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HWAN, Robert Jong-chin, 1947-
INTERACTION OF LYSINE-RICH HISTONES AND DNA.

City University of New York, Ph.D., 1978
Biophysics, general

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INTERACTION OF LYSINE-RICH HISTONES AND DNA

by

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November, 1977

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

12/20/77
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ABSTRACT

INTERACTION OF LYSINE-RICH HISTONES AND DNA

by

Robert Jong-chin Hwan

Advisor: Professor Hsueh Jei Li

Lysine-rich histones H1 and H5 from chicken erythrocytes were used in the study of their interaction with DNA. Nucleohistone complexes were prepared using the procedure of continuous NaCl gradient dialysis in the presence or absence of urea. Two physical methods, namely thermal denaturation and circular dichroism (CD), were used to examine histone-DNA complexes and erythrocyte chromatin. Chemical methods, using tetranitromethane (TNM) as the nitration agent were also employed to study the conformational changes of histones induced by high salt. Interactions among histones as well as between histone and DNA in chromatin were studied.

Thermal denaturation of nucleohistones H5 with chicken or calf thymus DNA shows three melting bands: band I at 45-50°C corresponding to helix-coil transition of free base pairs; band II at 75-79°C, and band III at 90-93°C corresponding to those of histone-bound base pairs. Similar melting properties have been obtained in the nucleohistone complexes prepared from histone H5 and Micrococcus luteus DNA. For nucleohistone H1, free base pairs melt at 45-50°C, histone bound base pairs melt primarily at 73°C with a very minor band at 63°C.

Nucleohistone (H1+H5) prepared by complexing an equimolar mixture of purified histones H1 and H5 from chicken erythrocyte with homologous DNA showed a melting profile closer to that of nucleohistone H1 than of nucleohistone H5. Based on thermal denaturation studies, it is calculated that in histone-bound regions, 1.5 amino acid residues/nucleotide are involved in forming the complex nucleohistone H5. In contrast, a value between 2.9 and 3.3 was determined for nucleohistone H1 (Li, 1973).

The binding of histone H5 to DNA induces only a slight transition from a B-type to a C-type CD spectrum. H5 in a histone-DNA complex also contains little α-helical structure. Trypsin treatment of nucleohistone H5 reduces melting band III much more effectively than band II. Such a treatment also restores the DNA from a distorted structure to the B conformation in the free state as measured by CD. The reduction of melting bands of nucleohistone H5 by polylysine binding follows the order of I > II > III, accompanied by the increase of a new band at 100°C.

When two DNAs of varied A+T (adenine + thymine) content compete simultaneously for the binding of histone H5, the more (A+T)-rich DNA is selectively favored. Clostridium perfringens DNA with 69% A+T is bound by histone H5 in preference to chicken DNA with 56% A+T, although the latter has natural sequences for histone H5 binding.

Circular dichroism (CD) studies showed that, in solutions of low ionic strength, the two lysine-rich histones

interact with a 1:1 stoichimetry, similar to that found in H2A-H2B and H3-H4. Results obtained from the nitration study of histones showed that in high salt, under which condition a tetrameric complex exists among histones H2A, H2B, H3 and H4, the tyrosine groups of all histones are still accessible to tetranitromethane. These results also suggest the existence of interaction between histones (H1+H5) and histones (H2a+H2B+H3+H4).

This dissertation is dedicated
to my father, Tze-Hsiang Tsai,
M.D. and mother, Chin Hwan Tsai,
M.D. who led me to the wonder of
science and encouraged me to
search after the inner beauty
of science with love and care.

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ACKNOWLEDGEMENT

I would like to express my thanks to Dr. Hsueh Jei Li, my thesis advisor, for his help throughout the course of this study. His support and continuous encouragement will long be remembered. I have also benefited from Dr. P.C. Huang of the Department of Biochemistry, School of Hygiene and Public Health, the Johns Hopkins University for a substantial amount of my thesis done in his laboratory. The stimulating discussions with my colleagues in both laboratories, particularly Drs. I. Michael Leffak, Sharon S. Yu, and Casilda Mura are also acknowledged.

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CHAPTER I

INTRODUCTION

Histones are the major components of eukaryotic cells, complexing with DNA to form the chromatins. Chromatin is the relaxed form of chromosomes. Together with the non-histone proteins and certain RNA, histones are purported to play a role in the regulation of gene expression (Stedman and Stedman, 1950).

There are five major classes of histones, namely: H1, H2a, H2b, H3 and H4 in all eukaryotic chromatins. An additional histone H5 exists in the nucleated erythrocytes. Histones H1 and H5 are particularly rich in lysine with about 24 mole % of total amino acids (Table 1). The lysine rich histones constitute about one fifth of the total histones. Like all other histones, these proteins are basic in charge having an isoelectric point greater than 10 and are notably devoid of tryptophane.

Due to their basicity, histones can be readily extracted by mild acids such as 0.5N sulfuric or 1% hydrochloric from the chromatin which is usually purified from the nuclei. Electrolytes, such as sodium chloride, sodium perchlorate and guanidinum chloride also extract histones (Bonner et al., 1968). This latter dissociation characteristic indicates that histone-DNA interaction is not covalent but electrostatic in nature.

Histones H1 and H5 can be selectively extracted from the

Table 1. Amino Acid Composition of Lysine-Rich Histones (mole %)

	[a] Duck H1	[b] Rabbit Thymus H1	[c] Calf Thymus H1	[d] Adult Chicken H5	[d] Embryonic Chicken H5	[e] Chicken H5	[a] Duck H5	[f] Goose H5	[f] Quail H5
Lys	25.9	27.3	25.7	22.8	22.2	24.1	24.0	24.2	22.6
His	0.4	0	0.1	1.5	0.8	1.4	1.9	1.5	1.7
Arg	2.9	1.6	2.5	9.7	10.3	10.9	11.6	11.5	12.7
Asp	2.0	2.2	3.1	2.1	2.4	1.8	1.8	2.1	2.4
Thr	5.3	4.3	5.6	3.7	3.1	3.3	2.1	4.0	3.6
Ser	6.6	5.3	6.4	10.7	12.4	13.0	9.4	10.1	12.2
Glu	4.4	3.6	5.3	4.6	4.5	4.4	5.1	3.3	3.4
Pro	9.7	11.1	9.7	7.5	8.0	7.1	7.9	9.1	6.5
Gly	6.8	6.8	7.4	6.1	6.1	4.8	5.6	5.4	4.5
Ala	24.0	26.6	23.3	15.9	15.9	15.8	17.1	17.3	16.0
Cys	0	0	0	0	0	0	0	0	0
Val	4.3	5.0	4.5	4.9	4.1	3.9	3.2	2.8	4.5
Met	0	0	0	0	0.3	0.6	0.8	0.3	0.2
Ile	1.2	0.9	0.9	3.3	3.2	3.0	3.0	3.0	2.8
Leu	4.8	4.5	4.0	5.1	4.3	4.6	4.1	3.6	4.5
Tyr	0.7	0.4	0.6	1.4	1.2	1.7	2.2	1.4	1.5
Phe	0.7	0.4	0.7	0.6	0.8	0.6	0.2	0.4	1.1

[a] Sanders and McCarty, 1972.

[b] Buslin and Cole, 1969.

[c] Kinkade and Cole, 1966.

[d] Huang et al., 1977.

[e] Greenaway and Murray, 1971.

[f] Seligy et al., 1976.

chromatin with even milder conditions (Murray, Vidali, and Neelin, 1968). In fact, they are the first histones to be removed by either mild acid or low ionic strength of electrolytes from the chromatins. For instance, treatments with 5% trichloroacetic acid or perchloric acid preferentially extract both H1 and H5 which can be separated by Amberlite CG 50 ion exchange chromatography. Histone H1 can also be extracted from chick erythrocyte chromatin by repeated extraction with 0.5 M NaCl, 50mM sodium phosphate pH 7.0 (Bradbury et al., 1972a). Histone H5 is subsequently removed by 0.65 M NaCl in the same buffer. Other histones remain complexed with the DNA. These procedures allow a rapid purification of these two lysine rich histones for physicochemical studies.

The lysine histones are similar in size, containing 216 and 198 amino acid residues in H1 and H5 respectively. Being over 21,000 dalton in molecular weight, these are the largest histones among all species. Although both are rich in lysine, H1 and H5 are different in amino acid compositions (see Table 1). While most histone sequences have been determined (see review by Elgin and Weintraub, 1975), only partial sequence data are available for H1 and H5. These are summarized in Figure 1. Histones, unlike all other globular proteins, show a unique distribution of the amino acid residues in the polypeptide chains. The C-terminal

Figure 1. Comparison of the Lysine Rich Histone Partial Amino Acid Sequences

A

H1

Calf Thymus	Ac-Ser	Glu	Ala	Pro	Ala	Glu	Thr	Ala	Ala	Pro	10
Chicken	Ac-Ser	Glu	Ala	Pro	-	-	Thr	Val	Ala	-	
	Ala	Pro	Ala	Pro	Lys	Ser	Pro	Ala	Lys	Thr	20
	Ala	Pro	Ala	Val	Ser	Ala	Pro	Ala	Lys	Lys	
	Pro	Val	Lys	Ala	Ala	Lys	Lys	Lys	Lys	Pro	30
	Pro	-	Lys	Ala	Ala	Lys	Lys	Lys	-	Ala	
	Ala	Gly	Ala	Arg	Arg	Lys	Ala	Ser	Gly	Pro	40
	Ala	Gly	Gly	-	Ala	Lys	Ala	Arg	Lys	Pro	
	Pro	Val	Ser	Glu	Leu	Ile	Thr	Lys	Ala	Val	50
	Pro	Val	Ser	Glu	Leu	Ile	Thr	Lys	Ala	Val	
	Ala	Ala	Ser	Lys	Glu	Arg	Ser	Gly	Val	Ser	60
	-	Ser	Ala	Lys	(- 12 residues			-	-	-	
	Leu	Ala	Ala	Leu	Lys	Lys	Ala	Leu	Ala	Ala	70
	-	-)	Ala	Leu	-	-	Ala	-	Ala	-	
	Ala	Gly	Tyr	- - - - - - - - - - - - - - - - - - - -							80
	Gly	Gly	Tyr	Asp	Val	Glu	Lys	Gly	Asn	Arg - - -	

B

H5

Chicken	Thr	Glu	Ser	Leu	Val	Leu	Ser	Pro	Ala	Pro	10
Goose	Thr	Asp	Ser	Pro	Ilu	Pro	Ala	Pro	Ala	Pro	
	Ala	Lys	Pro	Lys	Glu, Arg	Val	Lys	Ala	Ser	Arg	20
	Ala	Lys	Pro	Lys	Arg	Ala	Arg	Ala	Pro	Arg	
	Arg	Ser	Ala	Ser	His	Pro	Thr	Tyr	Ser	Glu	30
	Lys	Pro	Ala	Ser	His	Pro	Thr	Tyr	Ser	Glu	
	Met	Ilu	Ala	Ala	Ala	Ilu	Arg	Ala	Glu	Lys	40
	Met	Ilu	Ala	Ala	Ala	Ilu	Arg	Ala	Asp	Lys	
	Ser	Arg	Gly	Gly	Ser	Ser	Arg	Glu	Ser	Ilu	50
	Ser	Arg	Gly	Gly	Ser	Ser	Arg	Glu	Ser	Ilu	
	Gln	Lys	Tyr	Ilu	Lys	Ser	His	Tyr	Lys	Val	60
	Gln	Lys	Tyr	Val	Lys	Ser	His	Tyr	Lys	Val	
	Gly	His	Asn	Ala	Asp	Leu	Gln	Ilu	Lys	Leu	70
	Gly	Gln	His	Ala	Asp	Leu	Gln	Ilu	Lys	Leu	
	Ser	Ile	Arg	Arg	Leu	Leu	Ala	Ala	Gly	Val	80
	Ala	Ile	Arg	Arg	Leu	Leu	Thr	Thr	Gly	Val	
	Leu	Lys	Gln	Thr	Lys	Gly	Val	Gly	Ala	Gly	90
	Leu	Lys	Gln	Thr	Lys	Gly	Val	Gly	Ala	Ser	
	Ser	Ser	Phe	Arg	Leu	Ala	Lys	Ser	Asp	Lys	100
	Gly	Ser	Phe	Arg	Leu	Ala	Lys	Gly	Asp	Lys	
	Ala	Lys	Arg	Ser	Pro	Gly	Lys	Lys	Lys	Ala	110
	Ala	Lys	Arg	Ser	Pro	Ala	Gly	Arg	Lys	Lys	
	Lys										
	Lys										

Source of Data: Calf Thymus H1 (Rall and Cole, 1971).
Chicken H1 (Cole and Hsiung, personal communication to P.C. Huang).
Chicken H5 (Sautiere et al., 1976).
Goose H5 (Seligy et al., 1976; Yaguchi et al., 1977).

Dash line (-) denotes displace amino acids, dotted line (---) uncertainty, underline (___) the basic amino acids, and blocks area of homology.
Position 15 in chicken H5 shows polymorphism.

half of histone H1 is more basic than the N-terminal half (Bustin and Cole, 1969b) which is just opposite to the other histones. H5 also shows an uneven distribution of their basic amino acid residues, as judged from the partial sequence data (Sautiere et al., 1976).

The genetics of H1 and H5 are poorly understood. There are estimates that each histone is coded by as few as ten or as many as several hundred genes (Keder, 1976). Histones H1-H4 genes are arranged in interdigitated clusters. Between each gene there are spaces ranging from about 300 to 800 base pairs. The location of the H5 gene is unknown.

It has been shown that chicken H5 is polymorphic in that its amino acid residue No. 15 exists as either glutamine or arginine (Greenaway and Murray, 1971). These two species of H5, however, are not readily separable and for all practical purposes seem to function similarly. Histone H1 has been shown to be more heterogeneous (Bustin and Cole, 1969a; Cole, 1977). There are three electrophoretic components of histone H1 in mouse thymus and mouse liver, and five in Ehrlich ascites tumor. Two of the electrophoretic components from the latter were minor bands, and disappeared after alkaline phosphatase treatment (Sherod, Johnson, and Chalkley, 1970) indicating that they are derived from one of the remaining three protein bands. By using a very shallow guanidinium-HCl gradient, Smerdon and Isenberg (1976a) separated calf thymus histone H1

into four subfractions and showed that these subfractions interact differently with non-histones HMG1 and HMG2 (Smerdon and Isenberg, 1976b). The five subfractions of H1 in rat thymus chromatin, although different electrophoretically share a common set of antigenic determinants (Zick, Goldblatt, and Bustin, 1975). It should be cautioned that histone H1 is more readily degradable *in vivo* during extraction and would produce apparent multiplicity in electrophoresis (Bustin and Cole, 1969b). Thus electrophoretic mobility alone is an insufficient criterion in assigning heterogeneity for the lysine rich histones.

Many histone sequences are thought to be highly preserved during evolution, particularly for histone H4. Only two amino acids in a sequence of over one hundred differ in H4 from pea and from calf thymus (DeLange *et al.*, 1969). The lysine rich histones on the other hand have undergone more changes during evolution (Kinkade, 1969; Bendnikov *et al.*, 1976). Comparing the N-terminal sequence of H1 from calf and rabbit thymus, seven to fourteen amino acid replacements could be found (Bustin and Cole, 1969b; Cole, 1977). As shown in Figure 1, there are substantial differences among calf thymus and chicken H1. Similarly, H5 purified from various species of birds have also shown differences in their N-terminal amino acids (Yaguchi *et al.*, 1977) (a comparison between chicken and geese is shown also in Figure 1), and

Nevertheless, sufficient stretches of sequences are conserved among the birds that their electrophoretic mobility, or charges and immunocross reactivity or antigenic determinants are identical (Mura, Seligy, and Huang, personal communication). Fish species, whose erythrocytes are nucleated like those of birds, also contain H5 histone (Vendrely and Picaud, 1968; Miki and Neelin, 1975). These fish H5 histones also cross-react with antibody for chicken H5, but to a varied extent (Goetz, Esmailzadeh, and Huang, 1977). The same anti H5 antibody does not crossreact with H1 (Mura, Huang, and Levy, 1974). These results would argue that although heterogeneity exists in the lysine rich histones, their overall chemical and biological properties remain so similar that studies with them as a group of proteins are, for the present time, justifiable.

Interests in the study of histones have been aimed at elucidating the nature of their interaction with DNA. Thus, their contribution to the structure and function of the chromatin could be understood.

In water, histones exist mostly as random coils. Increasing ionic strength in the solution would reduce charge repulsion and facilitate the formation of ordered structures and histone-histone interaction. For instance, histone H4 would form dimers, $(H_4)_2$, which have less ordered secondary structures than other histones. Histone H5, on the other hand, has the

least tendency to aggregate as compared to other histones (Diggle and Peacocke, 1971). Nuclear magnetic resonance studies of this histone showed that, at ionic strength above 0.1 M NaCl, the N-terminal half of the molecule had 14% α-helical structure but no β-sheet structure (Crane-Robinson et al., 1976). H1 also contains essentially no α-helix in aqueous solution and shows very little tendency to aggregate, less than do the other histones. Nuclear magnetic resonance studies show that histone H1 in solution contains a globular head with a highly basic random-coiled tail (Chapman, Hartman, and Bradbury, 1976).

Interactions between histones and DNA are mainly electrostatic as demonstrated by the decrease of binding affinity to DNA (or histone dissociation from DNA) over the salt concentration range, from 0.3 to 2.0 M, although other kinds of interaction (such as hydrophobic) can still play an important role in binding because an ionic strength at 1.0 M NaCl still cannot dissociate all the histones. Removal of histone H1 by 0.6 M NaCl has only a small effect on the CD spectrum of the chromatin, especially for $\Delta\varepsilon_{220}$ and $\Delta\varepsilon_{278}$, indicating that histone H1 per se can exert little conformational effect on the DNA in chromatin (Gottesfeld et al., 1972; Li et al., 1975). However, selective removal of both of the very lysine-rich histones H1 and H5 resulted in a significant increase in transcription activity in vitro of

the chromatin and in ethidium bromide binding to chromatin (Lurquin and Seligy, 1976). These findings suggest that these histones play a structural role in regulating the accessibility of the chromatin DNA to enzymes or ligands. X-ray diffraction patterns of partial dehistonized chromatin showed that removal of histones H1 and H5 did not destroy the super-coiled structure of chromatin; the chromatin structure collapses only if other histones are further removed (Garrett, 1971; Bradbury et al., 1972b; Billett and Barry, 1974).

Histone H1 interacts with DNA cooperatively (Renz, 1975) with a binding coefficient of 1.9 and 0.4 x 10^2 at 0.1 M and 0.95 M NaCl respectively (Akinrimisi, Bonner, and Ts'o, 1965). Fasman, Chou, and Adler (1977) showed that the basic C-terminal half of histone H1 molecule was the primary site of interaction with DNA while the N-terminal half might modify this interaction. Indeed, phosphorylation of histone H1 at serine-37 tends to reduce the conformational effect on the DNA rendered by this histone (Adler, Langan, and Fasman, 1972). On the basis of an analysis of melting profiles of histone H1-depleted chromatin it has been calculated that H1 binds to about 30 base pairs of DNA (Li, Chang, and Weiskopf, 1977). Using a procedure that would deplete the chromatin of all other histones except H1, Hayashi (1975) showed that H1 distributed evenly over the whole length of DNA. The value of 30 is in general agreement with that obtained by a number of experiments

using nuclease digestion as a probe (Hewish and Burgoyne, 1973; Rill and Van Holde, 1973; Kornberg and Thomas, 1974; Van Holde et al., 1974; Varsharsky, Bakayev and Georgiev, 1976; Shaw et al., 1976).

Studies with nuclease digestion (Clark and Felsenfeld, 1971; Itzhaki, 1974; Weintraub, Palter, and van Lente, 1975; Morris, 1976; Felsenfeld, Cameriniootero and Sollner-Webb, 1977), thermal denaturation (Li and Bonner, 1971; Li, 1977a), circular dichroism (Li, 1977b) and electron microscopy (Woodcock, Maquire, and Stanchfield, 1973; Olins and Olins, 1974; Oudet, Gross-Bellard, and Chambon, 1975) have all pointed to the existence of chromatin subunit structures (see review by Olins et al., 1976; Kornberg, 1977) in which about 200 base pairs of DNA are complexed with an octamer (Thomas and Kornberg, 1975) of four classes of histones but not H1 and H5. From neutron scatter and diffraction studies (Baldwin, Boseley, and Bradbury, 1975) a globular model of nucleosome was proposed, in which apolar segments of histones form the core surrounded by DNA. Crystals have recently been obtained of nucleosome cores, analyzed by x-ray diffraction (Finch et al., 1977). The results are consistent with the subunit models thus far advanced.

