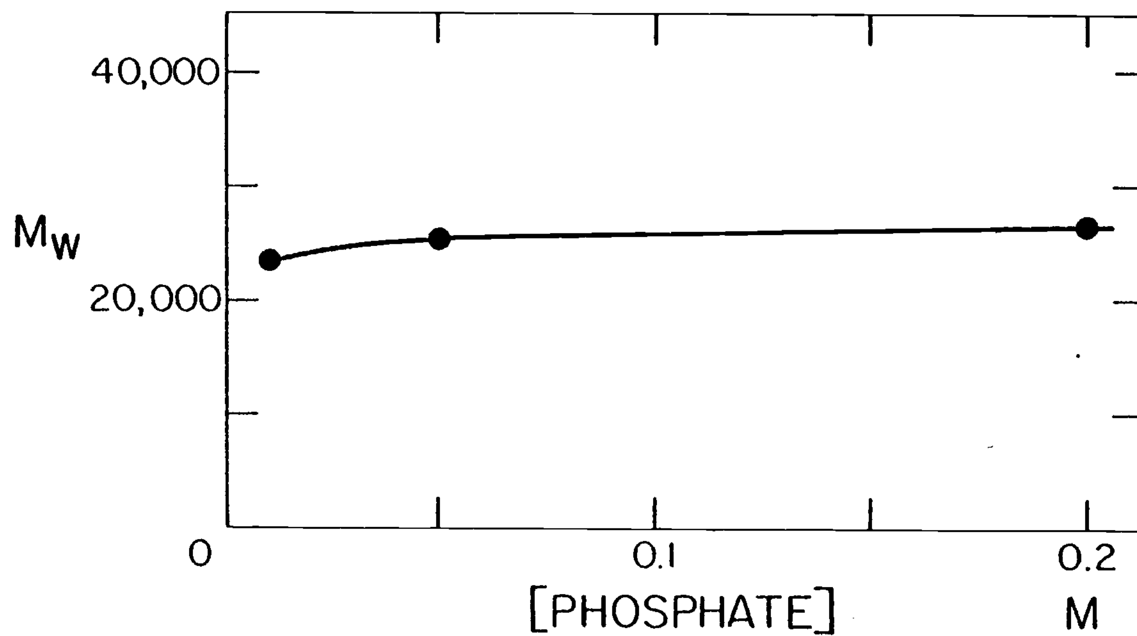

Figure 15. Weight average molecular weight of 4.1×10^{-5} M unfractionated H1, at pH 7.0, as a function of phosphate concentration. A rotor speed of 40,000 rpm was used.

ln C vs. Δr^2 plot gave a good straight line showing that only monomer was present. (The slight increase 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).)

Part C: Interactions Between the H1 Subfractions and NHC Proteins HMG1 and HMG2

Interactions of Unfractionated H1 With HMG1

To measure the interactions between the inner histones, our laboratory has, in the past, made use of the method of continuous variations (Job, 1928; Vosburgh and Cooper, 1941; Rossotti and Rossotti, 1961), where the concentrations of the individual histones were varied but the sum of the concentrations was constant (D'Anna and Isenberg, 1973, 1974b,d; Isenberg, 1976). In those studies the fluorescence anisotropy, and intensity, of the tyrosine residues of the individual histones served as sensitive parameters characterizing the different molecular species present in a mixture of two inner histones. However, a continuous variation method is not convenient in studying the interactions of H1 with either HMG1 or HMG2. These latter proteins contain tryptophan (Baker, et al., 1976) and tyrosine to tryptophan energy transfer is very favorable (Eisinger, et al., 1969; Longworth, 1971; Weinryb and Steiner, 1971; Berlman, 1973). Thus, it is better to use an excitation wavelength outside of the tyrosine absorbance, where only the tryptophans are excited. A continuous variation procedure is, therefore, not possible since at high concentrations of H1 relative to HMG, the signal to noise will become poor. Instead, the molar-ratio method of Yoe and Jones (1944) (see also Rossotti and Rossotti, 1961) was employed where the concentration of H1 is varied keeping

the concentration of HMG1 or HMG2 constant. It is noted that, in these studies, to avoid aggregation, I have chosen pH conditions that are far from the isoionic points of HMG1 and HMG2 (Baker, et al., 1976).

Figure 16 shows the molar-ratio data for unfractionated H1 and HMG1 in 0.02 M phosphate pH 7.6. As can be seen, there is a prounounced deviation in the data from the values expected for no interaction. The data follow two (approximately) straight lines with a non-integral break point at about 1.3. (Appendix II shows that two straight lines are good approximations to theoretical expections.) This result suggests that there is a strong interaction between whole H1 and HMG1, with a stoichiometry of 1.3:1.

Figure 17 shows the results of high speed sedimentation equilibrium (Yphantis, 1964) on a mixture of unfractionated H1 and HMG1 at a ratio of 1.3:1. As shown in the Figure, the mixture is heterogeneous in molecular weight. At low protein concentrations, mainly monomer is present. As the protein concentration goes up, the weight average molecular weight also increases. This result alone would suggest that H1 interacts weakly with HMG1. However, the anisotropy data showed that the interaction is very strong and the stoichiometry of the complex is 1.3 H1 molecules to one HMG1 molecule. This apparent dilemna was resolved upon studying the interactions between the individual H1 subfractions and HMG1.

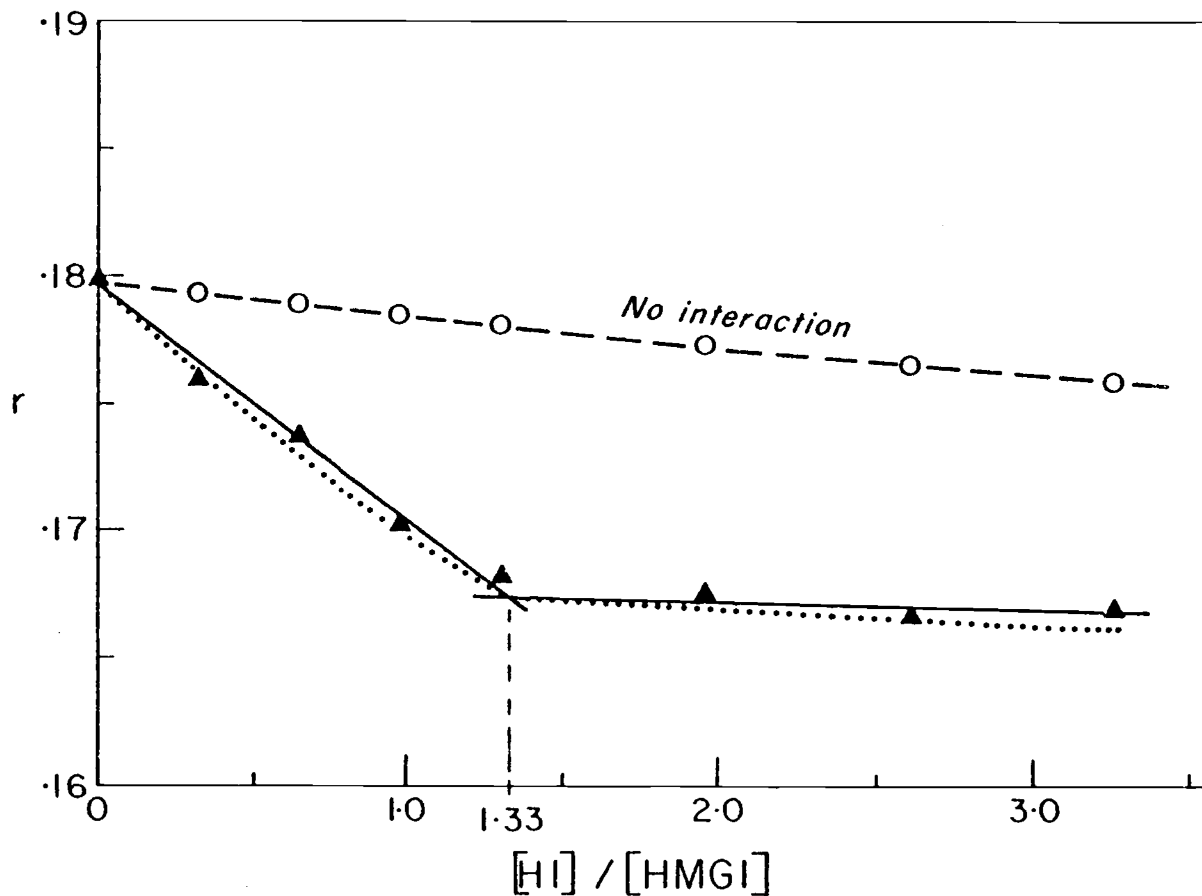

Figure 16. Molar-ratio curve for unfractionated H1 and 5.8 x 10^{-6} M HMG1 in 0.02 M phosphate, pH 7.6. r = Anisotropy. The Figure shows linear fit to the data (———) and curves given by equations (A1) and (A3) (·····). Note the good approximation of linear fits.

