I. CHROMOSOMAL RNA OF CALF THYMUS CHROMATIN

II. THE TEMPLATE PROPERTIES OF DNA-POLYPEPTIDE COMPLEXES

III. STUDIES ON DNA COMPLEXES WITH PURIFIED HISTONE FRACTIONS

Thesis by

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Dedicated to

My wife and parents

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ABSTRACT

Part I. Chromosomal RNA of Calf Thymus Chromatin

Calf thymus chromatin is shown to contain an associated chromosomal RNA as do the chromatins of other species. The chromosomal RNA of calf thymus chromatin is present in an amount of 1% of that of DNA. The purified material eluted from DEAE-Sephadex column at a NaCl concentration of 0.56 M as do the chromosomal RNA's of other organisms. Its average chain length by end-group assay is approximately 40 nucleotides and it contains approximately 3-4 dihydrouridylic acid residues per chain. Calf thumus chromosomal RNA is associated with chromosomal protein in a form not dissociable by high salt concentration.

ABSTRACT

Part II. The Template Properties of DNA-Polypeptide Complexes

DNA complexes with poly-L-lysine, poly-L-arginine and protamine were prepared by a salt gradient dialysis. The complexes possess the stoichiometry of one lysine or arginine residue per nucleotide residue as determined from the biphasic melting profiles. The template activity of a complex in support of RNA synthesis in the presence of excess RNA polymerase and required substrates is proportional to its fractional content of free DNA segments. The complexed DNA region is quantitatively blocked and does not act as template. Kinetic analysis of the template behavior reveals two different modes of inhibition by the polypeptides. If the template is in a finely dispersed state, it is available to the enzyme as shown by the fact that the equal template concentrations of complex and of pure DNA are required for half saturation of a given amount of enzyme (K). Inhibition of RNA synthesis is, we propose, due to interference with local untwisting of DNA. If the template is in a highly aggregated state, K is drastically increased and it is unavailable to the enzyme. The several species of histone molecules normally complexed with DNA in the eucaryotic organisms differ among themselves in content of lysine and arginine. The present studies show that the arginine residues are as effective as the lysine residues in abolishing DNA template activity.

ABSTRACT

Part III. Studies on DNA Complexes with Purified Histone Fractions

Well-defined DNA complexes with calf thymus histones Ia, Ib, IIb and IV have been prepared by a salt gradient dialysis in the presence of 5 M urea. The complexes with subequivalent histone/DNA ratio exhibit biphasic melting profiles. $T_{m,1}$ is the melting of free DNA segments, and $T_{m,2}$ that of the histone-complexed regions. $T_{m,2}$ is characteristic for each DNA-basic protein complex. $T_{m,2}$ (°C) of the complexes in 2.5×10^{-4} M sodium EDTA, pH 8.0 is as follows: DNA, 47.2; chromatin, 74.3; DNA-histone Ia, 75.4; DNA-histone Ib, 76.3; DNA-histone IIb, 81.5; DNA-histone IV, 83.7; DNA-protamine, 92.5; DNA-polyarginine, 98.0; and DNA-polylysine, 99.5. The stoichiometric ratio (histone lysine plus arginine to nucleotide) of the equivalent complexes as determined from the biphasic melting profiles is DNA-histone Ia and Ib, 0.8; DNA-histone IIb, 1.2 and DNA-histone IV, 1.5. The general shape of UV spectrum of DNA is not changed by complexing with various histone species. DNA-histone IV complex is inactive in priming RNA synthesis in *E. coli* RNA polymerase system. Possible structures of the DNA-binding parts of histone molecules have been discussed and illustrated with CPK molecular models in the case of histone IV (pp. 157, 158).

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General Introduction

DNA in eucaryotic organisms is organized to form the subcellular structure known as chromatin in the interphase cell nucleus and condensed to the morphologically well-defined metaphase chromosome during cell division. The recognition that both physical and biological properties of DNA is modified by other chromosomal components and the control of gene activity can occur at this level has stimulated intense interest in recent years. For the current status of the knowledge concerning the structure and function of chromosomes of eucaryotic organisms at the molecular level and the experimental approaches in this field, the reader will find the following review articles, essays and reports of a group of workers to be interesting. Recent development in the proteins of the cell nucleus has been reviewed by Hnilica (1967), Murray (1965) and nucleoprotamine in salmon testes by Dixon and Smith (1968). A proceeding of first world conference on biology and chemistry of histones has been edited by Bonner and Ts'o (1964). The role of histone in control of gene activity is discussed by a Ciba Foundation study group (deReuck and Knight, eds., 1966). The structure of chromosomal nucleohistone has been discussed by Bonner and Tuan (1968c) and physical chemistry of nucleoproteins by Peacocke (1960). Current research on the biology of isolated chromatin and the methods commonly used is described by Bonner et al. (1968a,b). The effect of histones on RNA synthesis in isolated nuclei has been discussed by Allfrey and Mirsky (1963), the ultrastructure of chromatin as studied by electron microscopy by Hyde (1965), and control of gene activity by histones and

problems of cellular differentiation by Ursprung and Huang (1967). Two excellent monographs by Bonner (1965) and Busch (1965) give general introduction and challenging prospects in this field.

The original research work presented in this Thesis is organized into three parts. The first part describes the presence of a special RNA species which appears to have the function of conferring specificity in DNA-histone interaction in calf thymus chromatin, the most commonly studied deoxyribonucleoprotein. The second part describes the template properties of DNA complexes with poly-L-lysine, poly-L-arginine and protamine. The third part discusses the thermal denaturation and template properties of DNA complexes with purified histone fractions. The molecular structure of nucleohistone is discussed with the illustration of the possible structure of DNA-binding part of histone IV molecule by the molecular model.

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PART I

CHROMOSOMAL RNA OF CALF THYMUS CHROMATIN*

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CHAPTER I

Introduction

Chromosomal RNA is a special class of RNA which is associated with chromosomal protein and which is of characteristic short chain length, sequence heterogeneity, and size homogeneity. It is complementary to a relatively large proportion of the genome, and possesses a high content of dihydropyrimidine. Such RNA has been found in tissues of pea bud (Huang and Bonner, 1965), pea cotyledon (Bonner and Widholm, 1967), chick embryo (Huang, Smith and Alexander, 1969), and rat liver and rat ascites tumor cells (Dahmus, 1968). It has been shown by studies on dissociation and reconstitution of chromatin that the function of chromosomal RNA is to introduce sequence specificity into chromosomal protein-DNA interaction (Bekhor, Kung and Bonner, 1969). Calf thymus chromatin is the longest, and in many respects the most intensively studied deoxyribonucleoprotein. We have therefore investigated the chromosomal RNA of calf thymus chromatin. It will be shown below that RNA with the properties of chromosomal RNA is associated with chromosomal protein of calf thymus chromatin.

CHAPTER II

Materials and Methods

Preparation of chromatin

Chromatin of calf thymus was prepared by a method modified from the procedures of Bonner et al. (1968). 20-25 g of calf thymus tissue was homogenized with 100 ml of grinding medium in a Waring blender at 50 V for 2 min. Three further portions of 100 ml each of grinding medium were added successively and homogenization continued at 60 V for 1 min, 70 V for 30 sec, and finally 80 V for 30 sec. The grinding medium consisted of 0.25 M sucrose, 3 mM $CaCl_2$, or 1 mM $MgCl_2$, and 0.01 M Tris (pH 8). The homogenate was filtered through two layers, and then through four layers of cheesecloth, and through two layers and then through four layers of Miracloth. The filtrate was then centrifuged at 4000 rev./min for 20 min in a Servall SS-34 rotor to pellet the nuclear fraction. The pellet was washed once with grinding medium and twice with 3 times diluted saline-EDTA [0.075 M NaCl and 0.024 M EDTA, (pH 8)] and then centrifuged at 4000 rev./min for 20 min. The nuclei were then lysed by suspending them in 60 ml of 0.01 M Tris (pH 8.0). In later experiments washing with saline-EDTA was omitted. The lysed nuclei were washed 2 times with 0.01 M Tris (pH 8) and pelleted each time at 12,000 x g for 20 min. The pelleted material will be referred to as crude chromatin. The crude chromatin was then layered on 1.7 M sucrose in 0.01 M Tris (pH 8). The upper one third of the centrifuge tube was stirred to form a rough gradient and then centrifuged at 22,000 rev./min for

2 hr in a No. 25 Spinco rotor. The pellets were dissolved in 0.01 M Tris (pH 8.0), dialyzed against this buffer to remove sucrose and constitute the purified chromatin.

Isolation of nucleic acid associated with chromosomal protein

Isolation of the chromosomal RNA from calf thymus chromatin was conducted essentially according to the method of Bonner and Widholm (1967). Chromatin, crude unless otherwise specified, was dissolved in 4 M CsCl containing 0.01 M Tris (pH 8) and stirred with a magnetic stirrer for 30 min at 0°C. The concentration of nucleoprotein was 20-50 $A_{260\ m\mu}$ per ml. The chromatin was then spun in the No. 40 Spinco rotor at 40,000 rev./min for 24 hr. In the high concentration of CsCl protein and DNA are dissociated. The protein floats to the top to form a well-defined skin or pellicle, while nucleic acids pellet. After centrifugation the pellicles were removed, washed with Tris, 0.01 M (pH 8), and homogenized. The homogenized material was then digested with pronase, 1 mg/ml (preincubated for 30 min at 37°C to destroy contaminating nucleases). The pronase-treated material was then deproteinized with phenol after which the aqueous fraction was made up to 2% in potassium acetate and 2 vol of absolute ethanol added. After storage overnight at -20°C the nucleic acid precipitate was collected by centrifugation. The precipitated nucleic acid collected by centrifugation was dissolved in 0.1 M NaCl and 7 M urea and applied to a DEAE-Sephadex column according to the procedure of Bonner and Widholm (1967). It was then eluted by a linear NaCl gradient, 0-1.5 M, all in 7 M urea. The

peak fractions as followed by $A_{260\ m\mu}$ were pooled, dialyzed to remove urea and lyophilized to dryness. The lyophilized materials were then dissolved in small volumes of 0.01 M Tris buffer (pH 8), and again precipitated by addition of 2 vol of ethanol. For preparation of RNA samples free of urea the material was rechromatographed on DEAE-Sephadex with a linear gradient of NaCl as described above but in the absence of urea.

Analytical methods

Protein was determined by the method of Lowry et al. (1951), RNA by the orcinol method of Dische and Schwartz (1937), DNA by the diphenylamine method of Burton (1956) or by the $A_{260\ m\mu}$ of the DNA hydrolysate (0.5 normal $HClO_4$, 80°C, 10 min, $\epsilon_{260\ m\mu}$ of 1 mg DNA hydrolysate per ml = 30). Nucleotide composition of RNA was determined by the method of Cohn and Volkin (1953) on Dowex 1X8 columns (CMP, $\epsilon_{260\ m\mu} = 6.8 \cdot 10^3$; AMP, $\epsilon_{260\ m\mu} = 14.2 \cdot 10^3$; UMP, $\epsilon_{260\ m\mu} = 10.0 \cdot 10^3$; GMP, $\epsilon_{260\ m\mu} = 11.8 \cdot 10^3$). Dihydrouridylic acid content of chromosomal RNA was determined both by conversion of the dihydrouracil residue to β-alanine by alkaline hydrolysis according to the method of Magrath and Shaw (1967) followed by determination of the β-alanine with the Spinco 120-B amino acid analyzer, and by the spectrophotometric method of Ceriotti and Spandrio (1963).

CHAPTER III

Results

Chemical composition of calf thymus chromatin

Chromatin isolated and purified according to the procedures described under Materials and Methods possesses the chemical composition in Table I. The overall protein to DNA ratio is 1.75. While the histone to DNA ratio is 1.0, the average ratio of RNA to DNA is 0.0093, although this ratio varied between separate preparations by as much as 2-fold. These variations are apparently due to uncontrolled losses of RNA during purification of the chromatin. The protein to DNA ratio of isolated calf thymus chromatin is thus essentially identical to that of rat liver, pea bud and ascites tumor chromatin. The RNA to DNA ratio is, however, lower than that of the chromatins of other tissues, as for example pea bud chromatin, 0.115 (Huang and Bonner, 1965), rat liver, 0.043 (Marushige and Bonner, 1966), and ascites tumor, 0.13 (Dahmus, 1968). The ultraviolet spectrum of purified calf thymus chromatin shown in Figure 1 is typical of nucleohistone from all tissues studied. The $A_{230\ m\mu}/A_{260\ m\mu}$ ratio of 0.705 is in agreement with the protein to DNA ratio of the nucleohistone as determined by analytical chemistry.

When calf thymus chromatin is dissolved in 4 M CsCl and centrifuged, the resulting DNA pellet contains but little protein, less than 2% as much as DNA. The protein pellicle, however, contains both DNA

TABLE I

Chemical Composition of Purified Calf Thymus Chromatin

Component	Mass ratio
DNA	1.00
RNA	0.0093 ± 0.0065*
Protein	1.75

*Five different preparations assayed.

FIGURE 1. Ultraviolet absorption spectrum of calf thymus nucleohistone. The purified chromatin was sheared with Virtis homogenizer at 75 V for 2 min in 0.01 M Tris buffer (pH 8.0) and centrifuged at 12,000 x g for 30 min. The ultraviolet spectrum is that of the supernatant.

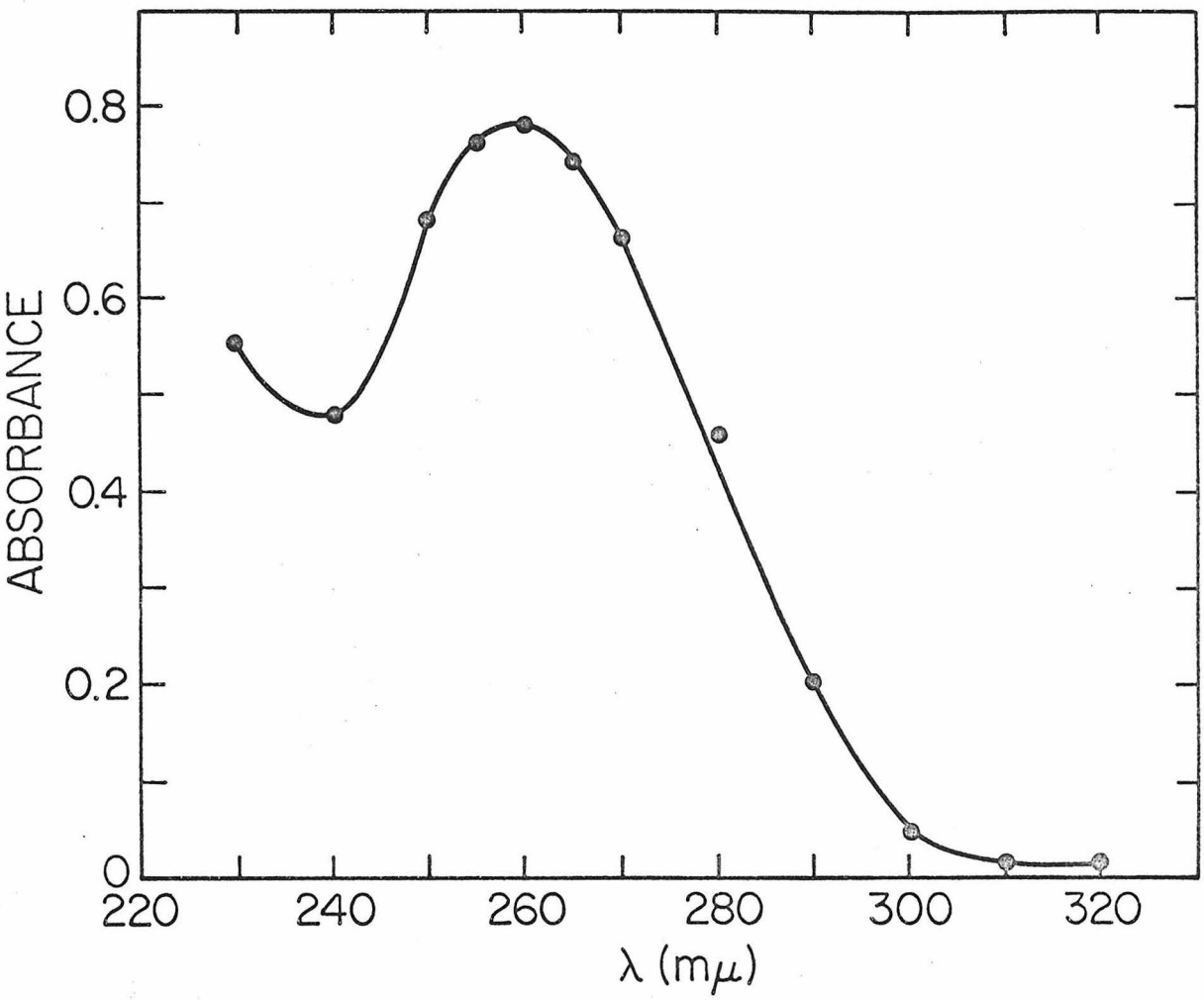

FIGURE 1

and RNA with a DNA to protein ratio of about 0.01 as is the RNA to protein ratio. The nucleic acids released from the pellicle proteins by pronase digestion and deproteinized by phenol extraction were purified by DEAE-Sephadex chromatography for which the elution profile is shown in Figure 2. Two major nucleic acid peaks appear, the first eluting at 0.56 M NaCl (Fraction I) and the second at 0.65 M NaCl (Fraction II). This elution pattern is highly reproducible. Qualitatively, the elution profile of Figure 2 is similar to that found for the protein-associated nucleic acid of pea bud chromatin (Bonner and Widholm, 1967) and for rat ascites tumor chromatin (Dahmus, 1968). The large peak of absorbance which appears at 0.15 M NaCl does not represent nucleic acid, but rather phenol left from the phenol extraction procedure.

Chemical characterization of Fractions I and II

Fraction I exhibits the ultraviolet spectrum of a nucleic acid with an absorption maximum at 260 mμ. It is completely hydrolyzed with 0.3 M KOH at 37°C in 18 hr and yields a positive orcinol reaction. Thus it is a polyribonucleotide. Fraction I also yields a positive ureido test by the method of Ceriotti and Spandrio (1963). It thus contains a dihydropyrimidine, and as shown in the data of Table II, the proportion of this base in Fraction I, 8.5 mole %, is similar to that found in other chromosomal RNA's (see Jacobson and Bonner, 1968, for summary). The dihydropyrimidine of calf thymus chromosomal RNA yields β-alanine on alkaline hydrolysis by the method of Magrath and Shaw (1967).

FIGURE 2. DEAE-Sephadex column chromatography of nucleic acid associated with calf thymus chromosomal proteins. The purified (see Materials and Methods), protein-free preparation, 3 ml of nucleic acid solution ($A_{260\ m\mu} = 26$), was applied to a column of DEAE-Sephadex and eluted with a linear NaCl gradient (0-1.5 M) in 7 M urea and 0.02 M Tris buffer (pH 7.6).

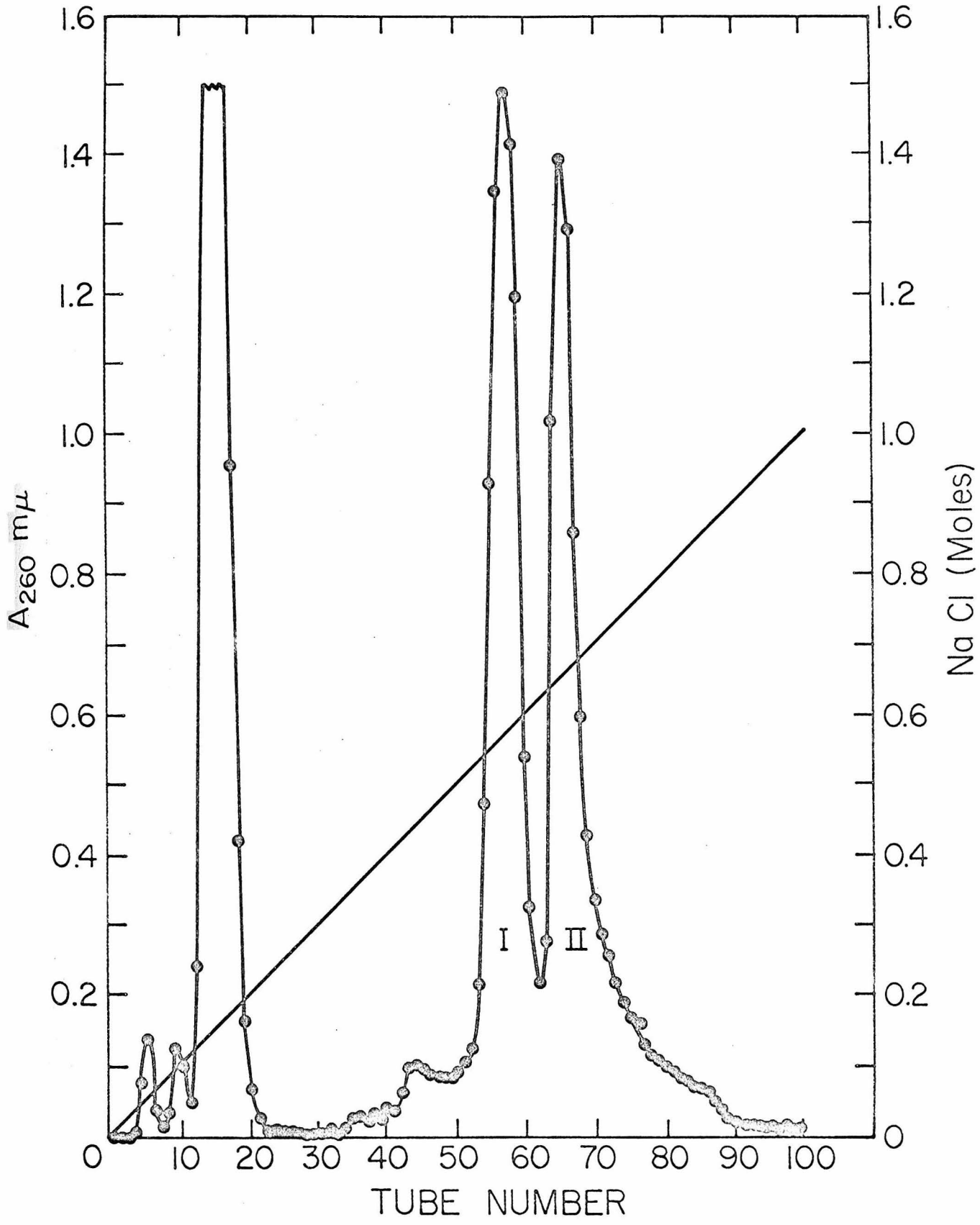
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A_{260} mμ
Na Cl (Moles)
I
II
10
20
30
40
50
60
70
80
90
100
TUBE NUMBER

FIGURE 2

It is therefore dihydrouridylic acid. This is in contrast to the chromosomal RNA of rat ascites tumor which contains dihydroribothymidylic acid (Jacobson and Bonner, 1968).

The alkaline digest products of calf thymus RNA were separated on a Dowex 1X8 column according to the method of Cohn and Volkin (1953). From the ratio of nucleosides to total nucleotides, as obtained from the elution profile, an average chain length of approximately 45 nucleotides is obtained (Table II). This is of the same order as the chain lengths of the chromosomal RNA's of other chromatins, approximately 40 for pea (Huang and Bonner, 1965) and approximately 60 for chick (Huang, Smith and Alexander, 1969) and rat (Dahmus, 1968). The base composition of calf thymus chromosomal RNA is summarized in Table III which includes comparative data on the compositions of other chromosomal RNA's. The composition of calf thymus chromosomal RNA is quite different from that reported for whole calf thymus RNA but is not significantly different from the composition of mammalian transfer RNA. The composition of calf thymus chromosomal RNA is somewhat similar to that of ascites tumor chromosomal RNA but quite different from that of pea bud chromosomal RNA.

Fraction II also exhibits the ultraviolet spectrum of a nucleic acid. It is however resistant to hydrolysis by 0.3 M KOH at 37°C for 18 hr and yields a positive diphenylamine reaction. Fraction II is therefore a polydeoxyribonucleotide. In the case of both pea bud chromatin and rat ascites chromatin small amounts of double-stranded DNA

TABLE II

Chemical Properties of Calf Thymus Chromosomal RNA

(1)	Ratio of nucleosides to nucleotides in alkaline hydrolysate	0.024
(2)	Chain length calculated from (1)	43
(3)	Ureido content of alkaline hydrolysate, μM/mg chromosomal RNA	0.266
(4)	Mole % dihydropyrimidine calculated from (3)	8.5

TABLE III

Nucleotide Composition of the Calf Thymus Chromosomal RNA in Comparison With That of Other RNA's

	Cytidylic acid (mole %)	Adenylic acid (mole %)	Uridylic acid (mole %)	Guanylic acid (mole %)	Dihydro-pyrimidine (mole %)
Chromosomal RNA of calf thymus	27.9	11.1	17.5	35.0	8.5
Calf thymus RNA (whole)[a]	25.7	18.4	12.0	43.8	-
tRNA of rat liver[b]	32.2	17.5	18.1	32.2	-
Chromosomal RNA of pea bud[c,d]	13.1	39.8	19.2	19.3	8.5
Chromosomal RNA of rat ascites tumor[e]	25.2	16.8	19.0	30.6	8.1

[a]Volkin and Carter (1951).

[b]Herbert and Canellakis (1960).

[c]Huang and Bonner (1965).

[d]Jacobson and Bonner (1968).