It is generally accepted that histone H1 and H5 are not involved in the globular structure of chromatin. Instead, their role appears to lie in the generation of higher

ordered structure of the chromatin. The binding strength of histone H1 to superhelical SV 40 DNA is about five to ten times greater than that of other histones (Vogel and Singer, 1975). Yet, when SV 40 DNA-histone complexes were isolated from infected cells, they were found to contain only H2a, H2b, H3, and H4 (Griffith, 1975; Germond et al., 1975). Van Holde and Isenberg (1975) and Noll (1977) were able to more precisely place the H1 binding site at the ends of the internucleosome DNA between base number 140 and 160. Bradbury et al., (1975) showed that the basic carboxyl half of H1 is involved in interacting with DNA in the binding. Whether H5 binds similarly to H1 in the erythrocyte chromatin is unclear.

The question of whether H5 interacts with DNA in the same way for the same site as H1 is particularly interesting in view of the established observation that H5 to H1 ratio changes in the chromatin during erythroid cell development (Pinilla and Huang, 1971; Moss, Joyce, and Ingram, 1973) and H5 is absent in tissues other than the nucleated erythrocytes (see review by Huang et al., 1977). While the synthesis of H1 is usually not coordinated with other histones (see review by Kedes, 1976), H5 is the last histone to be synthesized in the non-dividing reticulocytes (Sung et al., 1977). The accumulation of H5 is accompanied by a decrease in H1 and more significantly by an inactivation of the chromatin. Partial removal of the lysine rich histones

by salt extraction increases the template activity of the inactive erythrocyte chromatin (Gasaryan and Andreeva, 1972). Indirect immunofluorescence studies show that the distribution of H5 in the nucleus also changes with cell maturation, presumably reflecting the restructure and condensation of the chromatin (Mura, Craig, and Huang, 1977). Phosphorylation and dephosphorylation of the H5 protein have been noted to take place during the development of the erythroid cells and also a change in their chromatin structure and activity (Tobin and Seligy, 1975; Sung et al., 1977).

While it is clear from the amino acid sequence data that there cannot be a direct conversion from H1 to H5, (see Figure 1) it is unclear whether these two lysine rich histones interact, interfere, and intercede in each of their interactions with DNA.

The objective of this study is to understand more about the interaction of H5 with DNA in solution with or without the presence of H1. This study also examined the possible interaction between H1 and H5 in solution. Thermal denaturation profile (Subirana, 1973; Li, 1977a) and circular dichroism (review by Li, 1977b) two well established physical methods were used to measure conformational changes in DNA with H5 and/or H1 complexed to it. For the study of possible H5 and H1 interaction, the zero-distance cross linker tetranitromethane (TNM) was used.

TNM reacts specifically with tyrosyl residues of a protein to form dityrosine (William and Lowe, 1971), 3-nitrotyrosine and nitroformate (Solkolovsky, Riordan, and Vallee, 1966) which are readily detectable spectrophotometrically. A charge transfer complex and tyrosine free radical had been proposed as intermediates for the reaction (Bruice, Gregory, and Walters, 1968). Such a reaction had been used in the studies of several proteins, such as carbonic anhydrase B (Nilson and Lindskog, 1967), Actin (Mühlard, Corsi, and Granata, 1963), Aldolase (Riordan and Christen, 1968), Staphylococcal nuclease (Cuatrecasas, Fuchs, Anfinsen, 1968), Human α-Lactalbumin (Prieels, Dolmans, and Leonis, 1975) as well as histone H1 (Bustin, 1971; Shlyapnikov et al., 1976) histone H4 (Pekary and Chan, 1977; Shlyapnikov et al., ibid) and histone H2B-H4 (Martinson and McCarthy, 1975).

This thesis is to show by physical measurements such as CD and thermal denaturation, how the lysine rich histones interact with DNA and with each other, and what are the possible roles of these histones in maintaining the structure of chromatin.

CHAPTER II

MATERIALS AND METHODS

(1) Preparation of Histones from Chicken Erythrocyte Chromatin

A. Erythrocytes were obtained freshly from exsanguinated adult Leghorn chickens. During collection of the blood, sodium citrate (10% w/v) was added to minimize clotting. Bottles of blood were transferred to the lab on ice. All experiments were done in a cold room at 4°C and centrifugation at 2°C. The blood was centrifuged (Sorvall, SS 34 rotor) at 1,800 rpm for 30 min. Serum was discarded. Erythrocytes were washed by stirring with a sufficient amount of cold 0.14 M NaCl-0.01 M Na-citrate, pH 7.0-7.6, strained through 8 thickness of cheesecloth and centrifuged again. Both washing and centrifugation were repeated once. The erythrocytes were then lysed by vigorous stirring with an equal volume of Saponine (0.6%, w/v in 0.14 M NaCl).

The lysate was allowed to stand for two hours with occasional stirring, then diluted with an equal volume of 0.14 M NaCl, 10 mM Na-citrate, and then centrifuged at 1,800 rpm for 1 hour. In later experiments, centrifugation was prolonged up to 4-5 hours to improve recovery of nuclei. The nuclei were then washed 10-12 times with 0.14 M NaCl, 10 mM Na-citrate. Initially the pellet was dispersed and stirred with a glass rod, but after four washes, the suspension was blended for 2 min at half speed in a Thomas homogenizer. The pellet recovered from the final washing by centrifugation corresponded to "crude deoxyribonucleoprotein" and was used as

washed nuclei, the intactness of the football-shaped nuclei could be checked easily under a microscope. Nuclei were either stored natively at -20°C as suspension in 50% glycerol or in their dehydrated form after washing 3 times in absolute ethanol, and stored at -20°C.

B. Chromatin was prepared from the isolated nuclei using the methods of Paoletti and Huang (1969). Nuclear pellets from 30 gm wet weight of tissue were suspended in 40 ml of 75 mM NaCl-24 mM M Na-EDTA, pH 8.0, and centrifuged at 7,700 x g for 15 min. This step was repeated 3 more times. The resulting pellet was resuspended in 40 ml of 50 mM Tris, pH 8.0 and followed by two washes each in 10 mM, 2 mM and 0.4 mM Tris all adjusted to the same pH 8.0. The chromatin was then allowed to swell overnight in a total volume of 100 ml of ice-cold distilled H_2O, pH 8.0 in cold and stored in a refrigerator. The recovery of DNA, as determined by Burton's method (1956), in the chromatin prepared by this procedure was 90-99%.

C. Total histones were extracted from chromatin with 0.5 N H_2SO_4. The extracted histones were recovered from the supernatant by centrifugation at 12,000 rpm for 20 min at 4°C. The extraction was repeated once more, pooling the supernatants. The histones were precipitated with 20% trichloroacetic acid from the supernatant, washed once with the same acid, once with ether/ethanol (1:1) and dried in vacuum.

The yield was determined by the method of Lowry et al., (1951).

D. Mixtures of histones H1 and H5 were obtained by slowly adding trichloroacetic acid to erythrocyte chromatin to a concentration of 5%; the supernatants after a high speed centrifugation (10,000 rpm, 20 min) were pooled and exhaustively dialyzed against distilled water and lyophilized. Histones H1 and H5 were separated from an Amberlite CG-50 column (25 x 30 cm). Mixtures of histone H1+H5 in 8% guanidium chloride, 10 mM sodium phosphate, pH 6.8 were applied on top of the column and eluted with a GuCl gradient from 12% to 20%. At times, the histones were further purified by passing through a short CM-cellulose column (1 x 15 cm) with a salt gradient from 0.3 to 0.75 M NaCl in 10 mM phosphate buffer, pH 6.8 (Billett and Barry, 1974) or through a column of Bio-Gel P20 and eluted with 0.01 N HCl to remove impurities. The purity of histones were tested by gel electrophoresis.

E. Gel electrophoresis of histones was performed as described by Panyim and Chalkley (1969). Polyacrylamide gels were prepared by mixing the following solutions: one part of 43.2% glacial acetic acid (v/v), 4% TEMED (N,N,N',N'-tetramethylethylenediamine), two parts of 60% acrylamide (w/v) in H_2O, 0.4% bis-acrylamide and five parts of freshly prepared 0.2% ammonium persulfate in 10 M urea. All solutions were deaerated before polymerization. Glass tubings of 0.5 x

7.5 cm were soaked in RBS cleaning solution (Fisher Scientific Co.) overnight and rinsed thoroughly with distilled water and air dried before using. Polymerization was completed in an hour upon the addition of persulfate and overlayered with distilled H_2O on top of the 6 cm gel to make the surface flat. Gels were prerun for two hours at constant current of 2 mA/tube until the voltage reached 120 volts in 0.9 N acetic acid, which was replaced after preelectrophoresis. Samples containing 10-20 µg of histones were usually applied in a volume of 20-30 µl of 0.9 N acetic acid and 15% sucrose or glycerol, using pyronine-Y as the tracking dye. The gels were run at a water cooling tank for two hours and stained overnight in 0.1% amido black, 20% ethanol, 7% acetic acid. Destaining was done by several changes with 0.9 N acetic acid until the background cleared. Gels were scanned at 650 nm with a Gilforn Spectrophotometer Linear Transport device to record the pattern.

(2) DNA Preparations and Determinations

Calf thymus DNA was purchased from Sigma Chemical Co. and was further purified by phenol extraction. For the extraction, 100 mg of calf thymus DNA were dissolved in 50 ml of 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM Na_2EDTA, overnight, with a slow stirring at 4^oC. It was extracted with an equal volume of buffer-saturated phenol. This step was

repeated until no detectable denatured proteins were found at the water-phenol interphase. Phenol was then removed from the DNA solution by extensive dialysis against 2.5×10^{-4} M Na_2EDTA before use.

Chicken erythrocyte DNA was purified from pellets of chromatin after histones were extracted with 2 M NaCl by banding with CsCl isopynic-centrifugation and preserved by precipitation with ethanol. Clostridium perfringens DNA from Sigma Chemical Co. and Micrococcus luteus DNA from Miles Laboratory were used directly without further purification. The molar extinction coefficient at 260 nm was 7,400 for Cl. perfringens DNA, 7,000 for M. luteus DNA (Felsenfeld and Hirschman, 1965), and 6,500 M^{-1} cm^{-1} for calf thymus and chicken DNA, where M is mole/liter of nucleotide.

(3) Preparation of Histone DNA Complex

A. By Direct Mixing

Calf thymus DNA stock solution at nucleotides concentration of 10^{-4} M was prepared in 2.5×10^{-4} M EDTA, pH 8.0. To this solution, histone solution of 10^{-3} M in amino acid residues was added dropwise while stirring. The protein-DNA complexes were left at room temperature for at least an hour before measurements were taken.

B. By NaCl Gradient Dialysis in the Presence of Urea (Huang and Huang, 1969).

Reconstitution of histone to DNA was also made by

dialysis in a continuously decreasing NaCl gradient (2.0 to 0.1 M) in the presence of 5 M urea, 10 mM Tris, pH 8.0 for 2 days. The urea was then removed by continuous dialysis against 2.5×10^{-4} M EDTA, pH 8.0.

C. By NaCl Gradient Dialysis in the Absence of Urea (Sponar and Sormova, 1972).

Reconstitution of histone to DNA was done as in B by a continuously decreasing linear gradient of NaCl (2.0 to 0.1 M) in 10 mM Tris, pH 8.0 but without urea for two days. The complexes were then extensively dialyzed against 2.5×10^{-4} M EDTA, pH 8.0 before use.

(4) Thermal Denaturation and Circular Dichroism Measurements

A. Thermal Denaturation Measurements

Thermal denaturation of chromatin and protein-DNA complexes were performed at 260 nm using a Gilford spectrophotometer model 2400-S, at a constant temperature increase of Ca. $2/3^{o}$ per min. The changes in both temperature and absorbance at 260 nm temperature were recorded continuously.

Derivative melting profiles were calculated according to the equation (Li and Bonner, 1971):

$$\frac{h_{260}\ (T)}{dT} = \frac{h_{260}\ (T+1) - h_{260}\ (T-1)}{2} \qquad (2\text{-}1)$$

where h_{260} (T) is the hyperchromicity at 260 nm at temperature T degree Celsius.

B. Circular Dichroism (CD)

The CD spectra of DNA and histone-DNA complexes were taken on a Durrum-Jasco or Cary 60 Spectropolarimeter Model J-20 at room temperature in 25 x mM EDTA (pH 8.0). The CD results are reported as $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$, where ε_l and ε_r are respectively molar extinction coefficients for the left- and the right-handed circularly polarized light.

The units of $\Delta\varepsilon$ are M^{-1} cm^{-1} in terms of nucleotide concentration in DNA and histone-DNA complexes were determined spectrophotometrically by assuming the appropriate extinction coefficients (see 2, above). Light scattering in these complexes is negligible with A_{320}/A_{260} lower than 0.04

(5) Trypsin Digestion of Chicken Erythrocyte Chromatin and Nucleohistone V

For trypsin digestion of the chromatin and nucleohistone H5, salt-free crystal of trypsin (A grade) from Calbiochem. was used. It was prepared at a concentration of 0.1 mg/ml and dialyzed against Na_2-EDTA pH 8.0 before use. Trypsin inhibitor from Soybean (type 1-S) was purchased from Sigma Chemical Co. Trypsin was added to the chromatin and nucleohistone H5 solutions at 5 µg/ml or 20 µg/ml at room temperature. The reaction was stopped by adding 20 µl of trypsin inhibitor (1 mg/ml) at 1/2 hr. or 1-1/2 hr. after the reaction was started. The samples were then examined by CD and thermal denaturation.

(6) Tetranitromethane (TNM) Nitration of Histones and Chromatin

Procedures used for nitration of the histones with TNM (purchased from Sigma Co.) follow that of Pekary and Chan (1977) with some modifications. In brief, stock solutions of chicken erythrocyte chromatin (A_{260} ~1.5) and 1 mg/ml solutions of total histones, total histones minus H1 and H5, and histone H1 plus H5 were prepared in 0.5 M Tris, pH 8.3 with or without 2 M NaCl. They were then transferred to a matched set of Savant cuvettes and TNM (Sigma) added in such a way that readings can be taken at one minute intervals, for example, readings at 428 nm were taken at 1', 3', 5', etc. and 350 nm at 2', 4', 6', etc. The reaction completed after 15 min, and from then on readings were recorded every other 5 min.

$$OD_{428} = \varepsilon^{NF^*}_{428} \times C_{NF^*} + \varepsilon^{NO_2\text{-Tyr}}_{428} \times C_{NO_2\text{-Tyr}} \qquad (2\text{-}2)$$

$$OD_{350} = \varepsilon^{NF^*}_{350} \times C_{NF^*} + \varepsilon^{NO_2\text{-Tyr}}_{350} \times C_{NO_2\text{-Tyr}} \qquad (2\text{-}3)$$

Since the colored reaction products, nitrotyrosine and nitroformate, have spectral maximum in the near UV-visible region and these two spectra overlap, the following simultaneous equations of Pekary and Chan (1977) were used to calculate the concentration of these products. The extinction coefficients were given as follows (Bruice, Gregory, and Walters, 1968):

λ(nm)	$\varepsilon_{(NO_2\text{-Tyr})}$	$\varepsilon_{(Nitroformate)}$
350	1,000	14,418
428	4,100	577

*NF stands for Nitroformate.

(7) Alkaline Phosphatase Treatment of Histone H1

Alkaline phosphatase (*E. coli*, BAPC, Worthington) was incubated with the lysine-rich histones at 1 mole of enzyme per 4 moles of histones H1 to yield a weight ratio of 1:1.

The solutions were incubated in 0.01 M Tris, pH 8.0 at 37°C for 30 min and terminated by exhaustive dialysis against cold 0.9 N acetic acid.

CHAPTER III

RESULTS

A. Characterization of Sample

a) Chromatin

The purity of the chromatin preparation from chick erythrocytes was examined spectrophotometrically as shown in Figure 2. There was little absorbance in the range 320-360 nm indicating little light scattering due to aggregation. The thermal melting profile (Figure 3) is comparable to those reported by others (e.g., Tsai, Ansevin, and Hnilica, 1975). Circular dichroic spectrum of this chromatin is also typical of a native sample (Figure 4). The spectrum is comparable to that observed earlier (Williams, Lurquin, and Seligy, 1972; Williams and Seligy, 1973; Hjelm and Huang, 1974; Senior and Olins, 1975; Lewis, DeBuysere, and Rees, 1976)

b) Histone

The lysine rich histones H1 and H5 were purified on Amberlite CG 50 columns. A typical elution profile is shown in Figure 5. Histones H1 and H5 were eluted respectively at 13% and 15% GuCl as expected. Rechromatography of the H1 fraction resulted in a single peak on CM-cellulose column (Figure 6). This peak fraction, however, yielded two bands on the acid-area gel electrophoresis system, unless treated with alkaline phosphatase, suggesting that one of the bands was phosphorylated. A electrophoretogram depicting H1 and H5 histones as isolated by mild acid extraction from chromatin is shown in Figure 7. In this Figure, the presence

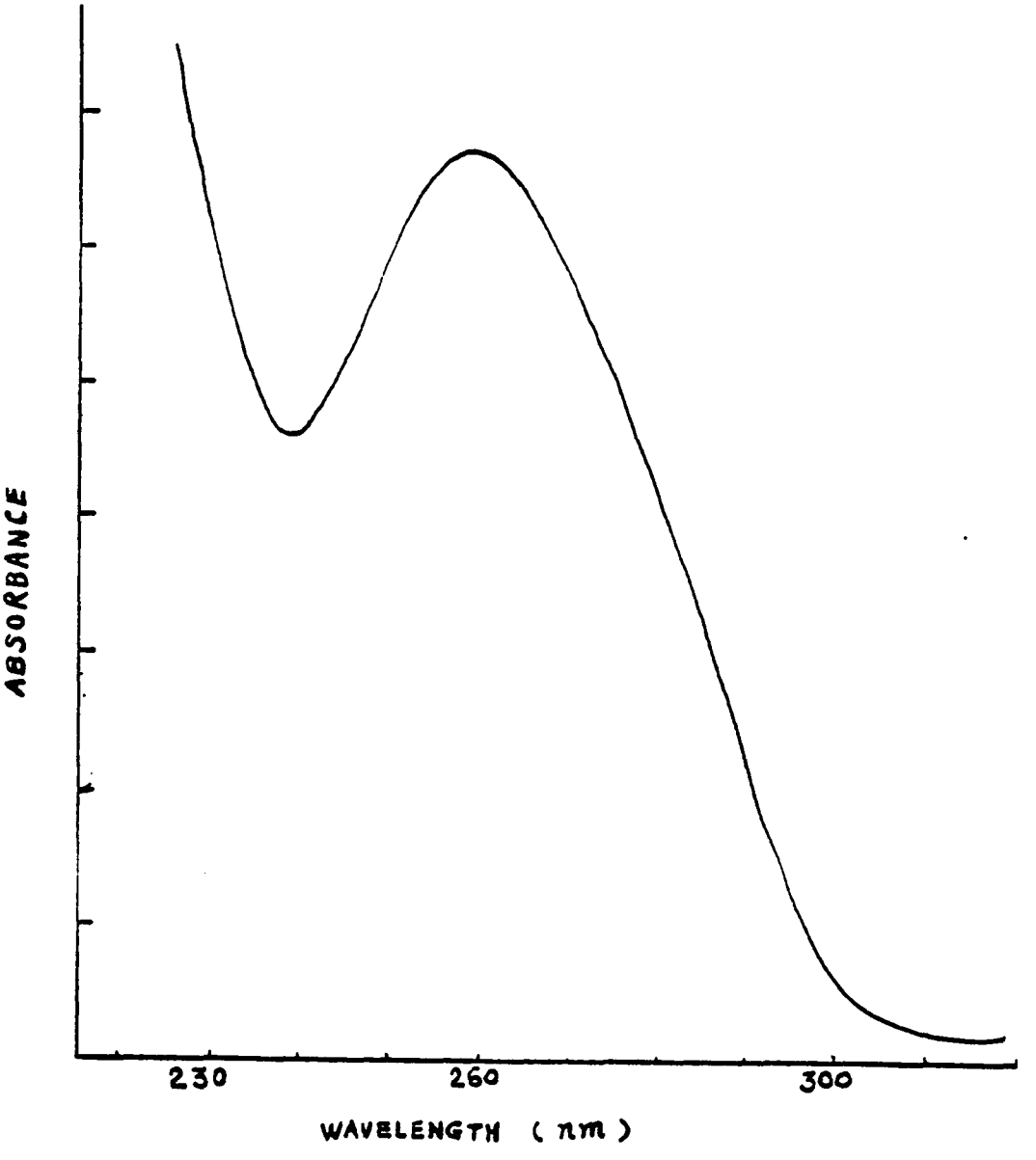


Figure 2. UV absorption spectra of chicken erythrocyte chromatin in 25 mM EDTA pH 8.0

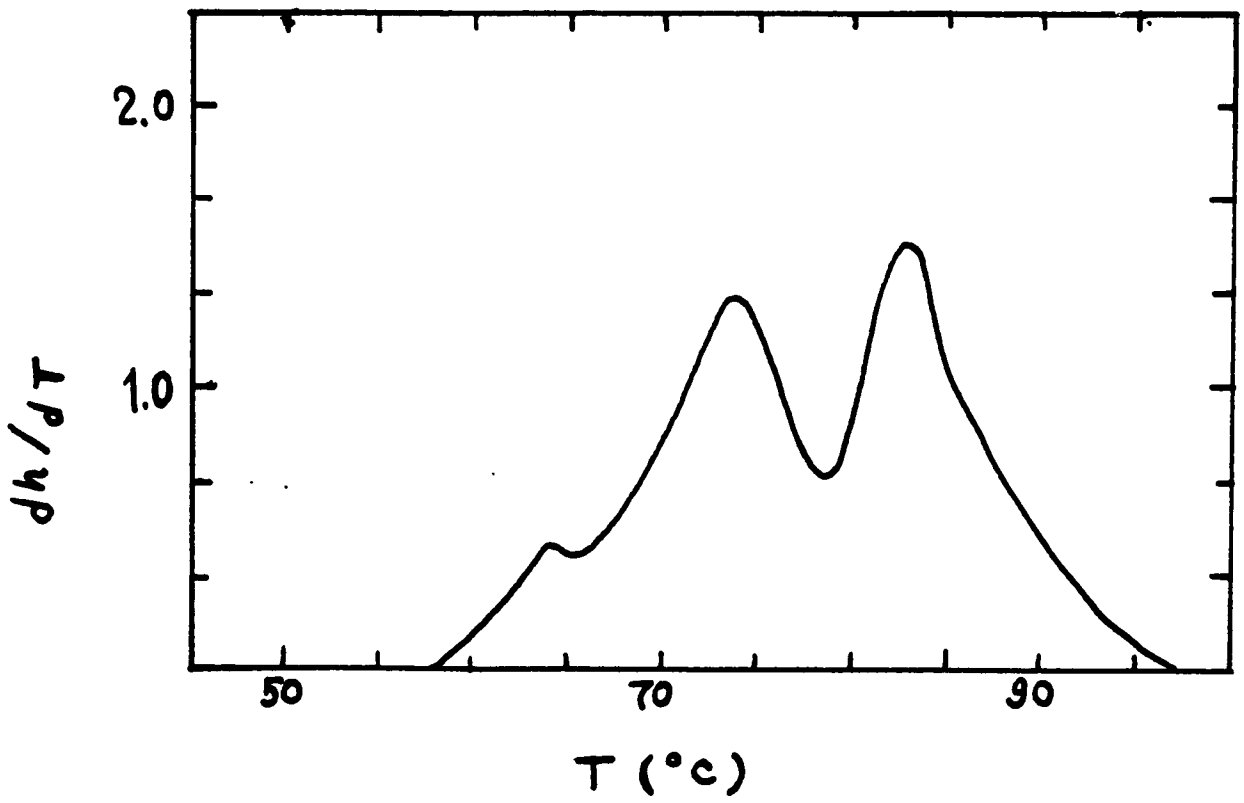


Figure 3. Derivative melting profile of chicken erythrocyte chromatin in 25 mM EDTA pH 8.0.