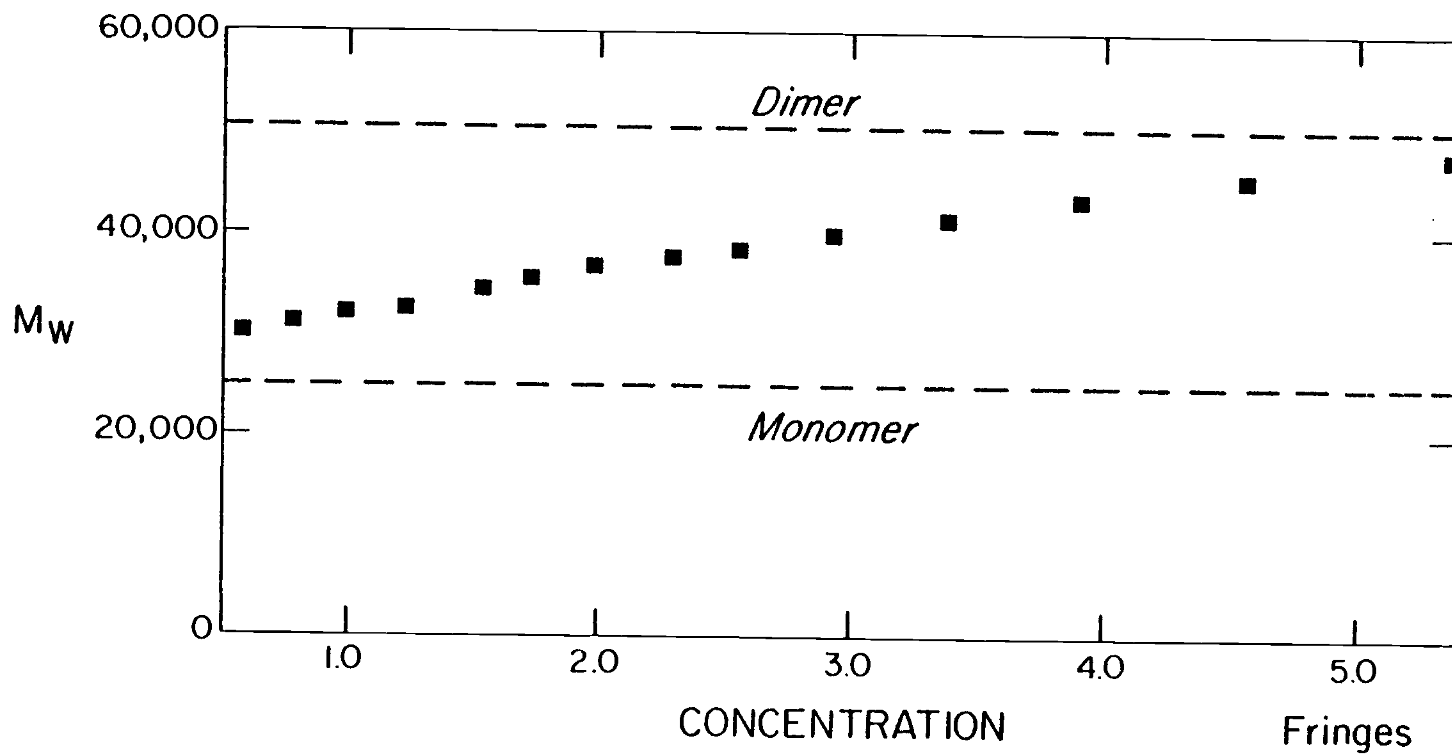

Figure 17. Weight average molecular weight as a function of protein concentration for a 1.3:1 mixture of unfractionated H1 and HMG1 in 0.02 M phosphate, pH 7.6.

Interactions of the H1 Subfractions With HMG1

Figure 18 shows the molar-ratio data for each of the four major H1 subfractions and HMG1. Subfractions 1b and 2 show the same strong interaction found using unfractionated H1. However, the stoichiometry has shifted to the integral value of 1:1. In contrast, subfractions 3a and 3b interact much more weakly. For 3a and 3b the titration data does not have a break point, and does not become linear even out to ratios of 5:1.

As was found for unfractionated H1, the intensity is essentially constant over this titration range for each of the subfractions (Figure 19). This means that the difference between the anisotropy observed and that calculated for no interaction is directly proportional to the concentration of the complex (D'Anna and Isenberg, 1973). Thus, in the cases of 1b and 2, under the conditions shown for Figure 18, at a molar-ratio of 1:1, essentially all of the molecules are complexed.

By integration of the elution profile in Figure 2, the amounts of protein in each peak are estimated to be: 38% in peak 1, 27% in peak 2, 33% in peak 3, and 2% in peak X. Peak 3 contains subfractions 3a and 3b. If these two subfractions did not interact at all with HMG1, and all of the others interacted very strongly, one would predict the break point of a titration using unfractionated H1 to be $(0.38 + 0.27 + 0.02)^{-1} = 1.49$. The measured value is 1.3. The deviation from 1.49 may be due, in part, to the experimental error in determining the fraction of H1 in each elution peak, but,