[e]Dahmus and McConnell (1969).

also accompany chromosomal RNA and are separated from it by DEAE-Sephadex chromatography just as is shown in Figure 2 for calf thymus chromatin.

CHAPTER IV

Discussion

The present study establishes the fact that calf thymus chromatin contains an RNA which in physical and chemical properties resembles the chromosomal RNA of other chromatins. Thus, the RNA of calf thymus chromatin accompanies protein upon centrifugation in an appropriate concentration of CsCl. This is not true of nascent messenger RNA which is known to accompany DNA to the pellet under these circumstances (Bonner and Widholm, 1967). The RNA of calf thymus chromatin exhibits chromatographic properties similar to those of the chromosomal RNA's of other organisms. The calf thymus chromatin material does however differ from other chromosomal RNA's so far studied in one principal respect. This is its small amount in chromatin, approximately 1% as much as DNA rather than approximately 5% as in other cases studied. Whether the 1% found in the present instance is real or whether the chromosomal RNA of calf thymus chromatin is subject to loss from chromatin during preparation has not been established.

Rigorous identification of chromosomal RNA of calf thymus chromatin as described in the present paper as functionally identical to the chromosomal RNA's of other organisms studied would require demonstration that the calf thymus chromosomal RNA is sequence-heterogeneous and that it hybridizes with a substantial proportion of the genome, 3-5% in the case of pea bud, chick embryo and rat ascites tumor. Such demonstration has not yet been attempted.

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PART II

THE TEMPLATE PROPERTIES OF DNA-POLYPEPTIDE COMPLEXES*

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*Manuscript to be submitted for publication.

CHAPTER I

Introduction

The several species of histone molecules found complexed with the nuclear DNA of all higher organisms differ among themselves in content of lysine and arginine. Histones of group I contain lysine as their principal basic amino acid (lysine/arginine 8.5 to 15.4) while histones of groups III and IV contain arginine as their principal amino acid (lysine/arginine 0.3 to 0.7). As one step in the direction of finding out why histones differ in the above respect, we have investigated complexes of DNA with polylysine or polyarginine. The physical properties of such complexes have already been studied by Olins, Olins and von Hippel (1967, 1968), by Tsuboi, Matsuo and Ts'o (1966), and by Leng and Felsenfeld (1966) among others. We direct our primary attention toward the properties of the complexes as templates for RNA polymerase. We include for comparison the properties of DNA complexes with protamine.

CHAPTER II

Materials and Methods

Materials

Calf thymus DNA, type 1 Lot 86B-1250 obtained from Sigma Chemical Co. (St. Louis, Missouri) was used for DNA-polylysine, -polyarginine complex studies. Another lot of calf thymus DNA (highly polymerized), Lot 1381 from Nutritional Biochemicals Corp. (Cleveland, Ohio) was used for DNA-protamine complex studies. The sedimentation coefficient ($s_{20,w}$) of these two DNA samples are, respectively, 25.22 and 26.44 corresponding to molecular weights of 1.26×10^7 and 1.44×10^7 as determined by band sedimentation (Vinograd, Bruner, Kent and Weigle, 1963) in 0.1 M NaCl, 0.01 M Tris, pH 8.0 and 90% D_2O. The RNA contents of the two DNA samples are 0.13% and 0.15% as determined by the orcinol method. Protein content is 0.4% for the DNA from Nutritional Biochemicals and is higher (2%) for DNA from Sigma as determined by the Lowry method.

Poly-L-lysine·HBr, M.W. 38,000 Lot L-39 spec. and poly-L-arginine·HCl, M.W. $\sim$ 40,000, Lot AG-2 were obtained through Pilot Chemicals, Inc., Watertown, Massachusetts. Protamine chloride (salmine) type V Lot 58B-4330 was purchased from Sigma. It is completely soluble in water and was used without further purification. ATP-8-C^{14} disodium salt (Lot 6704 and 6801) and UTP, GTP and CTP were all products of Schwarz BioResearch, Inc., Van Nuys, California.

Preparation of DNA-polypeptide complexes

DNA-polypeptide complexes were prepared by the salt gradient dialysis procedure according to Huang, Bonner and Murray (1964). DNA and polypeptide were mixed at various input ratios in 2 M NaCl, also 1.0×10^{-3} M in sodium citrate, pH 7.0. The final DNA concentration was 5.0×10^{-4} M or 2.5×10^{-4} M in phosphate concentration. The mixture was then salt gradient dialyzed successively against 30 to 50 vol of 0.4 M, 0.3 M, 0.15 M and 0.015 M NaCl in 1.0×10^{-3} M citrate buffer pH 7.0, each for 4 hr, and finally dialyzed overnight against a further change of 0.015 M NaCl in the same buffer. For melting experiments, NaCl was removed by further dialysis against 1.0×10^{-3} M citrate buffer alone for 2 to 3 changes. Any coarse precipitates of the resulting complexes were removed by low speed centrifugation (clinical centrifuge at 1500 to 2000 rev./min for 15 min). For template activity studies, the solution was further sheared by a Virtis homogenizer at 40 V for 90 sec. The recoveries of DNA in the supernatant fraction are presented in Table I. The recovery for DNA-polylysine complex is almost complete up to an input ratio (lysine/nucleotide) of 0.6. DNA-polyarginine complexes prepared by salt gradient dialysis alone exhibit very poor recovery even at an input ratio (arginine/nucleotide) of 0.6. Interactions between the complex and/or polyarginine itself can be diminished by carrying out the salt gradient dialysis in the presence of 5 M urea, followed by subsequent removal of urea by dialysis against several changes of citrate buffer. This results in marked improvement

of the recovery since, as shown in Table I, a 92% recovery was obtained at an input ratio of 0.6. The recovery of the DNA-protamine complex is satisfactory up to an input ratio of 0.6. DNA-polypeptide complexes prepared at input ratios higher than 0.6 exhibit a drastically decreased recovery, and all experiments were therefore carried out at input ratios of this value or lower.

Dialysis tubing size 8 was first boiled for 10 min in 1 M NaCl, rinsed, and boiled for 10 min in 0.02 M disodium EDTA, rinsed again, and finally boiled for 10 min in distilled water. The treated tubing was stored in 50% glycerol until use. Dialysis was carried out in glass-stoppered, graduated cylinders mounted on a slowly rotating carrier. A small air bubble in the dialysis tubes provided constant stirring. All operations were carried out at 0°C ~ 4°C.

Chemical analysis

Concentration of stock solutions of polypeptides was determined by the ninhydrin method of their 6 N HCl hydrolysates (Spies, 1957). Crystalline L-lysine·HCl (Sigma, Lot 116B-1730) and L(+)-arginine·HCl (Sigma, Ⓢ grade Lot 113B-0370) stored one week or more over anhydrous $CaSO_4$ were used as standards. For protamine, arginine was used as standard. A color yield relative to pure arginine of 0.918 was calculated on the basis of the amino acid composition of the protamine.

The concentration of nucleotide residues in DNA stock solutions was determined by inorganic phosphate analysis according to the procedure of Ames and Dubin (1960). Analytical reagent grade KH_2PO_4

TABLE I

Recovery of DNA from Salt Gradient Dialysis

Input ratio (+/-)	DNA-poly-L-lysine	DNA-poly-L-arginine		DNA-protamine
		Salt alone	Salt + 5 M urea	
0	100%	100%	100%	100%
0.40	98	92	97	96
0.60	99	65	92	87

DNA-polypeptide complex was prepared by salt gradient dialysis followed by low speed centrifugation to remove the coarse precipitate. DNA-polyarginine was also prepared in the presence of urea.

(Mallinckrodt, New York) stored one week over anhydrous $CaSO_4$ served as standard. Other DNA concentrations were determined by the diphenylamine method (Burton, 1956).

Assay of RNA polymerase activity

The incubation mixture contained 10 μmoles of Tris buffer, pH 8.0, 1 μmole of magnesium acetate, 0.25 μmole of $MnCl_2$, 3 μmoles of β-mercaptoethanol, 0.1 μmole each of ATP, GTP, CTP and UTP. ATP was labeled with C^{14} to a specific activity of 1 μc/μmole. Total volume of reaction mixture was 0.25 ml. Varying amounts of DNA or of DNA-polypeptide complexes were added to the reaction mixtures as template for RNA synthesis. Reaction was initiated by the addition of suitable amounts of RNA polymerase. Incubation was carried out at 37°C for 10 min. Over this time interval, the time course of RNA synthesis is linear under the present conditions. Reaction was terminated by the addition of 3 ml of 10% trichloroacetic acid. The samples were allowed to sit for at least 30 min in ice and the acid-insoluble material then collected by filtration through membrane filters (Bac-T-Flex, type B-6, 25 mm size from Carl Schleicher & Schuell Co., Keene, New Hampshire). The filters were washed further with 4 to 5 portions of 3 ml 5% trichloroacetic acid. Filters, after drying, were counted with a Beckman LS-200B liquid scintillation system. The counting cocktail contained 5 gm of PPO and 100 gm of naphthalene made to one liter with dioxane. Counting efficiency is 85%.

Preparation of E. coli RNA polymerase

DNA-dependent RNA polymerase was prepared by a modification of the procedure of Chamberlin and Berg (Bonner et al., 1968a). The preparation used corresponds to the f_4 fraction of Chamberlin and Berg (1962). Its specific activity ranged from 246 to 357 μμmoles incorporation of C^{14}-ATP per μg enzyme per 10 min with a saturating amount of DNA and under our standard reaction conditions. The purified enzyme had nearly complete DNA dependency. Enzyme concentration was calculated by the relation $\epsilon_{280}^{1\%} = 6.5$ (Richardson, 1966).

Statistical analysis of enzyme kinetic data

The template saturation curves, rate of RNA synthesis with increasing amounts of DNA in the presence of a constant amount of RNA polymerase, were fitted by the least-squares method with a weighting factor of v^4 for reaction velocity. This takes into account the fact that the principal inaccuracies are at low velocities. Data for both DNA and DNA-polypeptide complexes at low concentration were found to fit satisfactorily to the Michaelis-Menten equation. Fitting of the kinetic data to this equation was performed by a CITRAN program translated from the FORTRAN program of Cleland (1967) and on an IBM System/360 Model 50 time-sharing computer. V_{max}, K, and V_{max}/K with their standard errors were obtained.

Melting profile of DNA-polypeptide complexes

Thermal denaturation profiles were performed with a Gilford multiple sample recording spectrophotometer Model 2000 equipped with a linear temperature programmer. Samples in stoppered cuvettes were heated at a rate of 0.5 ~ 1°C/min after 15 min of degassing in a vacuum desiccator. Melting profiles were normalized by a CITRAN program and on the same computer system as in the previous section. The melting temperature of each particular transition was determined by making linear extrapolations of the plateau regions through linear approximations of each transition. T_m is the temperature for half-melting between these two intersections. Because of the turbidity of solutions of DNA-polypeptide complexes, the absorption at 260 mμ was corrected with turbidity by chemical determination of DNA concentration.

CHAPTER III

Results

Thermal denaturation studies and estimation of free DNA segments of DNA-basic polypeptide complexes

The melting profiles of DNA-poly-L-lysine complexes are presented in Figure 1. The melting temperature of the complex is high, and melting is not complete at 100°C in 5.0×10^{-4} M citrate buffer at pH 7.0. In order to see the entire melting profile, the buffer was made up with 50% methanol (v/v) (Olins, Olins and von Hippel, 1967). This lowers the melting temperature to convenient level. The melting profiles are sharply two-step for all lysine/nucleotide ratios suggesting that the binding is highly cooperative. Thus polylysine would appear to bind to DNA adjacent to previously bound polylysine rather than randomly along the DNA strand. The T_m of the higher melting step is identical for lysine/DNA input ratios of 0.40 and 0.60 as shown in Table II. Hence a definite molecular complex of polylysine and DNA is formed under the conditions of salt gradient dialysis. The T_m of the lower melting transition is also relatively constant and is approximately identical to that of pure DNA. This suggests that all of the polylysine is bound to DNA without significantly altering the ionic strength of the solution. The turbidity of the preparation as monitored at 350 mμ does not change during the first step but does during the second step. The hyperchromicity of the first step is due to melting of uncomplexed DNA. The fraction of such free DNA in the preparation

FIGURE 1. Melting profile of DNA-poly-L-lysine complex. DNA-polylysine with input ratio of lysine/nucleotide of 0 (O—O), 0.40 (Δ—Δ), and 0.60 (□—□) was melted in 5.0×10^{-4} M sodium citrate, pH 7.0 and 50% methanol (v/v). Thermal expansion of the solvent during the whole course of experiment is corrected.

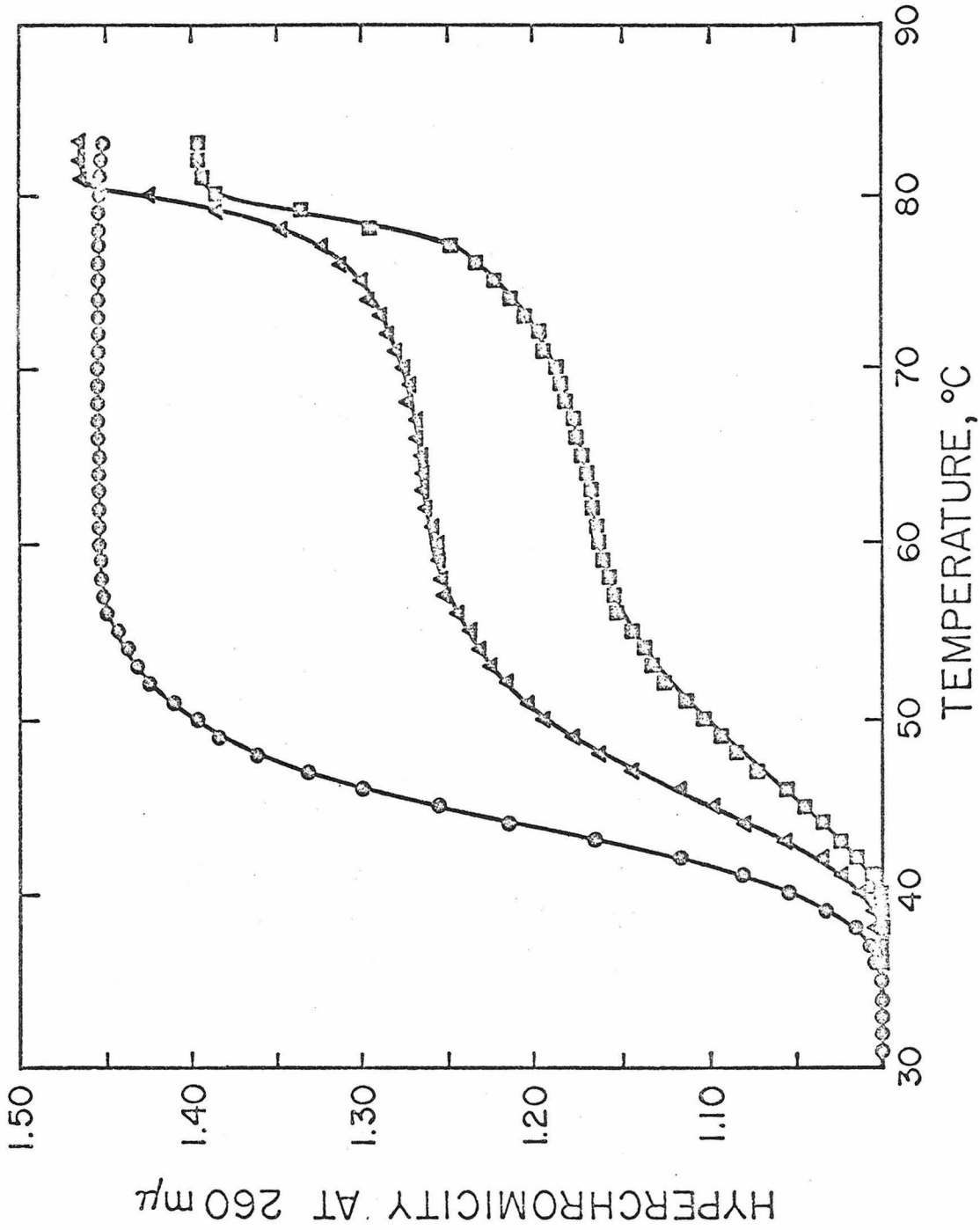
HYPERCHROMICITY AT 260 mμ
1.50
1.40
1.30
1.20
1.10
30
40
50
60
70
80
90
TEMPERATURE, °C

FIGURE 1

TABLE II

Melting Temperature of DNA-Poly-L-Lysine and Decrease of the Hyperchromicity of Free DNA Melting

Input ratio of $\frac{\text{lysine}}{\text{nucleotide}}$	$T_{m,1}$ °C	$T_{m,2}$ °C	% Hyperchromicity at $T_{m,1}$ relative to DNA
0	44.0	-	100
0.40	47.3	80.8	56
0.60	49.5	80.9	36

a. Melting buffer is 5.0×10^{-4} M sodium citrate, pH 7.0 and 50% methanol (v/v).

b. The last column is the per cent of hyperchromicity relative to pure DNA melting up to the midpoint between these two transitions.

c. Average values of three independent determinations are presented.

is therefore estimated from the hyperchromicity over the first transition. Such data are presented in Table II. At an input lysine/nucleotide ratio of 0.40, 56% of the complex melts like free DNA; at an input ratio of 0.60, 36%. The fraction of free DNA in the preparation therefore decreases in proportion to increase of lysine/nucleotide ratio. The complex therefore possesses a stoichiometry of one lysine residue per nucleotide residue. These results agree generally with those of Tsuboi, Matsuo and Ts'o (1966) and of Olins, Olins and von Hippel (1967).

Preparation of DNA-poly-L-arginine complexes by gradient dialysis in salt alone results in poor recovery especially at higher input ratios. Aggregation of the complex and/or polyarginine occurs during the dialysis process. Dialysis in the presence of 5 M urea was therefore introduced to decrease such interaction. The use of urea results not only in improved recovery but also in complexes of more sharply two-step melting behavior as shown in Figure 2. Binding of polyarginine to DNA is therefore more cooperative if carried out in the presence of urea. Table III shows that the T_ms of the first and second steps are similar for all arginine/nucleotide ratios. The fraction of total hyperchromicity contributed by the first step is also proportional to the fraction of free DNA expected on the basis of input arginine/DNA ratio. Polyarginine therefore also forms a well-defined molecular complex with DNA and also exhibits a stoichiometry of one arginine residue per nucleotide residue. The data of Table III also show the complex prepared in the presence of 5 M urea exhibits a

FIGURE 2. Melting profile of DNA-poly-L-arginine. DNA-poly-arginine was prepared by salt gradient dialysis in the presence of 5 M urea with the input ratio of arginine/nucleotide of 0 (O—O), 0.40 (Δ—Δ), and 0.60 (□—□). Melting buffer is 5.0×10^{-4} M socium citrate, pH 7.0.

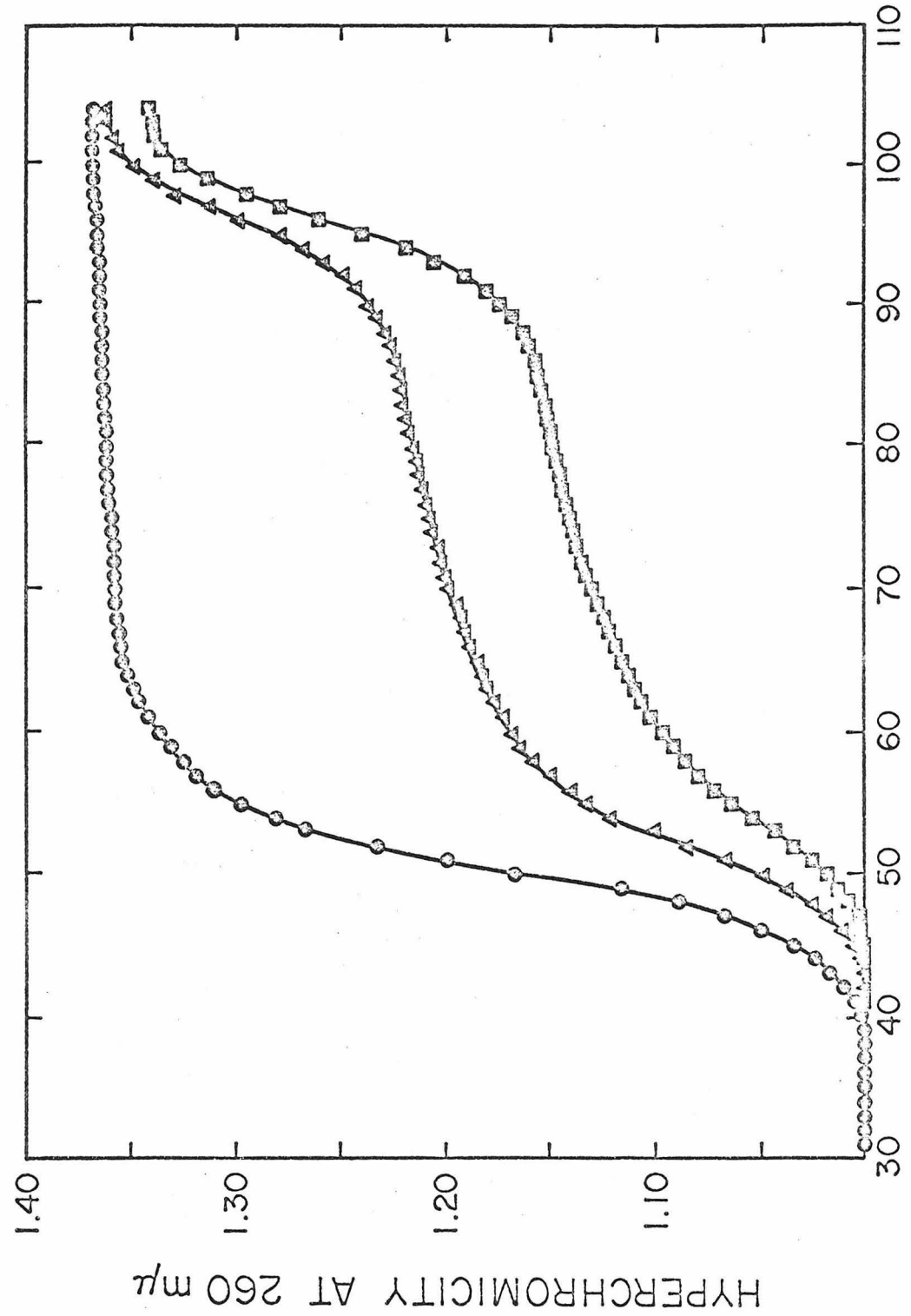
HYPERCHROMICITY AT 260 mμ
1.40
1.30
1.20
1.10
30
40
50
60
70
80
90
100
110
TEMPERATURE, °C

FIGURE 2

TABLE III

Melting Temperature of DNA-Poly-L-Arginine and Decrease of the Hyperchromicity of Free DNA Melting

Input ratio of $\frac{\text{arginine}}{\text{nucleotide}}$	Salt alone		Salt plus 5 M urea		% Hyperchromicity at $T_{m,1}$ relative to DNA
	$T_{m,1}$ °C	$T_{m,2}$ °C	$T_{m,1}$ °C	$T_{m,2}$ °C	
0	50.6	-	50.3	-	100
0.40	53.3	93.2	51.5	96.4	56
0.60	53.8	92.8	52.1	95.3	38

a. Melting buffer is 5.0×10^{-4} sodium citrate, pH 7.0.

b. Melting temperatures of complex prepared by plain salt gradient dialysis and also by salt gradient dialysis in the presence of 5 M urea are presented.

c. The last column is the per cent of hyperchromicity relative to pure DNA melting up to the midpoint between these two transitions.

d. Average values of two (salt alone column) and three (5 M urea column) independent determinations are presented.

melting temperature 3 degrees higher than that of the complex prepared in the absence of urea. At the neutral pH used, the cationic polypeptide is present in random-coil form, presumably due to charge repulsion (Applequist and Doty, 1962). It is unlikely therefore that the effect of urea is upon the secondary structure of polyarginine. It more probably decreases the weak interactions between polyarginine molecules themselves. In addition it may be noted however that DNA in 5 M urea is partially denatured as shown by its decreased T_m and hyperchromicity (Bekhor, Bonner and Dahmus, 1969). This may also have some effect on complex formation.

Figure 3 shows the melting profiles of DNA-protamine complexes. Arginine constitutes 69% of the amino acid residues of protamine and these are grouped in clusters of one to four residues (Ando, 1966). DNA-protamine complexes have a melting temperature of 92.6°C (Table IV) as does DNA-polyarginine prepared by gradient dialysis in salt alone. A similar result has been obtained by Olins, Olins and von Hippel (1968). The melting profiles of the DNA-protamine complexs are not as clearly two-step as are those of the homopolypeptide-DNA complex. This is due to the considerable melting between 55° and 85°C as can be readily seen in derivative curves. It is therefore not possible to estimate the fraction of free DNA from the melting profile in this case. Several explanations can be thought of in connection with this type of melting behavior: (1) The present annealing procedure may not allow all of the protamine molecules to form their most stable complexes with DNA. Complexes of intermediate stability may persist

FIGURE 3. Melting profile of DNA-protamine complex. DNA-protamine prepared by salt gradient dialysis with input ratio of protamine arginine to DNA phosphate of 0 (O—O), 0.40 (Δ—Δ), and 0.60 (□—□) was melted in 5.0×10^{-4} M sodium citrate, pH 7.0. Upper, the melting profile. Lower, the derivative curves of the hyperchromicity with respect to temperature. Arginine/nucleotide ratio is 0 (No. 1), 0.40 (No. 2), and 0.60 (No. 3).