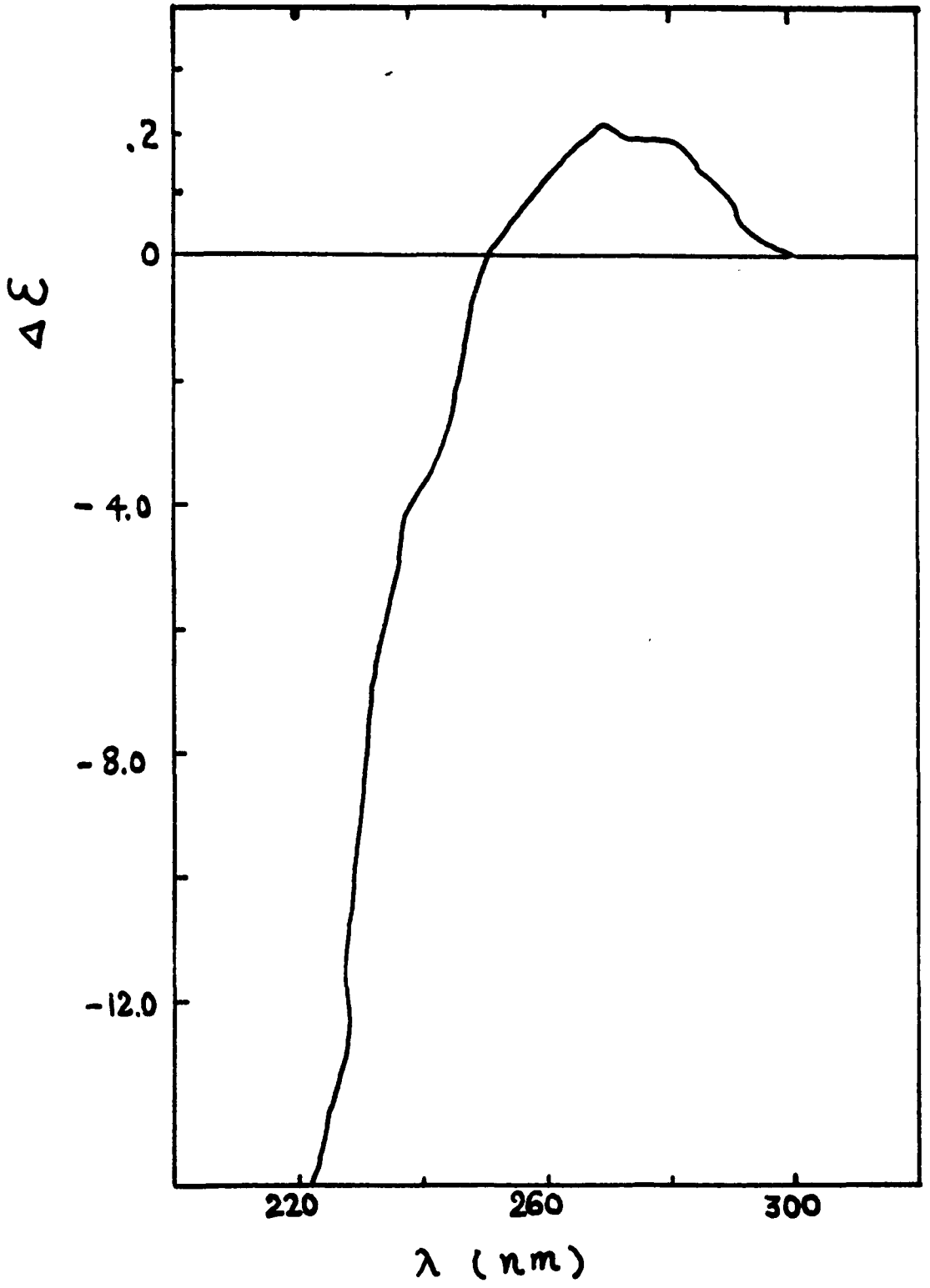


Figure 4. CD spectra of chicken erythrocyte chromatin in 25 mM EDTA pH 8.0.

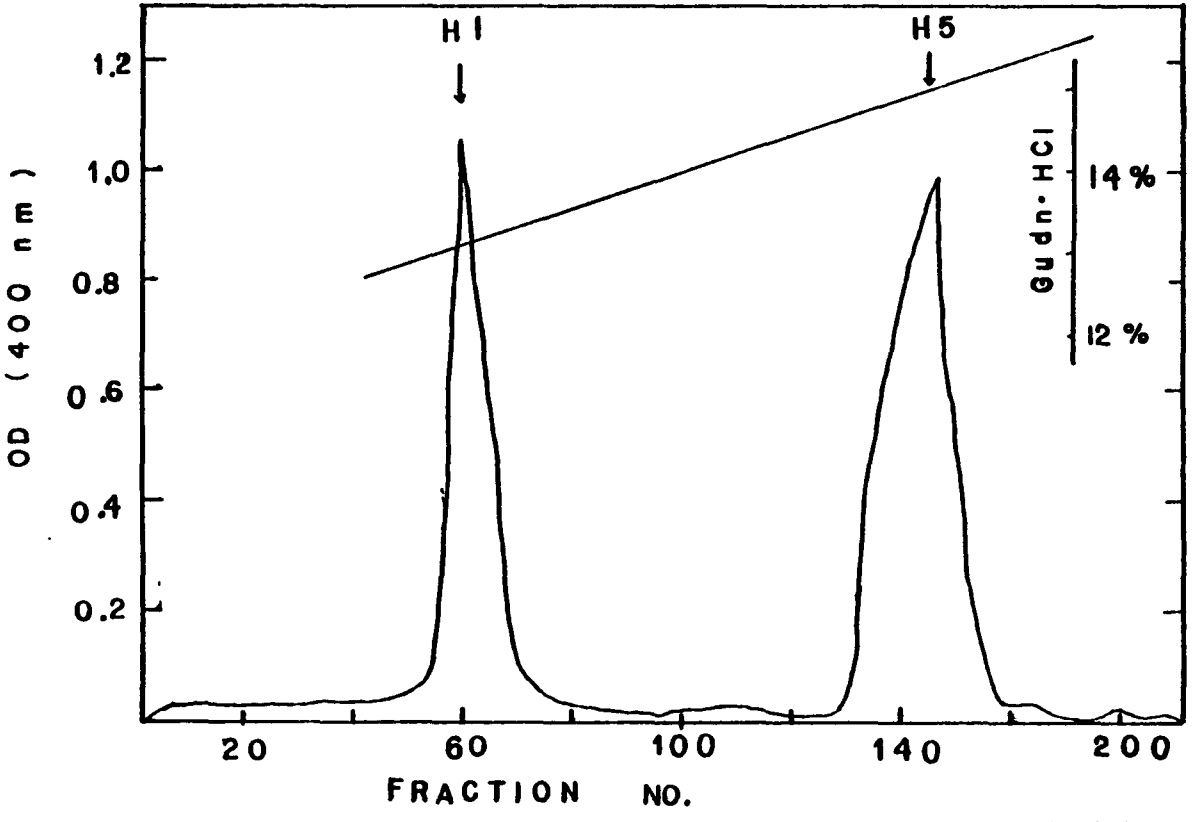


Fig. 5. Elution profile of histones H1 and H5 from Amberlite CG-50 column (30 x 2.5) with a linear gradient of Guanidium hydrochloride from 12% to 40%.

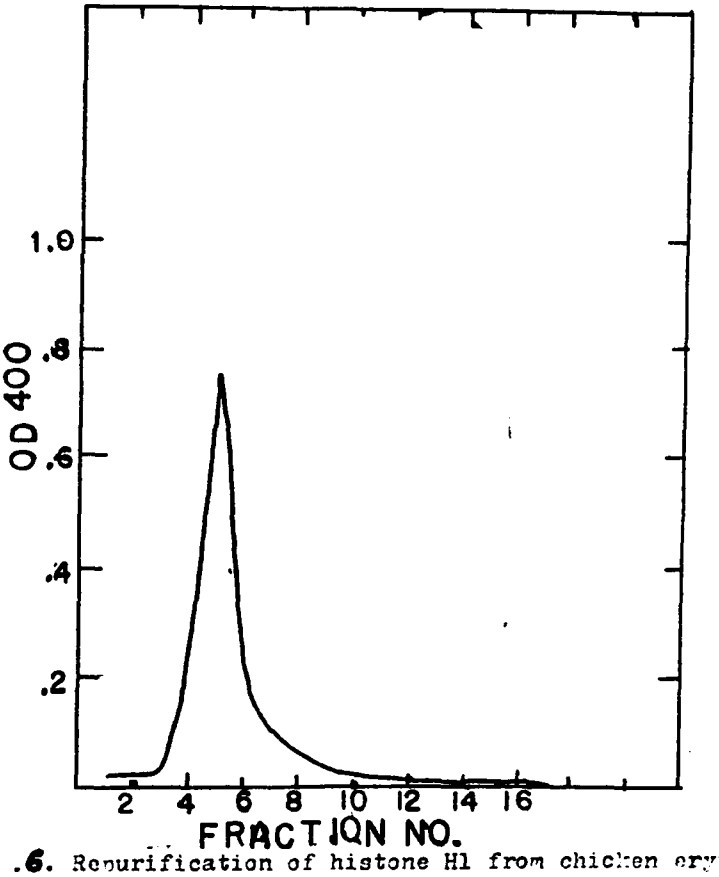


Fig. .6. Repurification of histone H1 from chicken erythrocyte by CM-cellulose chromatography (1x15) in a salt linear gradient from 0.3 M to 0.75 M.

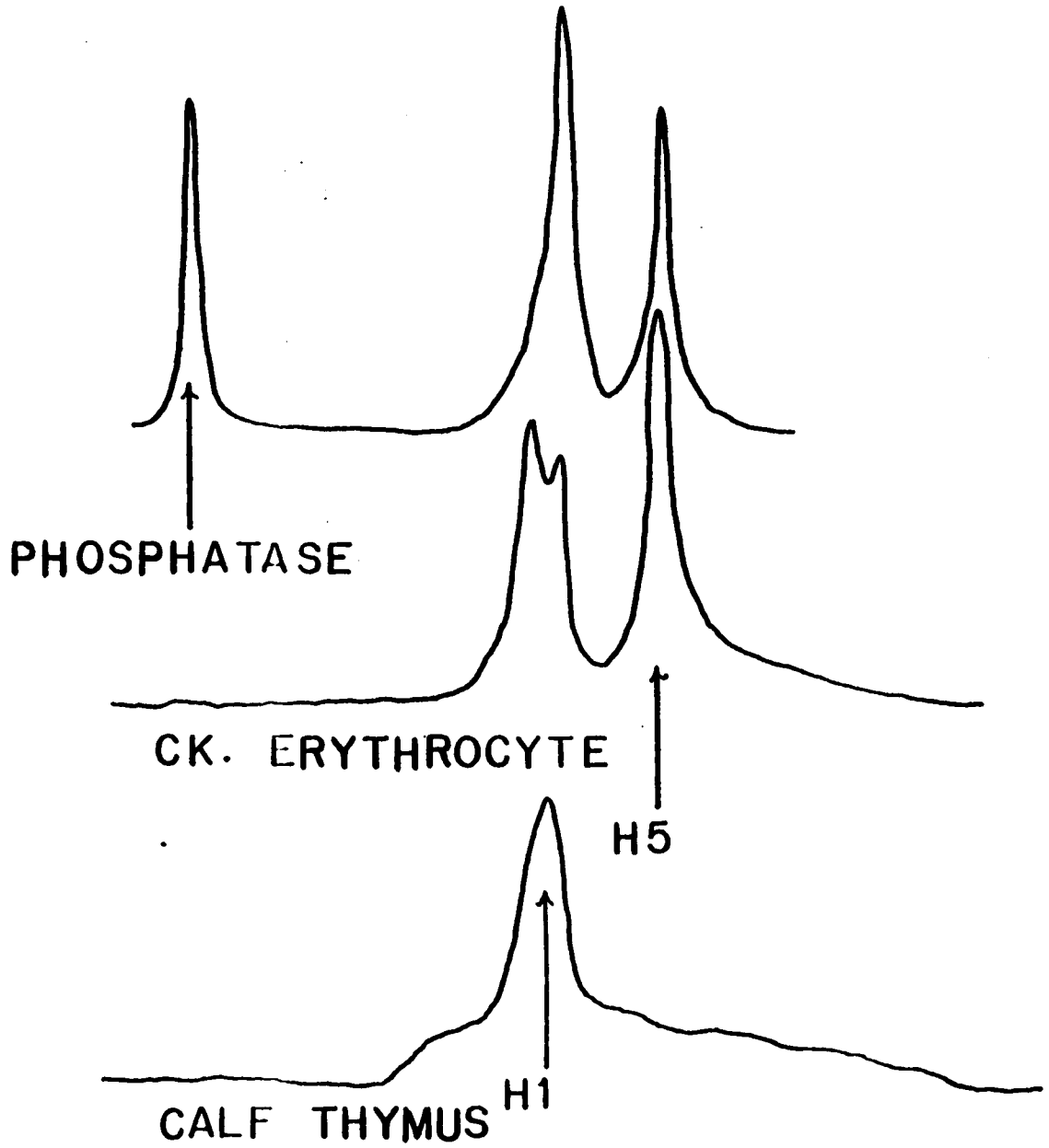


Fig. 7. Acid-urea gel patterns of histones H1 and H5 after *E. coli* Alkaline Phosphatase treatment

of single H1 band after alkaline phosphatase treatment is compared to that of double bands without such a treatment. Calf thymus histone H1 shows only one H1 band.

B. Histone H5-DNA Interaction: T_m and CD Studies

Thermal Denaturation of Nucleohistone H5

Figure 8 shows derivative plots of melting profiles of histone H5-chicken DNA complexes, as increasing amount of histone H5 was bound to DNA, the hyperchromicity at the melting temperature (T_m) of free base pairs, 45°C (band I), was shifted to higher temperatures, one at 75 to 79°C (band II) and 90 to 93°C (band III) corresponding to the melting of histone-bound base pairs. These two new melting bands were similar to those found in pea bud (Li and Bonner, 1971), calf thymus (Li, Chang, and Weiskopf, 1973), and rat liver and ascites hepatoma chromatins (Suzuki and Iwanami, 1975) except that in nucleohistone H5 these two melting temperatures were 5 to 10° higher than those in chromatin.

In order to test whether or not the characteristic melting properties of nucleohistone H5 depend upon the specific base sequence of chicken DNA, calf thymus DNA was used for reconstitution with histone H5. The melting profiles of these complexes shown in Figure 9 are essentially the same as those in Figure 8 when chicken DNA was used. Previously, Li, Brand, and Rotter (1974) showed that the

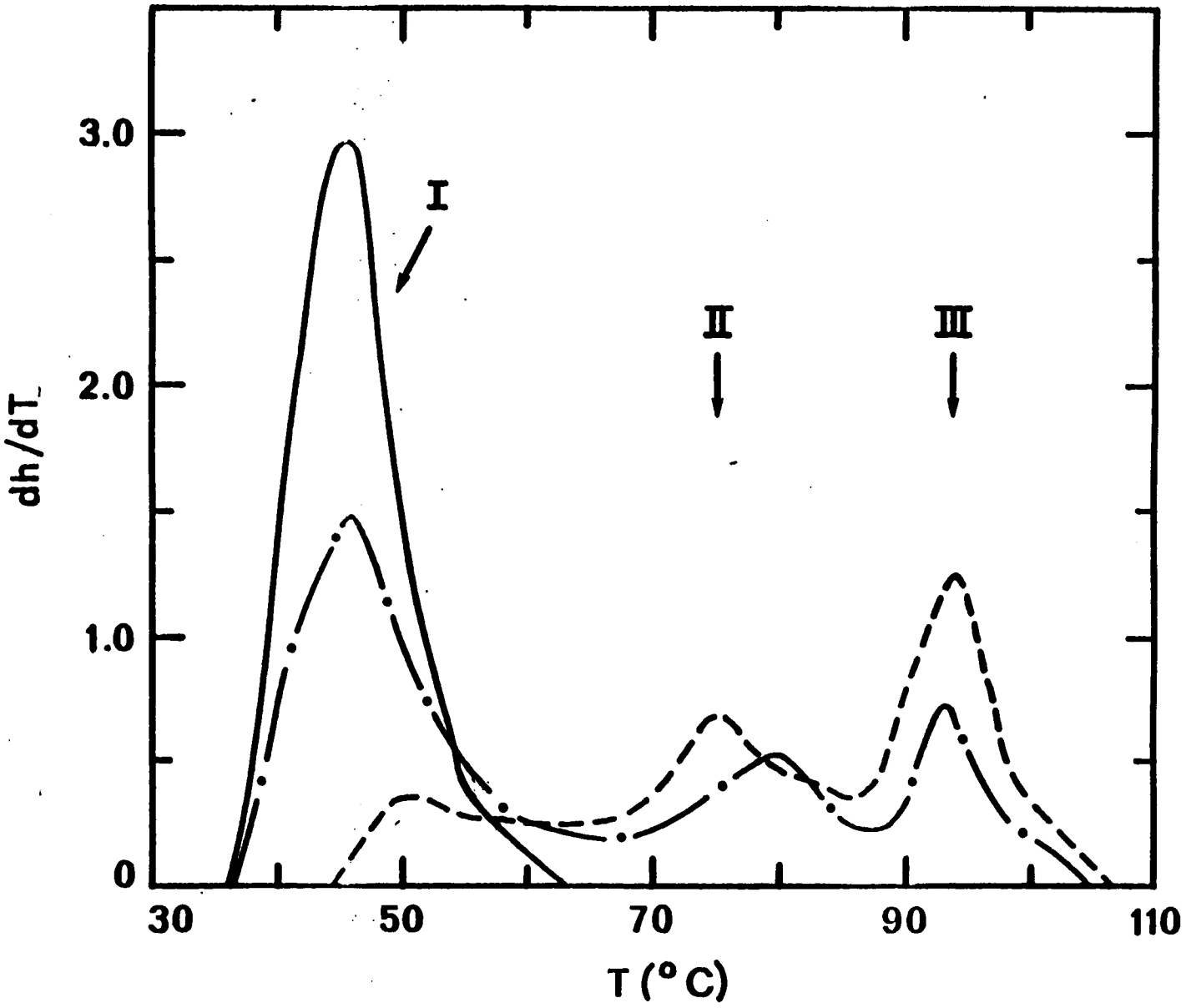


Fig. 8. Derivative melting profiles of histone H5-chicken DNA complexes. r = 0 (——), 0.5 (—•—) and 1.0 (----).

unbound calf thymus DNA melted at about 47° with a shoulder at 54°. The latter was interpreted as a more (G+C)-rich fraction in a heterogeneous population of calf thymus DNA molecules, possibly the satellite fraction. As shown in Figure 9, increasing the ratio of histone to calf thymus DNA in the complexes diminished the 45°C band with a concomitant increase of the two higher melting bands, while the shoulder at 54°C remained unaffected. As demonstrated in reconstituted poly-L-lysine-calf thymus DNA complexes, this phenomenon is explained as a selective binding of histone H5 to the more (A+T)-rich fractions of a heterogeneous DNA population. Later on it will be shown that this is indeed the case. Also included in Figure 9 is the melting curve of nucleohistone H5 with an input ratio of 1.5 amino acid/nucleotide. In this sample the area of the melting band at 55°C is still about 20% of the total area under the whole melting curve. The two melting bands, II and III, are also about 3 to 5° lower than the corresponding bands from complexes of lower r values.