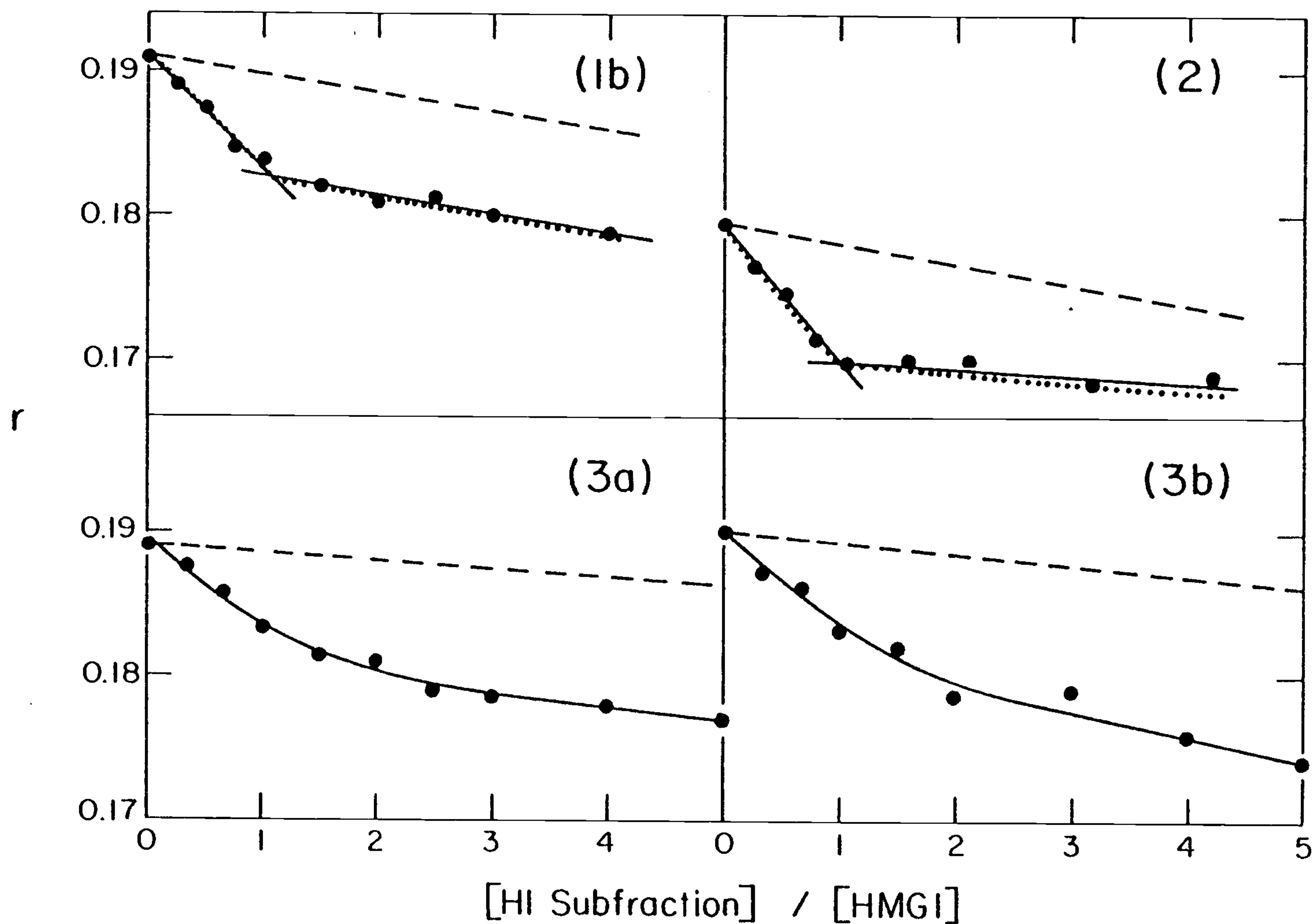

Figure 18. Molar-ratio curves for each of the four major H1 subfractions with 4.0 x 10^{-6} M HMG1 in 0.02 M phosphate, pH 7.6. Solid and dotted lines for subfractions 1b and 2 are defined the same as those for Figure 16. The dashed lines represent curves expected for no interaction.

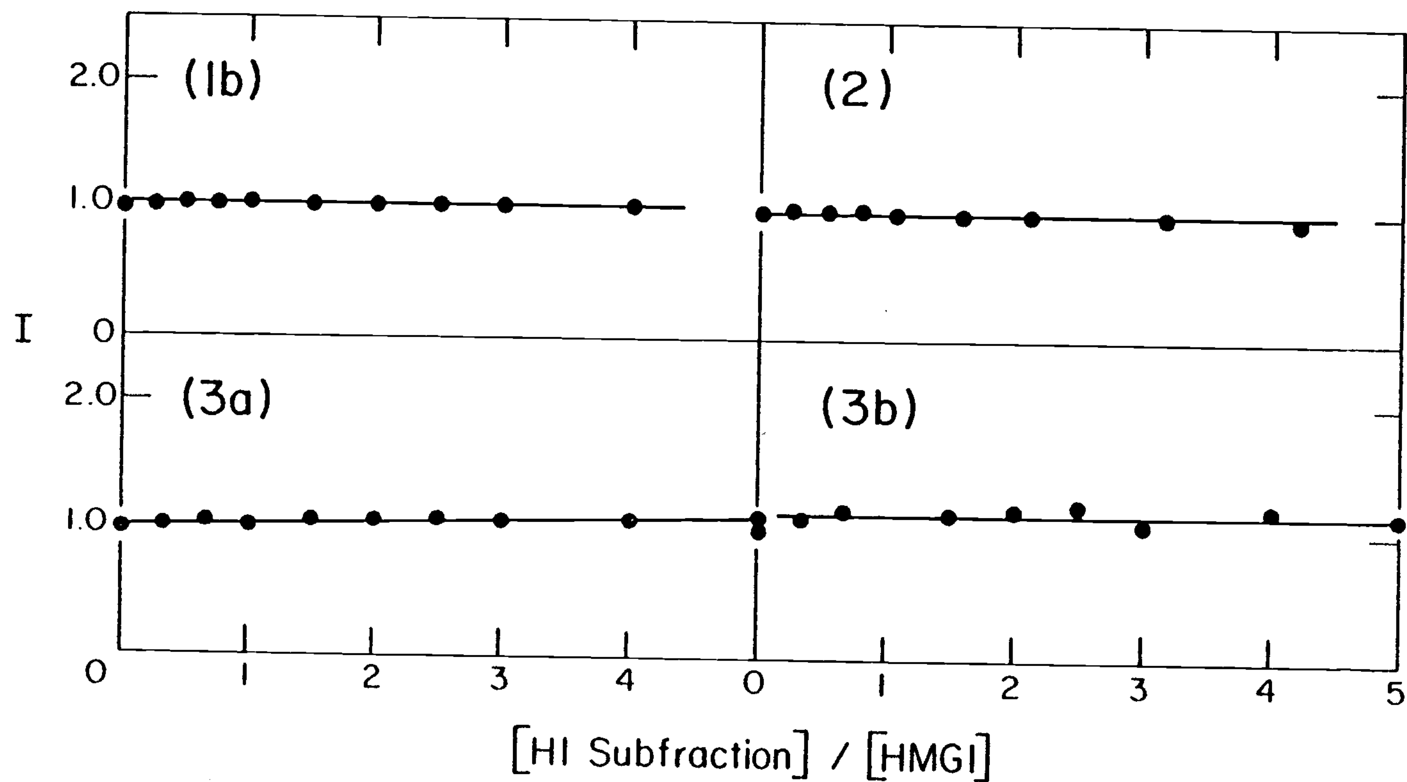

Figure 19. Molar-ratio curves of the fluorescence intensity of HMG1 with each of the four major H1 subfractions. Solutions are the same as those for Figure 18.

in addition, it may be due, in part, to the complexing of a small amount of HMG1 by the weaker interaction of subfractions 3a and 3b.

Molecular weight determinations of 1:1 mixtures of each of the subfractions and HMG1 were made, again using high speed sedimentation equilibrium. Figure 20 shows the ln C vs. Δr^2 data for the mixture of 3b and HMG1. The data is linear over most of the concentration range and the slope of the line shown yields a molecular weight of 25,180 daltons. The calculated molecular weight for a non-interacting 1:1 mixture of H1 (24,000 daltons) and HMG1 (26,500 daltons) is 25,250 daltons. Also, since H1 and HMG1 are so close in molecular weight, a non-interacting mixture of these two proteins would yield a near linear ln C vs. Δr^2 plot and appear to be homogeneous in molecular weight. This is what is observed. Thus, in agreement with the anisotropy data, subfraction 3b interacts only weakly with HMG1.

Figure 21 shows the ln C vs. Δr^2 data for a 1:1 mixture of subfraction 2 and HMG1. In this case the data is also linear. However, the slope yields a molecular weight of 53,800 daltons. The calculated molecular weight for a heterodimer of H1 and HMG1 is 50,500 daltons. Thus, this result shows that subfraction 2 and HMG1 interact very strongly to form heterodimers.

Figure 22 shows the values of M_w vs. concentrations of protein for each of the sedimentation equilibrium runs. In complete agreement with the anisotropy data, these data show that subfractions 1b and 2 interact strongly with HMG1 to form heterodimers, while subfractions 3a and 3b interact much more weakly. (It is noted that,