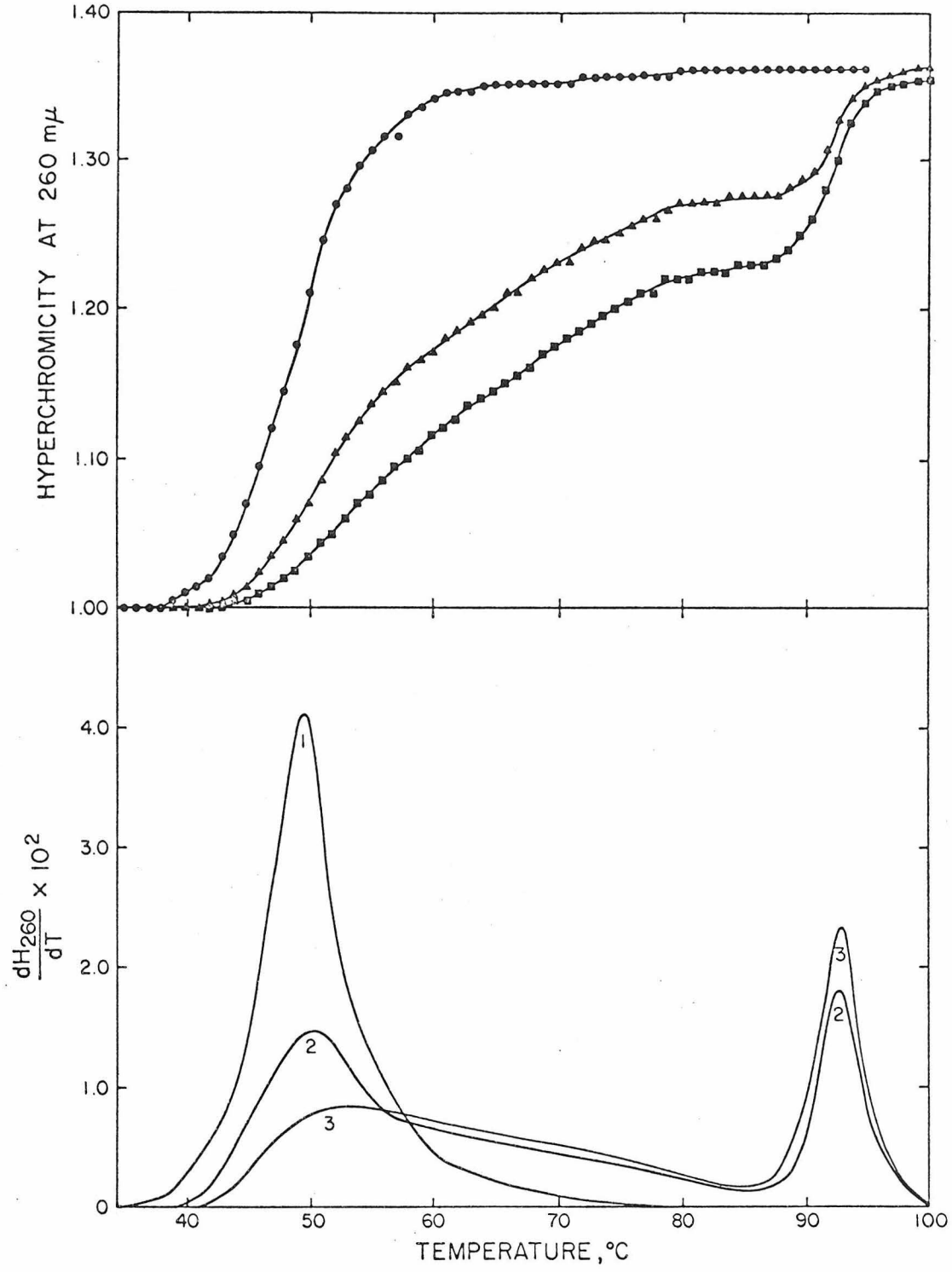
HYPERCHROMICITY AT 260 mμ
1.40
1.30
1.20
1.10
1.00
$\frac{dH_{260}}{dT} \times 10^2$
4.0
3.0
2.0
1.0
0
1
2
3
3
2
40
50
60
70
80
90
100
TEMPERATURE, °C

FIGURE 3

TABLE IV

Melting Temperature of DNA-Protamine Complex

Input ratio of $\frac{\text{arginine}}{\text{nucleotide}}$	$T_{m,1}$ °C	$T_{m,2}$ °C
0	49.7	-
0.40	55.7	92.6
0.60	60.1	92.6

a. Melting buffer is 5.0×10^{-4} M sodium citrate, pH 7.0.

b. Average values of three independent determinations are presented.

because of the difficulty of achieving the equilibrium state. DNA-polyarginine annealed by salt gradient dialysis in the absence of urea has a slightly lower melting temperature and a greater amount of material of intermediate melting temperature as compared to the complex prepared in the presence of 5 M urea. We have attempted to carry out DNA-protamine annealing in 5 M urea, but without success. The T_m of the first-step melting remains higher than that of DNA and the second-step melting usually disappears; (2) There may be two types of protamine interactions--"irreversible" and "dynamic"--as suggested by Olins, Olins and von Hippel (1968). According to this view irreversible complex formation contributes melting at the temperature characteristic of the complex while dynamic complexing results only in general stabilization of the free DNA fraction; (3) The binding may not be so cooperative as with polyarginine. In this case the lengths of the free DNA stretches would be highly variable and thus contribute to a diffuse melting profile. No decision as to which is correct can be made at present state.

In the course of the present studies, it has been found that purity of DNA importantly influences DNA-protamine complex formation. Some commercial DNA samples do not form well-defined complexes with protamine unless further purified by phenol deproteinization. For such DNA complexes the melting profile is monophasic and T_m is merely increased by increasing amounts of added protamine. Thus T_ms of 50.4, 52.8, 59.4, and 67.8 in 5.0×10^{-4} M citrate, pH 7.0 have been observed for protamine arginine/nucleotide input ratios of 0, 0.2, 0.4, and 0.6.

The resulting solutions are perfectly clear in contrast to the turbidity observed with complexes which exhibit two-step melting. Such stabilization is similar to that contributed by cations such as Mg^{+2} or Co^{+2} (Dove and Davidson, 1962). What the factor is that interferes with the irreversible cooperative binding of protamine to pure DNA is not clear although DNA that fails to form such complexes always possesses a high protein content.

Definition of template activity in support of RNA synthesis

Template activity has been studied quantitatively by measuring rate of RNA synthesis supported by DNA-polypeptide complexes and catalyzed by highly purified E. coli RNA polymerase. Template activity is defined as follows:

$$\text{Template activity} \equiv \lim_{A \to 0} \left(\frac{dv}{dA}\right) \qquad (1)$$

where v is the initial rate of RNA synthesis and A is the template concentration. This expression defines the inherent capability of the template to prime RNA synthesis in the presence of infinite excess enzyme and required substrates as A approaches zero, the limiting concentration. Such template activity refers to a set of selected reaction conditions, e.g. temperature, pH, ionic strength, etc. RNA polymerase under the present low ionic strength incubation condition has a very high affinity for and binds irreversibly to DNA (Berg, Kornberg, Fancher and Diechmann, 1965). It is logistically difficult to have a saturating amount of enzyme and a sufficient amount of template so that

the reaction velocity can be reliably measured. Therefore template saturation curves with a constant amount of enzyme per reaction mixture are performed. Such saturation curves are hyperbolic (Marushige and Bonner, 1966). Computer fitting of the data to the Michaelis-Menten equation,

$$v = \frac{V_{max} \cdot A}{K + A} \qquad (2)$$

in which V_{max} is the velocity of RNA synthesis in the presence of infinite template, and K, the template concentration required to half saturate the enzyme, yields quite satisfactory fitting as may be seen in Figures 4, 5, and 6. The standard error of the resulting rate constants are generally less than 10% of their values. Differentiation of equation (2) with respect to A and taking the limit, $A \rightarrow 0$ yields

$$\lim_{A \rightarrow 0}\left(\frac{dv}{dA}\right) = \frac{V_{max}}{K} = \text{Template activity} \qquad (3)$$

In case K remains unchanged, template activity is proportional to V_{max}. Since binding of RNA polymerase to DNA is not reversible, K is not interpretable as an ordinary Michaelis constant and is in fact not a constant. It is proportional to enzyme concentration (Wood and Berg, 1964; Marushige and Bonner, 1966). In the presence of finite amount of enzyme, it is a titration of the enzyme binding sites of the template and is related to the physical state of the template as will be shown below.

The template activity of DNA-polypeptide complexes

Figure 4 shows template saturation curves for DNA-poly-L-lysine complexes and Table V lists the derived rate constants and their standard errors. K remains constant for all lysine/nucleotide ratios within experimental uncertainty. Enzyme therefore binds equally well to complex and to free DNA. Template activity of the complex is less than that of pure DNA. The decrease is linearly correlated with lysine/nucleotide ratio. The residual fractional template activity is in fact exactly equal to the residual fractional free DNA of the complex as estimated from its melting profile (see first section of this chapter). It can therefore be concluded that only the free DNA portion of the complex supports RNA synthesis, and that the complexed regions are inactive. Since K does not change with lysine/nucleotide, V_{max} decreases proportionally to coverage of DNA by polylysine.

Figure 5 shows template saturation curves for DNA-poly-L-arginine complex, and the derived rate constants are presented in Table VI. Again K does not change with increasing coverage of DNA and template activity decreases with increase in arginine/nucleotide ratio. The conclusion can again be drawn that the complexed DNA regions are inactive and only the free DNA portion serves as template for RNA synthesis. The arginine residue is as effective as the lysine residue in abolishing the ability of the complexed DNA to act as template in support of RNA synthesis.

Template saturation curves for DNA-protamine complexes are presented in Figure 6 and the derived rate constants in Table VII. Because

FIGURE 4. Template saturation curves of DNA-poly-L-lysine. DNA-polylysine prepared by salt gradient dialysis was incubated for RNA synthesis as described in the text. 18 μg of *E. coli* polymerase was added to each incubation mixture. Lysine/nucleotide ratio is 0 (O—O), 0.40 (Δ—Δ), and 0.60 (□—□). Each point represents average of a duplicate experiment. Computer fitted curves are shown.

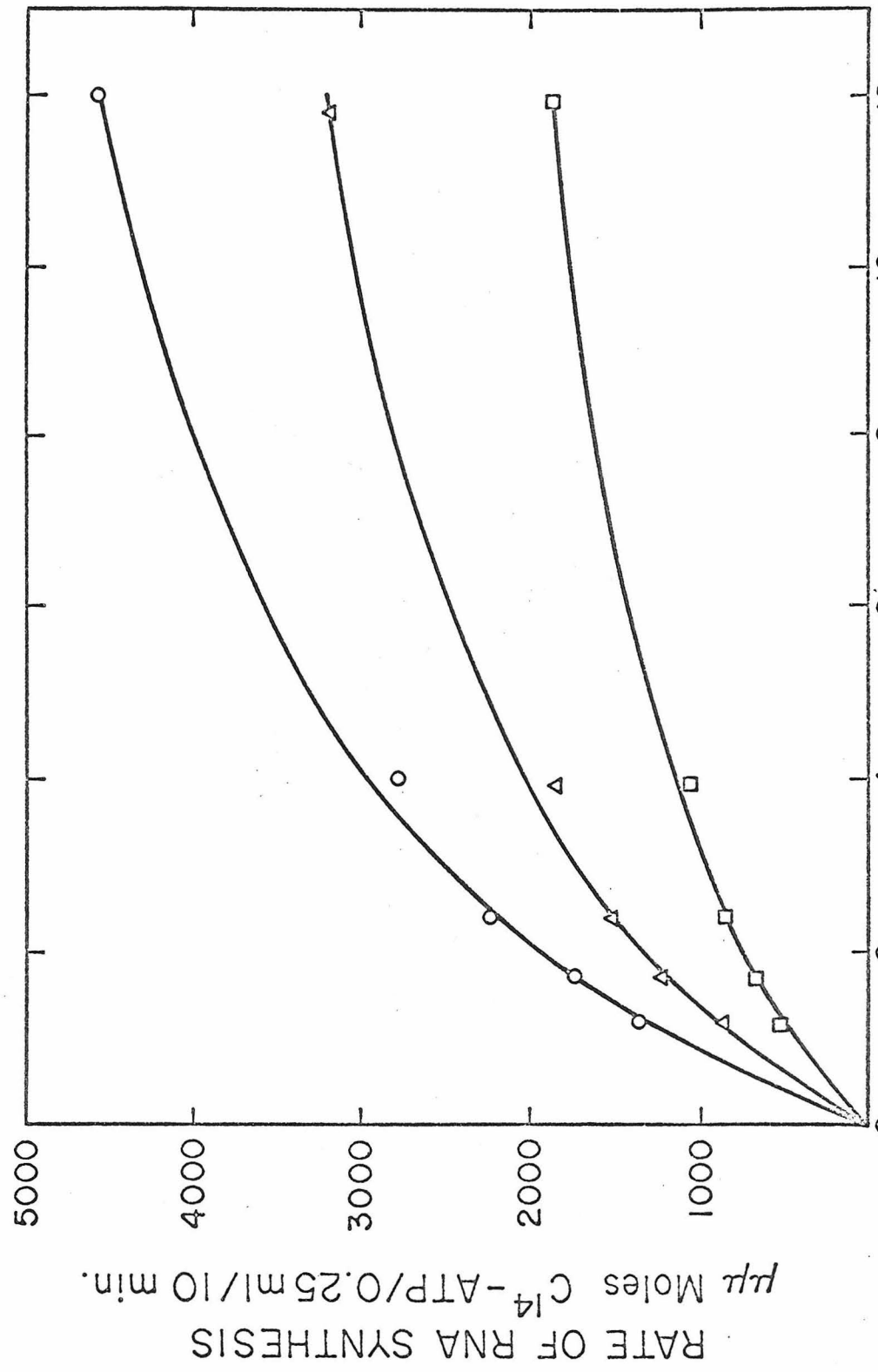
RATE OF RNA SYNTHESIS
μμ Moles C14-ATP/0.25ml/10 min.
5000
4000
3000
2000
1000
0
2
4
6
8
10
12
DNA CONCENTRATION, μg/0.25 ml

FIGURE 4

TABLE V

Template Activity of DNA-Poly-L-Lysine Complex

Ratio of $\frac{\text{lysine}}{\text{nucleotide}}$	V_{max} (μμmoles ATP/ 10 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 10 min/μg DNA)	%
0	6,281 ± 267	4.55 ± 0.41	1,380 ± 74	100
0.40	4,496 ± 281	4.86 ± 0.63	926 ± 69	67
0.60	2,710 ± 154	5.44 ± 0.62	498 ± 32	36

*Incubation condition is described in the text.

FIGURE 5. Template saturation curves of DNA-poly-L-arginine. DNA-polyarginine prepared by salt gradient dialysis in the presence of 5 M urea was incubated for RNA synthesis as described in the text. 37 μg of *E. coli* polymerase was added to each incubation mixture. Arginine/nucleotide ratio is 0 (O—O), 0.40 (Δ—Δ), and 0.60 (□—□) respectively. Each point represents average of a duplicate experiment. Computer fitted curves are shown.

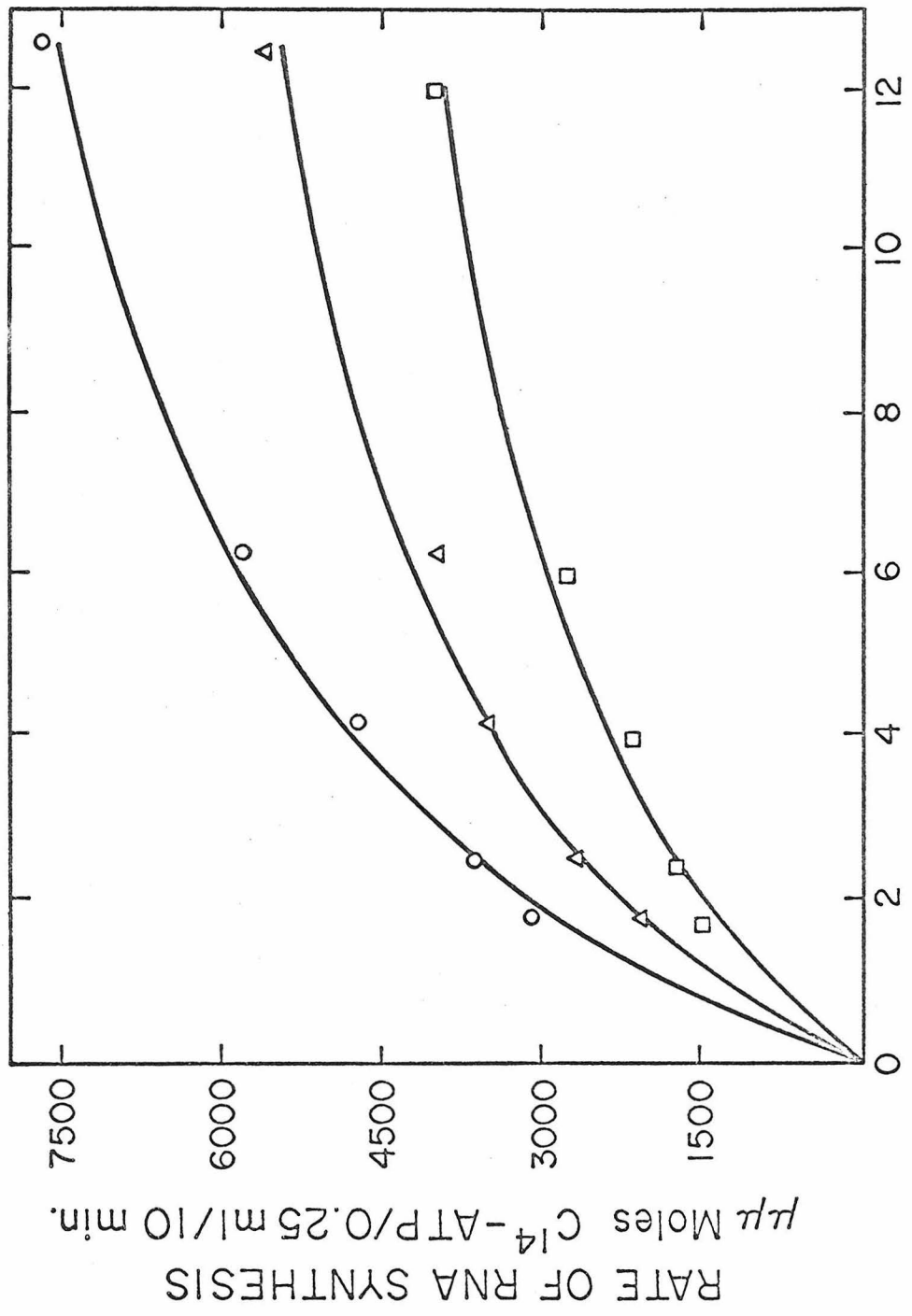
RATE OF RNA SYNTHESIS
μμ Moles C14-ATP/0.25 ml/10 min.
7500
6000
4500
3000
1500
0
2
4
6
8
10
12
DNA CONCENTRATION, μg/0.25 ml

FIGURE 5

TABLE VI

Template Activity of DNA-Poly-L-Arginine Complex

Ratio of $\frac{\text{arginine}}{\text{nucleotide}}$	V_{max} (μμmoles ATP/ 10 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 10 min/μg DNA)	%
0	10,330 ± 548	4.63 ± 0.55	2,231 ± 161	100
0.40	7,490 ± 508	4.66 ± 0.71	1,609 ± 147	72
0.60	5,870 ± 670	5.84 ± 1.36	1,004 ± 129	45

a. DNA-polyarginine was prepared by salt gradient dialysis in the presence of 5 M urea.

b. Experimental condition is described in the text.

FIGURE 6. Template saturation curves of DNA-protamine. DNA-protamine prepared by salt gradient dialysis was incubated for RNA synthesis as described in the text. 30 μg of *E. coli* RNA polymerase was added to each incubation mixture. Protamine arginine to nucleotide ratio is 0 (O—O), 0.37 (Δ—Δ), and 0.55 (□—□). Each point represents average of a duplicate experiment. Computer fitted curves are shown.

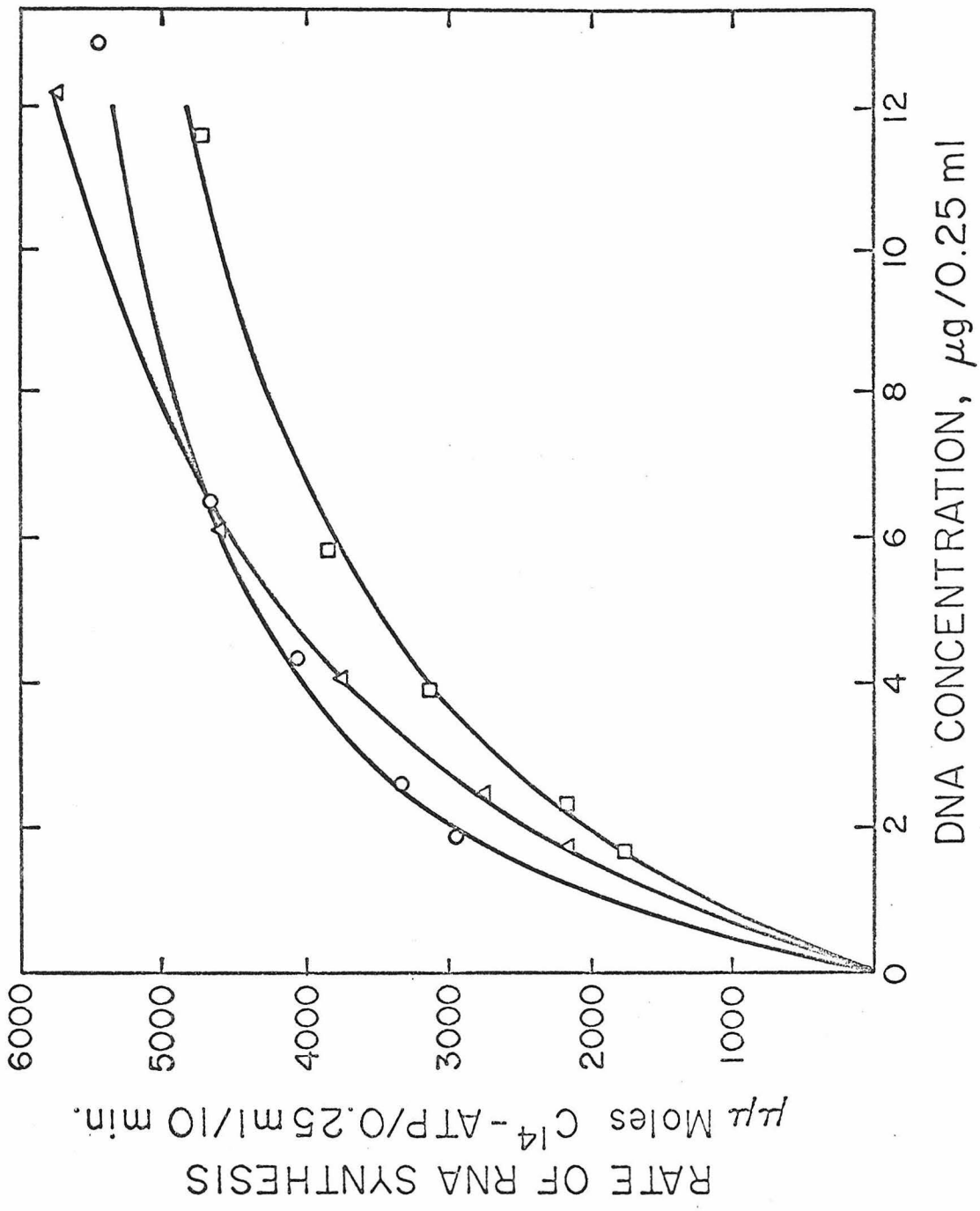
RATE OF RNA SYNTHESIS
μμ Moles C14-ATP/0.25ml/10 min.
6000
5000
4000
3000
2000
1000
0
2
4
6
8
10
12
DNA CONCENTRATION, μg/0.25 ml

FIGURE 6

TABLE VII

Template Activity of DNA-Protamine Complex

Arginine/nucleotide		V_{max} (μμmoles ATP/ 10 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 10 min/μg DNA)	%
Input	Recovered				
0	0	6,334 ± 156	2.27 ± 0.17	2,788 ± 152	100
0.40	0.37	7,873 ± 154	4.49 ± 0.20	1,756 ± 47	63
0.60	0.55	6,614 ± 218	4.45 ± 0.33	1,487 ± 65	53

*Experimental condition is described in text.

of the poor recovery of the complex from the annealing process (Table I), the arginine/nucleotide ratio of the recovered material is also presented. Template activity of the preparation decreases with increasing DNA coverage by protamine. An estimate of the fraction of free DNA from melting data is not possible as discussed in the first section of this chapter. Imperfect complexing of DNA by protamine no doubt accounts for the slightly higher than expected template activity found for the complex of input ratio 0.60. In general, however, protamine also seems to form a well defined one arginine to one nucleotide complex with DNA, and the conclusion can be safely drawn that the complex is inactive in support of RNA synthesis. Unlike the behavior of DNA-polylysine and polyarginine complexes, the DNA-protamine complex binds polymerase poorly or not at all. This is shown by the increase of K with increasing coverage of the DNA and by the relative constancy of V_{max}.