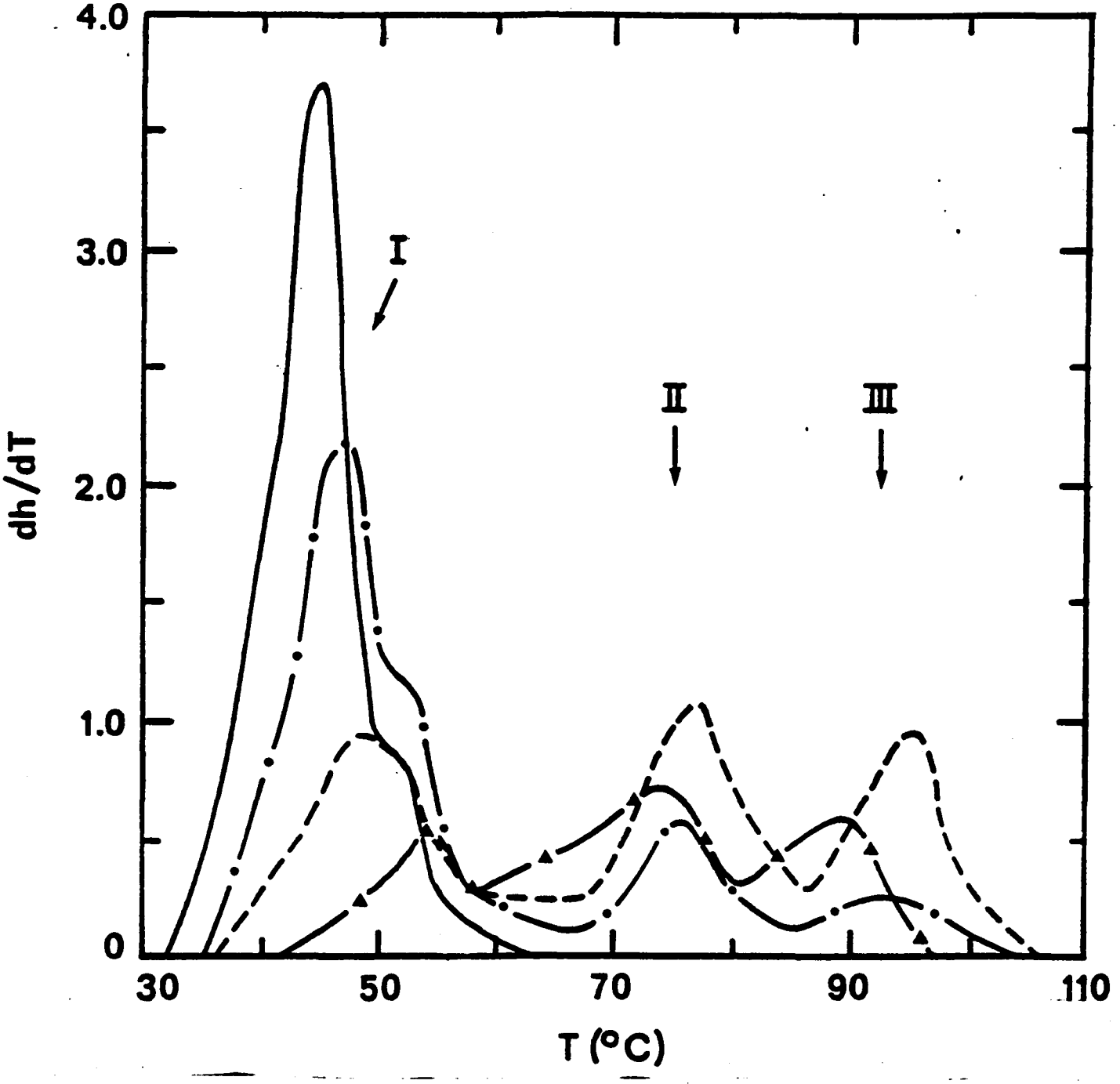


Fig. 9. Derivative melting profiles of histone H5-calf thymus DNA complexes. r = 0 (——), 0.5 (—·—), 1.0 (----) and 1.5 (—▲—)

Besides chicken and calf thymus DNA, procaryotic M. luteus DNA was also used for complex formation. Their melting profiles shown in Figure 10 are similar to those of complexes found with eucaryotic DNA with three melting bands except that the corresponding melting temperatures in M. luteus complexes are higher than the counterparts of calf thymus or chicken complexes. This is expected since M. luteus DNA has a higher G+C content (about 70%) than the other two eucaryotic DNAs (about 42%).

As shown by Li (1973), melting profiles can be used to calculate the ratio of amino-acid residues per nucleotide (β) in the histone-bound regions of a nucleohistone complex by the use of the equation:

$$r = \beta \frac{A_{T_{m,II}} + A_{T_{m,III}}}{A_T} \qquad (3\text{-}1)$$

where $A_{T_{m,II}}$ and $A_{T_{m,III}}$ are the melting areas under bands II and III, respectively, and A_T is the total melting area which is equal to maximum hyperchromicity (h_{max}).

The linear plots of equation (3-1) for histone H5 complexed with DNA from chicken, calf thymus and M. luteus are shown in Figure 11. β is 1.5 amino-acid residues per nucleotide for all three DNAs. Quantitatively the melting properties of histone H5 complexed with DNA are identical in all three cases. The value of 1.5 amino acids per nucleotide in nucleohistone H5 is much lower than the value of 3.0 to 3.5

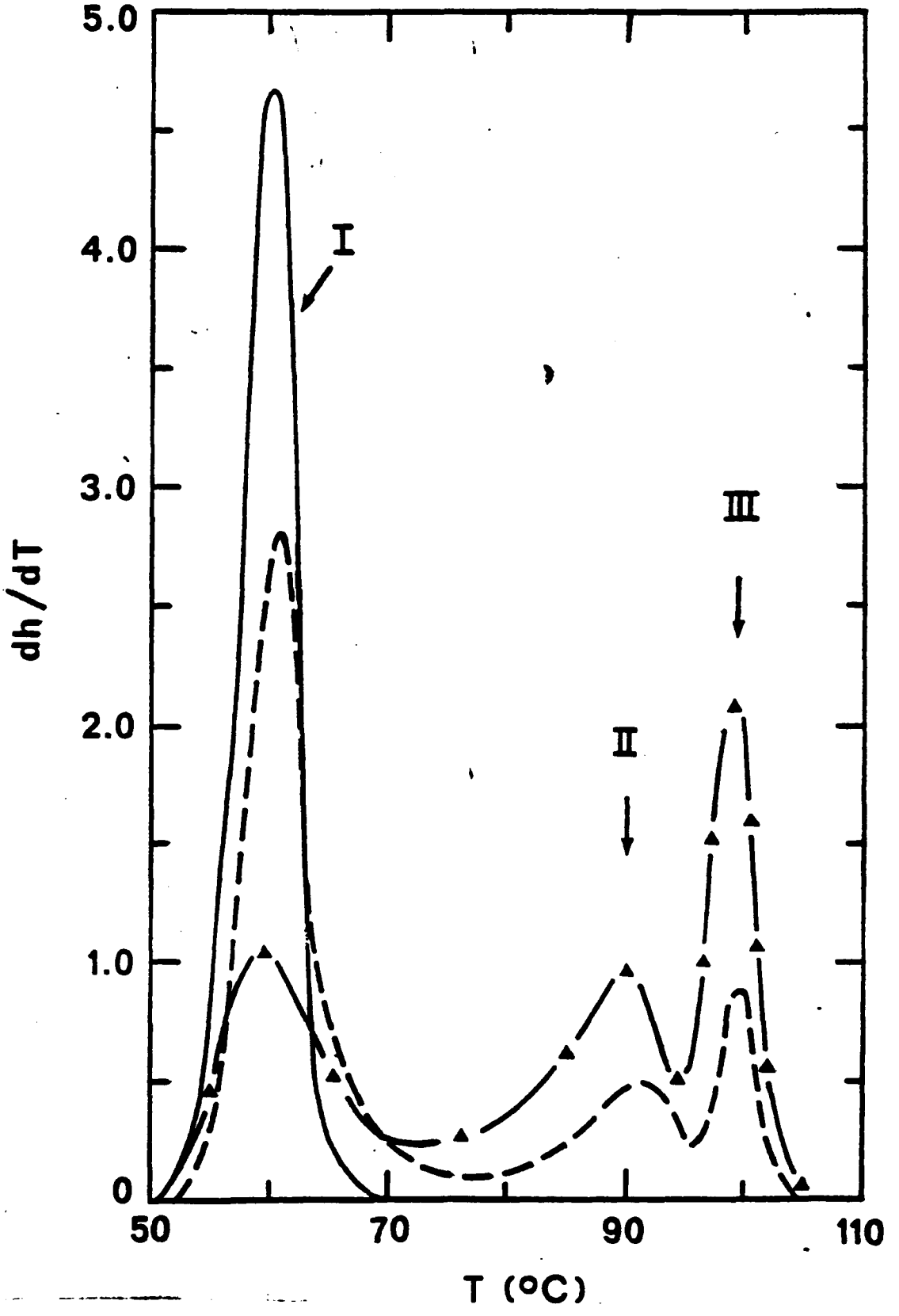


Fig.10. Derivative melting profiles of histone H5-M. luteus DNA complexes. r = 0 (——), 0.5 (----) and 1.0 (—▲—).

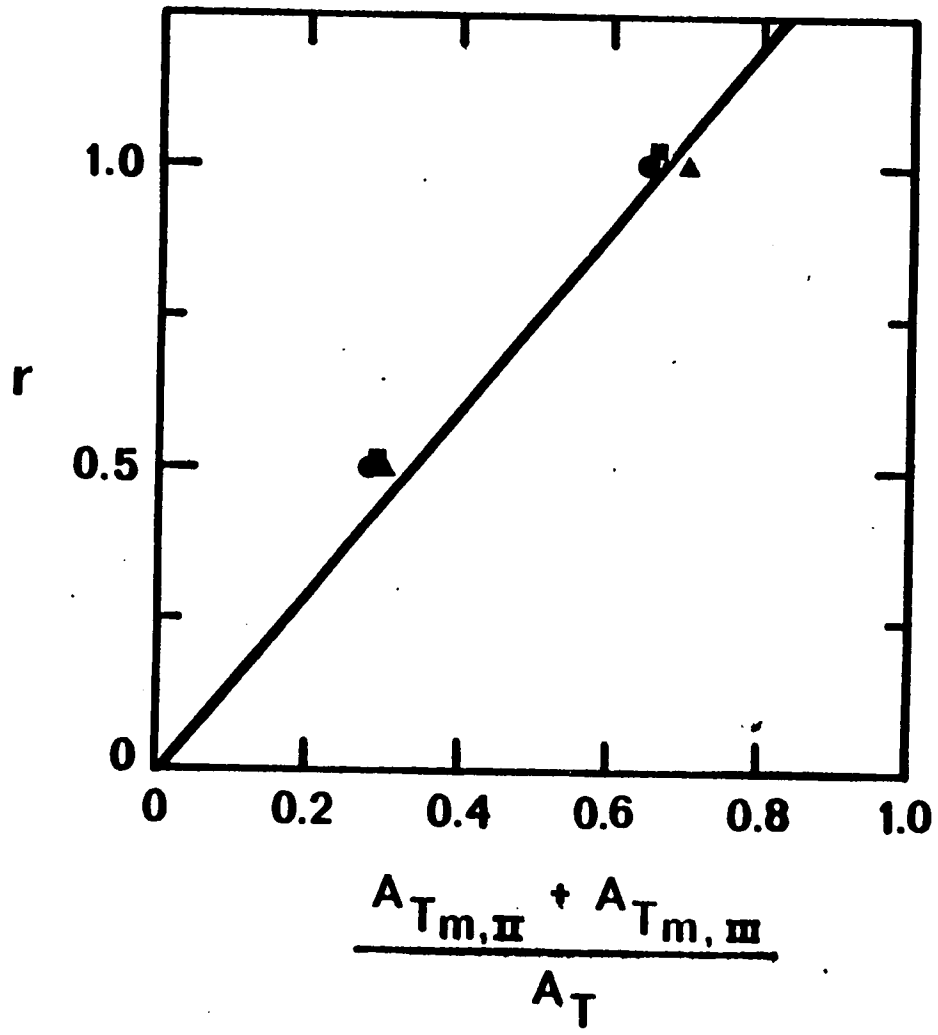


Fig. 11. Linear plot of equation (1). Chicken DNA (▲), calf thymus DNA (●) and M. luteus DNA (■).

obtained with chromatin or the value of 2.9 to 3.3 with nucleohistone H1. Some pertinent melting results of nucleohistone H5 using chicken or calf thymus DNA are summarized in Table II. It shows that the fraction of melting area under band II is close to that under band III. There is also a significant light scattering for all the complexes.

Circular Dichroism Spectra of Nucleohistone H5

CD and NMR studies of histone H5 in solution showed that, upon addition of salt, histone H5 underwent a conformational change which is reversible (Bradbury et al., 1972b; Williams and Seligy, 1973). Figure 12 shows CD spectra of histone H5-chicken DNA complexes. When more histone H5 was complexed to the DNA, there were slight red shifts for both the positive band at 275 nm (λ max) and the crossover at 256 nm (λ c) and a reduction of $\Delta\varepsilon_{278}/-\Delta\varepsilon_{246}$ ratio. In nucleohistone H5, histone H5 also makes a negative CD contribution near 220 nm. Similar CD results were observed for histone H5-calf thymus DNA complexes. The pertinent CD characteristics of nucleohistone H5 are summarized in Table III.

Based upon the red shift for λ_{max} and λ_c and the reduction of $\Delta\varepsilon_{278}/-\Delta\varepsilon_{246}$, histone H5 binding to DNA seems to induce a transition from a B-type CD spectrum to a C-type spectrum; this effect was small, however, when compared with that induced by protamine (Yu and Li, 1973), histone H2B (Leffak et al., 1974) or histones H3 and H4 (Yu, Li, and Shih, 1976).

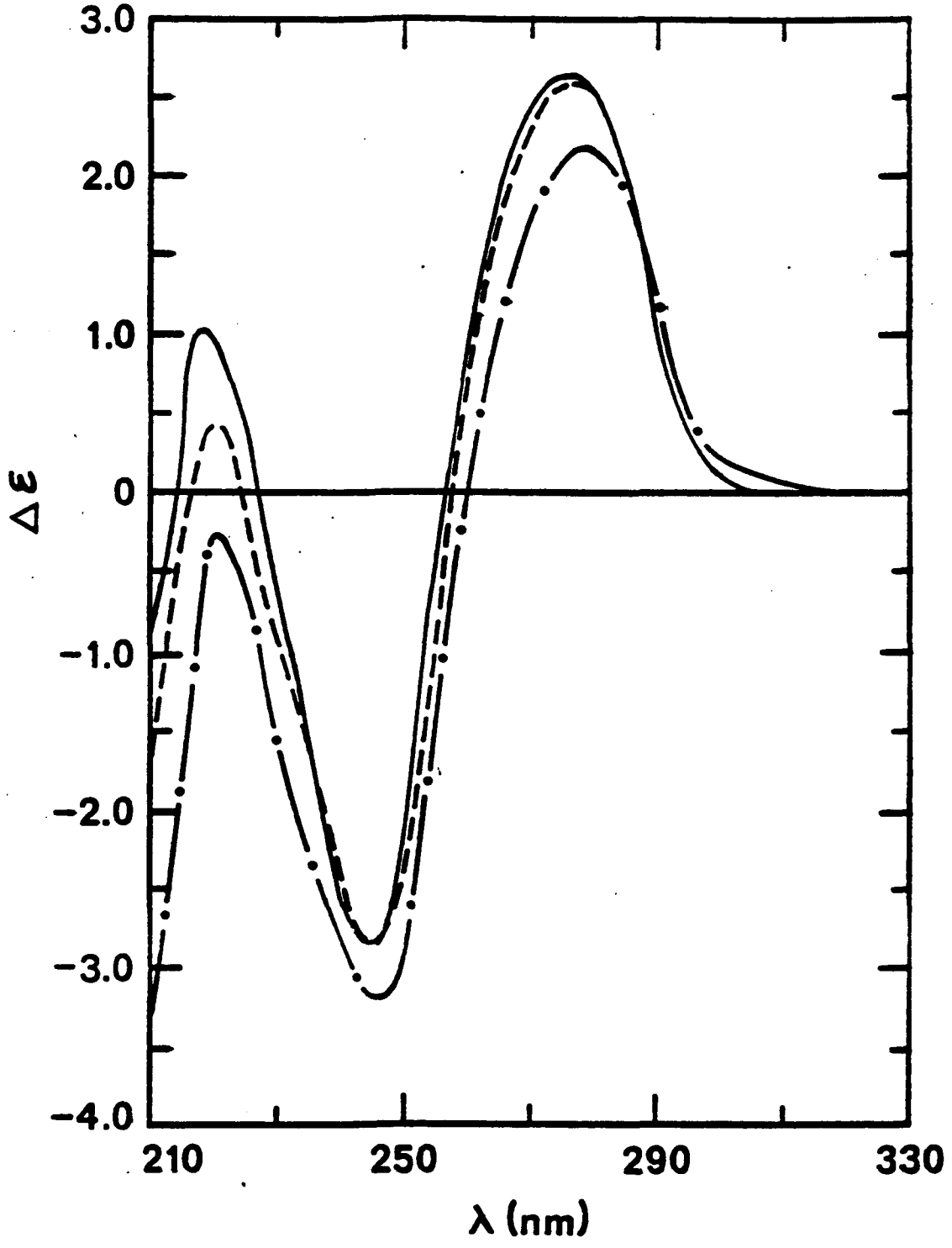


Fig.12. CD spectra of histone H5-chicken DNA complexes. r = 0 (——) 0.5 (- - -) and 1.0 (—·—).

Table II. Melting Characteristics of Nucleohistone H5

DNA	B	$T^a_{m,I}$	$T^a_{m,II}$	$T^a_{m,III}$	h_{max}	A_{320}/A_{260} 30°	A_{320}/A_{260} 110°
Chicken[b]	0	45.0 (100%)	--	--			
	0.5	45.5 (70%)	78.0 (14%)	92.0 (16%)	35.6		
	1.0	55.0 (30%)	76.0 (30%)	91.5 (40%)	27.1	0.154	0.159
Calf Thymus[c]	0	45.5 (100%)	--	--	35.8		
	0.5	49.5 (70%)	75.5 (18%)	91.0 (12%)	32.0	0.140	0.113
	1.0	52.5 (36%)	75.0 (32%)	93.0 (32%)	33.0	0.160	0.199

[a] (%) under each melting temperature is the percent of melting area of that melting band, namely $A_{T_{m,X}}/A_T \times 100\%$, where X is I, II or III and A_T is the total melting area which is equal to h_{max}.

[b] Average values from two preparations.

[c] Average values from three preparations.