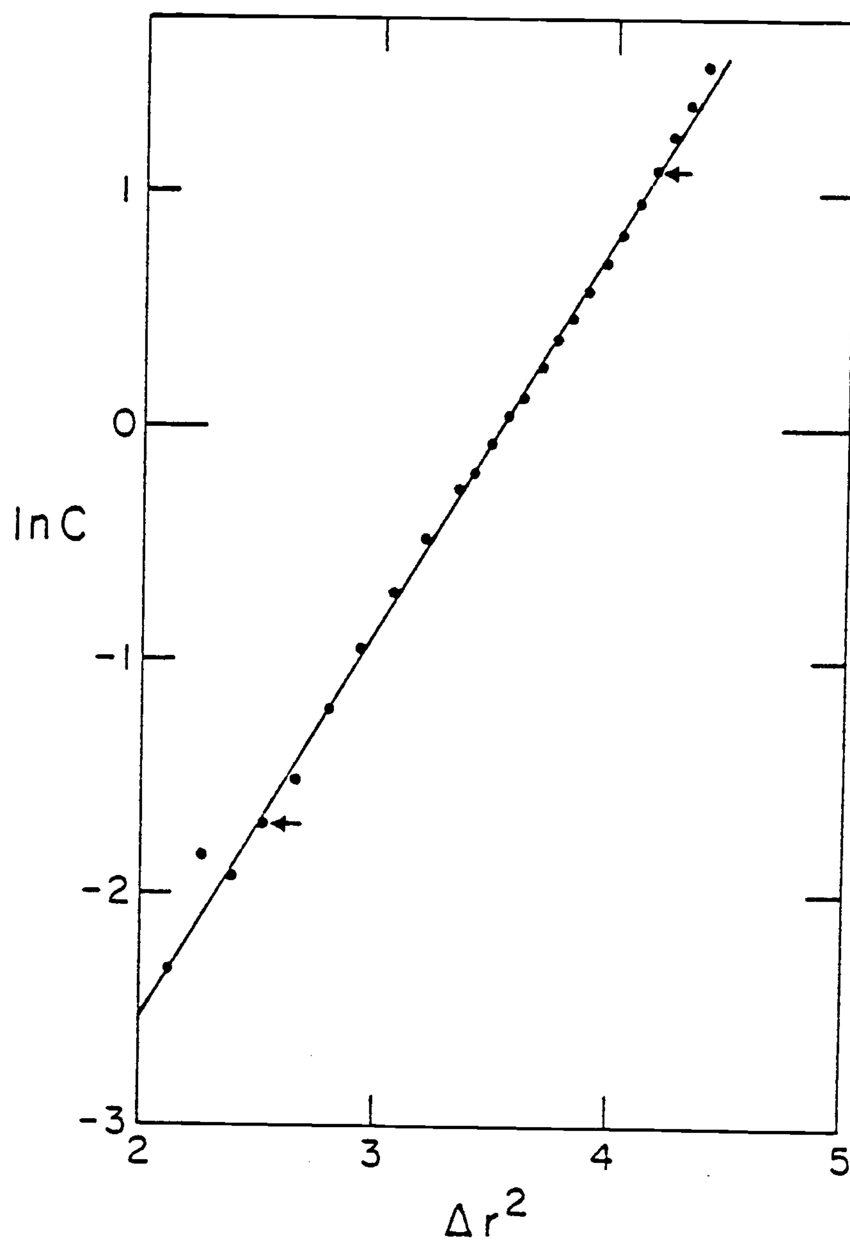

Figure 20. Sedimentation equilibrium: ln C vs. Δr^2 for a 1:1 mixture of subfraction 3b and HMG1 in 0.02 M phosphate, pH 7.6. Initial concentration of each was 4.0×10^{-6} M and the rotor speed was 34,000 rpm. Least squares fit was made for data points between arrows.

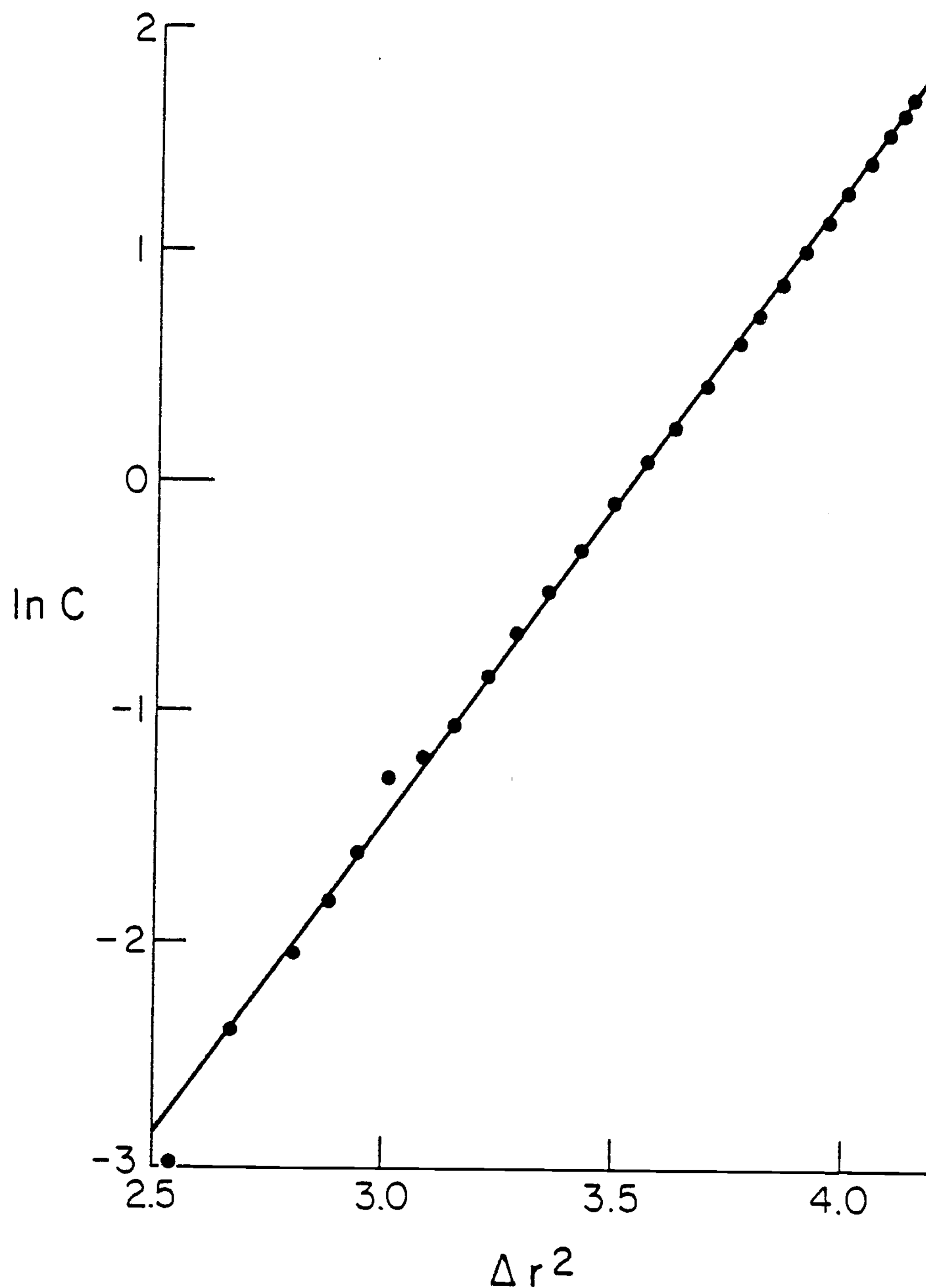

Figure 21. Sedimentation equilibrium: ln C vs. Δr^2 for a 1:1 mixture of subfraction 2 and HMG1. Solution conditions are the same as those for Figure 20. A rotor speed of 30,000 rpm was used.