Change of K as related to the physical state of the template

The fact that binding of basic polypeptides to DNA is cooperative results in a nonrandom assortment of polypeptide along and among the DNA fragments. This is dramatically illustrated by the fact that the DNA molecules which are but little complexed can be physically separated from those more nearly covered with polypeptide. This is accomplished by centrifugation of the preparation at 78,000 x g for 20 min. The pellet contains the complexed material and exhibits a T_m close to that of the second-step melting with little melting at the T_m

of free DNA. Resuspension of the pellet in buffer by shaking in the cold for one hour results in a homogeneous suspension. Data on the template behavior in support of RNA synthesis together with chemical determinations of peptide cation/nucleotide ratios are summarized in Table VIII. The template activity of such pelleted and resuspended complex is low and close to that expected on the basis of its content of uncomplexed DNA. The template concentration required for half saturation of the amount of enzyme present is, however, increased by 8- to 10-fold as compared to DNA. Figure 7 shows the effect of centrifugation and resuspension of DNA-polylysine on K. DNA-polylysine (lysine/nucleotide = 0.60) was centrifuged at 78,000 g for 20 min, the pellet resuspended in the original supernatant. This treatment resulted in doubling of K, although template activity was not greatly affected (33% that of DNA). This change of template behavior is associated with packing of the complex. The treatment only affects DNA molecules which are nearly completely complexed with polypeptide and does not affect uncomplexed DNA. Template activity, which is related to the free DNA fraction is, therefore, not greatly changed, while K, related to the availability of the template, is drastically changed. Centrifugation and resuspension presumably increases the aggregation of the complexed molecules, and thus renders them unavailable to the enzyme.

The DNA-polyarginine complex prepared by salt gradient dialysis in absence of urea also shows an increase of K (Table IX), although the template activity is still proportional to arginine/nucleotide ratio. This situation is similar to that of DNA-protamine (Figure 6 and

TABLE VIII

Template Activity of Pelleted DNA-Polypeptide Complexes

	Peptide cation / DNA phosphate	V_{max} (μμmoles ATP/ 10 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 10 min/μg DNA)	%
DNA	0	2,650 ± 144	2.41 ± 0.50	1,101 ± 178	100
DNA-polylysine	0.8	2,837 ± 671	21.4 ± 8.0	132 ± 19	12
DNA-polyarginine	0.8	2,385 ± 226	16.1 ± 2.7	148 ± 11	14
DNA	0	3,149 ± 44	3.33 ± 0.14	947 ± 29	100
DNA-protamine	0.9	255 ± 10	29.3 ± 2.7	9 ± 1	9
DNA-polyornithine	0.8	2,127 ± 54	10.1 ± 0.74	212 ± 11	22

a. DNA-polypeptide complexes prepared by salt gradient dialysis with input ratio of 0.5 were fractionated by centrifugation at 78,000 g for 20 min. The material from the pellet was resuspended in fresh buffer for one hour of shaking.

b. Assay of template activity is described in the text.

FIGURE 7. Effect of centrifugation-resuspension on template behavior of DNA-polylysine. DNA-polylysine prepared by salt gradient dialysis with input ratio of 0.60 was centrifuged at 78,000 g for 20 min. The pellet was then resuspended with the original supernatant and shaken in cold for 1 hr. Incubation for RNA synthesis is described in the text. Each point represents average of a duplicate experiment. DNA (O—O) has V_{max}/K of 1698 $\pm$ 140 and DNA-polylysine (Δ—Δ) has V_{max}/K of 553 $\pm$ 57 and is 33% that of DNA.

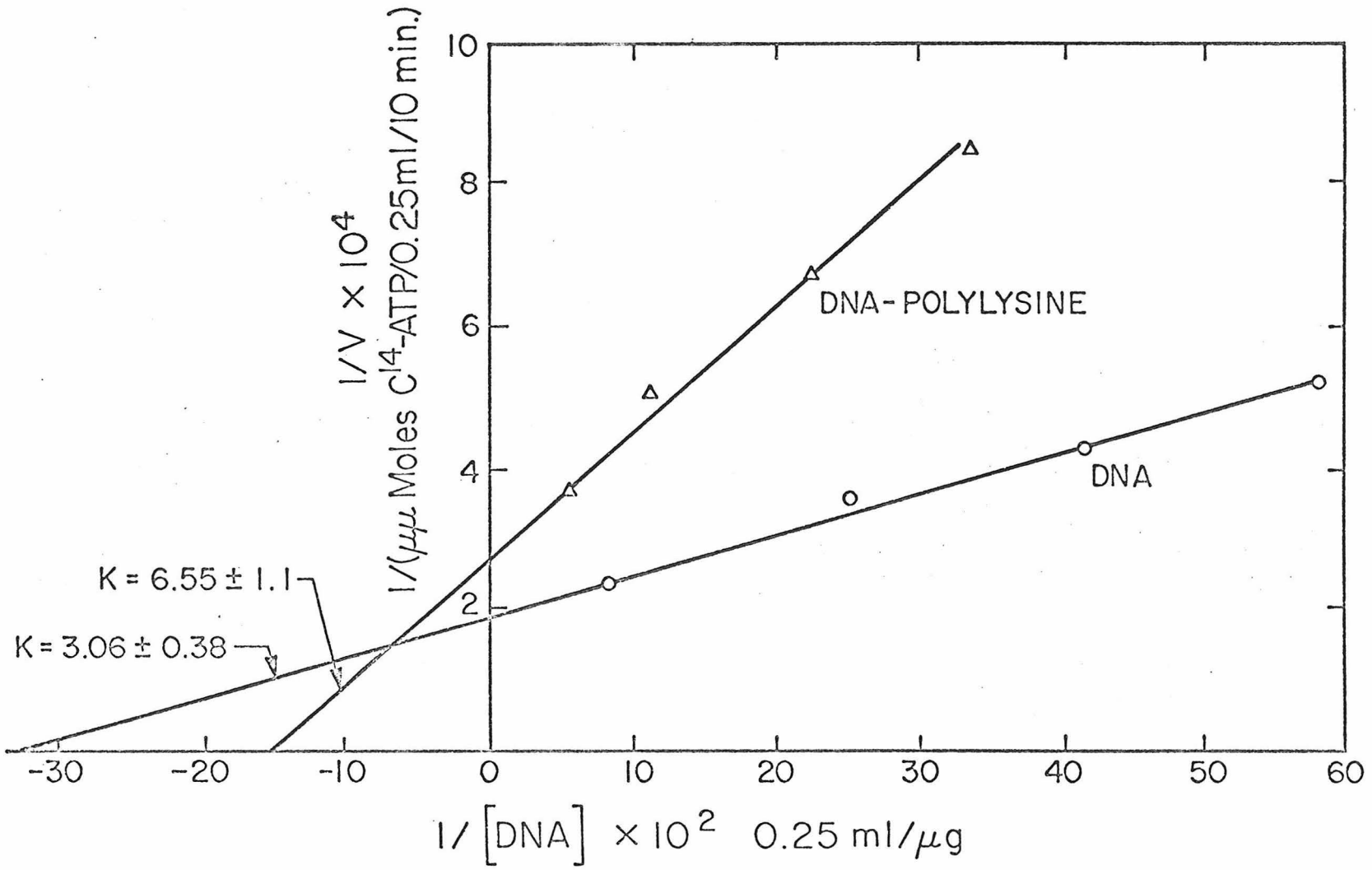
1/V × 10⁴
1/(μμ Moles C¹⁴-ATP/0.25ml/10 min.)
10
8
6
4
2
DNA-POLYLYSINE
DNA
K = 6.55 ± 1.1
K = 3.06 ± 0.38
-30
-20
-10
0
10
20
30
40
50
60
1/ [DNA] × 10² 0.25 ml/μg

FIGURE 7

TABLE IX

Template Activity of DNA-Poly-L-Arginine Prepared Without Urea

Input ratio of $\frac{\text{arginine}}{\text{nucleotide}}$	V_{max} (μμmoles ATP/ 10 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 10 min/μg DNA)	%
0	5,285 ± 130	3.24 ± 0.21	1,629 ± 70	100
0.3	5,656 ± 136	5.15 ± 0.27	1,098 ± 33	67
0.6*	3,972 ± 284	4.41 ± 0.71	901 ± 86	55

‡DNA-polyarginine was prepared by salt gradient dialysis without urea.

*Precipitation can be noted at this input ratio, the recovery is also not complete (Table I).

Table VII) prepared by gradient dialysis from salt in the absence of urea. These observations together with the poorer recovery of the complex from salt gradient dialysis (Table I), cause us to suspect that molecular aggregation of the complex occurs.

CHAPTER IV

Discussion

The experimental evidence presented in this paper supports the conclusion that DNA complexed with polylysine, polyarginine or protamine is quantitatively blocked from acting as template in support of RNA synthesis. Only the free DNA portion of such preparations serves as template. The template concentration required for half saturation of a finite amount of enzyme (K) is essentially the same for DNA-polypeptide complexes as for free DNA, provided the template is in a finely dispersed state (Figures 4 and 5 and Tables V and VI). It appears, therefore, that polymerase binds to complexed DNA as well as to free DNA. This situation is similar to that of chromatin (Marushige and Bonner, 1966) for which polymerase binds equally well to histone covered and to histone not covered regions. The interaction of polymerase and DNA is strong and enzyme can apparently either replace basic polypeptide at the binding site or can bind in the presence of basic polypeptide. Blockage of template activity is therefore not due to inaccessibility of the complexed DNA region to RNA polymerase. Elongation of the RNA chain requires local untwisting of the DNA double helical structure. The DNA-polypeptide complexes have very high melting temperatures as compared to free DNA and the double helical structure is therefore stabilized by complexing with polypeptide. The neutralization of phosphate charge by cationic amino acid side chain, and more importantly, the formation of interstrand ionic bonds by the cationic

polypeptide, would make more difficult the process of local unwinding. This may well be, we think, the reason why DNA complexed with cationic polypeptide is inactive in RNA synthesis.

The present evidence also favors the notion that the inhibition of DNA template activity is a genuine one and is not simply due to precipitation of DNA. The availability of polymerase binding sites in the DNA-polypeptide is essentially unchanged as compared to pure DNA. The finely dispersed template is therefore available to the enzyme. Aggregation into larger particles, as is caused by pelleting, however, does decrease availability of the template to the enzyme. The enzyme is clearly not able to completely penetrate a highly aggregated template. Two different modes of inhibition of DNA template activity by basic polypeptides can therefore be identified.

Chromatin isolated from cell nuclei possesses low template activity in comparison with pure DNA. Histone is the principal component responsible for depression of template activity (Bonner et al., 1968). Histone is normally complexed with DNA in nuclei of eucaryotic organism. Different histone fractions differ among themselves in the nature of their cationic amino acid residues, histone I possessing principally lysine, and histones III and IV principally arginine. The present result shows the arginine residue is as effective as the lysine residue in blocking template activity of the complexed DNA region. Differences among histone species in their effects on template activities must therefore result from other structural features, such as the grouping and distribution of the cationic residues in the primary

structure and the contributions of nonpolar amino acid residues to nonionic DNA-protein or protein-protein interactions.

Marushige and Dixon (1968) have found that native nucleoprotamine isolated from mature trout sperm is totally inactive in support of RNA synthesis. Our observations on the reconstituted DNA-protamine complex are in accord with theirs on the native complex.

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PART III

STUDIES ON DNA COMPLEXES WITH PURIFIED HISTONE FRACTIONS

CHAPTER I

Introduction

One of the approaches to study the structure and function of nucleohistones, especially the role of individual histones, is examination of the interactions of DNA with purified histone fractions and of the physical and biological properties of the DNA-histone complexes (Barr and Butler, 1963; Hindley, 1963; Huang, Bonner and Murray, 1964; Hnilica and Billen, 1964; Johns and Butler, 1964; Akinrimisi, Bonner and Ts'o, 1965; Olivera, 1966; Touvet and Champagne, 1966; Skalka, Fowler and Hurwitz, 1966; Boublik, Sponar and Sormova, 1967; and Wood, Irvin and Holbrook, 1968). Recent advances in histone chemistry have made it possible to purify some histone fractions to homogeneous molecular species (Fambrough and Bonner, 1968; Bustin and Cole, 1968; Starbuck et al., 1968; and Fambrough and Bonner, 1969), and the complete amino acid sequence of calf thymus histone IV has been worked out (DeLange et al., 1969). Thus reconstitution of nucleohistones becomes a more fruitful approach. A major obstacle in this kind of study is the preparation of well-defined DNA-histone complexes. The DNA-histone interaction is a complex one and both nucleohistones and histones exhibit aggregation behavior. This is especially the case with the arginine-rich histones in high ionic strength solution. In the present studies, well-defined molecular complexes of DNA with purified histone fractions are prepared by salt gradient dialysis in the presence of urea. The complexes were characterized by thermal denaturation

and UV absorption and the stoichiometry of each DNA-histone complex determined from its melting profile. Studies were also made of DNA complexes formed from combinations of purified histone fractions. The molecular structure of nucleohistones implied by the experimental evidence will be discussed and the structure illustrated by CPK molecular models. It will be shown that the DNA-histone IV complex lacks template activity in support of RNA synthesis in the RNA polymerase system.

CHAPTER II

Preparation of DNA-Histone Complexes

A. Salt gradient and urea in DNA-histone complex formation

A crucial step in the study of the structure and function of a DNA-histone complex is the preparation of the complex. If DNA and histone are simply mixed at low ionic strength, an aggregate is formed. Sudden mixing of DNA and histone results in imperfect complex formation and at low ionic strength, it is difficult for the components to resort to a stable equilibrium state. A major advancement in the reconstitution procedure is the use of salt gradient dialysis (Huang, Bonner and Murray, 1964). DNA-histone interaction is mainly through ionic reaction of DNA phosphates and the lysine, arginine and histidine residues of histone. The binding coefficient of histone is decreased by increasing ionic strength of the solution. As shown by the method of equilibrium dialysis (Akinrimisi, Bonner and Ts'o, 1965), a sharp decrease in the binding coefficient for histone Ib occurs at 0.4 M NaCl, for histone III-IV at around 0.5 M NaCl. In 2 M NaCl, histone and DNA are virtually completely dissociated. Soluble complexes can therefore be prepared by mixing DNA and histone at high ionic strength, and then gradually dialyzed against a decreasing salt gradient. The weak and reversible binding under these conditions permit DNA and histone to resort to a more stable conformation. Such reconstitution procedure has already yielded much information about the

structure and function of nucleohistones. Reconstituted nucleohistone Ib and IIb show stabilization of DNA against thermal denaturation, although they exhibit no characteristic T_m, which on the contrary is a function of histone/DNA ratio. Nucleohistone III-IV confers only slight stabilization on DNA (Huang, Murray and Bonner, 1964; Olivera, 1966).

Nucleohistone is insoluble and precipitated at NaCl concentrations of about 0.15 M. Histones, especially histone IV, undergo molecular aggregation at high salt concentration. This may be the reason why nucleohistone III-IV reconstituted by salt gradient dialysis confers no stabilization on DNA. The complexes prepared appear to be complexes of histone aggregates with DNA (Olivera, 1966). Nucleohistone is soluble in urea solution and histone aggregation is diminished in such solution (Trautman and Crampton, 1959). The use of urea has therefore been introduced into the reconstitution procedure to overcome this difficulty. As described in Part II of this thesis, DNA-polyarginine complex prepared by salt gradient dialysis in the presence of 5 M urea shows higher recovery, a sharper two-step melting profile, and a higher T_m of the complex than that produced by salt gradient dialysis alone. Nucleohistone has therefore been reconstituted by salt gradient dialysis in the presence of 5 M urea.

B. DNA and purified histone fractions

Calf thymus DNA (highly polymerized), lot 1381, was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). It possesses

$s_{20,w}$ of 26.44 corresponding to a molecular weight of 1.44×10^7. The RNA content is 0.15% and protein content, 0.4%. This is the same lot as used in Part II of this thesis.

Calf thymus histones Ia, Ib and IV were prepared by Miss M. Thomas. The author is indebted to her for these very clean preparations. Histones Ia and Ib were prepared by extraction of calf thymus chromatin with 5% perchloric acid which selectively removes these lysine-rich histones; the two components Ia and Ib, were then resolved by chromatography on Amberlite CG-50 column eluted with a GuCl-PO_4 gradient (Fambrough, 1968). Histone IV was separated from III and a trace of II by BioGel P-60 column chromatography (Fambrough and Bonner, 1969) from the III-IV fraction obtained from the Amberlite CG-50 column. Calf thymus histone IIb was prepared by Amberlite CG-50 column chromatography from crude histone (Bonner et al., 1968a). Identification of these purified histone fractions was carried out by the disc electrophoresis on polyacrylamide gel (Bonner et al., 1968a). Microdensitometer tracings of these gels are presented in Figure 1. The electrophoretic mobilities as compared with those of whole histone, establish the authenticity of the purified fractions. Mixtures of Ib and IIb, Ib and IV, IIb and IV were also clearly resolved by the gel. Histones Ia and Ib have similar mobilities and are not resolved. No contaminants can be detected by disc electrophoresis for the preparations of Ia, Ib and IV. This represents a purity of more than 95%. The preparation of IIb, when applied to the gel in large quantity,

FIGURE 1. Disc electrophoresis of purified calf thymus histone fractions. Microdensitometer tracings of polyacrylamide gels are shown in this figure. Left tracings are those of purified histones in comparison with whole calf thymus histone. Tracings at the right are electrophoretic separation of mixtures of individual histones.

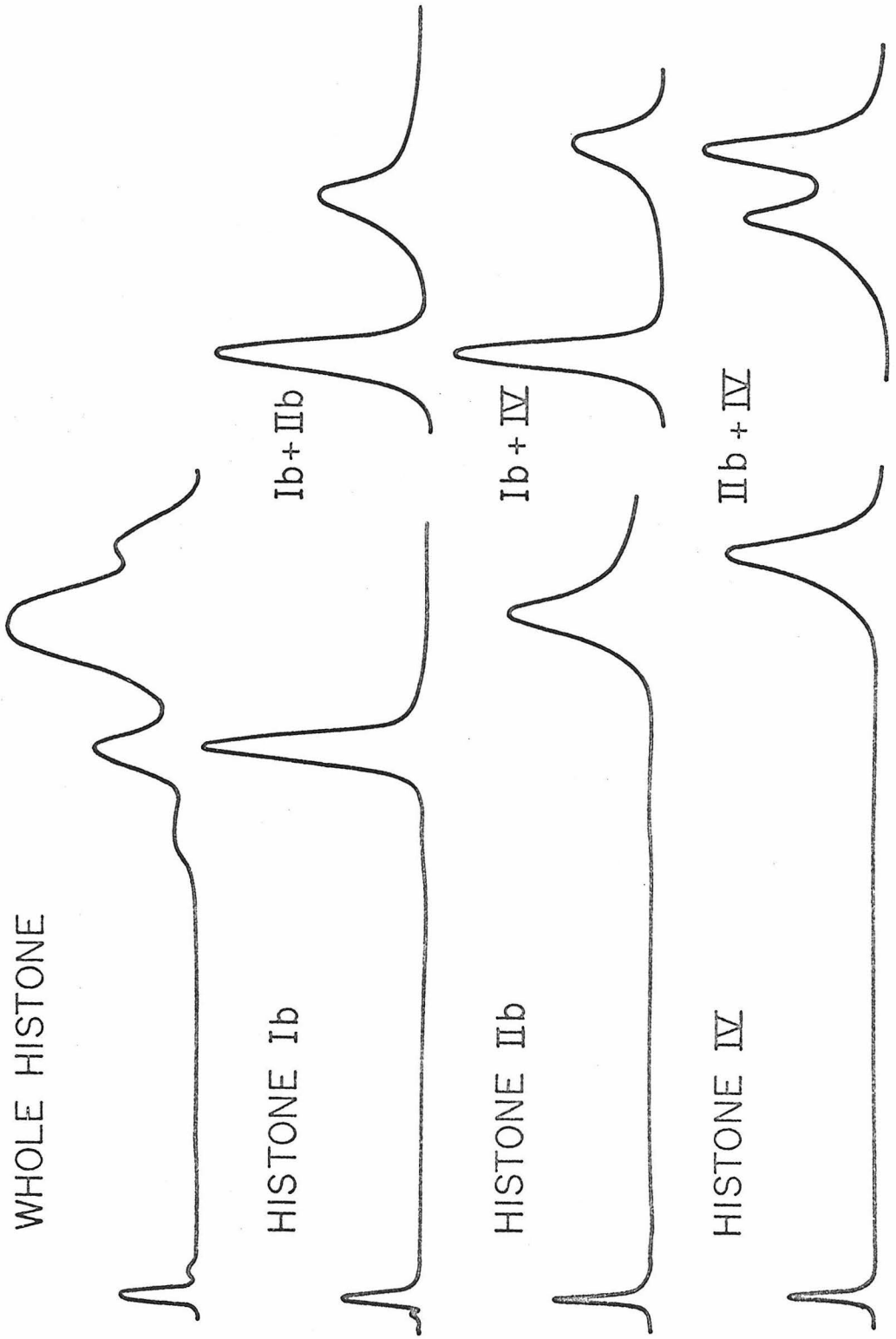
WHOLE HISTONE
HISTONE Ib
Ib + IIb
HISTONE IIb
Ib + IV
HISTONE IV
IIb + IV
+
-
+
-

FIGURE 1

exhibits trace contamination by III and IV. Its purity is ca. 90% as estimated from the gel tracing.

Histone IV is a single homogeneous molecular species (Fambrough and Bonner, 1968, 1969). IIb is composed of at least two molecular species. Although Ia and Ib may be slightly cross contaminated as prepared by the present method, they are extremely similar as judged from their amino acid composition and tryptic fingerprints. They may differ in their primary structure by only a few amino acids (Fambrough, 1968). In this study, only DNA complexes with highly purified histone fractions were used. Each fraction represents a single or only a very limited number of molecular species. The amino acid composition of each histone fraction is compiled in Table I for a convenient reference.

C. Chemical determinations of concentration of DNA and histones

Concentration of the DNA stock solution was determined by inorganic phosphate analysis (Ames and Dubin, 1960). Analytical reagent grade KH_2PO_4 (Mallinckrodt, New York) stored one week over anhydrous $CaSO_4$ was used as standard. An $\epsilon_{(Pi)}$ at 260 mμ of 9.9×10^3 for 0.5 N perchloric acid hydrolysate of DNA (boiling water bath for 10 min) has also been established for convenient determination of DNA concentration.

Concentration of histone stock solutions was determined by the ninhydrin method on their 6 N HCl hydrolysates (Spies, 1957). L-leucine (Σ grade, Sigma Chem. Co., St. Louis, Missouri) stored more than one week over anhydrous $CaSO_4$ was used as standard. The color

TABLE I

Amino Acid Compositions of Purified Calf Thymus Histone Fractions

Amino acid	Ia[2]	Ib[3]	IIb[3]	IV[2]
Lys	27.7	26.2	13.5	9.7
His	-	0.2	2.8	1.7
Arg	1.8	2.6	7.9	14.1
Asp	2.1	2.5	5.6	5.9
Thr	5.7	5.4	5.2	7.2
Ser	6.6	6.5	7.0	3.2
Glu	3.6	4.3	8.7	6.6
Pro	9.2	9.1	4.7	1.2
Gly	6.8	7.3	8.2	15.3
Ala	25.6	24.2	11.5	7.7
Val	4.9	4.1	6.7	7.4
Met	-	0.1	0.8	-
Ile	1.0	1.2	4.5	5.3
Leu	4.1	5.0	8.6	8.1
Tyr	0.5	0.7	3.0	3.5
Phe	0.6	0.6	1.3	1.9

(1) Amino acid compositions are expressed in mole per cent.

(2) Data taken from D. M. Fambrough, Ph.D. Thesis, California Institute of Technology, Pasadena, Calif. 1968, pp. 71 and 87.

(3) Data taken from P. S. Rasmussen, K. Murray, and J. M. Luck, 1962, Biochemistry 1, 79.

yield relative to L-leucine was calculated from the amino acid composition of histones as follows: Ia, 0.94; Ib, 0.94; IIb, 0.96; and IV, 0.97. Concentration of lysine and arginine residues in histones was then simply calculated from the determined total amino acid concentration and their mole per cent in Table I.

D. Procedure for preparation of DNA-histone complexes

DNA in 4 M NaCl and 5 M urea was mixed under vigorous stirring with an equal volume containing an appropriate amount of histone in 5 M urea. The final DNA concentration was 1.50×10^{-3} M of phosphate. For histones Ia and Ib, satisfactory results can be also obtained at DNA concentration as low as 5.0×10^{-4} M. The mixture was then dialyzed against a step-gradient of sodium chloride (analytical reagent, Mallinckrodt, New York) of concentration 0.6 M, 0.4 M, 0.3 M, 0.15 M and 0.015 M all in 2.5×10^{-4} M sodium EDTA and 5 M urea (analytical reagent, Mallinckrodt, New York). Solutions were all adjusted to pH 8.0. Each step was for 3 hr. The urea was finally removed by dialysis against 0.015 M NaCl and 2.5×10^{-4} M EDTA, pH 8.0 overnight. Trace amounts of NaCl were then removed by exhaustive dialysis against 2.5×10^{-4} M sodium EDTA, pH 8.0. It is recommended to dialyze urea out before complete removal of NaCl in order to keep the DNA native. T_m of DNA in 0.01 M NaCl, 5 M urea and 2.5×10^{-4} M EDTA, pH 8.0 has been found to be 47°C with a hyperchromicity of 1.37 at 260 mμ. The entire dialysis was carried out at 0° to 4°C with constant stirring of the outside solution. It has earlier been found beneficial in the

reconstitution of DNA-polypeptide and protamine complexes to carry out dialysis in a cylinder mounted on a slowly rotating carrier in order to allow a small air bubble inside the dialysis tubing to move back and forth, and thus provides constant stirring of the solution. This has been proven to be harmful for DNA-histone complex preparation since histones undergo surface denaturation under these conditions.