Table III. CD Characteristics of Nucleohistone H5

DNA	B	$\frac{\Delta\varepsilon_{278}}{-\Delta\varepsilon_{246}}$	$\Delta\varepsilon_{220}$	λ_{max}	λ_c
Chicken	0	0.93	+0.95	275	256.5
	0.5	0.85	+0.30	277.5	258.5
	1.0	0.66	-0.63	279.5	260
Calf Thymus	0.5	0.88	+0.37	276	256.5
	1.0	0.83	-0.40	278	258
	1.5	0.48	-1.4	280	260.5

Average values from two preparations except the complex of calf thymus DNA with B = 1.5 where values were from one preparation.

Trypsin Digestion of Nucleohistone H5

Thermal denaturation properties of nucleohistone H5 with r = 1.0 treated by trypsin at different time courses and enzyme to substrate ratios are shown in Figure 13. Similar to the results from calf thymus chromatin (Ansevin and Brown, 1971; Li et al., 1975), trypsin reduces the highest melting band (band III) before affecting the second highest band (band II). In this experiment, digestion was performed by adding appropriate amount of trypsin stock solution directly to the nucleohistone. The mixtures were then allowed to stand at room temperature for the indicated periods of time before the reactions were terminated with Soy bean trypsin inhibitor. As shown in the figure, half an hour's digestion by 5 μg/ml of trypsin diminishes the melting band III completely. Melting band II is substantially reduced with the appearance of more melting between 50 and 70°. At higher trypsin concentration (20 μg/ml), band II was reduced further, accompanied by an increase of melting at about 57°. This melting was further shifted to 51° when the digestion was extended to 1-1/2 hour periods.

An obvious explanation for these observations is that the bound histone H5 was digested, thus freeing the DNA and lowering the melting temperature. This explanation can be applied also to the CD results (Figure 13) that upon trypsin digestion, the negative CD at 220 nm originated from bound

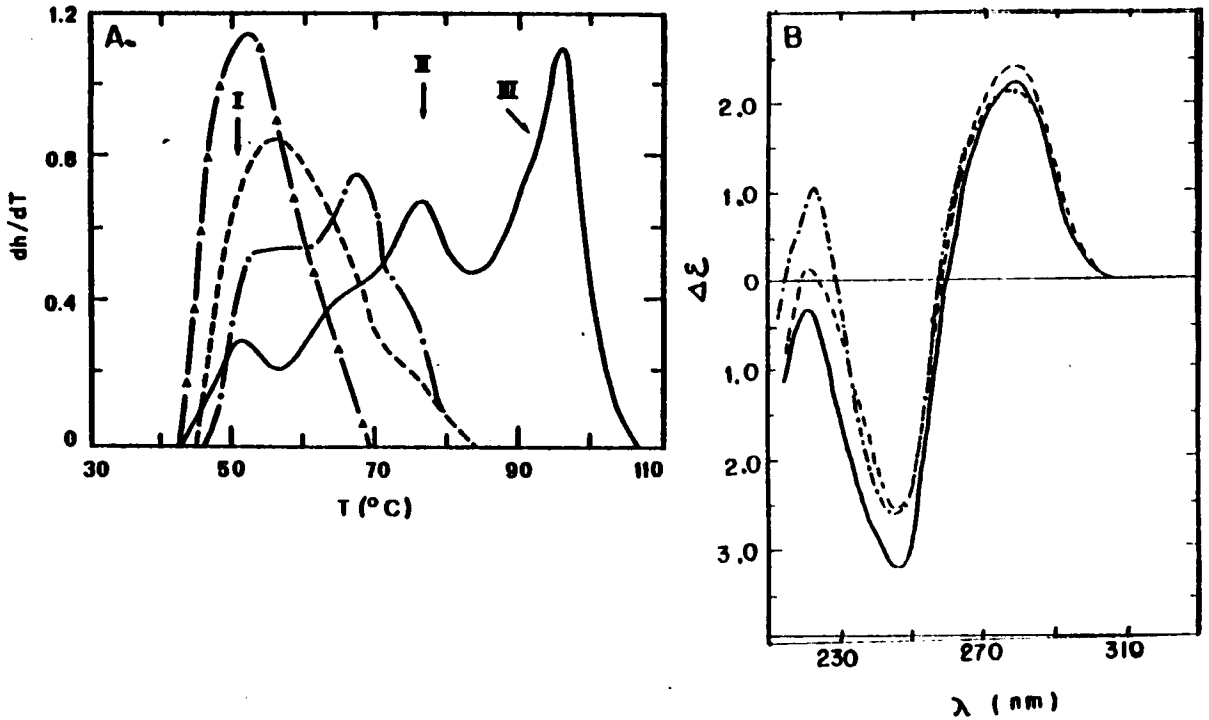


Fig. 13. Effect of trypsin treatment on derivative melting profile (A) of histone H5-chicken DNA complex (r = 1.0), control (—), trypsin treatment with 5 μg/ml for ½ hr. (—•—), 20 μg/ml for ½ hr. (- - - -) and 20 μg/ml for 1½ hr. (—▲—), and on the CD spectra of the nucleohistone (r = 1.0), control (—), trypsin treatmentwith 5 μg/ml for ½ hr. (- - - -) and 20 μg/ml for ½ hr. (— • —)(B).

histone H5 was reduced and both λ_{max} and λ_c of the DNA CD spectrum blue shifted slightly. It is concluded that the breaking down of bound histone H5 at lysine and arginine residues by trypsin slightly restores the DNA conformation back to that of pure DNA. Trypsin digestion also reduces the negative CD contribution at 220 nm by bound histone H5. Trypsin-treated histone H5-calf thymus DNA complexes yield thermal denaturation and CD results similar to those of histone H5-chicken DNA.

Polylysine Binding to Nucleohistone H5

Figure 14 shows the effect of polylysine binding on the derivative melting profiles of histone H5-calf thymus DNA complex with r equal to 0.5. When the input ratio of polylysine to nucleohistone H5 increased, melting band I decreased accompanied by a slight decrease of band II and an increase of a new band IV at 98 to 100°. This phenomenon was also observed in polylysine-chromatin complexes (Li, Brand, and Rotter, 1974). Although the overlap of band III and IV made it difficult to examine the effect of polylysine on melting band III, a trend is still clear that the reduction in the amplitude of melting bands in nucleohistone H5 caused by polylysine follows the order: band I < II < III.

Figure 15 shows the CD spectra of polylysine binding to nucleohistone H5 with r = 0.5. The CD effects on nucleohistone H5 induced by polylysine binding are similar to

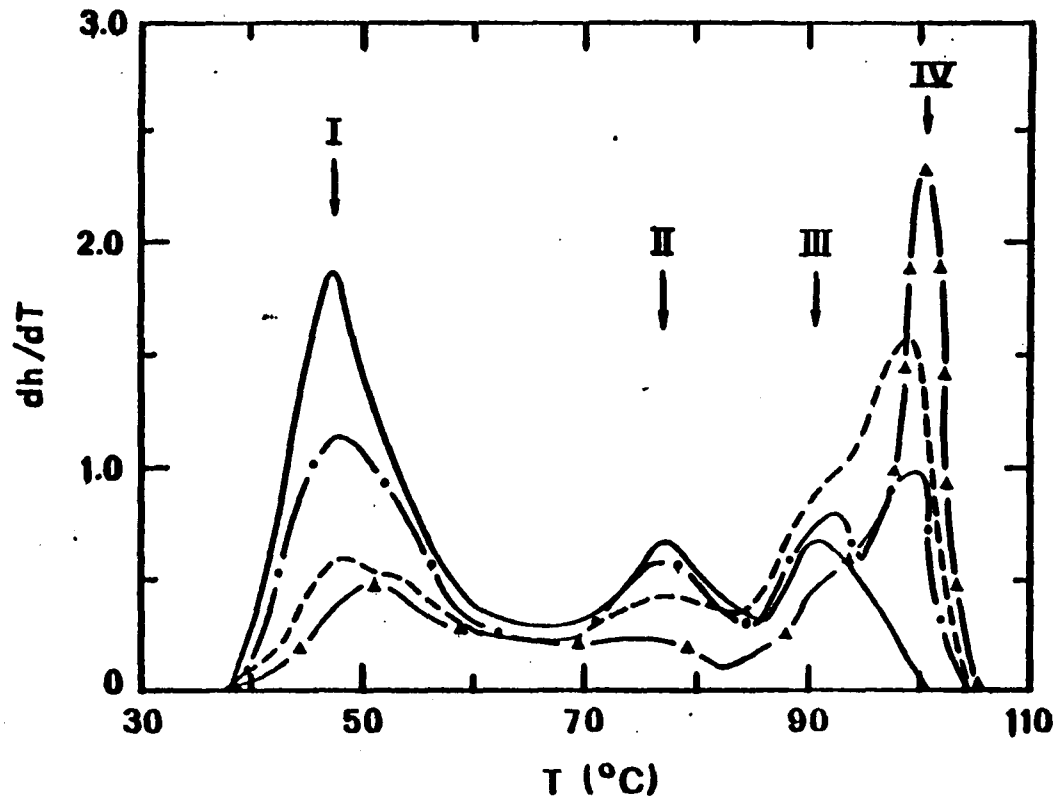


Fig. 14. Effect of polylysine binding on derivative melting profile of histone H5-calf thymus DNA complex ($r = 0.5$). Input lysine/nucleotide is 0 (——), 0.2 (-•-), 0.4 (----) and 0.5 (—▲—).

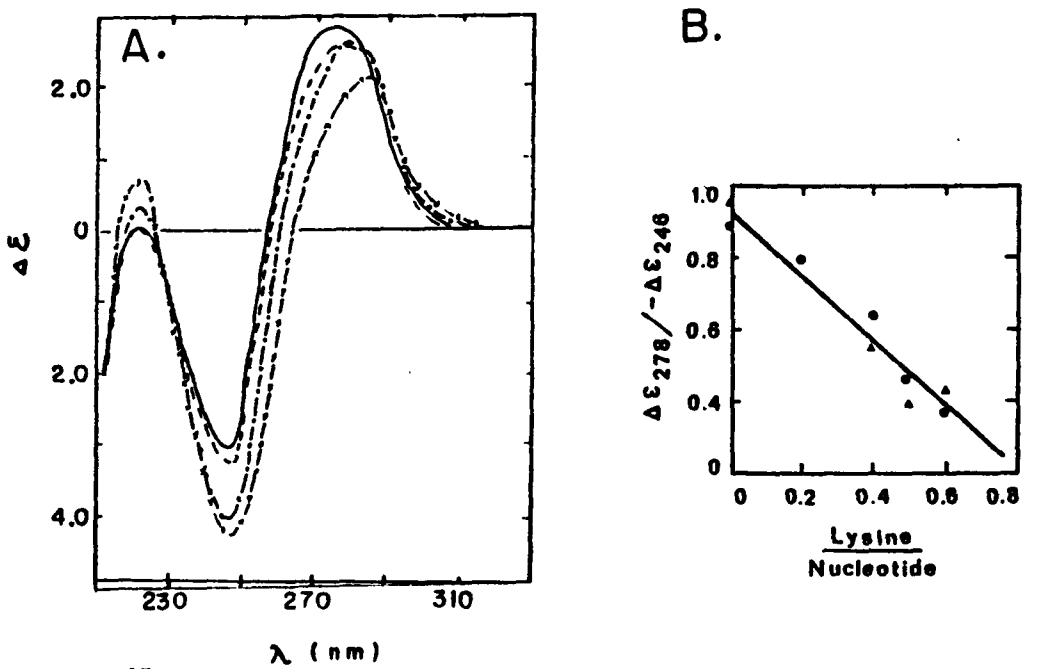


Fig 15A. Effect of polylysine binding on the CD spectra of histone H5-calf thymus DNA complex (r = 0.5), control (——), amino per nucleotide equals to 0.2 (----), 0.4 (— • —) and 0.5 (—▲—)

Fig 15B. Comparison of effect of polylysine binding on CD spectra of DNA and histone H5-DNA complex. Calf thymus DNA (▲), histone H5-calf thymus DNA complex with r = 0.5 (●).

those found in complexes of polylysine to pure DNA (Chang, Weiskopf, and Li, 1973). Also shown in Figure 15 is the dependence of $\Delta\varepsilon_{278}/-\Delta\varepsilon_{246}$ on the input ratio of polylysine to DNA or nucleohistone H5. Within the accuracy of measurement, there is no significant difference between these two systems. Similar thermal denaturation and CD measurements were found in polylysine-histone H5-chicken DNA complexes.

Selective Binding of Histone H5 to (A+T)-rich DNA

Previously it was shown that, at 1.0 M NaCl, polylysine favored (A+T)-rich DNA for binding (Leng and Felsenfeld, 1966; Sponar and Sormova, 1972) and that arginine-rich histone-bound regions in chromatin were slightly (G+C)-rich (Clark and Felsenfeld, 1972). It is therefore interesting to see whether or not histone H5 prefers (A+T)-rich DNA for binding. The melting temperature of a DNA is known to be a linear function of its base composition. An equimolar mixture of Cl. perfringens (69% A+T) and M. luteus (30% A+T) DNA would show two characteristic bands in the derivative melting profile. Complexing histone to either DNA would diminish one of the two bands depending upon which DNA is selected for histone H5 binding. Figure 16 shows that, at r = 0, there were two melting bands at 40.5 and 61.5°, corresponding to the melting band of Cl. perfringens and M. luteus DNA, respectively. At r = 0.5, the melting band of free Cl. perfringens DNA was greatly reduced, but that of

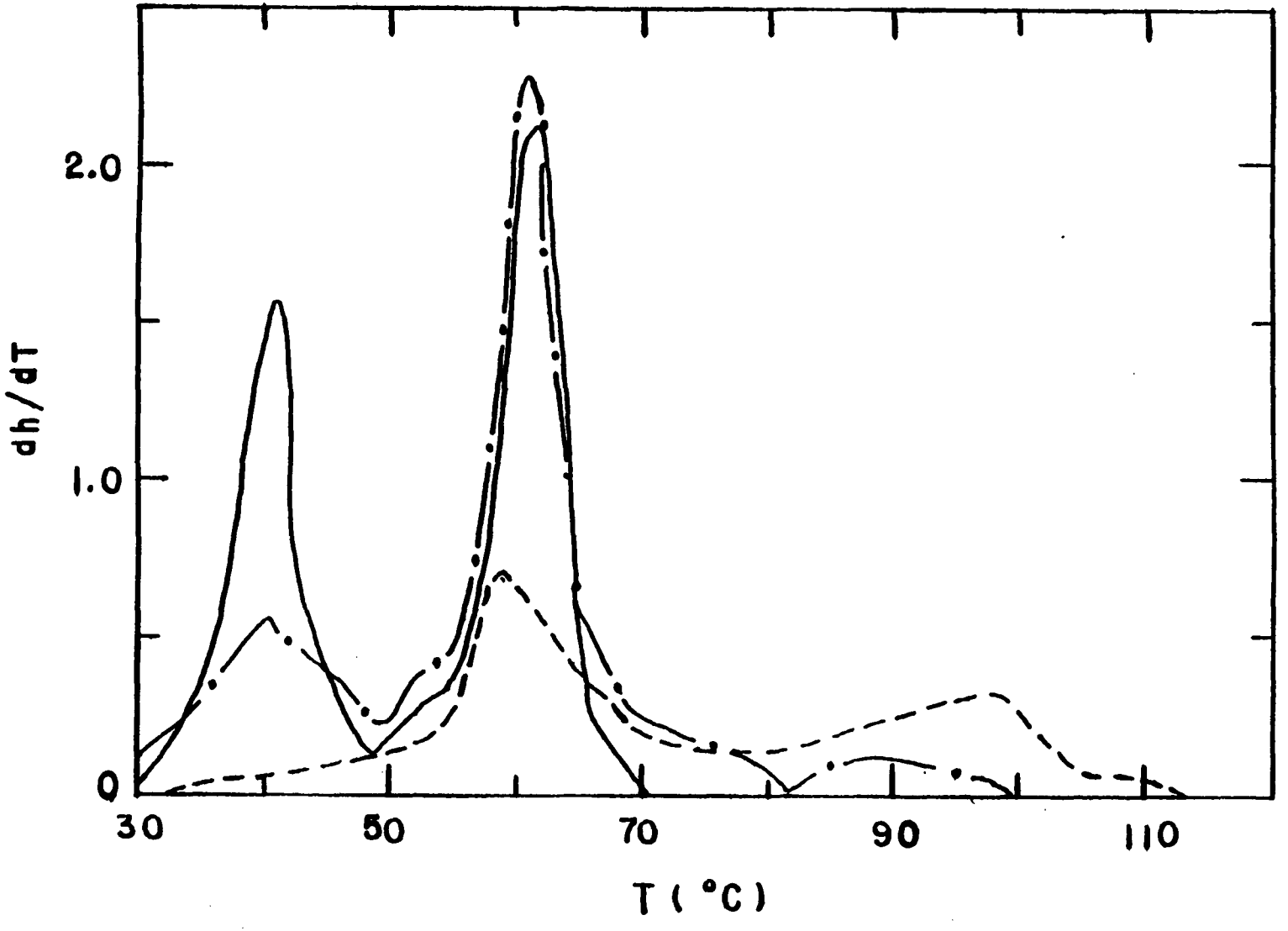


Fig. 16 Selective binding of histone H5 to (A+T)- rich DNA. An equimolar mixture of Cl. perfringens DNA and M. luteus DNA was complexed with histone H5 by reconstitution. r = 0 (——), 0.5 (– . –) and 1.0 (---).

M. luteus remained unchanged and there was a corresponding increase in hyperchromicity at higher temperature. At r = 1.0, the Cl. perfringens DNA band disappeared entirely, and the M. luteus band was reduced by about 50%. These results from reconstitution in the presence of urea are similar to those observed for selective binding of polylysine to (A+T)-rich DNA (Li et al., 1974). However, if the same experiment was done by reconstitution in the absence of urea, this preference became less pronounced. As shown in Figure 17, at r = 0.5, both bands decreased accompanied by the appearance of new melting bands at higher temperature. At r = 1.0, the Cl. perfringens band was greatly reduced and the M. luteus band was also decreased but only to a lesser extent. It is therefore concluded that histone H5 strongly prefers (A+T)-rich DNA for binding in the presence of urea and still favors (A+T)-rich DNA even in the absence of urea.

Importance of A+T Content vs. Base Sequence in DNA for Histone H5 Binding

The above studies showed that, when two bacterial DNAs without any natural base sequence for histones compete for the binding of histone H5, (A+T)-rich DNA is strongly favored over the (G+C)-rich DNA. It would be interesting to see whether chicken DNA with its natural base sequence for histone H5, but having a lower (A+T)-content than Cl. perfringens DNA, would be favored for binding

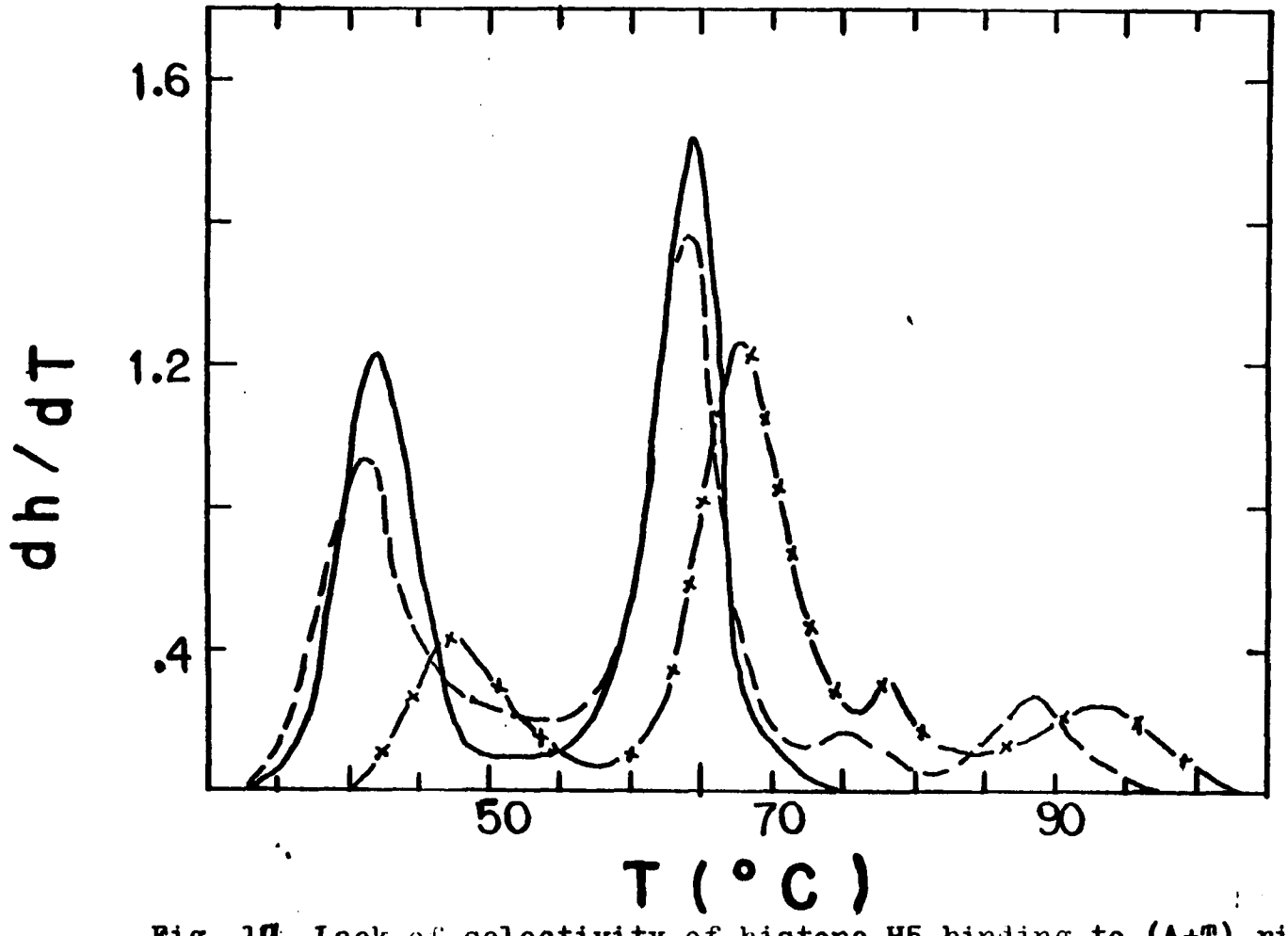


Fig. 17. Lack of selectivity of histone H5 binding to (A+T)-rich DNA. Anequimolar mixture of *Cl. perfrigens* DNA and *M. luteus* DNA was complexed with histone H5 by reconstitution without urea. r= 0 (—), 0.5 (- - -) and 1.0 (-·-·-).

when competing with Cl. perfringens DNA. A mixture of equimolar Cl. perfringens (69% A+T) and chicken DNA (56% A+T) was used for reconstitution with histone H5. Since the percentage of A+T content of these two DNAs is close, their melting bands overlapped and appeared as a broad band (Figure 18). When histone H5 was bound to this mixture of DNAs, it was seen that a greater amount of hyperchromicity was reduced at lower temperature side of this band accompanied with the appearance of new melting bands above 60°. The results became clearer when the derivative melting curves $\Delta(dh/dT) = (dh/dT)_{complex} - (dh/dT)_{DNA}$, were presented. As shown in Figure 19, the negative peak of the curve was located at 39 to 40°, the melting temperature of Cl. perfringens DNA (39°). Since the melting band at this temperature was affected to a larger extent by histone H5 binding, it appeared that Cl. perfringens DNA was more favored than chicken DNA for the binding of histone H5.