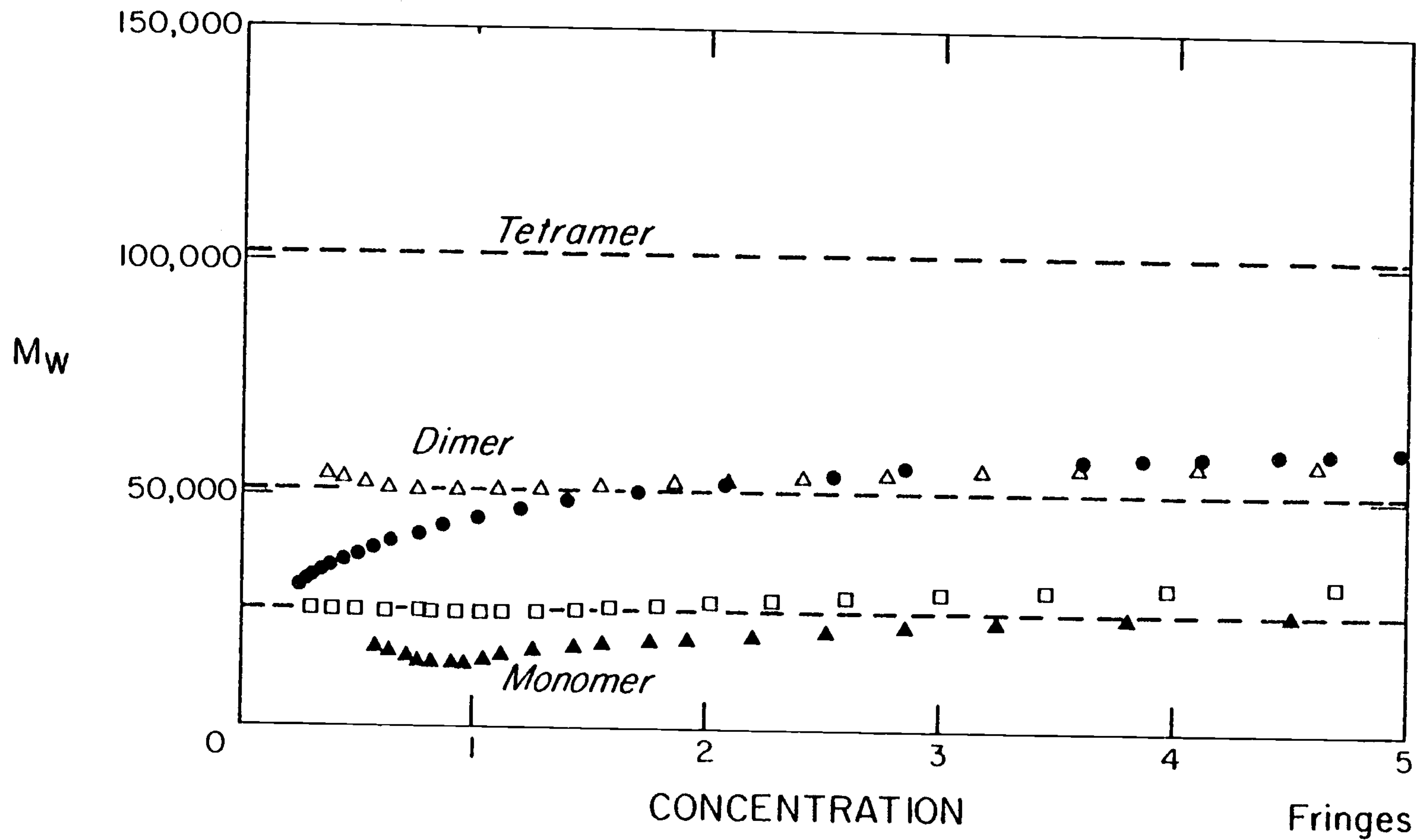

Figure 22. Weight average molecular weight as a function of protein concentration for 1:1 mixtures of HMG1 and subfractions 1b (●), 2 (△), 3a (▲), and 3b (□). Solution conditions are the same as those for Figure 20. A rotor speed of 30,000 rpm was used for 1b and 2, 40,000 rpm for 3a and 34,000 rpm for 3b.

in the case of subfraction 3a, some lower molecular weight species is present and is probably due to proteolysis during the run.)

From Figure 22, it is estimated that the association constant, for the H1(2) · HMG1 complex, is greater than 2 x 10^6 M^{-1}.

Figure 23 shows CD spectra for the two strong complexes. As can be seen, these data show that there is no appreciable change in CD upon complex formation. This is in marked contrast to what is found in complexes of the inner histones (D'Anna and Isenberg, 1973, 1974b,d). When the inner histones form strong complexes, the α-helical content rises.

Table V shows the results of sedimentation velocity measurements on subfraction 2, HMG1, and the complex of these two. The frictional ratio (f/f_0=1.37) for HMG1 is not very different from what is observed for typical globular proteins (Sober, 1968) while the f/f_0 value for subfraction 2, 1.72, shows that it is asymetrical. Upon complexing, f/f_0 drops to 1.41. This could mean that subfraction 2 changes its structure upon complexing; it is also possible that the H1 molecule does not change its structure, but that the more globular HMG1 interacts along the side of the H1 molecule, thus giving the complex a more spherical shape.

Interactions of H1 With HMG2

HMG2 interacts with H1 in a fashion markedly different from HMG1. There is no sharp break point in the molar-ratio data using unfractionated H1 (contrast Figure 16 with Figure 24). Furthermore, subfractions 1b and 2, measured individually, show no observable

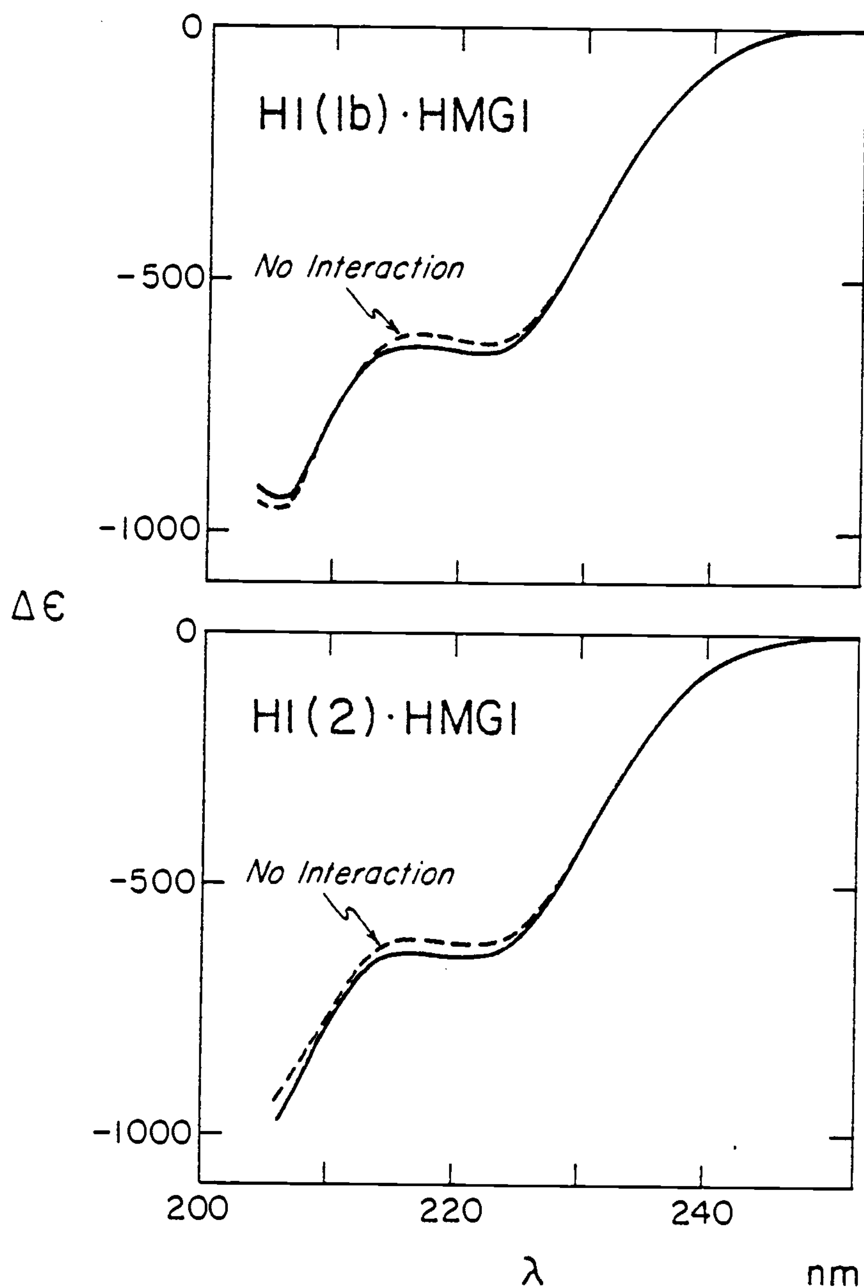

Figure 23. Circular dichroic spectra of 1:1 mixtures of HMG1 with subfractions 1b or 2 in 0.02 M phosphate, pH 7.6. In each case, the concentration of each component was 4.0×10^{-6} M. A 2 mm path length cell was used.