Dialysis tubing size #8 was obtained from Union Carbide, Chicago, Illinois. It was treated with anhydrous acetic anhydride overnight and washed exhaustively with distilled water to reduce the hydrophilic character of cellulose hydroxyl groups, thus to minimize the adherence of positively charged histone to the tubing. The loss of histone in the dialysis tubes is negligible except at very low histone concentration.

E. Recovery and physical appearance of the complexes

The complexes prepared as described are clear viscous solutions except at high histone/DNA ratios. DNA-histone IV complex for example is a transparent gel-like material at high concentration (1.5×10^{-3} M DNA phosphate). The material was then diluted with stirring to the desired concentration. Any precipitate was centrifuged down with clinical centrifuge. The recovery of the DNA in the supernatant is presented in Table II. Except at input ratios higher than 0.8 (NH IIb, 0.6), there is no appreciable precipitate formed by the preparation procedure. This is much better than has been achieved

TABLE II

Recovery of DNA in 2000 Rpm Supernatant

Input ratio $\frac{\text{(Lysine + Arginine)}}{\text{Nucleotide}}$	NH Ia	NH Ib	NH IIb	NH IV
0	100%	100%	100%	100%
0.2	100	99	100	100
0.4	100	99	100	100
0.6	100	98	95	100
0.8	95	95	35	98
1.0	-	46	-	-

for complexes prepared by salt gradient dialysis alone. Urea thus does have an effect in preventing aggregation and precipitation.

The turbidity of the resulting solutions of complexes is presented in Table III. There is a general tendency for turbidity to increase with increasing histone/DNA ratio. Even so turbidity is low as compared with that of DNA complexes with polylysine, polyarginine and protamine. The DNA complex formed from a combination of IIb and IV does however have a high turbidity. This may suggest that IIb and IV have some special mutual interaction.

TABLE III

A_{320}/A_{260} Ratio of DNA-Histone Complexes

Lysine + Arginine / Nucleotide	0	0.2	0.4	0.6	0.8
Nucleohistone Ia	0.002	-	0.004	0.008	0.010
Nucleohistone Ib	0.001	-	0.006	0.010	0.010
Nucleohistone IIb	0.004	0.004	0.013	0.080	-
Nucleohistone IV	0.002	0.009	0.013	0.020	0.053
Nucleohistone Ia/IIb	0.005	-	0.005	0.030	-
Nucleohistone Ia/IV	0.006	-	0.006	0.008	-
Nucleohistone IIb/IV	0.005	-	0.050	0.138	-
Nucleohistone Ia/IIb/IV	0.005	-	0.010	0.044	-

*Histones in the DNA complexes with histone combinations are all of one to one ratio.

CHAPTER III

Thermal Denaturation Profiles of DNA Complexes with Purified Histone Fractions

A. Method of obtaining absorbance melting profiles of DNA complexes

Melting profiles of DNA complexes were accomplished with a Gilford multiple sample recording spectrophotometer Model 2000 equipped with a linear temperature programmer. Thermal denaturation of DNA double helix structure was followed by the absorbance change as monitored at 260 mμ. After 10 to 15 min of degassing in a vacuum desiccator, samples in stoppered cuvettes were heated at a rate of 0.5 to 1°C per min. Melting was always done in 2.5×10^{-4} M sodium ethylenediaminetetraacetate (EDTA), pH 8.0 (Sigma Chem. Co., lot 114B-0270). Demineralized distilled water was used throughout. In this low ionic strength medium, wide separation of nucleohistone melting from that of free DNA segments can be achieved. EDTA also removes any trace contamination of multivalent cations and this is important since these have great effects on the T_m of DNA (Dove and Davidson, 1962). Melting profiles were normally obtained in the DNA concentration range 0.6 to 1.2 A_{260} unit. In cases in which turbidity changes of the solution occurs during melting, the melting was carried out at a lower concentration such as 0.3 A_{260} unit. Changes of turbidity of the solution during the course of heating were monitored at 320 mμ. In most cases, there is no change in A_{320}.

Melting profiles were normalized by a CITRAN program on an IBM System/360 Model 50 time sharing computer. The derivative curves of the melting profiles were also accomplished by calculating the H_{260} increment for each temperature. The author is indebted to Mr. J. Smart for allowing me to use his program for the normalization. The melting temperature of each transition was determined by the graphical method involving linear extrapolation of the plateau regions through linear approximation of each transition. T_m is the temperature for half-melting between these two intersections. This method is more accurate than that of using the maxima of the derivative curves.

B. Melting profiles of DNA complexes with purified histone fractions

1. Nucleohistone Ia

Melting profiles and their derivative curves of DNA-histone Ia complex are presented in Figure 2. Histone/DNA ratio is expressed as the ratio of the molar concentration of lysine and arginine residues of histone to concentration of nucleotide residue of DNA. The two-step nature of the melting profiles can be clearly seen at subequivalent histone/DNA ratio. Melting temperatures of the first and second transition obtained by the graphical method are presented in Table IV. $T_{m,2}$ is essentially constant, and independent of the coverage of DNA by histone. $T_{m,1}$ is very close to that of pure DNA although a slight shift to higher temperature is found. $T_{m,1}$ represents therefore the

FIGURE 2. Melting profiles of DNA-histone Ia complex. Nucleohistone Ia with (lysine + arginine)/nucleotide ratio of 0 (No. 1), 0.20 (No. 2), 0.40 (No. 3), 0.60 (No. 4) and 0.80 (No. 5) were melted in 2.5 x 10^{-4} M of sodium EDTA, pH 8.0. At the top are melting profiles and at the bottom, their derivative curves.

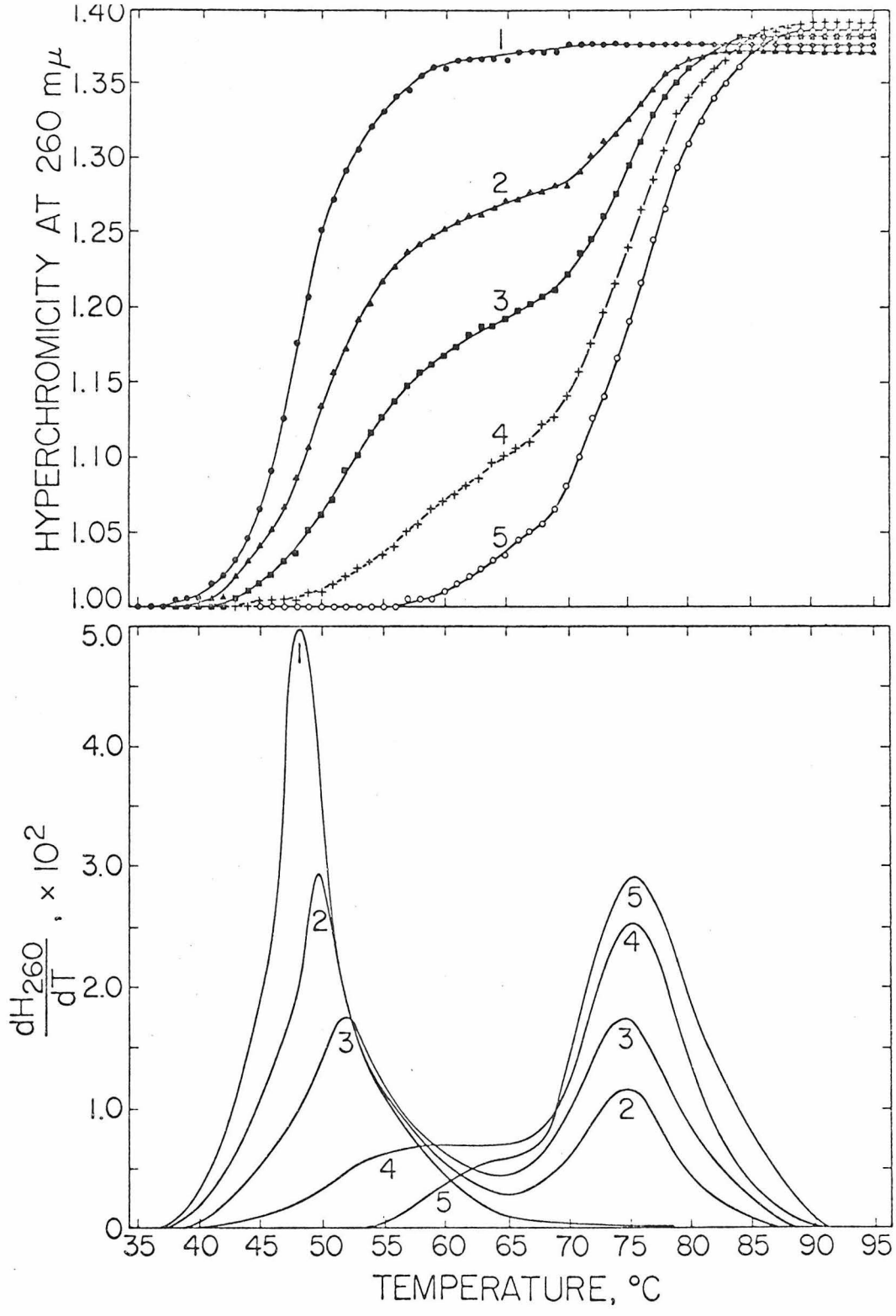
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
1
2
3
4
5
$\frac{dH_{260}}{dT}$, x 10^2
5.0
4.0
3.0
2.0
1.0
0
35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 2

TABLE IV

Melting Temperature of DNA-Histone Ia Complex

Lysine + Arginine / Nucleotide	$T_{m,1}$ °C	$T_{m,2}$ °C	H_{260}
0	49.1	-	1.373
0.20	49.6	75.2	1.375
0.40	51.4	75.2	1.380
0.60	-	75.6	1.385
0.80	-	75.9	1.386

a. Melting buffer is 2.5×10^{-4} M sodium EDTA, pH 8.0.

b. DNA-histone Ia complex was prepared by salt gradient dialysis in the presence of 5 M urea.

c. Average values of two independent determinations are presented.

melting of essentially free DNA segments of the complex, while $T_{m,2}$ represents the melting of DNA region complexed with histone molecules.

2. Nucleohistone Ib

Melting profiles and the derivative curves of DNA-histone Ib complex are presented in Figure 3. Melting temperatures obtained by the graphical method are shown in Table V. The general features of the melting profiles and melting temperature are very similar to those of nucleohistone Ia. The similarity of the primary structures of these two histone fractions (section B of the previous chapter) is reflected in melting profiles of their DNA complexes.

3. Nucleohistone IIb

Nucleohistone IIb has higher turbidity than DNA complexes with other histones as is shown by the A_{320}/A_{260} ratios of Table III. Histone IIb used in this study has about 10% contamination by histones III-IV. Since the DNA complex with the IIb-IV combination has the highest turbidity and lowest recovery, it may be that the higher turbidity found for nucleohistone IIb is due to its contamination by III-IV. The turbidity also poses a serious problem for obtaining good melting profiles. Turbidity of the solution increases during melting, especially at high histone/DNA ratio (Figure 4). This complication can, however, be minimized by carrying out melting at low nucleohistone concentration, such as 0.3 A_{260} unit, and by correcting for the slight turbidity change. An identical run monitored at 320 mμ was done after

FIGURE 3. Melting profiles of DNA-histone Ib complex. Nucleohistone Ib with (lysine + arginine)/nucleotide ratio of 0 (No. 1), 0.22 (No. 2), 0.44 (No. 3) and 0.66 (No. 4) were melted in 2.5×10^{-4} M sodium EDTA, pH 8.0. At the top are melting profiles and at the bottom, their derivative curves.

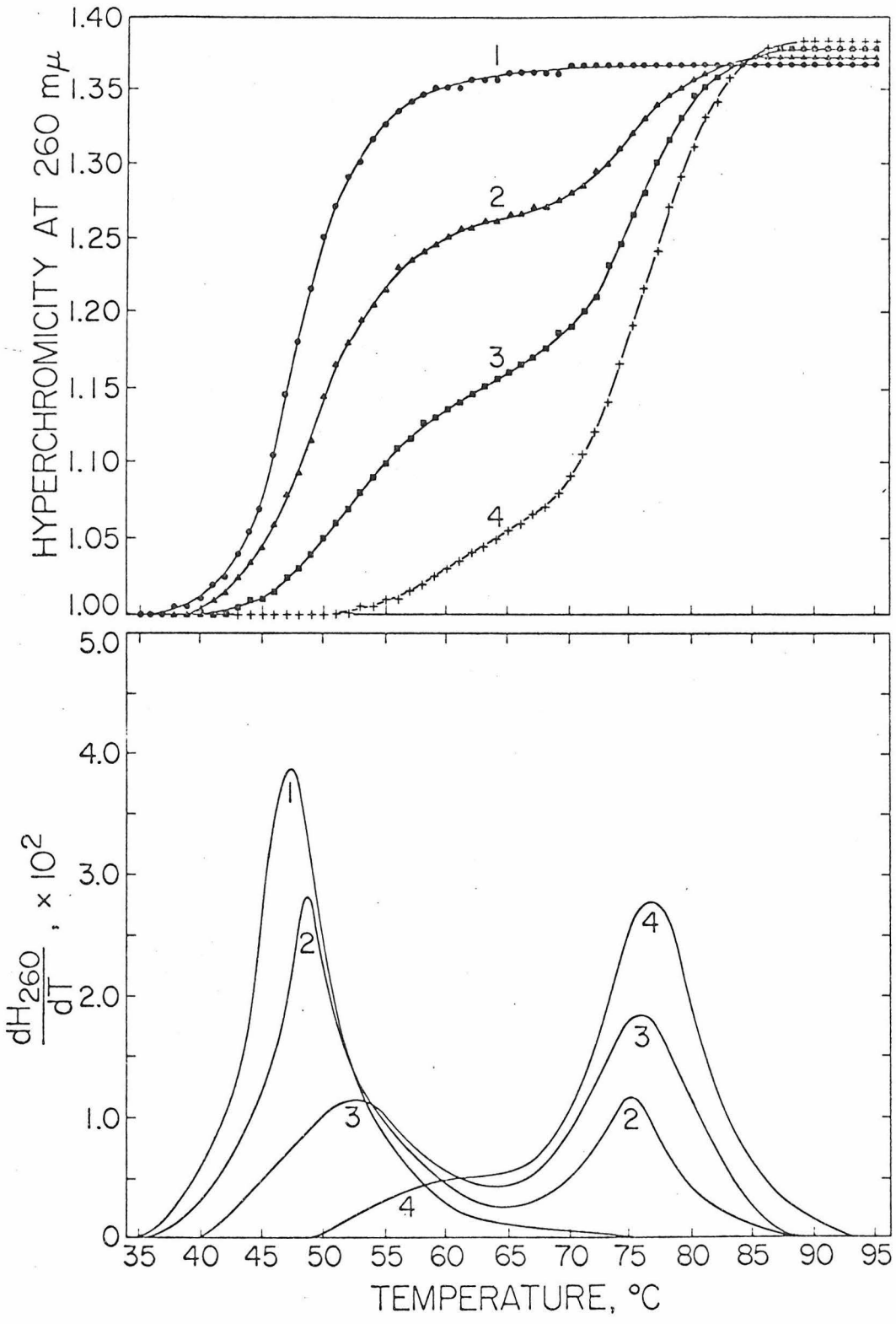
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
1
2
3
4
$\frac{dH_{260}}{dT}$, × 10²
5.0
4.0
3.0
2.0
1.0
0
35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 3

TABLE V

Melting Temperature of DNA-Histone Ib Complex

$\frac{\text{Lysine + Arginine}}{\text{Nucleotide}}$	$T_{m,1}$	$T_{m,2}$	H_{260}
0	49.5	-	1.372
0.20	50.4	75.7	1.375
0.40	52.3	76.0	1.372
0.60	-	76.5	1.385
0.80	-	77.6	1.388

a. Melting buffer is 2.5×10^{-4} M sodium EDTA, pH 8.0.

b. DNA-histone Ib complex was prepared by salt gradient dialysis in the presence of 5 M urea.

c. Average values of two independent determinations are presented.

FIGURE 4. Turbidity change of DNA-histone IIb complex during the melting experiment. Nucleohistone IIb with (lysine + arginine)/ nucleotide ratio of 0.20 (Δ——Δ), 0.40 (□——□), and 0.60 (+——+) were melted in 2.5×10^{-4} M EDTA, pH 8.0. Optical density at 320 mμ was monitored. A_{320} in relation to its original A_{260} is presented.

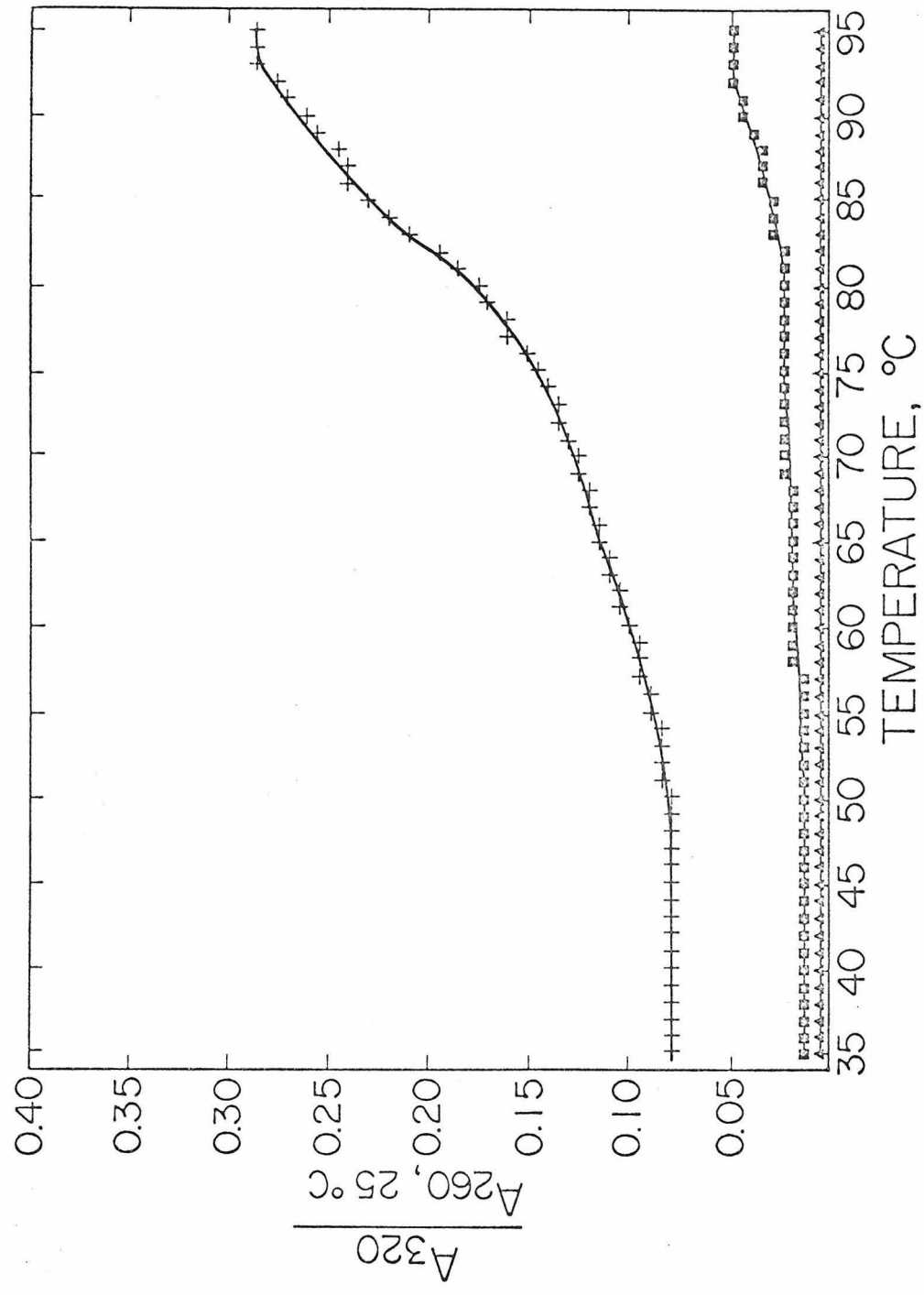
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
A260, 25 °C / A320
35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 4

a run at 260 mμ as is shown in Figure 4. At the end of the run, the turbidity dispersion between 320 mμ and 360 mμ was measured. Turbidity correction for A_{260} at each temperature was then made by extrapolation of A_{320} from the dispersion between 320 mμ and 360 mμ. Turbidity dispersion follows the equation, $\log A_\lambda = -a\log_\lambda + C$, where A_λ is optical density at wavelength, λ, and C is a constant. The slope, a, was found to be 2.9 in the present case. The melting profiles and their derivative curves are presented in Figure 5. Profiles for pure DNA and for a histone/DNA ratio of 0.2 show no turbidity change during the experiment, while correction for the slight turbidity changes of preparations No. 3 and No. 4 of Figure 4 was made as described above. The corrected curves are presented in Figure 5. The turbidity increase after 65°C for curve 4 was too large for reliable correction, and the true melting profile beyond this temperature is therefore obscure. The two-step nature of the melting profiles is again evident for nucleohistone IIb.

4. Nucleohistone IV

Figure 6 shows the melting profiles and their derivative curves for nucleohistone IV. The biphasic nature of the melting profiles is clear, although the transition at the higher temperature is broader at lower histone/DNA ratio. The melting profiles for complexes of histone/DNA ratio greater than 0.6 are not available because of serious turbidity change during heating. The melting temperature

FIGURE 5. Melting profiles of DNA-histone IIb complex. Nucleohistone IIb with (lysine + arginine)/nucleotide ratio of 0 (No. 1), 0.20 (No. 2), 0.40 (No. 3) and 0.60 (No. 4) were melted in 2.5×10^{-4} M EDTA, pH 8.0. At the top are melting profiles and at the bottom their derivative curves. Scattering correction was made for No. 3 and No. 4. For detail see the text.

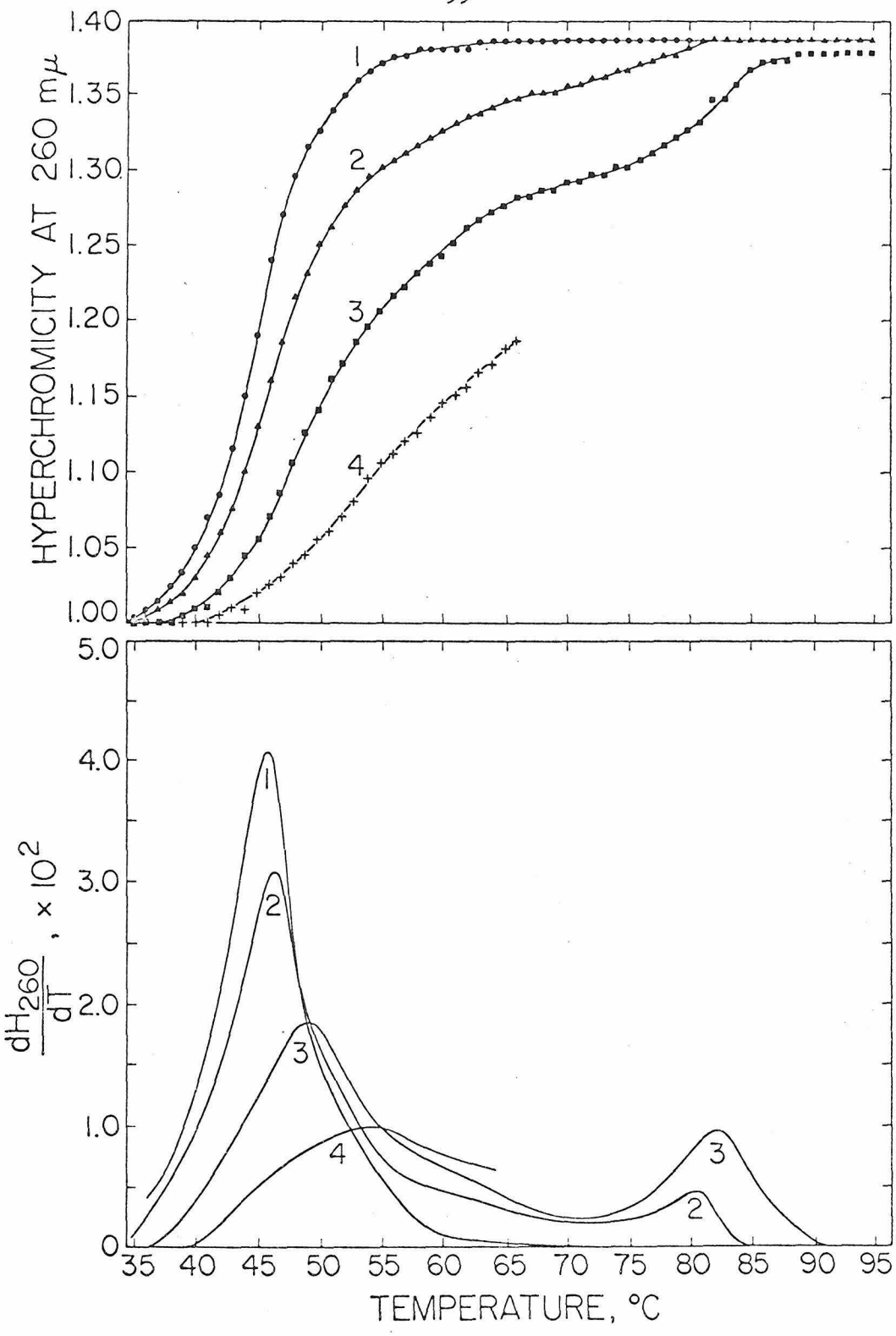
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
1
2
3
4
$\frac{dH_{260}}{dT}$, × 10^2
5.0
4.0
3.0
2.0
1.0
0
35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 5

FIGURE 6. Melting profiles of DNA-histone IV complex. Nucleohistone IV with (lysine + arginine)/nucleotide ratio of 0 (No. 1), 0.20 (No. 2), 0.40 (No. 3) and 0.60 (No. 4) were melted in 2.5×10^{-4} M sodium EDTA, pH 8.0. At the top are melting profiles and at the bottom, their derivative curves.