The results indicate that the affinity for histone H5 is primarily determined by the (A+T)-content rather than by the base sequence.

C. Histone H1-DNA Interaction in the Presence of H5.

Thermal Denaturation of Reconstituted Nucleohistone H1

Figure 20 shows the derivative melting profiles of reconstituted nucleohistone H1 prepared by salt gradient dialysis in the presence of urea. There are three phases of

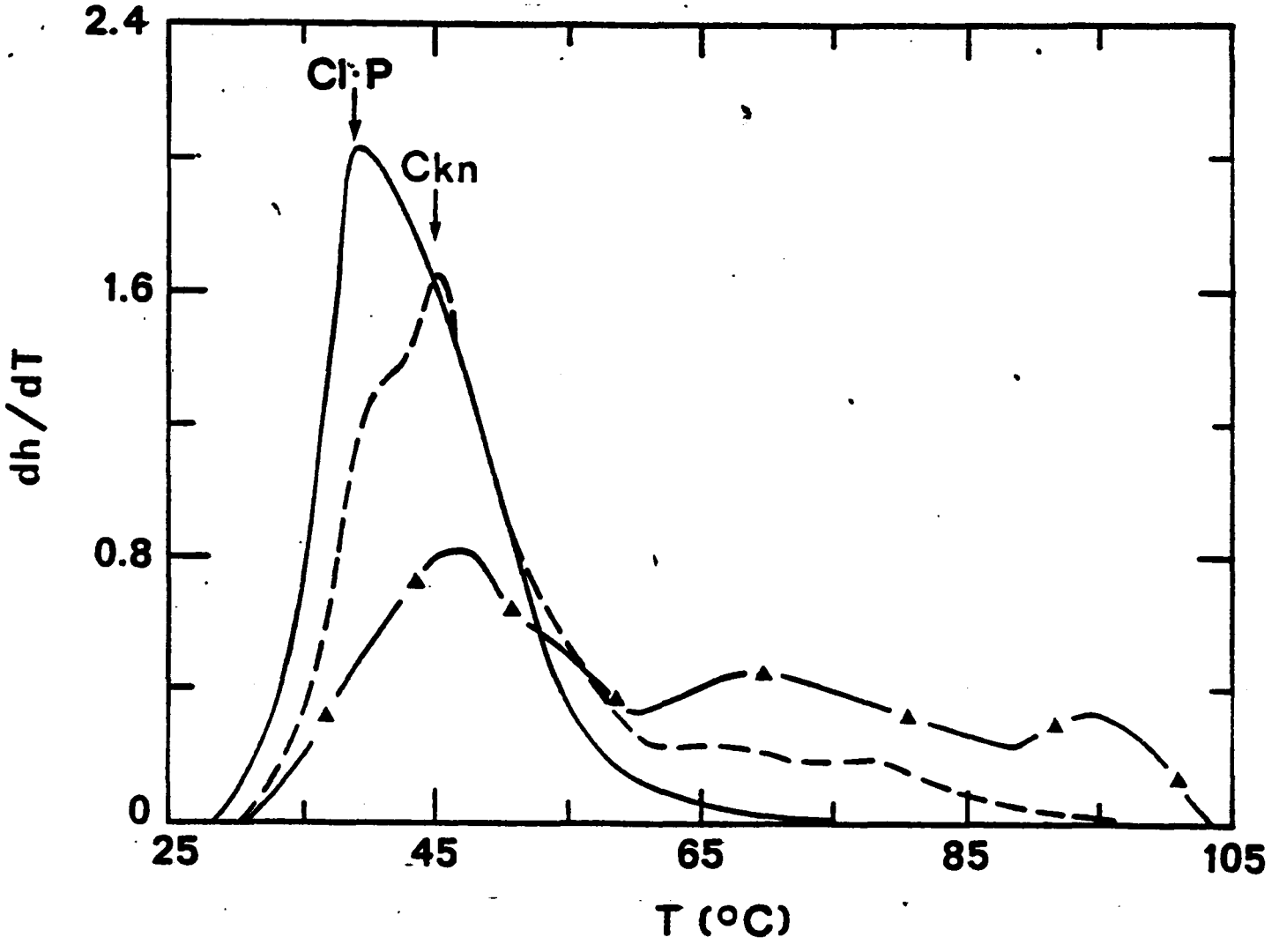


Fig. 18. Competition between chicken and Cl. perfringens DNA for histone H5 binding. The arrows indicate the melting temperatures for chicken and Cl. perfringens DNA. An equimolar mixture of chicken and Cl. perfringens DNA was complexed with histone V by reconstitution. r = 0 (——), 0.5 (---) and 1.0 (— ▲ —).

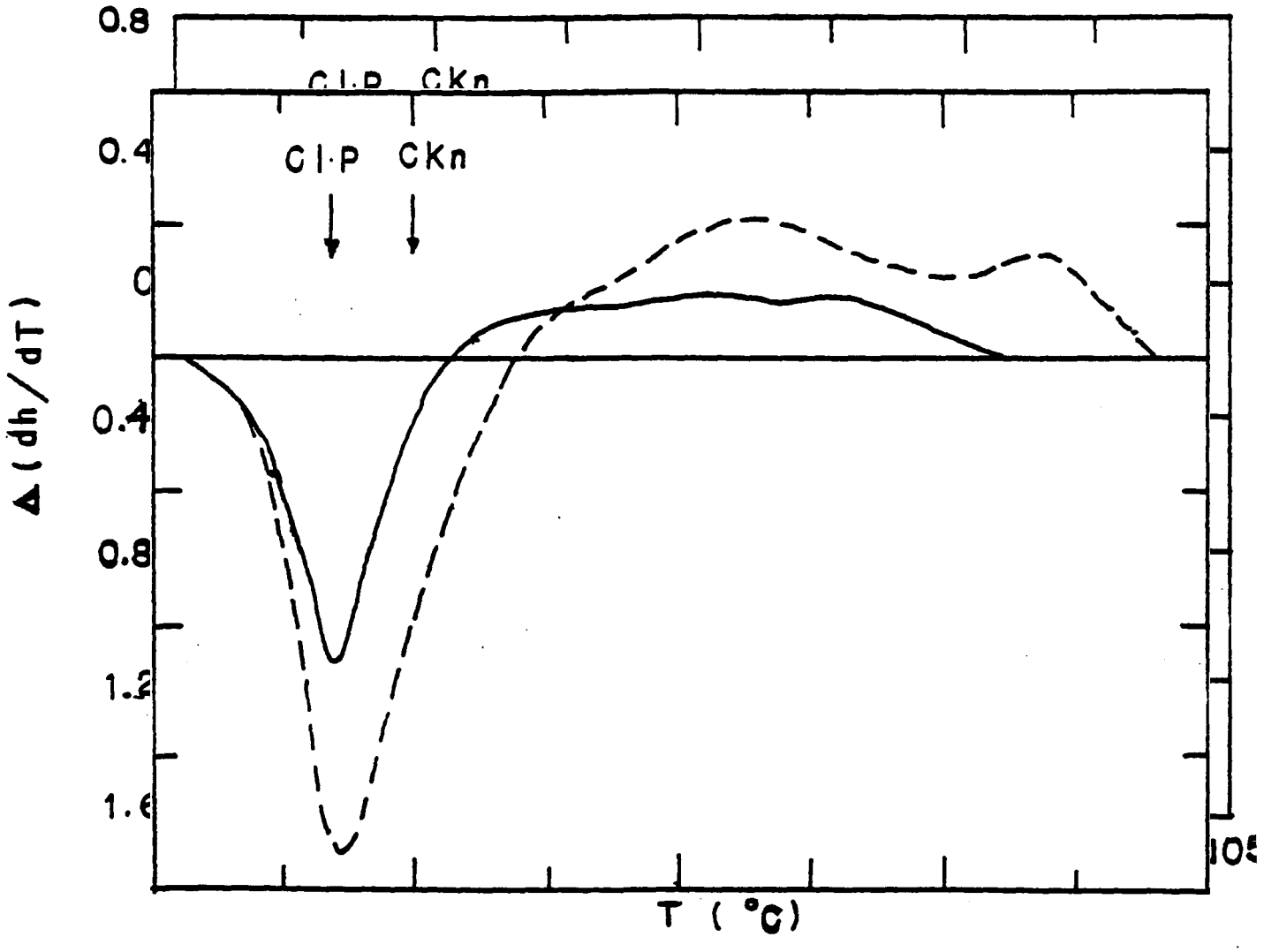


Fig. 19. Difference derivative melting profiles of Fig. 10. $\Delta\,(dh/_{dT}) = (dh/_{dT})_{complex} - (dh/_{dT})_{DNA}$. For the complex, r = 0.5 (——) and 1.0 (---).

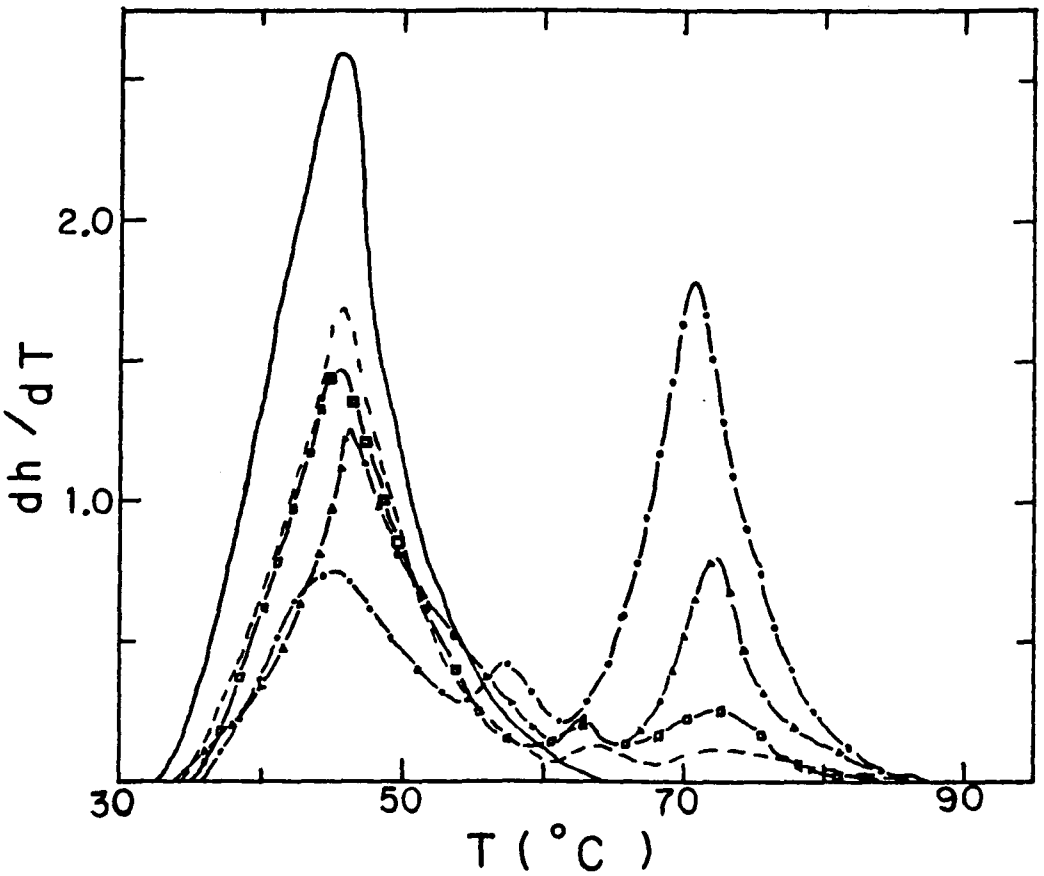


Fig. 20. Derivative melting profile for reconstituted nucleohistone H1, r=0 (——), r=0.12 (----), r=0.24 (—□—), r=0.5 (—▲—) and r= 1.0 (—•—).

melting: an increasing input ratio of histone H1 to DNA diminishes the first band at 45°C, corresponding to the melting of free DNA region, accompanied by an increase of two higher melting bands, a major one at 70-73°C and a minor one at 57-63°C. The temperature of the minor band shifted from 63°C to 57°C at r = 1.0. Our data indicate that, although histone H1 resembles histone H5 in many aspects, such as lysine content, extractability, etc., its binding characteristics with DNA and its capacity in stabilizing DNA against thermal denaturation are very different from that of histone H5.

Thermal Denaturation Studies of Nucleohistone H1 and Nucleohistone (H1+H5) Prepared by Direct Mixing

Figure 21 shows derivative melting profiles of chicken DNA complexed with chicken erythrocyte histone H1 or (H1+H5), using the method of direct mixing in EDTA buffer. At such low ionic strength (25 mM EDTA, pH 8.0), ionic bonding is expected to be the primary force to hold histone and DNA together. As shown in the figure, melting profile of nucleohistone H1 is similar to that of nucleohistone H5 except that the melting temperatures are a few degrees higher in nucleohistone H5 than in nucleohistone H1. When histone H1 was mixed with histone H5 first before complexing with the DNA, the melting profile differed from that of either nucleohistone H1 or nucleohistone H5. Nevertheless, the difference is small that it is

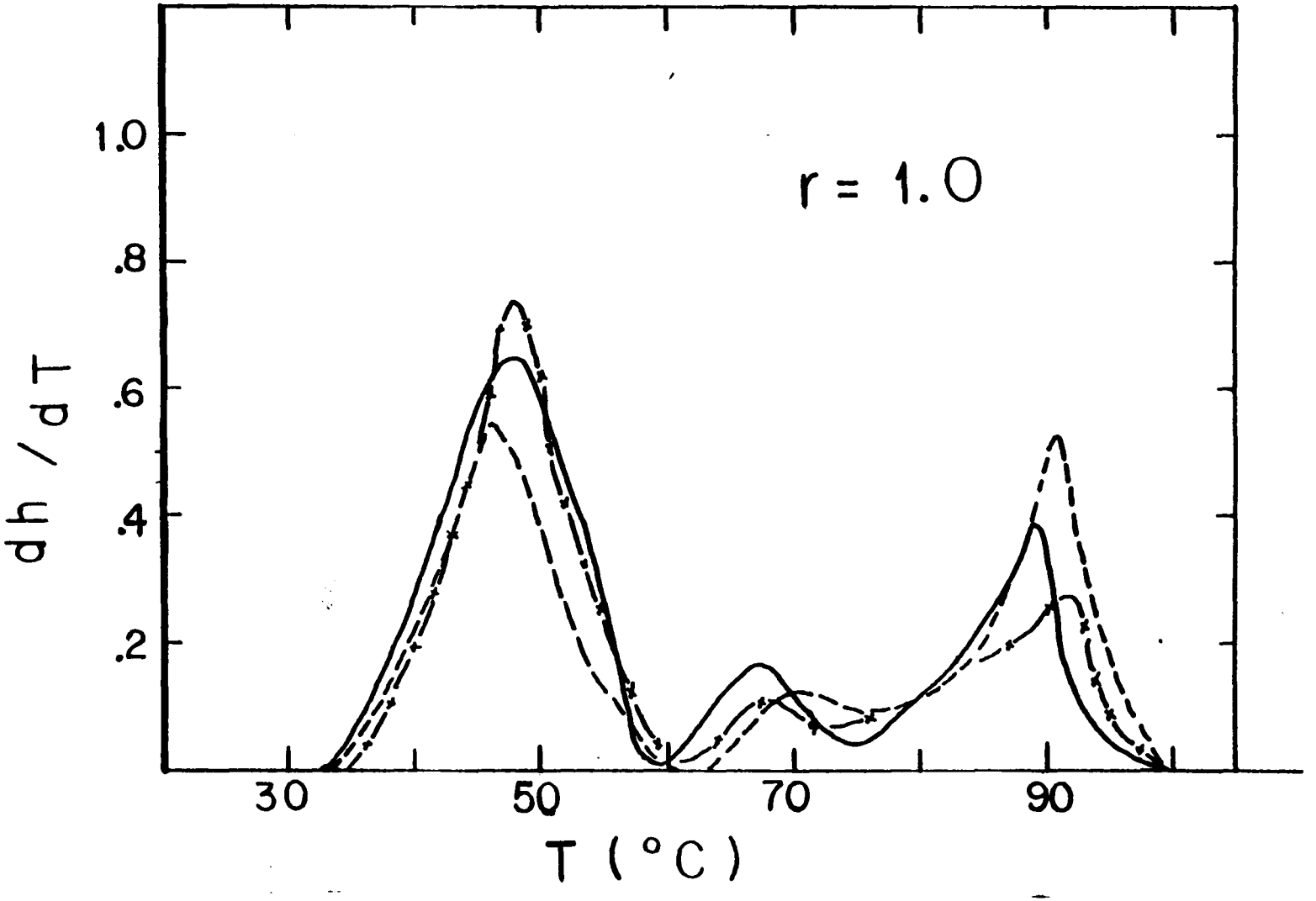


Fig. 18. Derivative melting profile for nucleohistones H1 (——), H5 (– – – –) and H1+H5 (– x – x –), prepared by direct mixing.

still difficult to conclude whether interaction between histone H1 and H5 occurs before complexing with the DNA.

Thermal Denaturation Studies of Nucleohistone H1 and Nucleohistone (H1+H5) Prepared by Salt Gradient Dialysis

Figure 22 and 23 show derivative melting profiles of nucleohistone (H1+H5) prepared by complexing an equimolar mixture of purified histone H1 and H5 with chicken DNA using NaCl-gradient dialysis in the presence of urea. In nucleohistone (H1+H5), the characteristic melting band of nucleohistone H5 at 93° was not observed and the whole melting profile shifted toward that of nucleohistone H1. Specifically, there are three phases of melting, with band I at about 45° for unbound DNA base pairs, band II at about 60° and band III at 72° corresponding to histone-bound regions. The amplitude of the two higher melting bands in nucleohistone (H1+H5) was lower than that of nucleohistone H1.

When the area ratio of the melting bands of histone-bound regions to total area is plotted vs. the input ratio of histone to DNA, r, the slopes of nucleohistones H5, H1 and H1+H5 follow the order of H5 < H1 < H1+H5, suggesting a decrease of histone binding efficiency in the mixture (Fig. 24). In other words, the results suggest that histone H1 and H5 interact with each other and this complex has a lower binding efficiency with DNA than when both histones bind DNA separately.