TABLE V: SEDIMENTATION VELOCITY RESULTS[a]

SAMPLE	$s_{20,w}$	f/f_o
H1(2)	1.47S	1.72
HMG1	2.35S	1.37
H1(2) + HMG1	3.24S	1.41

[a]Protein concentrations were: 5.0×10^{-5} M for H1(2), 4.0×10^{-6} M for HMG1, and 4.0×10^{-6} M for each component of the H1(2)·HMG1 complex. A rotor speed of 52,000 rpm was used for H1(2) and 48,000 rpm for HMG1 and the complex H1(2)·HMG1. Each protein solution contained 0.02 M phosphate, pH 7.6.

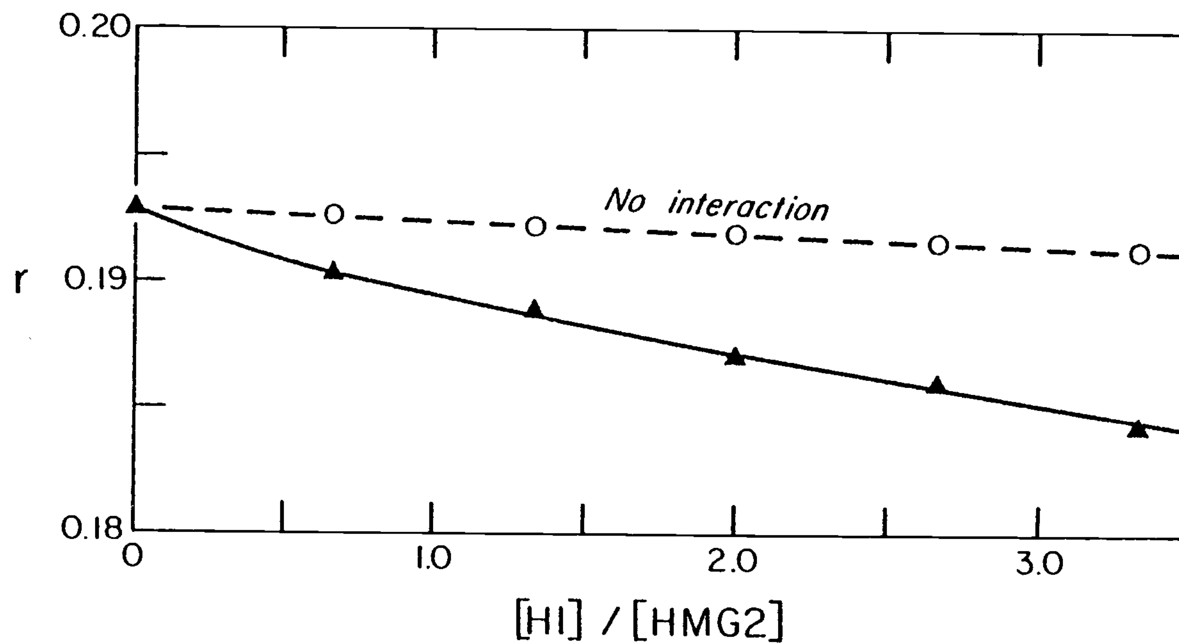

Figure 24. Molar-ratio curve for unfractionated H1 and 5.8 x 10^{-6} M HMG2 in 0.02 M phosphate, pH 6.8.

interaction with HMG2 whatsoever. Also, subfractions 3a and 3b do interact, but no break point is observed (Figure 25). As was found using HMG1, there is no change in the intensity of the HMG2 tryptophan fluorescence upon titration with the H1 subfractions (Figure 26).

Sedimentation equilbrium data on 1:1 mixtures of HMG2 with either subfraction 3a or subfraction 3b yield curved ln C vs. Δr^2 plots (Figure 27). Thus, in both cases there is a heterogeneity in molecular weight. This is probably due to the weak interactions observed in the anisotropy data (Figure 25), and shows that these interactions are much weaker than those observed for subfractions 1b or 2 and HMG1.

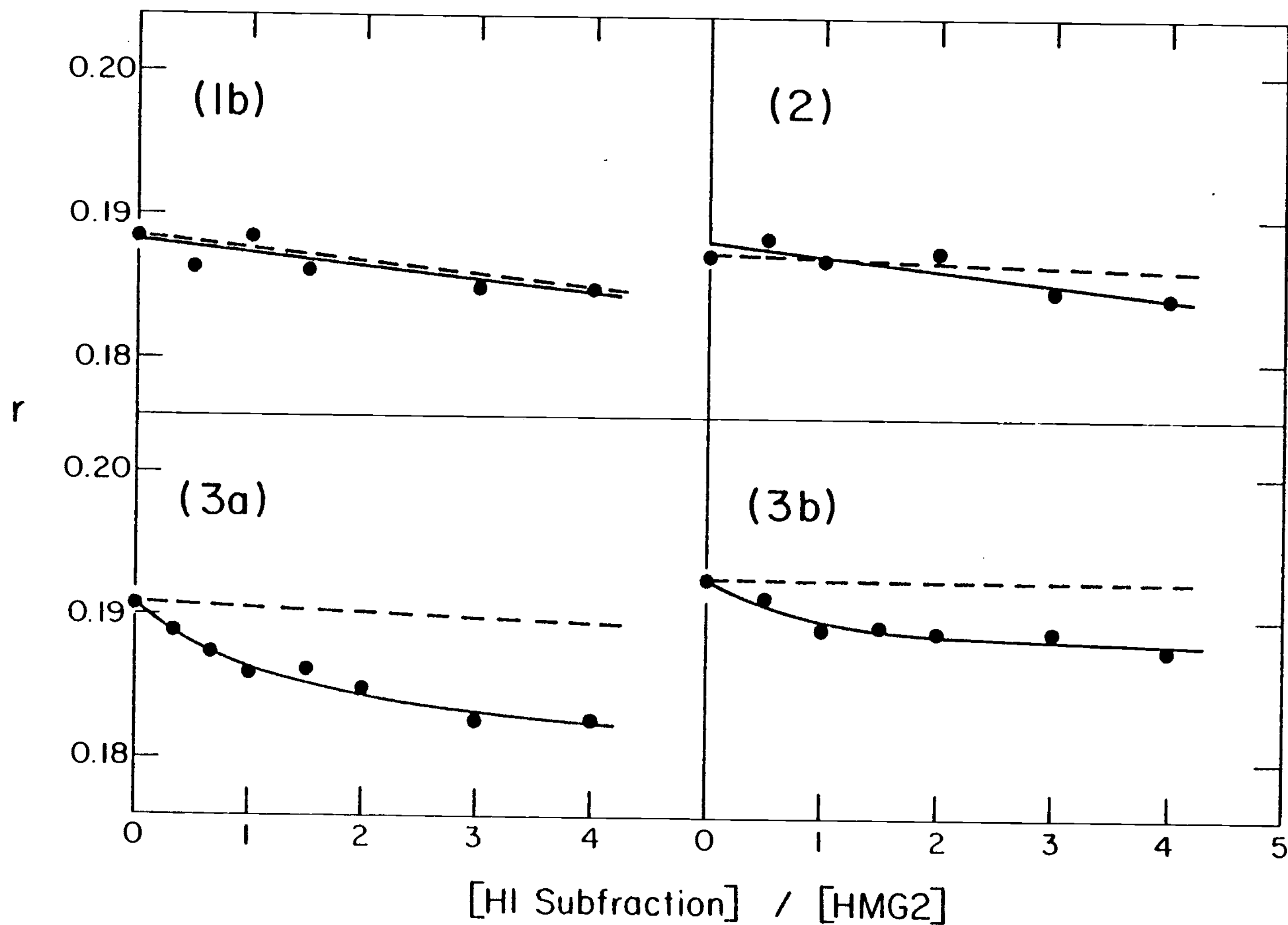

Figure 25. Molar-ratio curves for each of the four major subfractions with 4.0×10^{-6} M HMG2 in 0.02 M phosphate, pH 6.6. The dashed lines represent curves expected for no interaction.