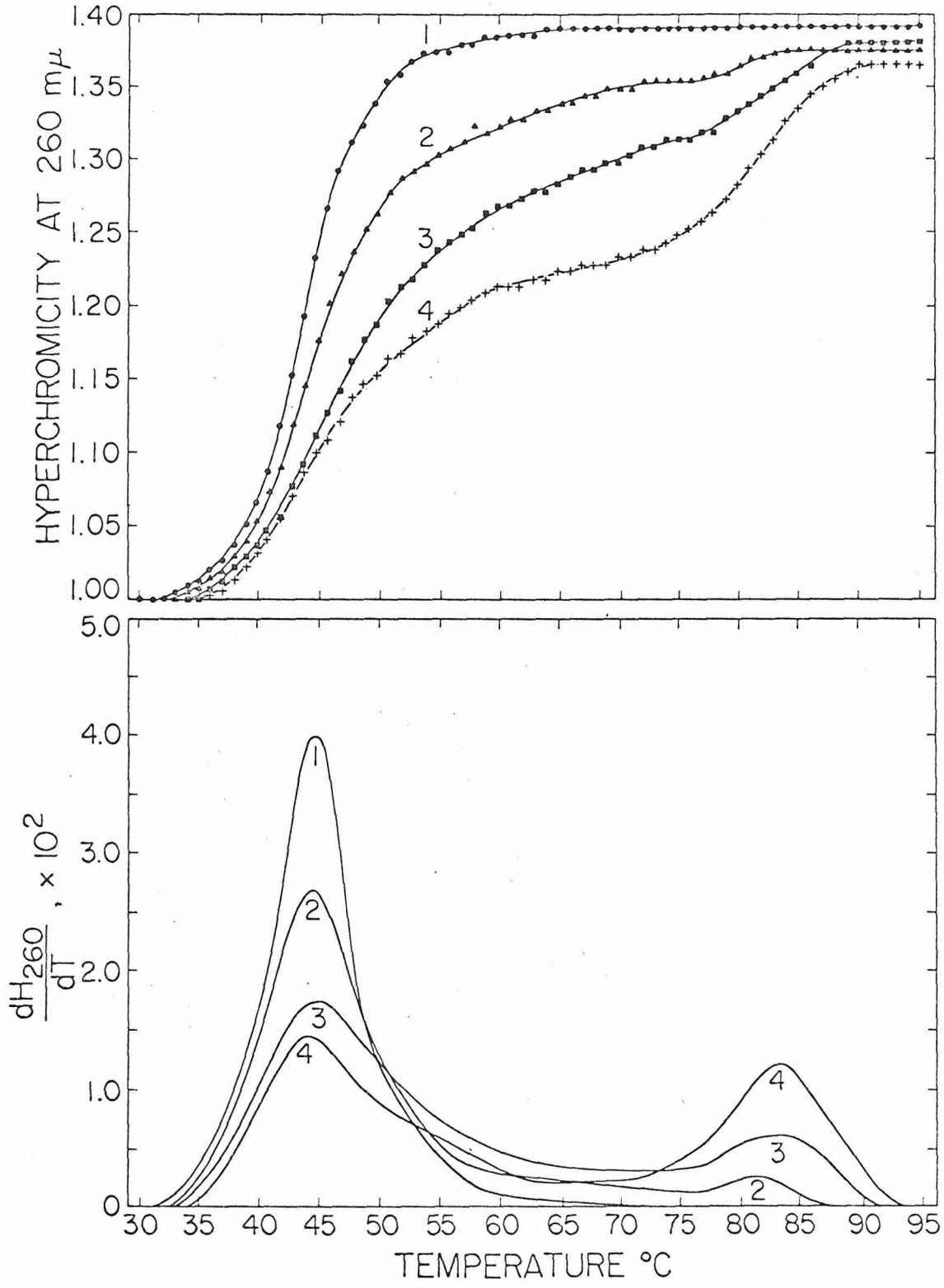
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
1
2
3
4
$\frac{dH_{260}}{dT}$, x 10^2
5.0
4.0
3.0
2.0
1.0
0
30 35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE °C

FIGURE 6

for the DNA-histone IV complex is evident from the profiles and is constant for all histone/DNA ratio.

C. Comparison of T_m of DNA complexes with various cationic proteins

Melting profiles of DNA complexes with cationic proteins as annealed by the present method are of the biphasic nature. The first-step transition is that of "naked" DNA region, and the second-step transition is that of the complexed region. The transition temperature of the second step is constant for all coverages, and is characteristic for each particular DNA-protein complex. Comparison of T_m of the various DNA complexes is therefore meaningful. The T_m of a complex is a characteristic parameter which indicates the stability of the double helix structure of the DNA component of the complex against thermal denaturation. Comparison of T_m of DNA complexes with various proteins thus yields information about the extent of stabilization of DNA structure by the cationic proteins. T_m of various DNA complexes are listed in Table VI together with their standard deviations. Meltings are all done in 2.5×10^{-4} M EDTA, pH 8.0. With such a low concentration of EDTA, buffer capacity is low, and accurate control of pH and thus ionic strength of the melting medium becomes difficult. At this low salt concentration, the actual concentration of sodium EDTA inside the dialysis tubing will be influenced by the concentration of DNA and/or DNA-histone complexes because of the Donnan phenomenon. The T_m of DNA thus fluctuates a few degrees as between separate experiments, but the T_m of DNA complexes are much less affected. Increasing number of

TABLE VI

Comparison of Melting Temperatures of DNA Complexes with Various Cationic Proteins

	$T_m \pm$ S.D. (°C)
DNA	47.2 ± 2.7
Chromatin	74.3 ± 1.5
DNA-histone Ia	75.4 ± 0.5
DNA-histone Ib	76.3 ± 0.9
DNA-histone IIb	81.5 ± 0.6
DNA-histone IV	83.7 ± 1.2
DNA-protamine	(92.5 ± 0.7)[b]
DNA-polyarginine	98.0 ± 1.4
DNA-polylysine	99.5 ± 2.1

a. Melting buffer is 2.5×10^{-4} M sodium EDTA, pH 8.0.

b. Complexes were prepared with salt gradient dialysis in the presence of 5 M urea (except DNA-protamine) at various input ratios. Average values of 3 to 12 determinations and the standard deviations are presented.

c. Calf thymus DNA, histones and chromatin were used.

experiments reduces the standard deviation to a satisfactory range. T_m of various complexes are of the following order:

DNA $\ll$ Chromatin, NH Ia, NH Ib $<$ NH IIb, NH IV $\ll$ DNA-protamine $<$ DNA-polyarginine, DNA-polylysine.

All the complexes were prepared by salt gradient dialysis in the presence of 5 M urea except DNA-protamine complex which is not successful by the procedure and was prepared by salt gradient dialysis alone. Olins, Olins and von Hippel (1967) have reported that the T_m of DNA-polylysine is slightly higher than that of DNA-polyarginine. The present data show they are similar. The apparent lower T_m of DNA-polyarginine is due to imperfect complex formation in the absence of urea during salt gradient dialysis as discussed in Part II of this thesis. The T_m of the DNA-protamine complex is very close to that of DNA-polyarginine, especially when the latter is prepared without urea (see Part II). Nucleohistones IIb and IV also have very similar T_m. They are considerably lower than those of complexes with polylysine, polyarginine or protamine. Nucleohistones Ia and Ib have the same T_m which is slightly lower than that of nucleohistones IIb or IV. The T_m of chromatin, a native complex of DNA with histone, non-histone proteins, and small amounts of RNA is of the same order as that of the DNA-histone I group. The reason for this will be discussed in Chapter VIII. The T_m of DNA is far lower than that of any of its protein complexes.

CHAPTER IV

Thermal Denaturation Profiles of DNA Complexes with Combination of Histones

DNA complexes with combinations of histones were studied to learn about possible interactions between different histones. The questions are asked: can the multiphasic melting profiles of DNA complexes with individual histone fractions be demonstrated with combination of histones; what will be the T_m of the complexes; is there increased cooperativity and sharpness of the melting transition?

A. Melting profiles of DNA complexes with combination of histones

1. Nucleohistone Ia/IIb

Melting profiles of nucleohistone Ia/IIb are presented in Figure 7. Histones Ia and IIb were mixed on the basis of equal molar concentration of their lysine and arginine residues. DNA complexes were prepared with total (lysine + arginine)/nucleotide ratio of 0, 0.40 and 0.60. Biphasic melting profiles are again obtained. The transition temperature of the first step is close to that of DNA and the second-step transition also occurs at constant temperature. Like nucleohistone IIb, complexes with combination of Ia and IIb also show slight turbidity changes during heating. Curve 3 has been corrected for the scattering effect as described in Chapter III, section B.

FIGURE 7. Melting profiles of DNA complex with histones Ia and IIb. Histones Ia and IIb are mixed with the same concentration of their lysine and arginine residues. The molar ratios of total lysine and arginine to nucleotide are 0 (No. 1), 0.40 (No. 2) and 0.60 (No. 3). Melting was done in 2.5×10^{-4} M EDTA, pH 8.0. Slight scattering correction has been made for curve 3.

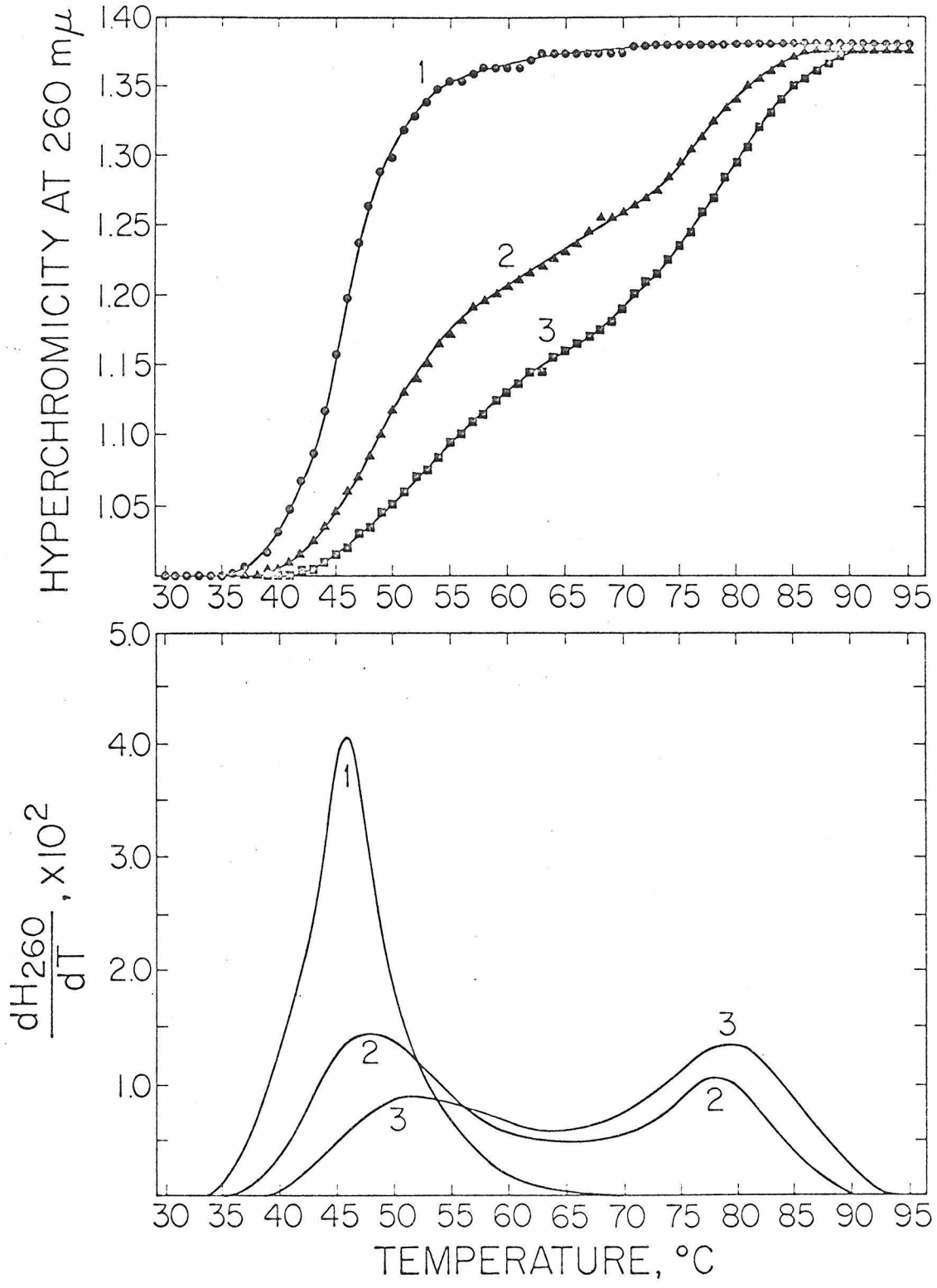
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1
2
3
30 35 40 45 50 55 60 65 70 75 80 85 90 95
$\frac{dH_{260}}{dT}$, x10²
5.0
4.0
3.0
2.0
1.0
1
2
3
3
2
30 35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 7

2. Nucleohistone Ia/IV

Melting profiles of DNA complexes with equal molar lysine and arginine mixture of histones Ia and IV are presented in Figure 8. The biphasic nature of the transition is evident. The second-step melting also occurs at constant temperature.

3. Nucleohistone IIb/IV

DNA complexes prepared with equal mixture of IIb and IV yield the lowest recovery and the highest turbidity of any complexes studied as can be seen in Tables II and III. Melting profiles are not obtainable by direct heating of the resulting complex because of serious turbidity change. The preparations were, therefore, subjected to centrifugation at 12,000 g for 20 min. The recovery of DNA in the supernatant is 76% for the complex with input ratio of 0.40 and 45% for ratio of 0.60. Melting profiles obtained with the supernatant are presented in Figure 9. Scattering corrections were made, although for curve 3, it is too serious for reliable correction above 67°C. Biphasic profiles are none the less clear in curve 2. Although it is not evident from the melting profile, the special interaction of IIb and IV can be noted by the ready precipitability of the complex.

4. Nucleohistone Ia/IIb/IV

The melting profiles of DNA complexed with histones Ia, IIb and IV are presented in Figure 10. Like nucleohistone IIb/IV, the complex shows high turbidity and the preparation of input ratio 0.60

FIGURE 8. Melting profiles of DNA complex with histones Ia and IV. Histones Ia and IV are mixed with the same concentration of their lysine and arginine residues. The molar ratios of total lysine and arginine to nucleotide are 0 (No. 1), 0.40 (No. 2) and 0.60 (No. 3). Melting was done in 2.5×10^{-4} M EDTA, pH 8.0.

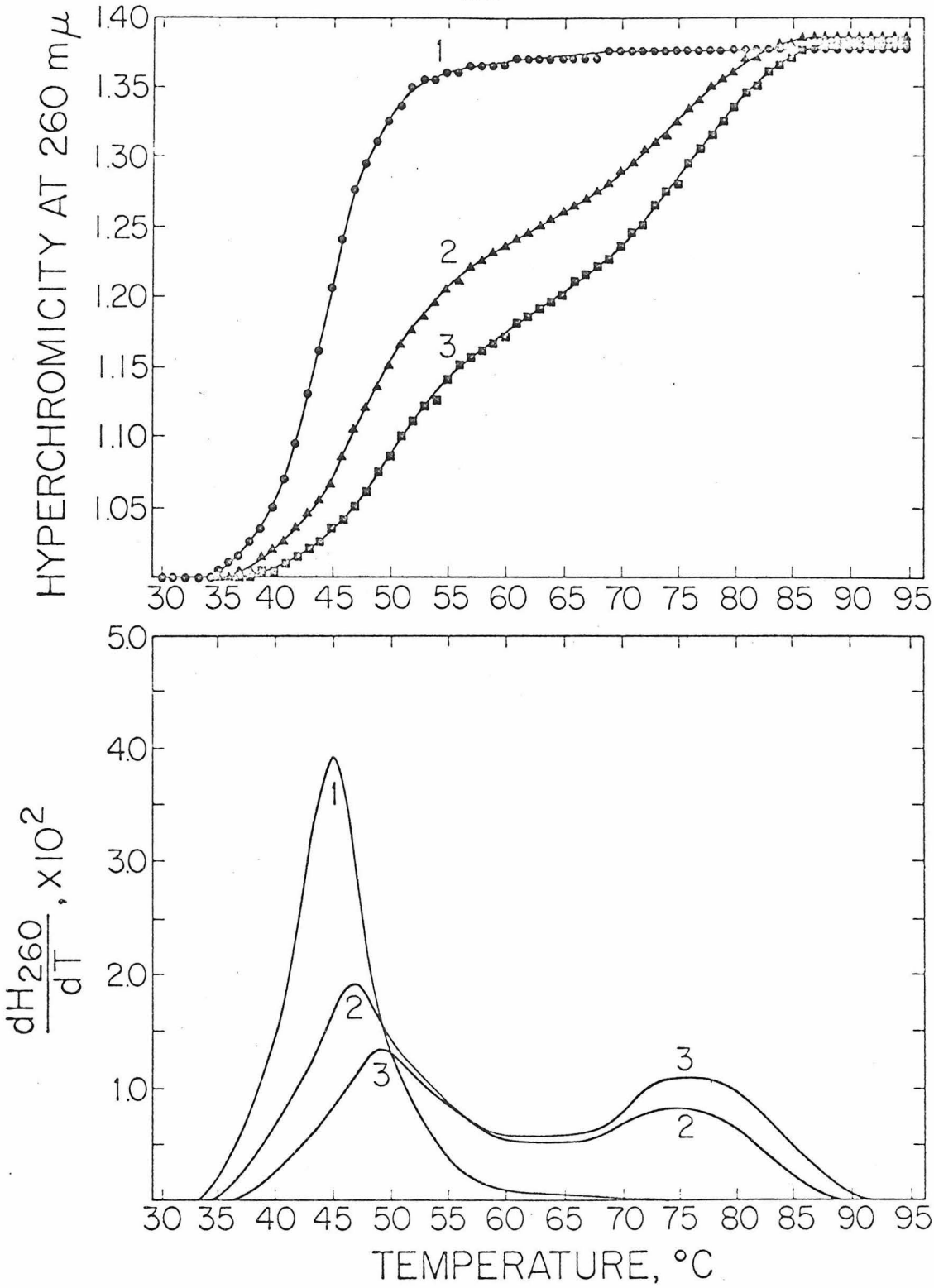
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1
2
3
30 35 40 45 50 55 60 65 70 75 80 85 90 95
$\frac{dH_{260}}{dT}$, x10²
5.0
4.0
3.0
2.0
1.0
1
2
3
3
2
30 35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 8

FIGURE 9. Melting profiles of DNA complex with histones IIb and IV. Histones IIb and IV are mixed with the same concentration of their lysine and arginine residues. The input ratios of total lysine and arginine to nucleotide are 0 (No. 1), 0.40 (No. 2) and 0.60 (No. 3). The resulting turbid complex was centrifuged at 12,000 g for 20 min, and the supernatant was melted in 2.5×10^{-4} M EDTA, pH 8.0. The slight scattering has been corrected. The turbidity change beyond 68°C of curve 3 is too serious for reliable correction and is not presented.

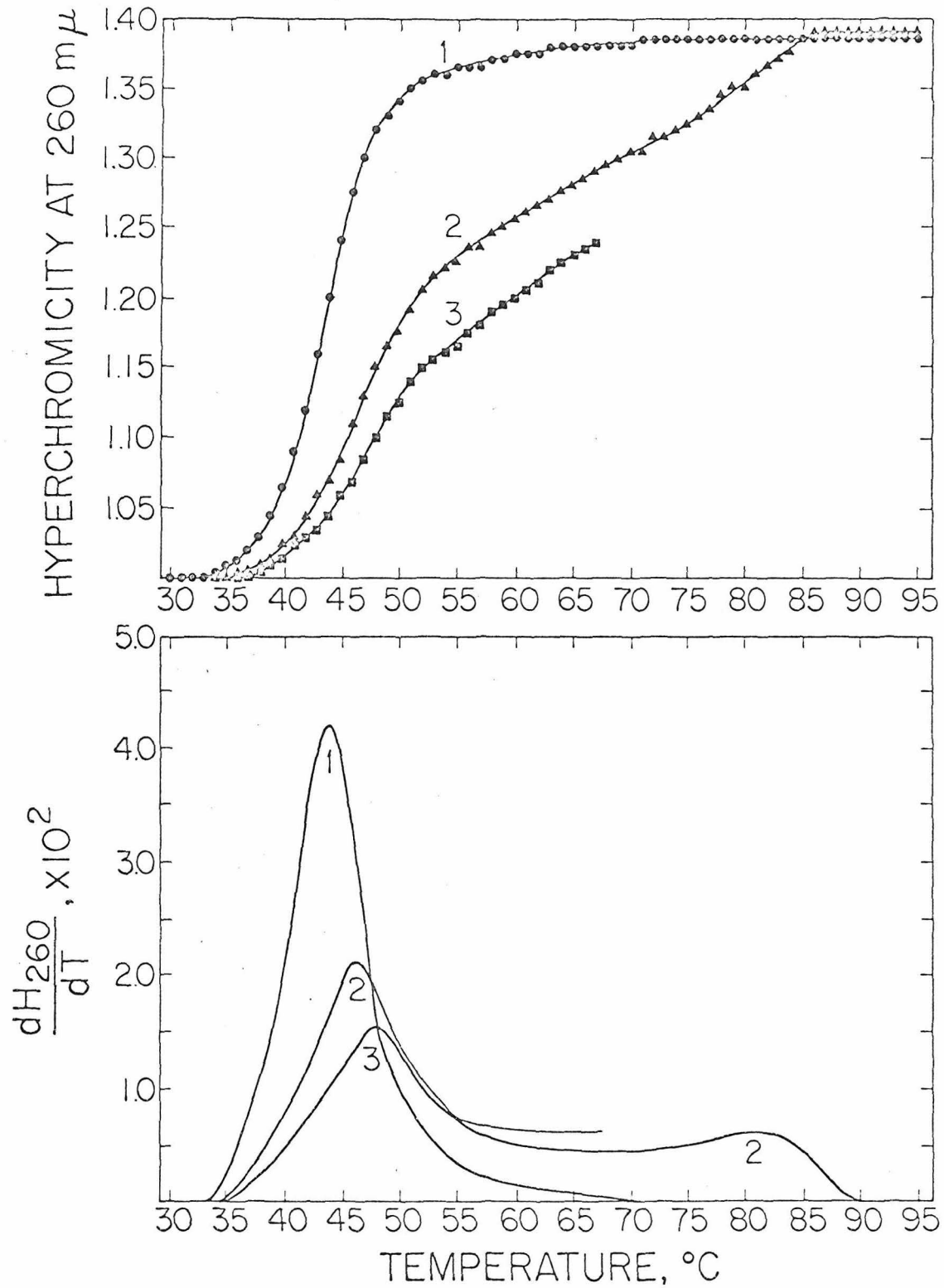
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1
2
3
30 35 40 45 50 55 60 65 70 75 80 85 90 95
dH260/dT, x10²
5.0
4.0
3.0
2.0
1.0
1
2
3
2
30 35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 9

FIGURE 10. Melting profiles of DNA complex with histones Ia, IIb and IV. Histones Ia, IIb and IV were mixed with the same concentration of their lysine and arginine residues. The input ratios of total lysine and arginine to nucleotide are 0 (No. 1), 0.40 (No. 2) and 0.60 (No. 3). Complex with ratio of 0.60 was turbid and was centrifuged at 12,000 g for 20 min. Slight scattering correction has been made, and the turbidity change beyond 68°C of curve 3 is too serious and the melting profile is not presented.