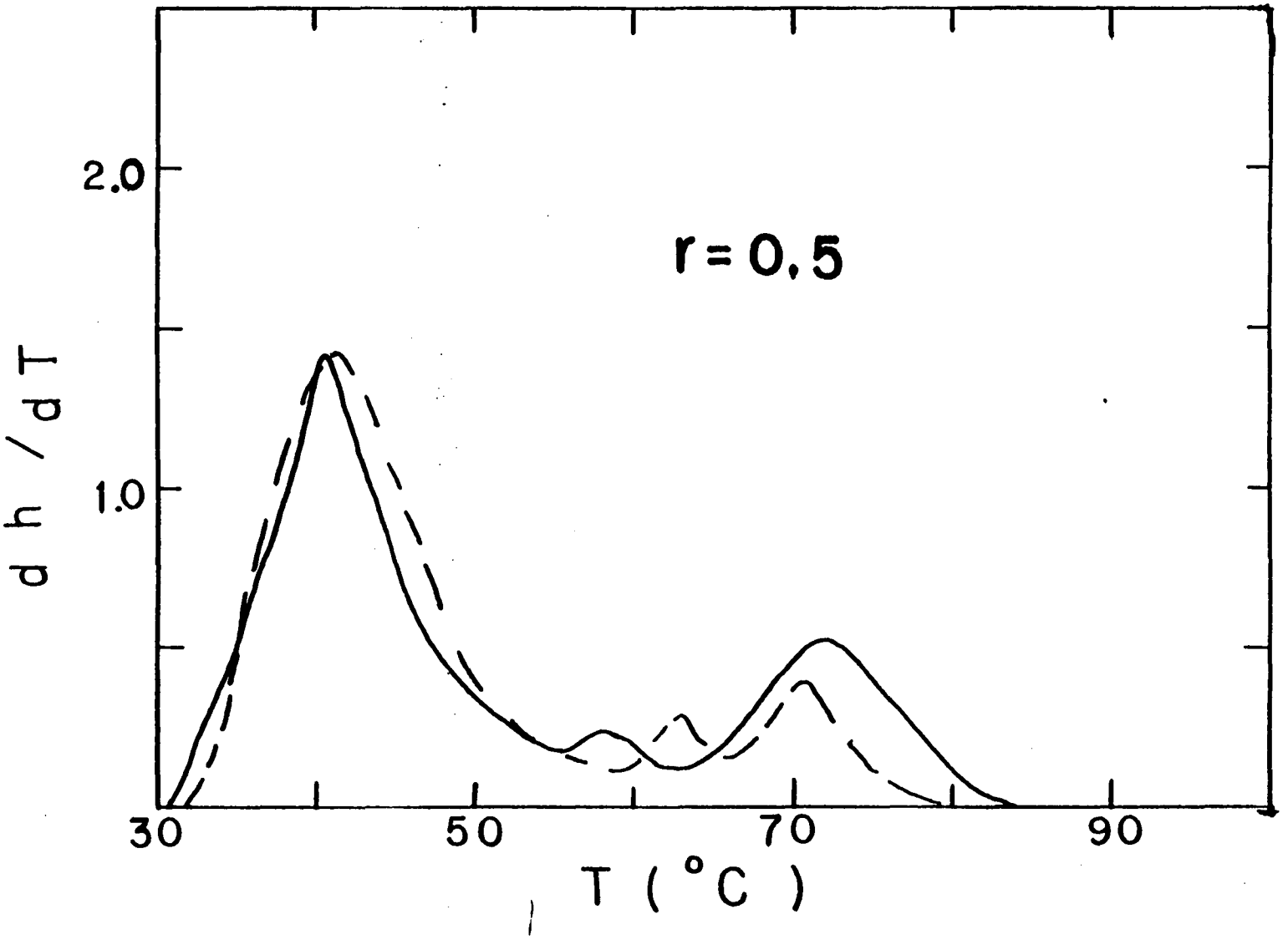


Fig. 22. Derivative melting plot of nucleohistone H1+H5 (— — —) and H1 (——) prepared by continuous salt gradient dialysis. r= 0.5.

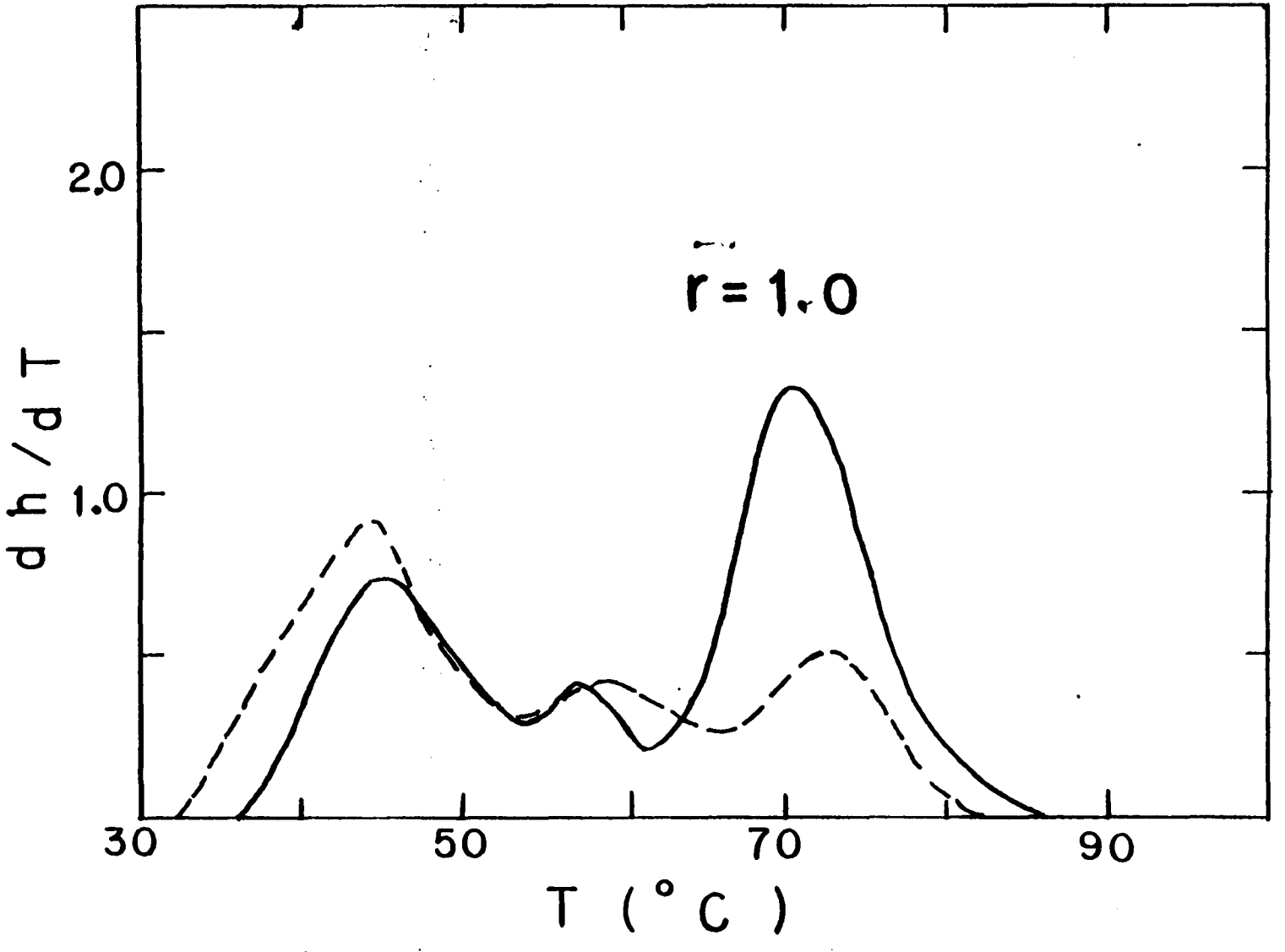


Fig. 23. Derivative melting plot of nucleohistone H1+H5 (— —) and H1 (———) , prepared as described in Fig. 22, r= 1.0.

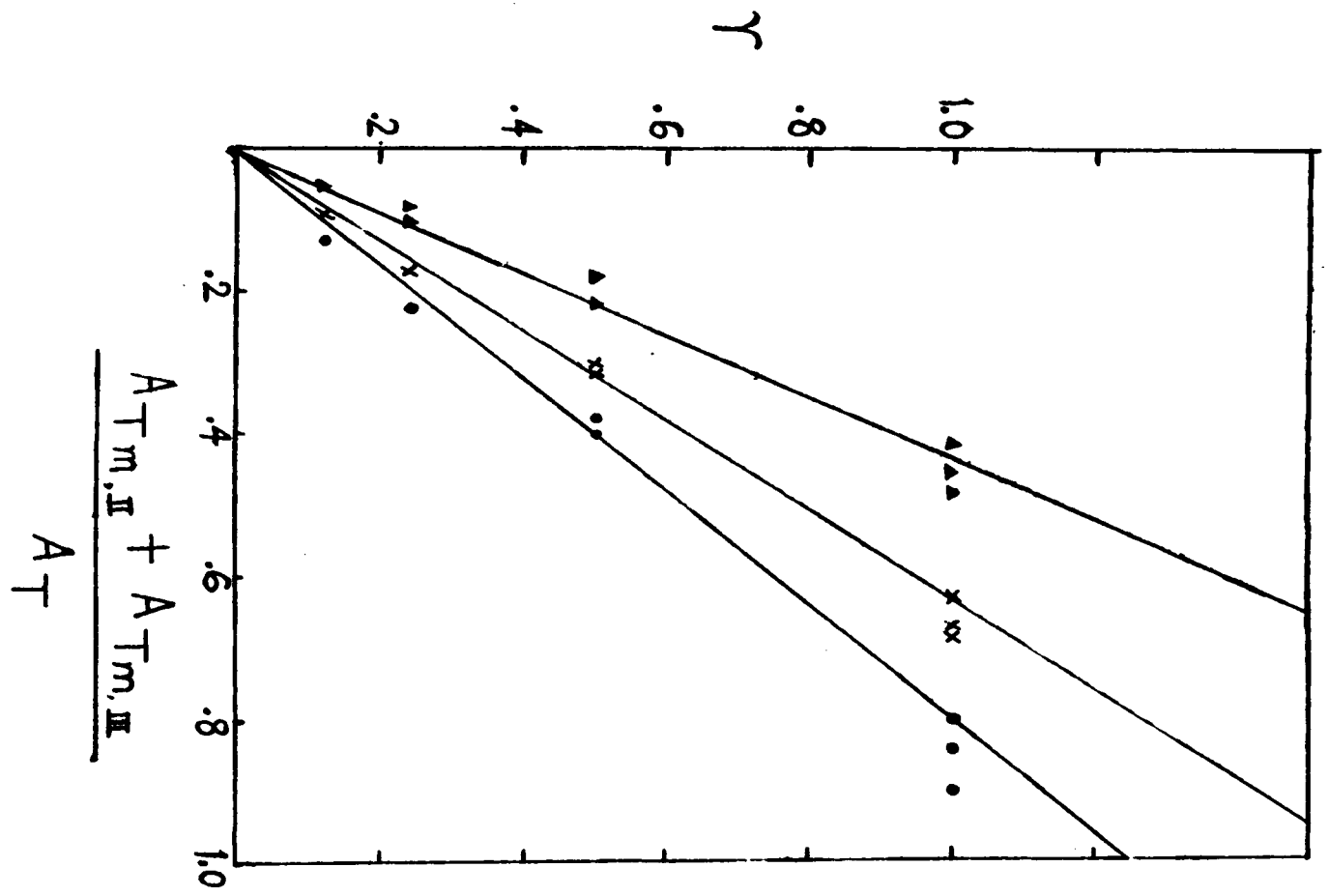


Fig. 24. Plot of areas under histone bound regions vs. the input ratio of amino acid per nucleotide (r). Nucleohistone H5 (—•—), H1 (--x--) and H1+H5 (—▲—). Straight lines were drawn by linear regression analysis.

Interactions Between Histone H1 and H5 in Solution

In order to study interactions between histone H1 and H5 in solution, CD spectra of histone H1, H5 and their mixtures were taken (Figure 25). The spectra of these two histones were similar to each other with a trough at about 200 nm in the $\pi \rightarrow \pi^*$ region and a shoulder at 220 nm in the $n \rightarrow \pi^*$ region. The amplitude of the negative ellipticity of histone H1 was larger than that of H5. The CD spectra of these two histones in solution agree with those reported by Crane-Robinson et al., (1976).

Assuming no interaction between histone H1 and H5 in solution, the CD of the mixture should be equal to the sum of the two individual histones, i.e.,

$$\Delta\varepsilon_{NI}(\lambda, X_{H1}) = \Delta\varepsilon_{H5}(\lambda)\ (1 - X_{H1}) + \Delta\varepsilon_{H1}(\lambda)\ X_{H1} \qquad (3\text{-}1)$$

where X_{H1} is the molar fraction of histone H1 and $\Delta\varepsilon_{NI}$ the CD of non-interacting mixture. At each given wavelength, $\Delta\varepsilon_{H1}$ and $\Delta\varepsilon_{H5}$ are constants. Therefore,

$$\Delta\varepsilon_{NI}(X_{H1}) = \Delta\varepsilon_{H5} + X_{H1}\ (\Delta\varepsilon_{H1} - \Delta\varepsilon_{H5}) \qquad (3\text{-}2)$$

If histone H1 interacts with H5 and if their CD in the complex differs from that of the non-interacting mixture, $\Delta\varepsilon_{NI}$ will differ from $\Delta\varepsilon_{measured}$. The differences, i.e., $\Delta\varepsilon_{measured} - \Delta\varepsilon_{NI}$ will indicate the extent of interaction. Figure 26 shows a plot of $(\Delta\varepsilon_{measured} - \Delta\varepsilon_{NI})$ vs. X_{H1}. At 200 nm, the maximum of this difference occurred at $X_{H1} = 0.5$, suggesting that the stoichiometric ratio for the interaction of

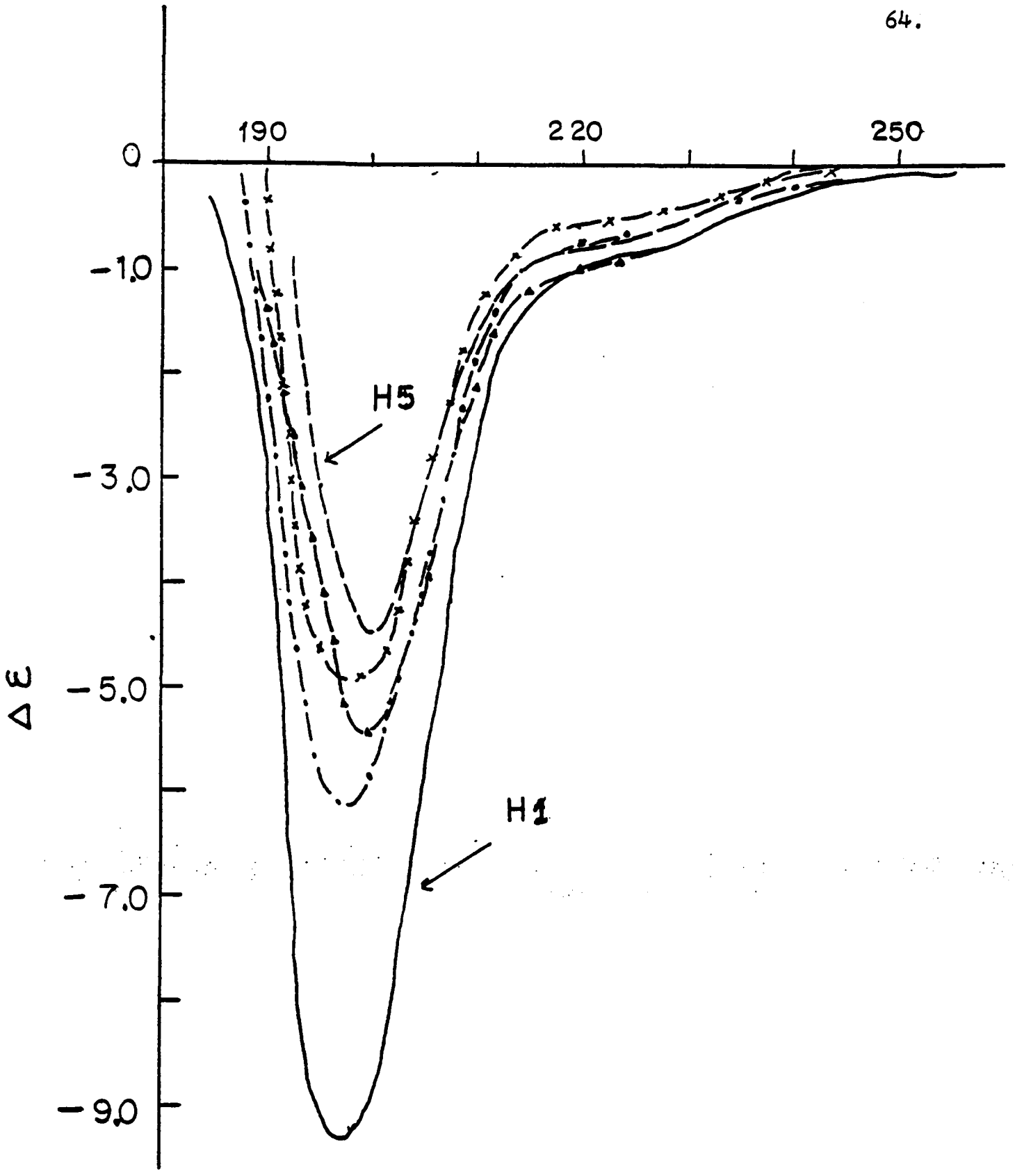


Fig. 25. CD spectra of histones H1, H5 and mixtures of histones H1 and H5 in different molar ratios, X_{H1} = 0.3 (— × —), X_{H1} = 0.5 (—▲—) and X_{H1} = 0.66 (—●—).

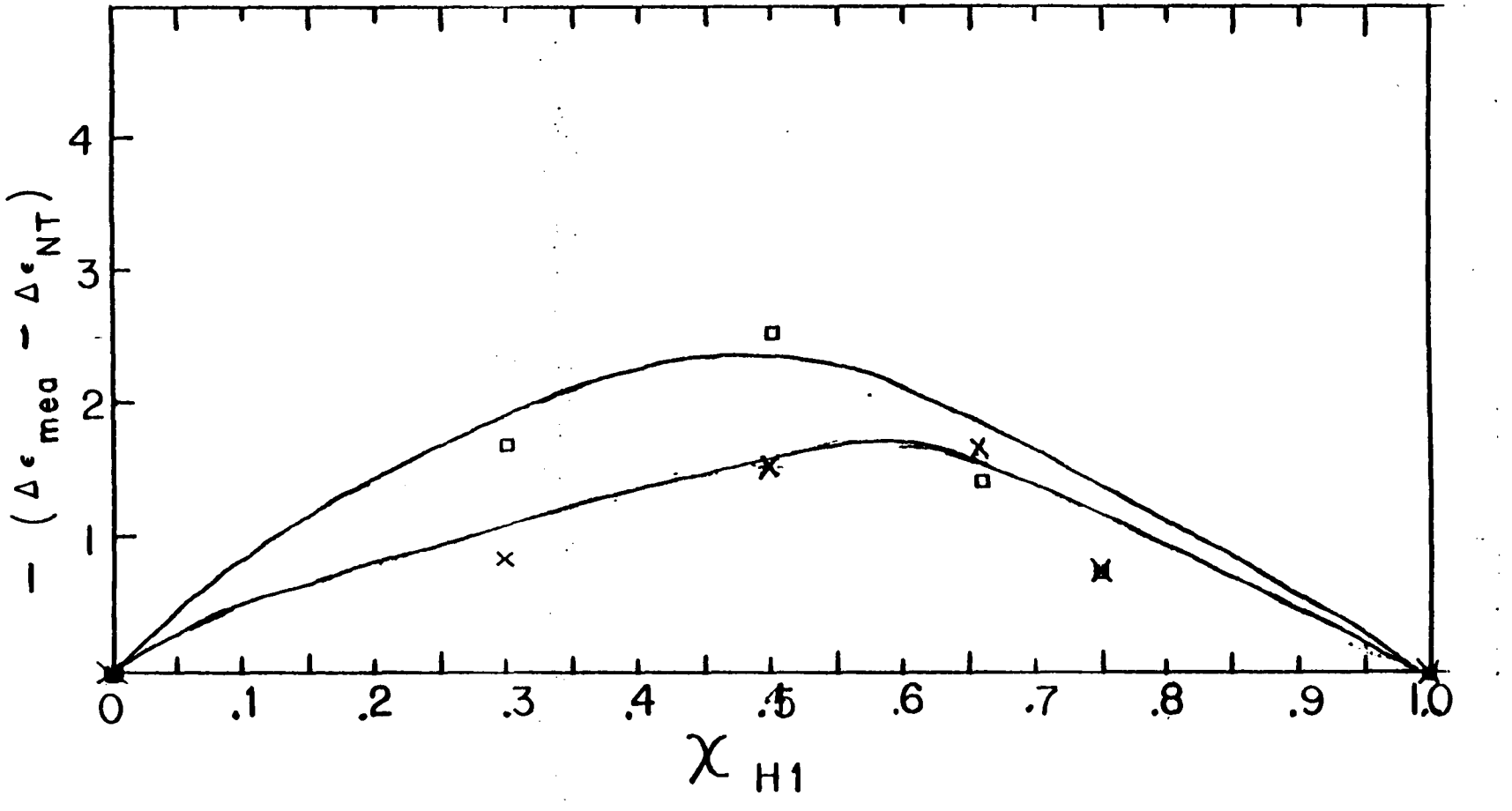


Fig. 26. CD continuous variation curves, difference of measured $\Delta\epsilon_{mea}$ and $\Delta\epsilon_{NT}$ assumming no interactions between histones H1 and H5, vs. X_{H1} at 197 nm (— × —) and 200 nm (— □ —).

histone is 1:1. However, if the CD at 197 nm was used, the maximum of the CD difference occurred at about $X_{H1} = 0.6$. The experimental imprecisions do not make this maximum significantly different from 0.5, as determined at 200 nm.