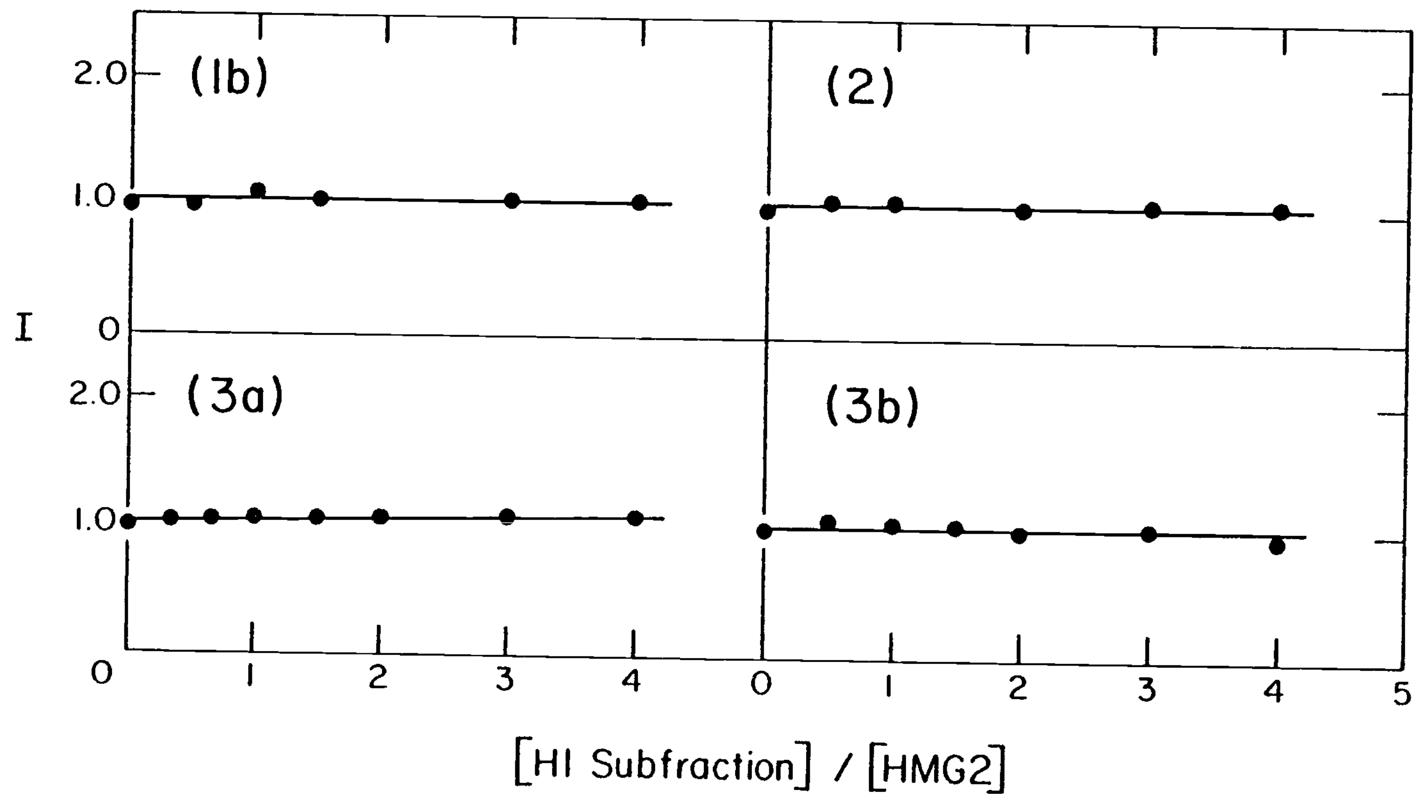

Figure 26. Molar-ratio curves of the fluorescence intensity of HMG2 with each of the four major H1 subfractions. Solutions are the same as those for Figure 25.

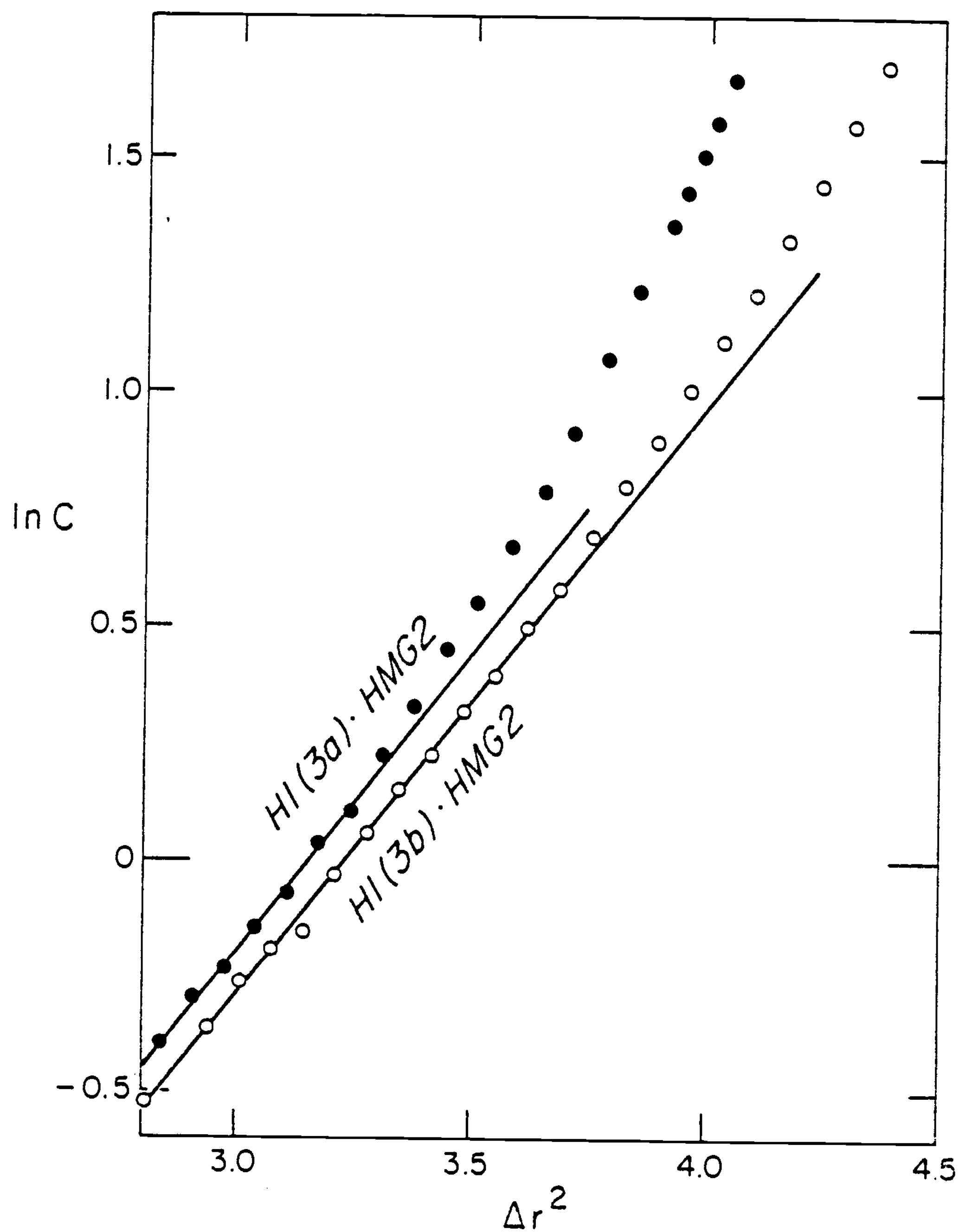

Figure 27. Sedimentation equilibrium: ln C vs. Δr^2 for 1:1 mixtures of HMG2 with subfractions 3a or 3b in 0.02 M phosphate, pH 6.6. Initial concentration of each component was 4.0×10^{-6} M and the rotor speed was 34,000 rpm.

IV. DISCUSSION

In the course of preparing calf thymus H1 subfractions for physical studies, it was found that perhaps more subfractions are present than previously reported (Kincade and Cole, 1966a,b). Of the possible new subfractions, the one that I have called 1a is present in the largest amount (5% - 8% of whole H1). I now ask if this is really a new subfraction of calf thymus 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.