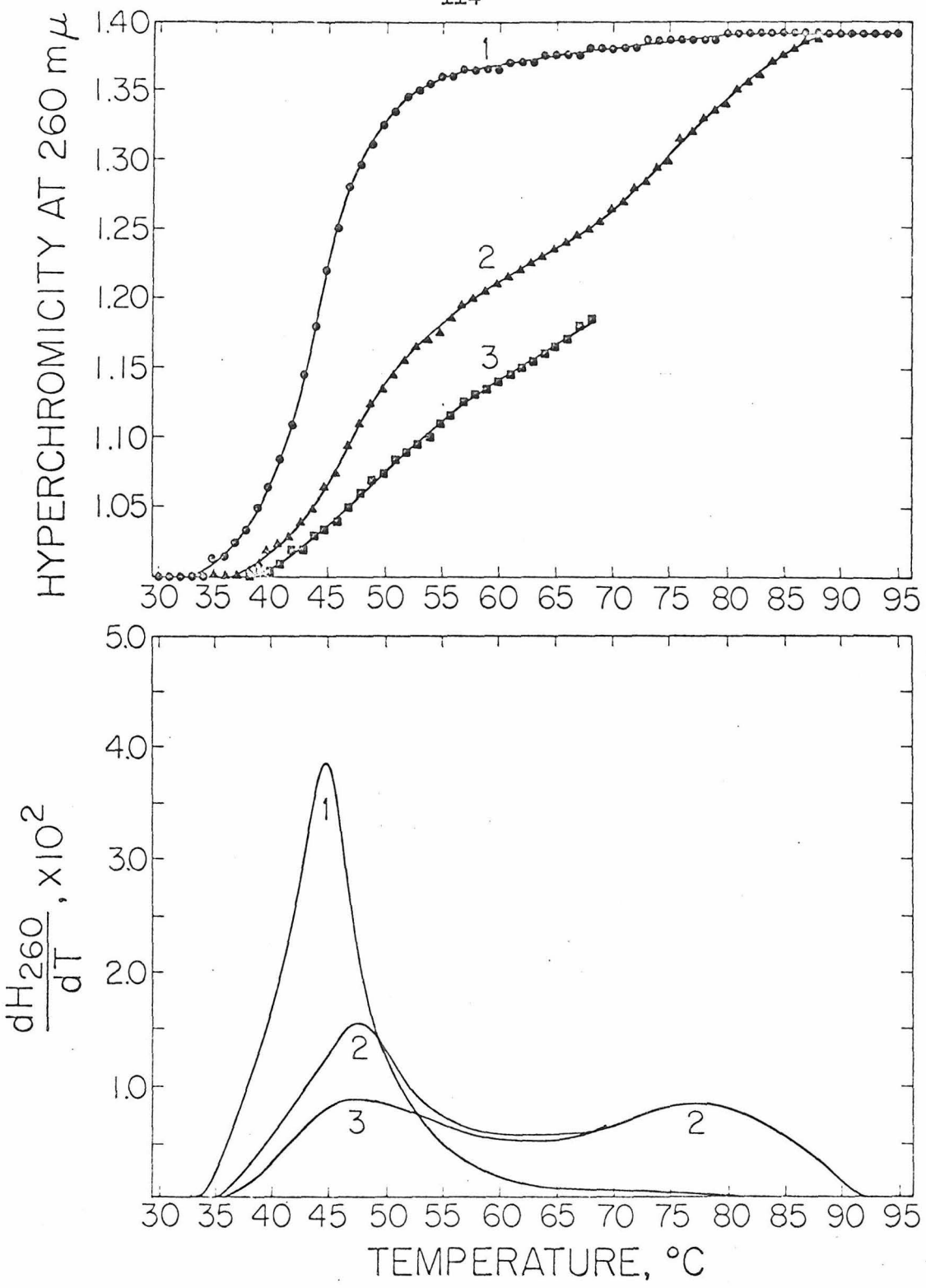
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1
2
3
30 35 40 45 50 55 60 65 70 75 80 85 90 95
$\frac{dH_{260}}{dT}$, x10^2
5.0
4.0
3.0
2.0
1.0
1
2
3
2
30 35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C

FIGURE 10

was subjected to 12,000 g centrifugation for 20 min. The melting profile is that of the supernatant. Both curves 2 and 3 have been corrected for scattering effects. Two-step nature of the melting profile can be seen in curve 2.

B. T_m of DNA complexes with combination of histones

T_m of nucleohistones IIb and IV is about $6 \sim 8°C$ higher than nucleohistones Ia and Ib. If DNA complexes with mixture of Ia and IIb or IV have very long stretches of DNA covered by identical histone molecules, say Ia or IIb or IV, it might be possible to see three-step melting. The melting profiles observed are, however, two-step. This suggests that the DNA complexes with histone mixtures involve heterogeneous assortment of different species of histone molecules on the DNA, although the presence of short stretches with a few identical histone molecules cannot be excluded. DNA complexes with combination of histones also have characteristic melting temperature. The T_m as determined by the graphical method from the melting profiles are presented in Table VII. The melting temperatures are in all cases intermediate between those of the two concerned species. This also indicates the heterogeneous nature of the assortment.

C. Sharpness of the melting transition

Thermal denaturation of DNA occurs over a very narrow temperature range and the denaturation profile is sigmoid. This implies that the melting of DNA is a highly cooperative process. The breadth

TABLE VII

Melting Temperatures of DNA Complexes with Mixture of Histones

	T_m, °C
DNA	47
Nucleohistone Ia/IIb	78
Nucleohistone Ia/IV	77
Nucleohistone IIb/IV	82
Nucleohistone Ia/IIb/IV	78

a. Mixtures are of histones of equal molar concentration of their lysine and arginine residues.

b. Melting was done in 2.5×10^{-4} M sodium EDTA, pH 8.0.

of the transition is a measure of the sharpness or cooperativity of DNA denaturation. Since the slope $(\frac{dH_{260}}{dT})$ of the melting profile follows a more or less Gaussian distribution, the maximal slope is therefore a measure of sharpness of the transition and is directly proportional to the transition breadth. Temperatures at the maxima of the derivative curves are the melting temperatures of the helix-coil transition. In this section, a phenomenological description and discussion of the sharpness of denaturation of DNA and DNA-histone complexes will be given, and the molecular nature of this will be discussed in Chapter VIII on the structure of nucleohistones.

As seen from all the melting profiles presented, the sharpness of the melting of DNA-histone complexes is generally less than that of pure DNA. For the melting of free DNA segments (first-step melting), the maximal slope is also related to the fractional hyperchromicity of that transition. Thus pure DNA has highest maximal slope, and with increasing histone/DNA ratio, the maximal slope is decreased. The same thing is also true for the melting of DNA-histone complexes (second-step melting). The higher the coverage by histone, the sharper the melting. Although it is very interesting to compare the intrinsic sharpness of the melting of DNA complexed with different histones and the effect of combination of histones on the transition breadth, for the above reasons, quantitative knowledge cannot be obtained. Qualitatively, it is however possible to compare the transition breadth for DNA complexes with the same fraction of total

hyperchromicity at this transition. Thus nucleohistones Ia and Ib of histone/DNA of 0.2 compare with other nucleohistones of histone/DNA of 0.4. For all about 30% of total hyperchromicity is due to the second-step transition. The maximal slope for nucleohistones Ia and Ib is 1.2 x 10^{-2} H_{260}/°C (1.8 x 10^{-2} H_{260}/°C for histone/DNA of 0.40), nucleohistone IIb, 1.0 x 10^{-2} H_{260}/°C, and nucleohistone IV, 1.2 x 10^{-2} H_{260}/°C. Thus the intrinsic sharpness of melting of DNA complexes with different histones is very similar. The maximal slopes for the complexes with mixture of histones with histone/DNA ratio of 0.4 is as follows: NH Ia/IIb, 1.0 x 10^{-2} H_{260}/°C, NH Ia/IV, 0.75 x 10^{-2} H_{260}/°C, NH IIb/IV, 0.6 and NH Ia/IIb/IV, 0.75 x 10^{-2} H_{260}/°C. Generally, the sharpness of melting of DNA complexed with a combination of histones is less than that of pure histone.

CHAPTER V

Stoichiometries of DNA-Histone Complexes

A. Determination of stoichiometry from melting profiles

An important parameter for characterization of nucleohistones is the stoichiometries of the complexes. Analytical determination of the histone/DNA ratio of the complex is not wholly satisfactory since, in most cases, it is difficult to obtain a completely histone-complexed DNA molecule. This is because the higher the histone/DNA ratio, the more difficult it is to prepare the complex. Since DNA-histone complexes have characteristic melting temperatures and since at subequivalent histone/DNA ratio the melting profiles are biphasic, it is possible to determine the amount of free DNA segments and the amount of complexed segments from the melting profiles. Determination of the ratio of free DNA to the complexed DNA from the melting profiles and the ratio of histone to total DNA thus constitutes one of the most direct ways of assessing the stoichiometries of DNA-histone complexes.

Two important assumptions are made in this kind of determination. Firstly, it is assumed that the reaction of histone with DNA is complete. There must be no considerable amount of free histone in the complex, so that the histone/DNA ratio is a measure of the ratio of histone complexed to DNA. Secondly, the melting of free DNA segments and of complexed segments is assumed to be independent. Thus the measure of the amount of first-step and second-step transition is assumed to be a measure of the amount of free DNA and of the

histone-DNA complex. The validity of these two assumptions will be examined in the following sections.

B. Completeness of DNA-histone complex formation

The amount of free histone in the reconstituted complex was determined by separation of free histone from DNA and DNA-histone complex by high speed centrifugation. The $s_{20,w}$ of DNA is 26.44 and that of histone ca. 1. Centrifugation at 130,000 g for 21 hr pellets DNA and the associated histone while free histone largely remains in the supernatant. Nucleohistone Ia at concentration of 1.3 A_{260} unit and in 0.005 M Tris, pH 8.0 and 0.005 M NaCl was centrifuged with Spinco Model L ultracentrifuge, #SW-39 rotor. The chamber temperature was set at 10°F. After centrifugation, the amount of histone in the supernatant and in the pellet was determined by the method of Lowry (Lowry et al., 1951), and of DNA by absorption at 260 mμ. The results are presented in Table VIII. The amount of histone in the supernatant is 2 to 5% and of DNA, 1 to 2% of the total. The few per cent of histone found in the supernatant is of the order of the error of this method of determination. It can be concluded that most histone accompanies DNA to the pellet, and that complex formation is very complete. Histone I is the most labile histone species in the sense that it is most readily dissociated from DNA. Thus it is the first histone species to be dissociated by increasing salt concentration (Ohlenbusch et al., 1967) or by decreasing pH (Johns, 1964), and has the lowest binding

TABLE VIII

Completeness of DNA-Histone Complex Formation

Nucleohistone Ia ($\frac{\text{Lysine + Arginine}}{\text{Nucleotide}}$)	DNA in		Histone in	
	Supernatant %	Pellet %	Supernatant %	Pellet %
0.6	1	99	2	98
0.8	2	98	5	95

*DNA-histone Ia complex (1.3 A_{260} unit) in 0.005 M Tris, pH 8.0 and 0.005 M NaCl was centrifuged at 130,000 g for 21 hr in the cold. Distribution of DNA and histone after centrifugation is presented. Under the same conditions, free histone remains in the supernatant.

coefficient (Akinrimisi, Bonner and Ts'o, 1965). Completeness of complex formation with other histone species may logically be expected. The validity of the first assumption of section A is therefore justified.

C. Independence of melting of the two concerned components

The irreversibility of the DNA-histone complex during the time and temperature range of the melting experiment is already evident from the biphasic nature of melting profiles. A further experiment was carried out to demonstrate this phenomenon. A known amount of DNA and of nucleohistone Ia were mixed and melted side by side with pure DNA and with nucleohistone Ia alone. The actual melting profile of the mixture follows closely the profile calculated from the profiles of the two components measured separately as shown in Figure 11. Therefore, the second assumption raised in section A is valid. The mid-point between the two melting temperatures may be taken as a fair separation point for the two transitions. The percentage of hyperchromicity relative to pure DNA at $T_{m,1}$ for the biphasic curve is 50%. This value minus 1.5% contributed by NH Ia is 48% and is in excellent accord with the 48% of pure DNA added to the mixture. Quantitative recovery of DNA mixed with nucleohistone from the melting profile was also obtained from mixtures of DNA and nucleohistone Ia of (lysine + arginine)/nucleotide of 0.60 which has itself 23% melting at $T_{m,1}$.

FIGURE 11. Melting profile of mixture of DNA and nucleohistone Ia. Nucleohistone Ia with (lysine + arginine)/nucleotide ratio of 0.80 was mixed with pure DNA (DNA/NH Ia = 48/52) and melted in 2.5×10^{-4} M EDTA, pH 8.0 (Δ——Δ). The dash line is the curve calculated from melting profiles of DNA (O——O) and nucleohistone Ia (□——□) according to their relative amount in the mixture.

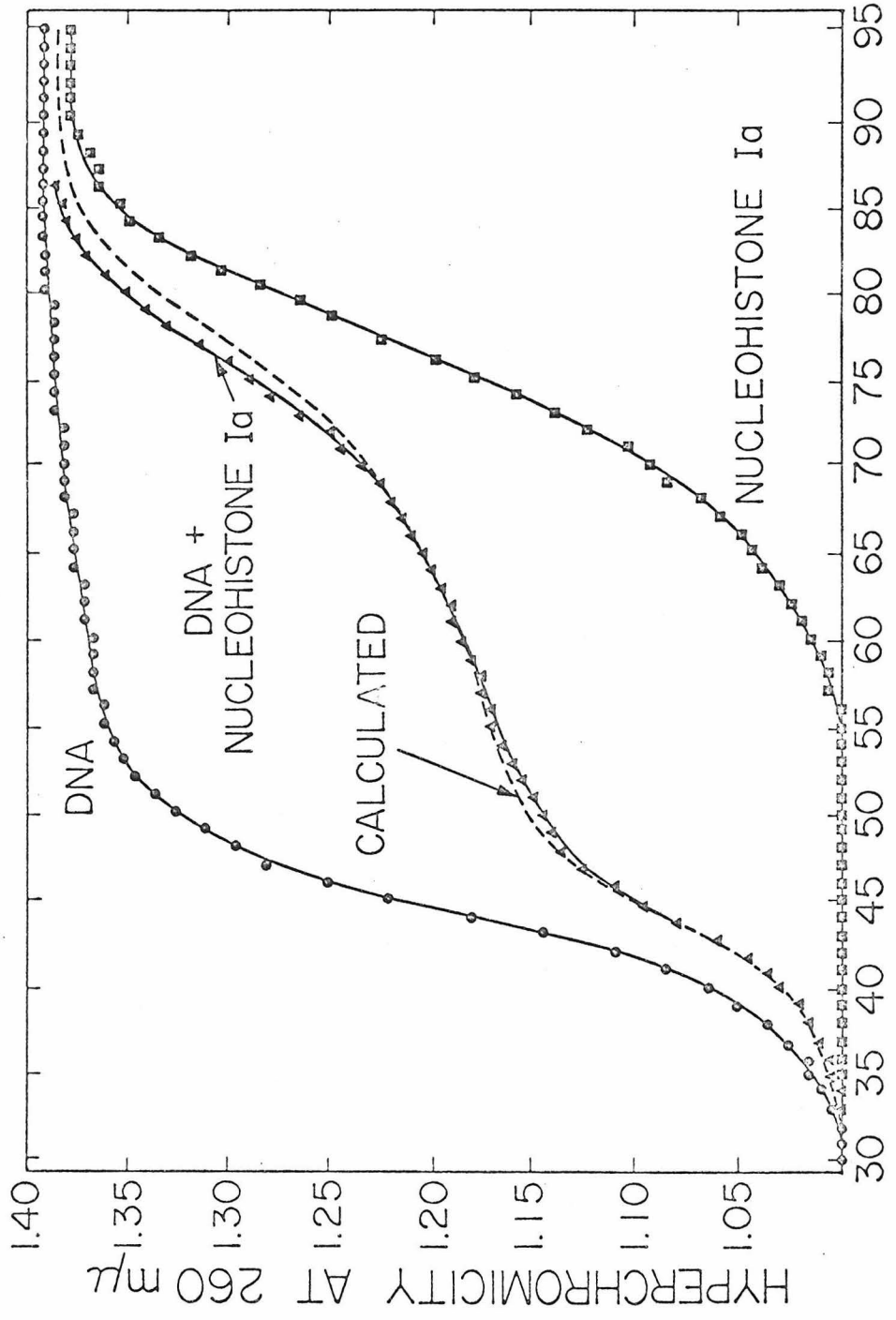
HYPERCHROMICITY AT 260 mμ
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
30 35 40 45 50 55 60 65 70 75 80 85 90 95
TEMPERATURE, °C
DNA
DNA + NUCLEOHISTONE Ia
CALCULATED
NUCLEOHISTONE Ia

FIGURE 11

D. Stoichiometries of DNA complexes with purified histone fractions

Since some complexes exhibit serious turbidity increase at high temperature, the magnitude of hyperchromicity at $T_{m,2}$ is not suitable for a measure of the amount of complexed DNA region. However, the fraction of total expected hyperchromicity at $T_{m,1}$ provides a good measure of the amount of free DNA segments and is inversely proportional to histone/DNA ratio. This result is shown in Figure 12. The histone/DNA ratio at the point of absence of free DNA melting is the stoichiometric ratio of the complex. The stoichiometric ratio in terms of their lysine and arginine to nucleotide ratio is as follows: nucleohistone Ia, 0.8; nucleohistone Ib, 0.8; nucleohistone IIb, 1.2; and nucleohistone IV, 1.5. In terms of total amino acid residues of histone to nucleotide residues of DNA, the stoichiometries are as follows: nucleohistone Ia, 2.7; nucleohistone Ib, 2.8; nucleohistone IIb, 5.6; and nucleohistone IV, 6.3.

In histones Ia and Ib, lysine and arginine are the only cationic amino acid residues (histidine only 0.2 mole %, see Table I). The stoichiometry of nucleohistones Ia and Ib of 0.8 would indicate that the phosphate group of DNA is not completely bound to cationic amino acid side chains in these nucleohistones. If it is assumed that all of the lysine and arginine groups of these two histone species are bound to phosphate groups, at least 20% of DNA phosphates are free in nucleohistones Ia and Ib. In contrast, the stoichiometries of nucleohistones IIb and IV are considerably higher than the unity. Taking further account of the histidine residue (Table I), the total cationic

FIGURE 12. Percentage of hyperchromicity at the first-step melting as a function of histone/DNA ratio. Percentage of hyperchromicity at $T_{m,1}$, the hyperchromicity up to the mid-point between $T_{m,1}$ and $T_{m,2}$, is plotted against the (lysine + arginine)/nucleotide ratio. □—□ is nucleohistone Ia; O—O, nucleohistone Ib; X—X, nucleohistone IIb; and Δ—Δ, nucleohistone IV. Each point represents average of 2 to 5 determinations.

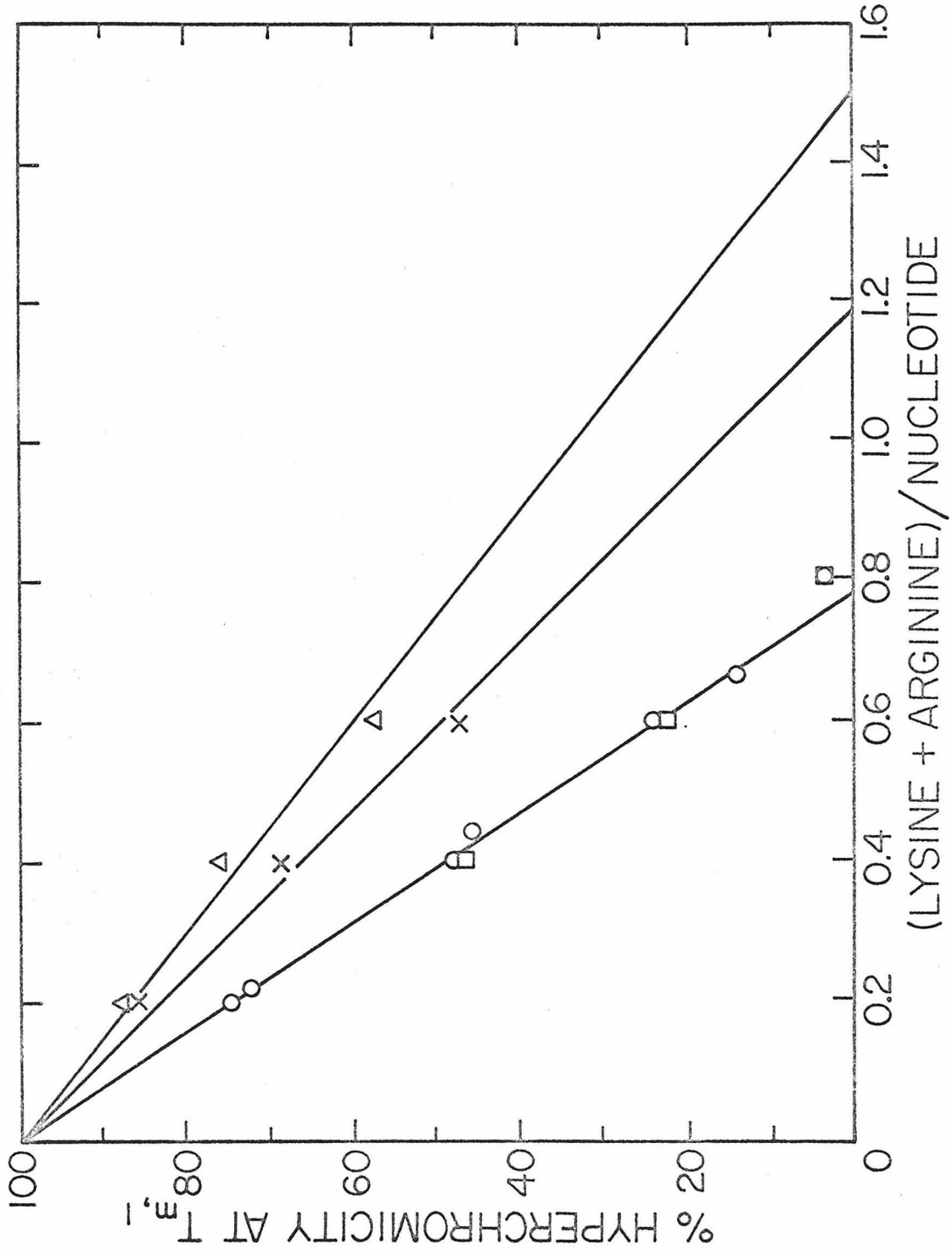
% HYPERCHROMICITY AT $T_{m,1}$
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
(LYSINE + ARGININE)/NUCLEOTIDE

FIGURE 12

group to nucleotide ratios are nucleohistone IIb, 1.35 and nucleohistone IV, 1.6. Although any molecular aggregation of these histones would tend to increase this value, it does suggest that not all of the cationic amino acid side chains are bound to DNA phosphates. In DNA complexes with homopolypeptides, such as poly-L-lysine and poly-L-arginine, the stoichiometric ratio is one to one (Tsuboi, Matsuo and Ts'o, 1966; Higuchi and Tsuboi, 1966; and Part II of this Thesis).

Study of the interaction of toluidine blue (a cationic dye) with calf thymus nucleohistone, indicates about half of the phosphates are free (Miura and Ohba, 1967). Electrometric and spectrophotometric titration study of native nucleohistone complex shows 80% of the lysine and arginine residues are not titratable, presumably bound to DNA phosphates, but 20% still accessible for titration as in free histone (Walker, 1965). There is, therefore, a considerable fraction of lysine and arginine residues of histones which are not bound to DNA phosphate groups in the native state. The implication of the present finding to the structure of nucleohistone will be further discussed in Chapter VIII.

E. Stoichiometries of DNA complexes with combination of histones

Is the stoichiometry of complexes of DNA with combination of histone fractions the same or different as with complexes of individual histone fractions to DNA? From Figure 13, the stoichiometric ratio of nucleohistone Ia/IIb with equal molar lysine and arginine of Ia and IIb is found to be 1.0. This is an exact average of NH Ia, 0.8 and

FIGURE 13. Per cent hyperchromicity at $T_{m,1}$ as a function of histone/DNA ratio of DNA complexes with histone combinations. O—O is nucleohistone Ia/IIb; Δ——Δ, nucleohistone Ia/IV; and X---X, nucleohistone IIb/IV. Each point is an average of two determinations. Nucleohistones were prepared with mixtures of two histones of equal molar lysine and arginine residues. Melting profiles of nucleohistone IIb/IV were obtained from 12,000 g, 20 min supernatant, so that the histone/DNA ratio is only tentative.