D. Histones H1 and H5, Interaction-Crosslink Studies

Nitration of Histone H1 with Tetranitromethane (TNM)

Fixation with glutaraldehyde induces a polymeric form of H1 in chromatin (Chalkley and Hunter, 1975). Subsequent reaction leads to multiple small oligomers of other histones. This result suggests that histones are organized in proximal arrays of entities and crosslinking reagents may detect their arrangements. Tetranitromethane reacts with tyrosine; this interaction can be more specifically used to probe the microenvironment of the tyrosine residues in a histone molecule under various conditions.

Figure 27 shows the kinetics of nitration of histone H1 with TNM. The reaction was fast within the first 10 min and gradually leveled off afterwards. In the presence of 2 M NaCl, the rate of nitration was the same as that for histone H1 at low salt (0.05 M Tris). This result suggests that the accessibility of the tyrosine residue in histone H1 molecule for TNM was not changed. Also included in the figure is the kinetics of nitration of histone H5 in 0.05 M Tris. The total amount of nitration was greater in histone H5 than in histone H1. This is expected, since histone H5 has three tyrosine residues as compared to one tyrosine residue in

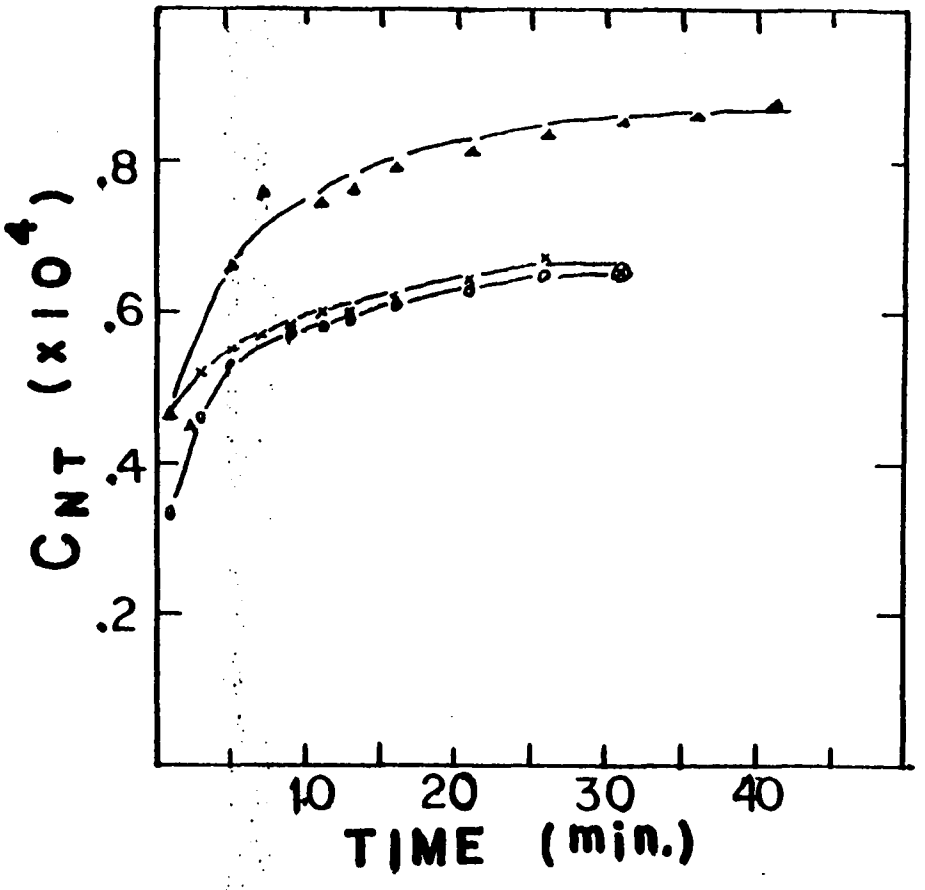


Fig. 27. Kinetic studies of nitration of histone H1 in 0.05 M Tris, pH 8.3, in the presence (— o —) and absence (— x —) of 2 M NaCl and of histone H5 (— ▲ —) in the absence of NaCl.

histone H1. Except the difference in amplitude, these curves were similar to each other. Figure 28 shows the kinetics of nitration of total histones in 0.05 M Tris, pH 8.3 with or without 2 M NaCl and Figure 29 that of total histones minus histones H1 and H5. For total histones, although the rate of reaction seems to differ right after the reaction starts, the final level of nitration remains the same no matter whether histones are left in 0.05 M Tris with or without 2.0 M NaCl. On the other hand, for total histones minus H1 and H5, the kinetic curves are essentially the same either with or without 2.0 M NaCl, the reaction medium. It is noted that, the reaction was completed within 10 min and the concentration of nitrotyrosine reached 10^{-4}M, while the reaction was completed in total histones only at about 20 minutes after the reaction started and the plateau concentration of nitrotyrosine was only 0.8×10^{-4}M. These differences between total histones and total histones minus (H1+H5) (Figures 28 and 29) probably are caused by histones H1 and H5 because the nitration rate and plateau values of nitrotyrosine are lower for these two histones (Figure 27) than for the others (Figure 29).

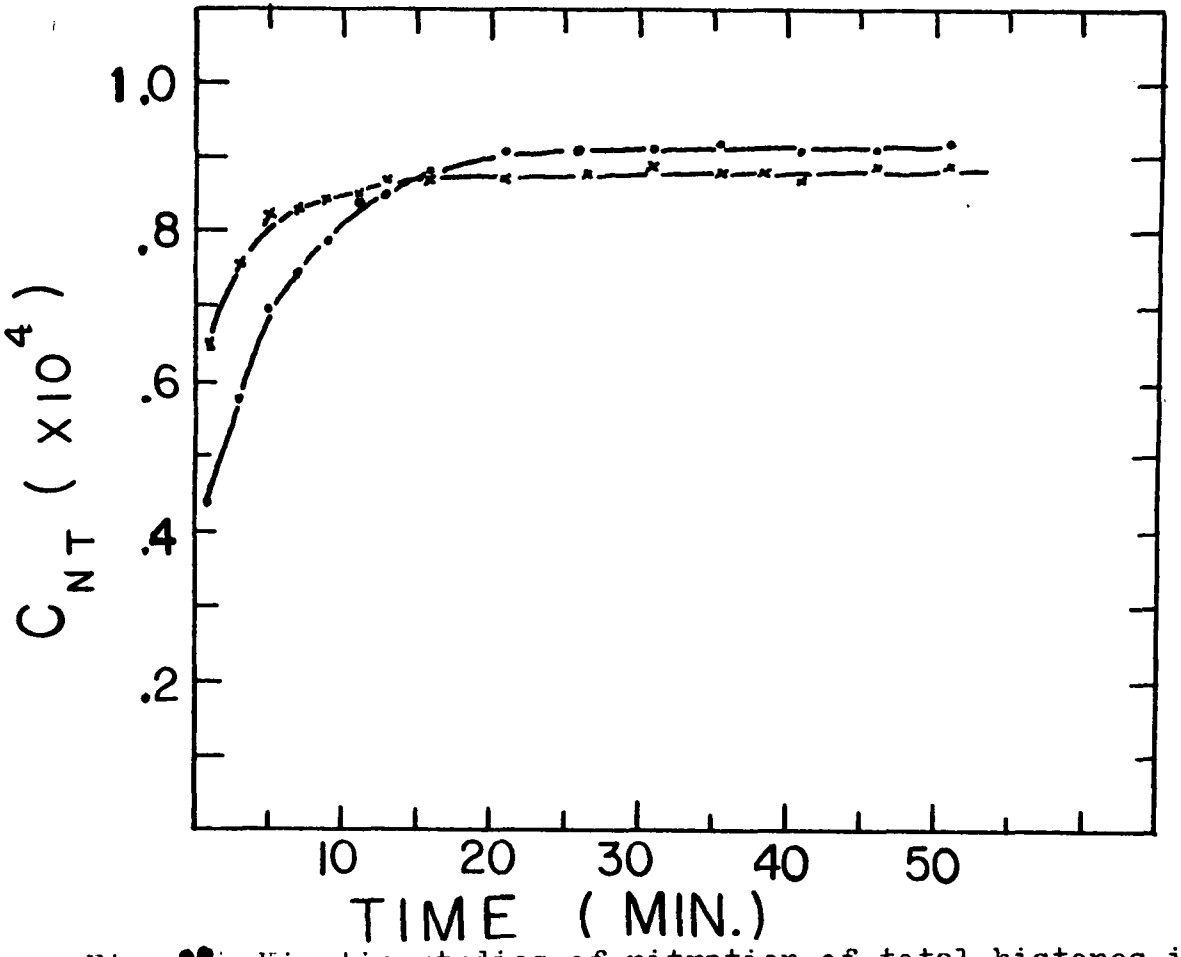


Fig. 28. Kinetic studies of nitration of total histones in the presence (— • —) and absence (— × —) of 2 M NaCl. Experimental condition is as that described in Fig. 27.

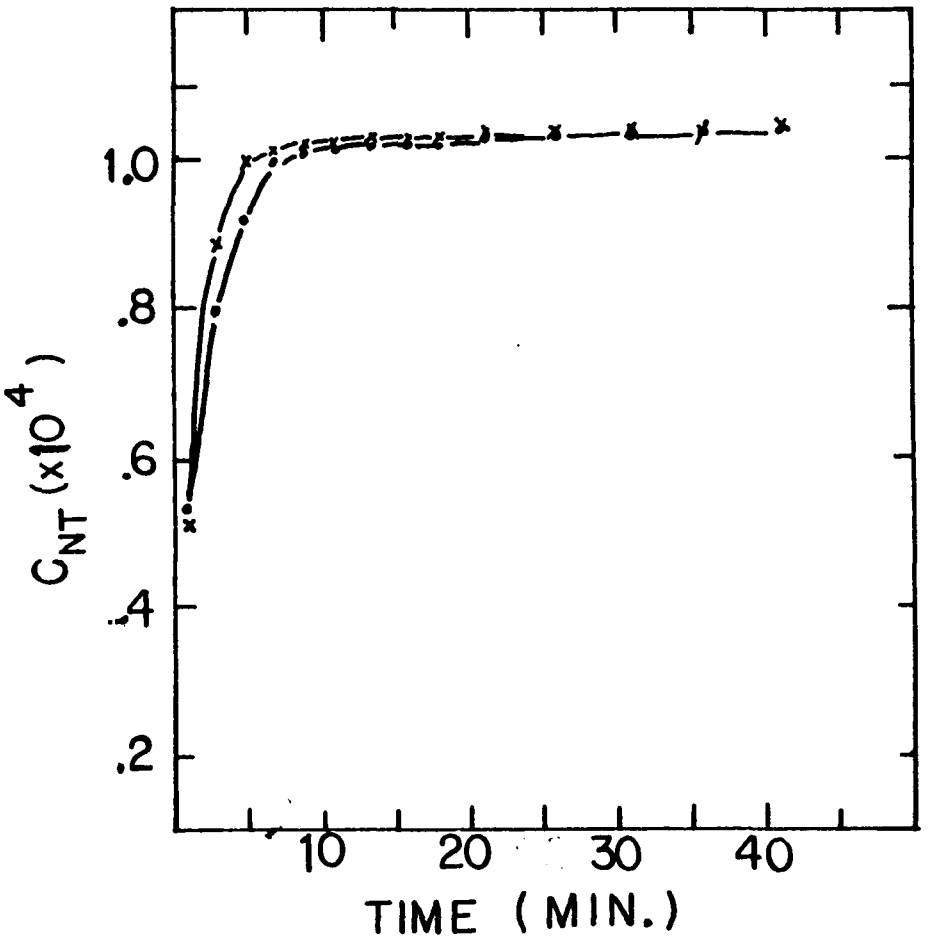


Fig. 29 Kinetic studies of nitration of total histones minus histones H1 and H5 in the presence (— • —) and absence (— x —) of 2 M NaCl.

CHAPTER IV

DISCUSSION

Based on thermal stabilization and conformational effect on DNA, the results from this study show strong interactions between histone H5 and DNA. The three phases of melting of nucleohistone H5 (Figures 8-10) are similar to those in chromatin (Figure 3) and other nucleohistones, except that the melting temperatures are higher which is probably due to the higher content of basic amino-acid residues (32-40%) in histone H5 than in any other histone from chicken, calf or pea (25-30%) (Hnilica, 1972).

As shown in this study, in histone-bound regions of reconstituted nucleohistone H5, there are only 1.5 amino-acid residues per nucleotide (Figure 11). This value is much lower than the 3.2 for pea bud chromatin and 3.0 for nucleohistone H1. This lower value implies that histone H5 suppresses the genome more effectively than do other histones, since the same amount of histone H5 can block a longer sequence of DNA than can the other histones. The partial sequence of histone H5 determined so far also showed an uneven distribution of basic amino-acid residues along the H5 molecule, as with the other histones (Sautiere et al., 1976). This could explain the two phases of melting in histone H5-bound DNA, i.e., one for the less basic and the other for the more basic regions of histone H5.

Trypsin has been shown to cleave off about 55 peptide bonds per 100 DNA base pairs in chromatin (Simpson, 1972)

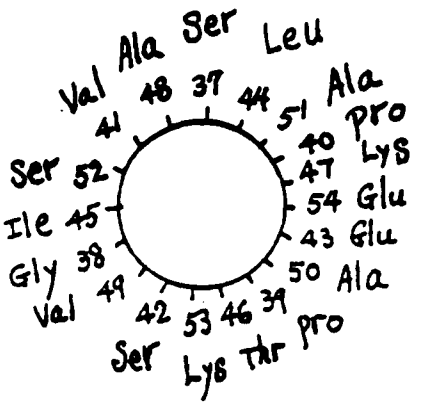
Since trypsin cleaves protein molecules at the carboxyl side of lysine and arginine, the higher vulnerability of band III than band II (Figure 13) could be explained as due to a more favorable digestion of the more basic regions by trypsin than the less basic regions (Li, Chang, and Weiskopf, 1973). This hypothesis is compatible with the recent finding that in chromatin, histones H2A, H2B, H3, and H4 were discretely cleaved by trypsin toward the N-terminal (the more basic regions) (Weintraub, 1975) while histones H1 and H5 were digested all over their molecules (Weintraub and Van Lente, 1974). It is also interesting to note that under the same conditions, trypsin digestion of calf thymus chromatin reduced only the highest melting band while the second highest melting band was not affected at all (Ansevin and Brown, 1971; Li et al., 1975). The lysine and arginine content of histone H5 is higher than that of histones in calf thymus chromatin. This higher content in lysine and arginine could contribute to the higher vulnerability of bound histone H5 to trypsin digestion.

Although histone H5 binds DNA strongly with higher thermal stabilization, the distortion of DNA conformation in nucleohistone H5 is only moderate compared to that induced by the binding of other histones in chromatin. Ionic

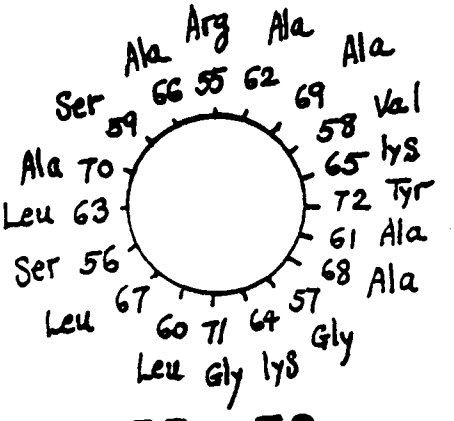
bonding itself perhaps cannot dictate the conformational effect in a nucleoprotein. The binding of histone H5 to DNA yields the same quantitative melting results irrespective of the DNA used, whether from chicken, from calf or from M. luteus. These results suggest that specific type of interaction between histone H5 and DNA is primarily determined by the histone itself, rather than by the A+T content or the base sequence in a DNA molecule.

Thermal denaturation studies of nucleohistone (H1+H5) suggest that histone H1 can affect the strong and direct binding of histone H5 to DNA. Thermal denaturation properties of nucleohistone (H1+H5) was found to be closer to that of nucleohistone H1 than to that of nucleohistone H5 (Figures 8, 21, and 22). These results further imply interaction between histone H1 and H5 before complexation with the DNA. Such interaction between these two histones in solution is supported by CD spectroscopic studies of histone H1, H5 and (H1+H5) in solution (Figure 25). CD results further suggest a stoichiometry of 1:1 in the complex. It is interesting that the Schiffer-Edmundson (1967) helical wheels of the two histones H1 and H5 yield about the same number of residues in the α-helical state (Figure 30). The helical wheels of histone H1 and H5 indicate a hydrophobic arc from residue 55 to 72 in histone H1 and from 19 to 36 in histone H5. These two regions

H1

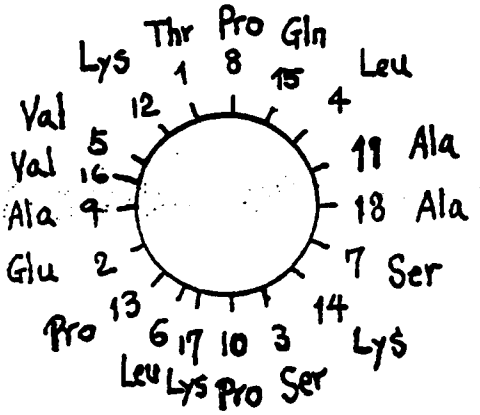


RESIDUES 37 – 54

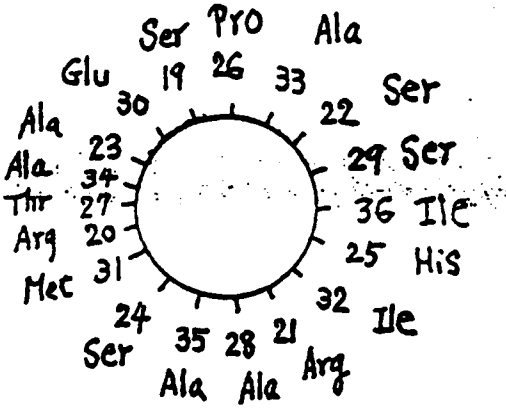


55 – 72

H5



RESIDUES 1 – 18



19 – 36

Fig. 30. Helical wheels of the very lysine-rich histone H1 according to Rall and Cole (1971) and the avian erythrocytes specific lysine- and serine-rich histone H5, according to Sautiere et al. (1976).

probably are the favorable sites for interaction between these two histones. This analysis rests upon the finding of α-helical regions which have a relatively large number of hydrophobic residues on one side of the α-helix. Interaction between histone H1 and H5 could possibly occur through hydrophobic interaction in these α-helical regions. The secondary structure of histone H1 has recently been predicted by Fasman, Chou, and Adler (1977).

It is noted that when probed with tetranitromethane (Figures 27 and 28), the tyrosine groups in histone H1, H5 total histones minus (H1+H5) and total histones were equally accessible even when the ionic strength of the media was increased to 2 M NaCl. Although it is known that 2.0 M NaCl could induce conformational changes in histones from a more flexible, uncoiled state to a more ordered, compact state, such structural transition does not seem to alter the final accessibility of tyrosine residues for reaction with TNM.

CHAPTER V

CONCLUSIONS

The results from thermal denaturation and circular dichroism studies presented here show that histone H5 interacts more strongly with DNA as compared to histone H1 and other histones. It also shows a preference towards (A-T)-rich DNA, irrespective of the source of DNA.

Spectroscopic evidence also showed a one to one interaction between the two lysine rich histones in solution. This interaction may interfere with the strong affinity between histone H5 and DNA. The interaction between DNA and lysine rich histones could relate to the loosening or condensing of the chromatin during the cell cycles.

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