I first note that there was no evidence, in the subfraction 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.) When H1, previously run through the Sephadex G-100 column, was used, only the peaks shown in Figure 2 were seen. Furthermore, the same profile was obtained with H1 prepared with either $NaHSO_3$ or PMSF, two different protease inhibitors. In addition, 1a is the slowest migrating band on an acetic acid-urea gel and migrates slower than 1b on an SDS 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. This latter possibility cannot be ruled out completely; a definitive evaluation must await sequence determinations. However, even now some conclusions can be drawn from the amino acid composition determinations (Table II). There is, for example, significantly

more serine and valine in the mixture of 1a and 1b than in 1b alone. Consequently, since the molecular weights of all the subfractions are almost the same (as judged by SDS mobilities and sedimentation equilibrium), 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 I cannot, at present, rigorously eliminate the possibility that 1a is a degradation product, it appears unlikely.

This 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. These 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 about 15% of the residues are in either α-helical or β-sheet form. There is probably additional tertiary structure, since the tyrosine could not be so firmly buried by secondary structure alone. The transition to the folded state is highly cooperative. In this state at least part of the molecule has a compact, folded structure.

There are 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 structure. In any case, subfraction 3a is clearly different from the others. It may therefore have structural features that are different, although these differences are not discernable by the techniques used.

The NHC proteins HMG1 and HMG2 interact with the H1 subfractions with a high degree of specificity. This is the first time that any differences in the binding properties of the H1 subfractions has been demonstrated (aside from the finding of Bustin and Stollar (1972) that the subfractions have distinct antigenic determinants) and opens up the possibility that the subfractions perform different functions in chromatin. It is noteworthy that very large binding differences are seen for the various subfractions, even though no striking differences are observed in the physical properties of the subfractions themselves. The high degree of binding specificity that was found rules out, of course, non-specific charge-charge interactions as the cause of complexing. Ultimately, the reason for the specificity must be found in the different structural properties of the H1 subfractions. A schmetic representation of the cross-complexing pattern is shown in Figure 28.

These results, incidentally, explain the anomolous findings of Shooter, _et al_., (1974). These authors obtained sedimentation

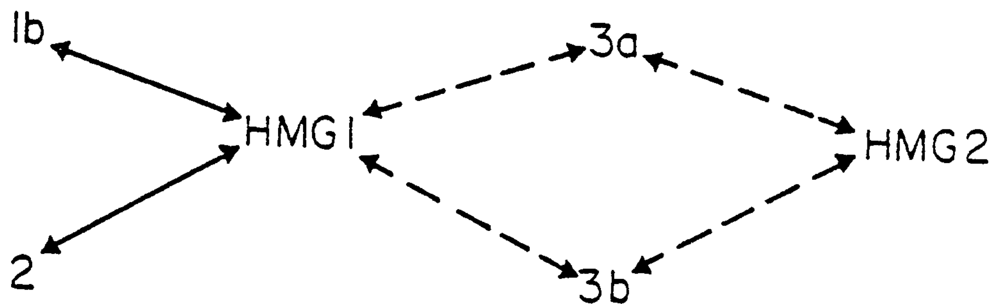

Figure 28. Schematic representation of the cross-complexing pattern for the four major subfractions of calf thymus H1 with non-histone chromosomal proteins HMG1 and HMG2. Solid lines represent strong interactions and dashed lines represent weaker interactions. The pattern shows only the manner in which the proteins interact with each other and is not meant to suggest any particular complexing arrangement in chromatin.

data showing that there was an interaction between HMG1 and unfractionated H1, but concluded that no specific type of complex was produced. This work shows that their conclusion was due to the use of unfractionated H1; with specific subfractions definite complexes are observed.

I now turn to the question of whether the H1·HMG complexes exist as such in chromatin. There is no evidence to settle this point at present, but the possibility is raised that H1·HMG complexes may already have been detected by cross-linking experiments. Figure 1 of the paper by Thomas and Kornberg (1975) shows an SDS gel pattern of proteins, cross-linked in chromatin by dimethyl suberimidate. Among the bands was a weak one corresponding to a molecular weight of about 50,000-60,000. Thomas and Kornberg marked this band "$(F1)_2$?" and stated that it might be a cross-linked dimer of H1. When H1-depleted chromatin was cross-linked, this band was no longer present, so it appeared that the band did contain H1.

Thomas and Kornberg found a similar band when proteins were first extracted from chromatin, and then cross-linked in solution. It is noted, however, that there is now convincing evidence that H1 does not dimerize (Teller, _et al_., 1965; Haydon and Peacocke, 1968; Edwards and Shooter, 1969; Kornberg and Thomas, 1974; the present report), whereas it has now been shown that certain subfractions of H1 and HMG1 do form heterodimers. The band observed by Thomas and Kornberg was a minor one compared to the amount seen at the H1 monomer position; it is noted that the ratio of the HMG proteins to H1 is about 0.03:1 to 0.06:1 (Johns, _et al_., 1975; Walker, _et al_.,

1975; the present report). All of these considerations make it more reasonable that the SDS gel band observed by Thomas and Kornberg is an H1·HMG heterodimer rather than an H1 dimer. However, at the present time, there is no direct evidence supporting this speculation, and its validity must await future work.

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APPENDICES

APPENDIX I

Development of Expressions for Extrapolation of CD Data to Infinite Salt Concentrations

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

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

Thus, by equating coefficients,

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

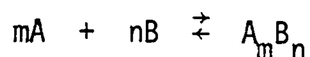
$$A_\beta = [\xi_\beta - (1-f)a_\beta]/f$$

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

APPENDIX II

Development of Theoretical Expressions for Fluorescence Anisotropy Applied to Molar-Ratio Data

Consider the complex formation



$$mA + nB \rightleftarrows A_mB_n$$

with an equilibrium constant

$$K = \frac{[A_mB_n]}{[A]^m[B]^n}$$

Let A_o and B_o be the total amount of A and B. Let $X = B_o/A_o$.

Let r_A, r_B, and r_C be the respective anisotropies of A, B, and A_mB_n and I_A, I_B, I_C be the respective intensities.

We consider a titration in which A_o is held constant and B_o is varied. By the Weber addition law (Weber, 1953; Dale and Eisinger, 1975),

$$r = \frac{I_Ar_A + I_Br_B + I_Cr_C}{I_A + I_B + I_C}$$

We also have, for dilute solutions,

$$I_A = k'_A\ [A]$$

$$I_B = k'_B\ [B]$$

$$I_C = k'_C\ [A_mB_n]$$

where k'_A, k'_B and k'_C are constants.

We first consider the titration region $0 \leq X \leq n/m$.

For very strong binding, $I_B r_B \ll r_A I_A + r_C I_C$, $I_B \ll I_A + I_C$, and $B_o \approx n[A_m B_n]$. Therefore,

$$I_C \approx \frac{k'_C}{n} B_o$$

$$= k_C X$$

$$I_A = k'_A A_o \frac{m}{n} \left(\frac{n}{m} - \frac{B_o}{A_o}\right)$$

$$= k_A \left(\frac{n}{m} - X\right)$$

and

$$r = \frac{I_A r_A + I_C r_C}{I_A + I_C}$$

From these,

$$r = \frac{\frac{k_A}{k_C} r_A \left(\frac{n}{mX} - 1\right) + r_C}{\frac{k_A}{k_C} \left(\frac{n}{mX} - 1\right) + 1} \qquad \text{(A1)}$$

which may also be written

$$\frac{1}{r_A - r} = \frac{\xi_1}{X} + \phi_1 \qquad \text{(A2)}$$

where

$$\xi_1 = \frac{nk_A}{mk_C}\left(\frac{1}{r_A - r_C}\right)$$

and

$$\phi_1 = \frac{1 - k_A/K_C}{r_A - r_C}$$

Knowing n/m, data fitted by equation (A2) will permit a determination of ξ_1 and hence k_A/k_C.

In a similar manner, for the titration region, $X > n/m$, we obtain the functional forms

$$r = \frac{\frac{k_B}{k_C} r_B (X - n/m) + \frac{n}{m} r_C}{\frac{k_B}{k_C} (X - \frac{n}{m}) + \frac{n}{m}} \tag{A3}$$

and

$$\frac{1}{r - r_B} = \xi_2 (X - \frac{n}{m}) + \phi_2 \tag{A4}$$

where

$$\xi_2 = \frac{mk_B}{nk_C (r_C - r_B)}$$

and

$$\phi_2 = \frac{1}{r_C - r_B}$$

Figures (16) and (18) show that, for strong binding, the expressions (A1) and (A3) can be approximated well by straight lines.