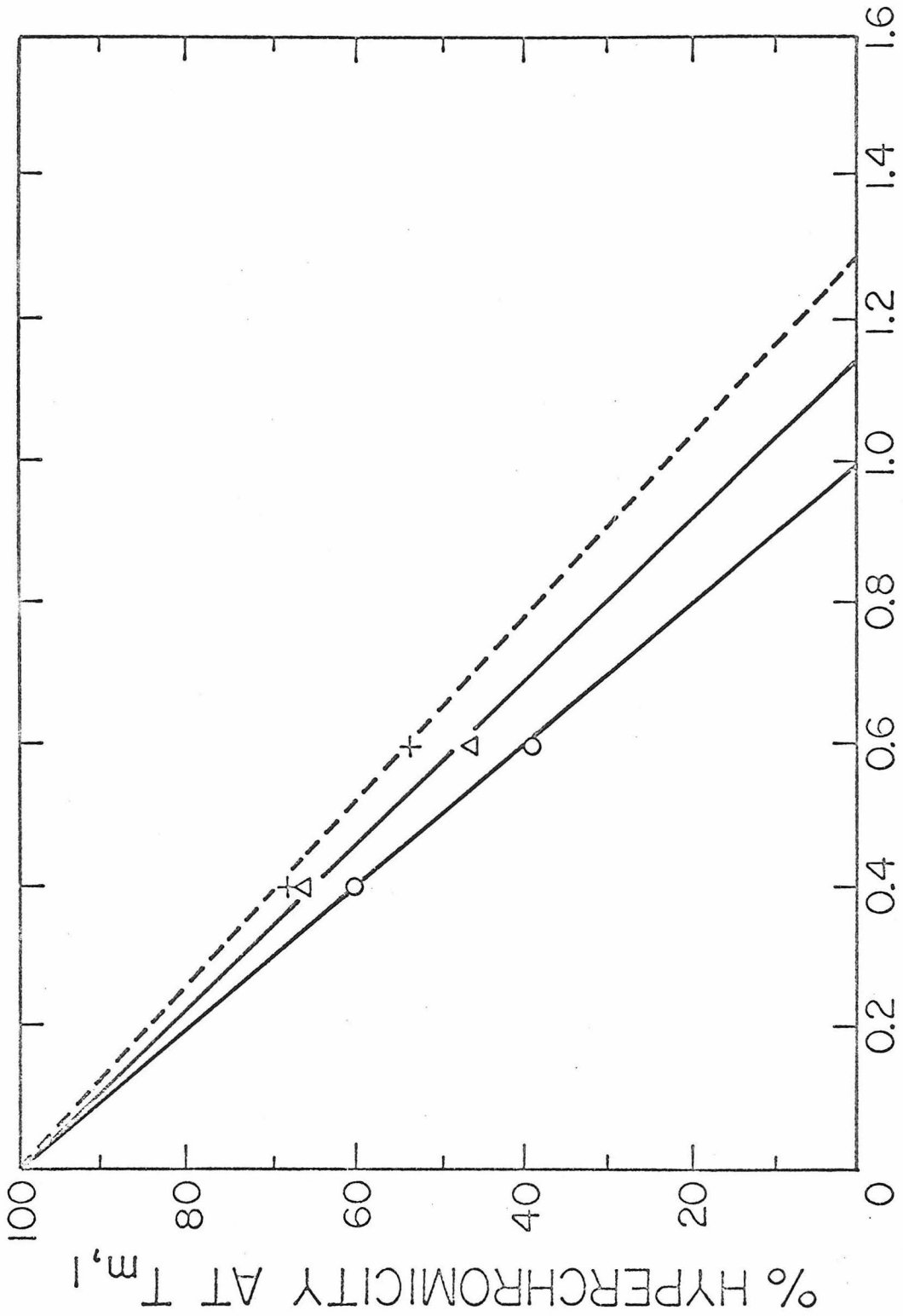
% HYPERCHROMICITY AT $T_{m,1}$
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
(LYSINE + ARGININE)/NUCLEOTIDE

FIGURE 13

NH IIb, 1.2. The stoichiometry of nucleohistone Ia/IV is 1.15, similar to the average of NH Ia (0.8) and NH IV (1.5). For nucleohistone IIb/IV, melting profiles can only be obtained from their 12,000 g, 20 min supernatant, and the recovery of DNA in the supernatant is not complete (Chapter IV, section A). The histone/DNA ratio is not exactly equal to the input, and the stoichiometric ratio of 1.3 obtained in Figure 13 is therefore only tentative. This value nonetheless lies between those of NH IIb and NH IV. The picture seems to be correct, that the characteristic stoichiometric ratios of individual nucleohistone are retained in the DNA complexes with histone mixtures.

CHAPTER VI

UV Absorption Spectra of Histones and DNA-Histone Complexes

Absorption spectra of DNA in the near UV region is mainly due to the conjugated heterocyclic ring systems of purine and pyrimidine bases of DNA. If the UV spectrum of DNA is changed by complexing with histone, it would suggest that the interaction of DNA and histone involves reaction with DNA bases. UV absorption spectra of DNA complexes with various histones and the spectra of these histone species will be presented in this chapter. The implication of these findings for the molecular structure of nucleohistone will be discussed in Chapter VIII together with evidence from other lines. All the spectra were taken with Cary 11 recording spectrophotometer.

A. UV absorption spectra of histones

The spectra of the purified histone fractions are presented in Figure 14. The spectrum of histone IIb taken at higher concentration shows a clear maximum at 277 mμ and possesses a molar absorptivity of 49 in terms of molar amino acid residues ($\varepsilon_{\text{amino acid}}$). The small UV absorption of histones at wavelength above 240 mμ is consistent with the fact, that they contain no tryptophan residues and only small amounts of tyrosine (0.7 mole % in Ib, 3.0 in IIb and 3.5 in IV), and phenylalanine (0.6 mole % in Ib, 1.3 in IIb and 1.9 in IV). The absorption at 260 mμ of all histone species is less than 0.4%

FIGURE 14. UV absorption spectra of histones. Histone Ib (-·-·-), IIb (———) and IV (-----) were dissolved in 0.01 M Tris buffer, pH 8.0 at the concentration of 2.0×10^{-3} M in amino acid residues. Molar extinction coefficients (in terms of molar amino acid residue) versus wavelength are shown.

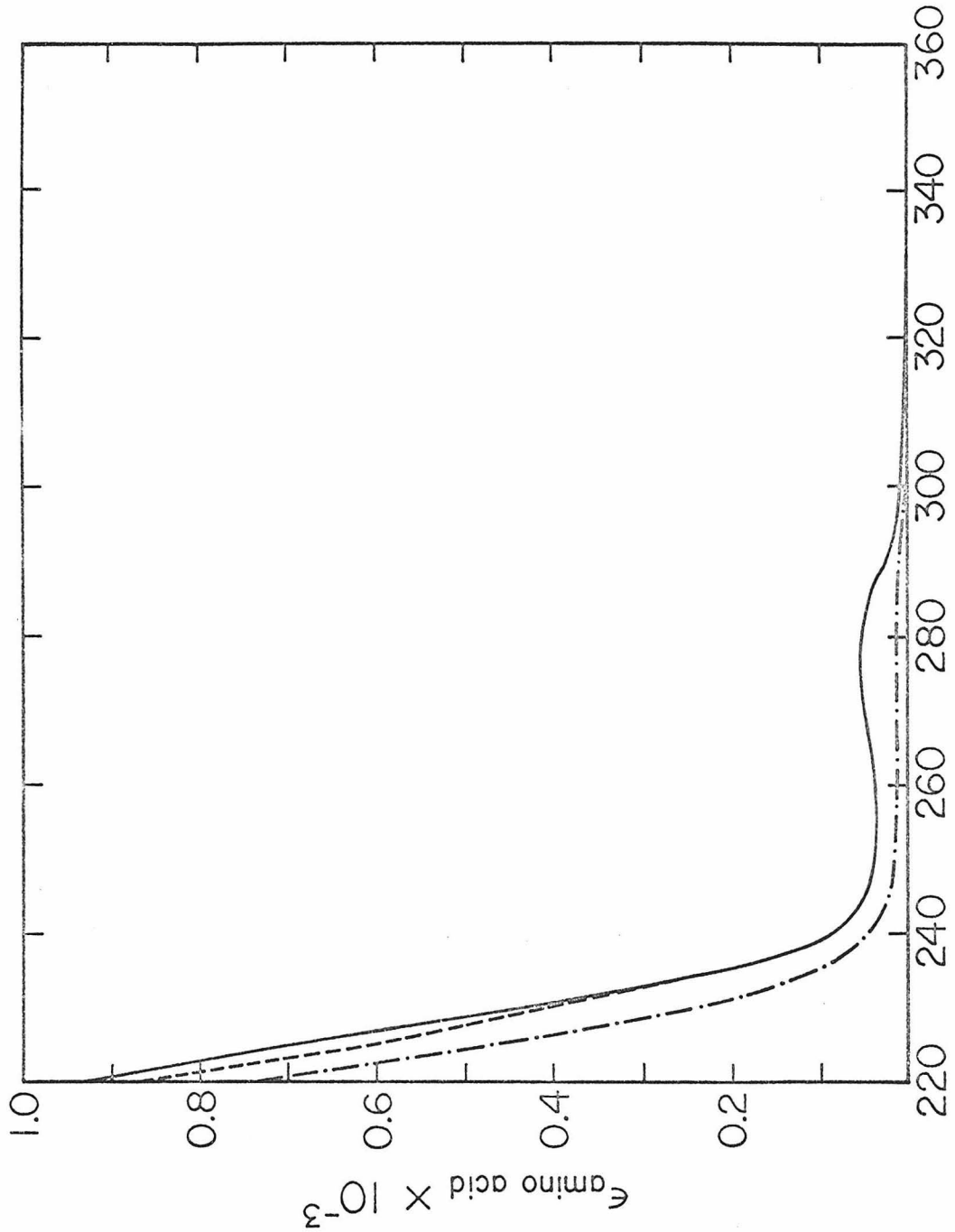
1.0
0.8
0.6
0.4
0.2
$\epsilon_{amino\ acid} \times 10^{-3}$
220
240
260
280
300
320
340
360
λ, mμ

FIGURE 14

that of DNA on equal molar monomer basis. However, strong UV absorption is found below 240 mμ.

B. Absorption spectra of nucleohistone Ia

Absorption spectra of DNA-histone Ia complexes of various coverage are presented in Figure 15. The spectra have all been normalized to the same maximum of 1.0 for convenience of comparison. The absorption maximum of DNA at 258 mμ is not changed by complexing with histone Ia. The minimum at 230 mμ is increasingly red-shifted by increasing coverage with histone Ia. The absorbance below 240 mμ also shows increased magnitude in nucleohistone. These two apparent changes can be accounted for by the superimposition of histone absorption on that of DNA. The general shape of the DNA spectrum is, therefore, not changed by complexing with histone Ia.

C. Absorption spectra of nucleohistone IIb

Absorption spectra of DNA-histone IIb complex are presented in Figure 16. There is a considerable scattering effect on the spectrum of histone/DNA complex at a ratio of 0.60. However, the scattering is not serious at lower histone/DNA ratios. The maximum of DNA absorption is also not changed by the presence of histone IIb. The apparent changes at shorter wavelength, can also be accounted for by superimposition of histone absorption and of the scattering effect.

FIGURE 15. UV absorption spectra of nucleohistone Ia. Nucleohistone Ia with (lysine + arginine)/nucleotide ratio of 0 (———), 0.40 (-----), 0.60 (– – – –) and 0.80 (–·–·–) are dissolved in 2.5×10^{-4} M EDTA, pH 8.0. Solvent absorption is substracted and spectra are all normalized to the same maximum.

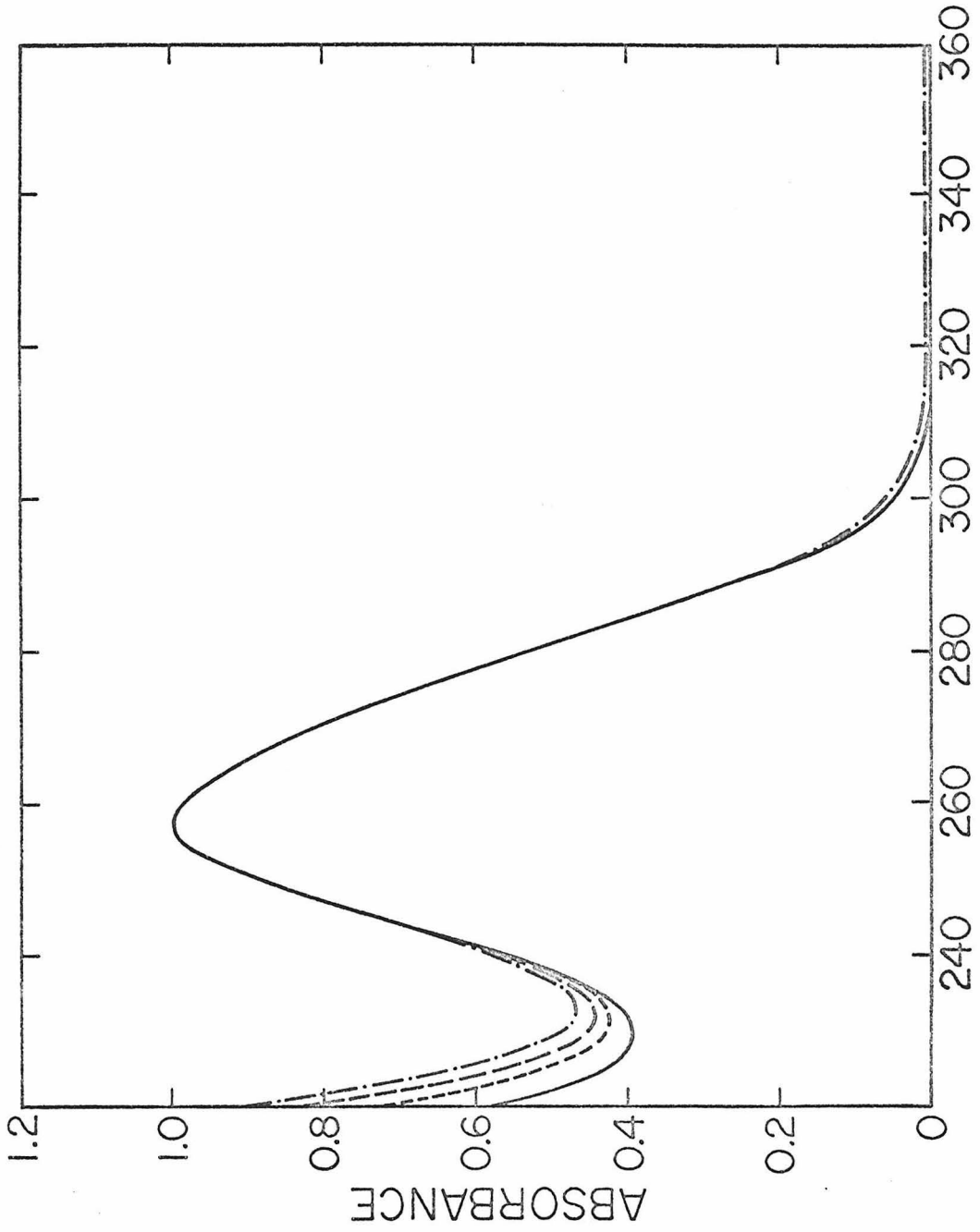

FIGURE 15

FIGURE 16. UV absorption spectra of nucleohistone IIb. Nucleohistone IIb with (lysine + arginine)/nucleotide ratio of 0 (———), 0.20 (-----), 0.40 (— — —) and 0.60 (—·—·—) were dissolved in 0.01 M Tris buffer, pH 8.0. Absorption of solvent is subtracted and spectra are all normalized to the same maximum.

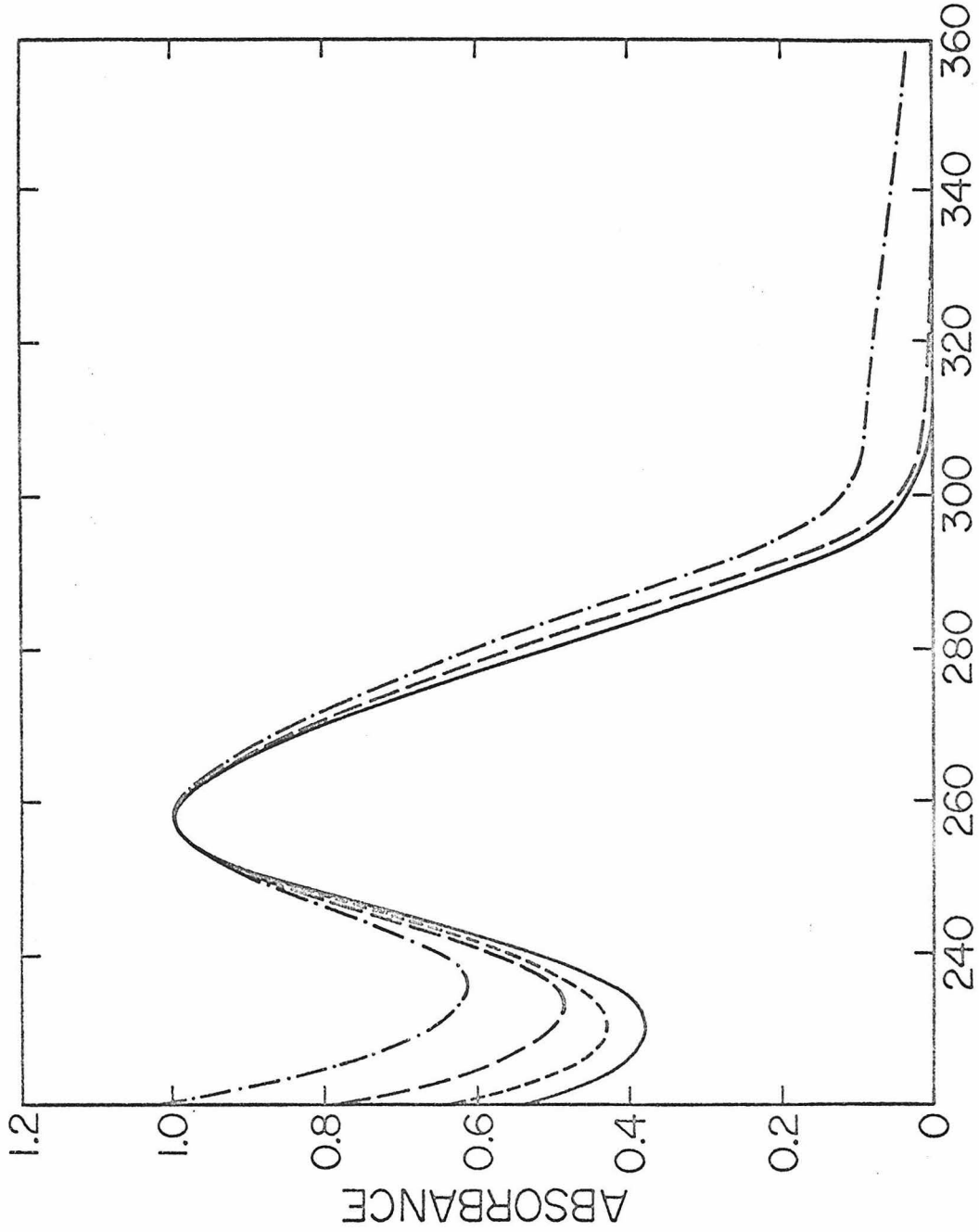

FIGURE 16

D. Absorption spectra of nucleohistone IV

Figure 17 shows the absorption spectra of DNA-histone IV complex with various histone/DNA ratio. Again, the changes of the spectra at shorter wavelength is due to histone absorption. The general shape of DNA spectrum is unaltered.

FIGURE 17. UV absorption spectra of nucleohistone IV. Nucleohistone IV with (lysine + arginine)/nucleotide ratio of 0 (———), 0.20 (-----), 0.40 (– – – –) and 0.60 (–·–·–) were dissolved in 0.01 M Tris buffer, pH 8.0. Absorption of solvent is subtracted and all spectra are normalized to the same maximum.

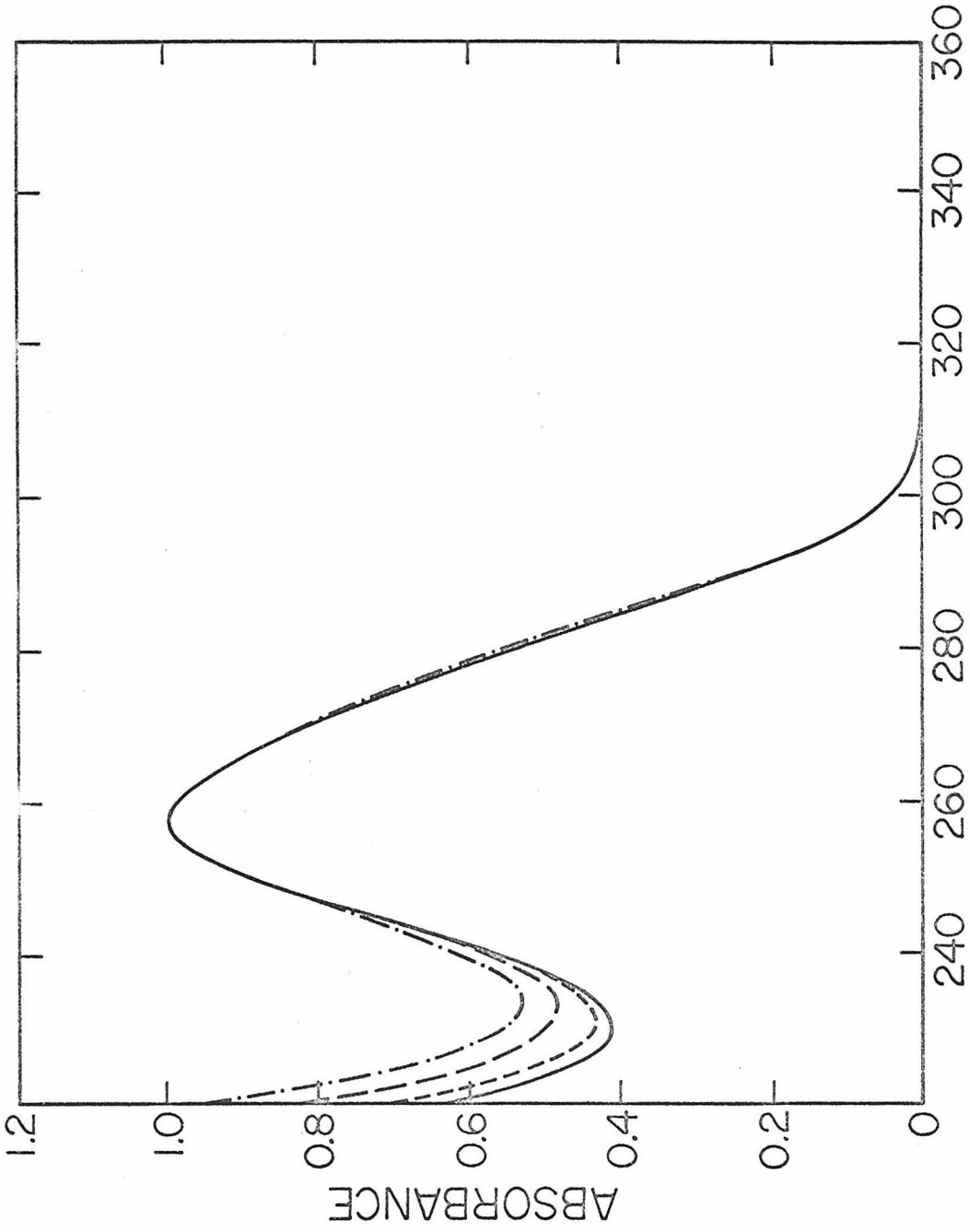

FIGURE 17

CHAPTER VII

Template Activity of DNA-Histone IV Complex in Support of RNA Synthesis

Histones Ib and IIb have been shown to inhibit the template activity of DNA in support of RNA synthesis in DNA-dependent RNA polymerase system (Huang, Bonner and Murray, 1964). The role of arginine-rich histones has however remained controversial. There are several speculations about the reason why different histones have such different behaviors. Histone-DNA interaction is mainly due to ionic reaction of lysine and arginine residues with DNA phosphates. Histone I contains most of its cationic amino acid residue as lysine (lysine/arginine, 8.5 to 15.4) and histone IV as arginine (lysine/arginine, 0.8). Is their different effect on DNA template activity due to differences in their basic amino acid composition? As shown in Part II of this Thesis, both polylysine and polyarginine quantitatively block the template activity of DNA. The arginine residue is as effective as lysine residue in inhibition of DNA template activity. The protamine complex of DNA in which 70% of the amino acids are arginine, is also inactive in support of RNA synthesis. Therefore, the differences are not due to different proportion of lysine and arginine content. Since the correlation between the effect of histone fractions on DNA template activity and the stabilization of DNA against thermal denaturation is a well-known phenomenon (Huang, Bonner and Murray, 1964; Hnilica and Billen, 1964), the less extent of inhibition by arginine-rich histones

may be due to imperfect DNA complex formation. In the present study, in which improved methods and pure histone preparations are used, histone IV has been shown to stabilize DNA against thermal denaturation. In fact, the T_m of DNA-histone IV complex is higher than that of DNA-histone I complex. The template activity of DNA-histone IV in support of RNA synthesis has, therefore, been examined. Figure 18 shows the template saturation curves of DNA-histone IV complex in support of RNA synthesis and the derived rate constants are presented in Table IX. Detailed description of experimental procedures and methods of data analysis are presented in Part II of this Thesis. It is clear that the inhibition of DNA template activity is quantitative, and that the template concentration required for half saturation of a finite amount of enzyme is not appreciably changed by complexing with histone. The last two columns of Table IX show the determined template activity (V_{max}/K) and the calculated percentage of free DNA segments. It is concluded that DNA complexed with histone IV is inactive in support of RNA synthesis.

FIGURE 18. Template saturation curves of DNA-histone IV complex. DNA-histone IV complex with histone lysine and arginine to DNA phosphate ratio of 0 (O—O), 0.60 (□—□) and 0.80 (Δ—Δ) were incubated for RNA synthesis. The reaction mixture contained 10 μmoles of Tris buffer, pH 8.0, 1 μmole of magnesium acetate, 0.25 μmole of $MnCl_2$, 3 μmoles of β-mercaptoethanol, 0.1 μmole each of C^{14}-ATP (1 μc/μmole), GTP, CTP and UTP, and indicated amount of template in a total volume of 0.25 ml. Reaction was initiated by addition of 30 μg of E. coli RNA polymerase (f_4 fraction) and incubated at 36°C for 5 min. Reaction was stopped by 10% TCA. After filtration and washing through membrane filters, radioactivity was counted by Beckman LS-200B liquid scintillation system. Computer fitted curves are shown.

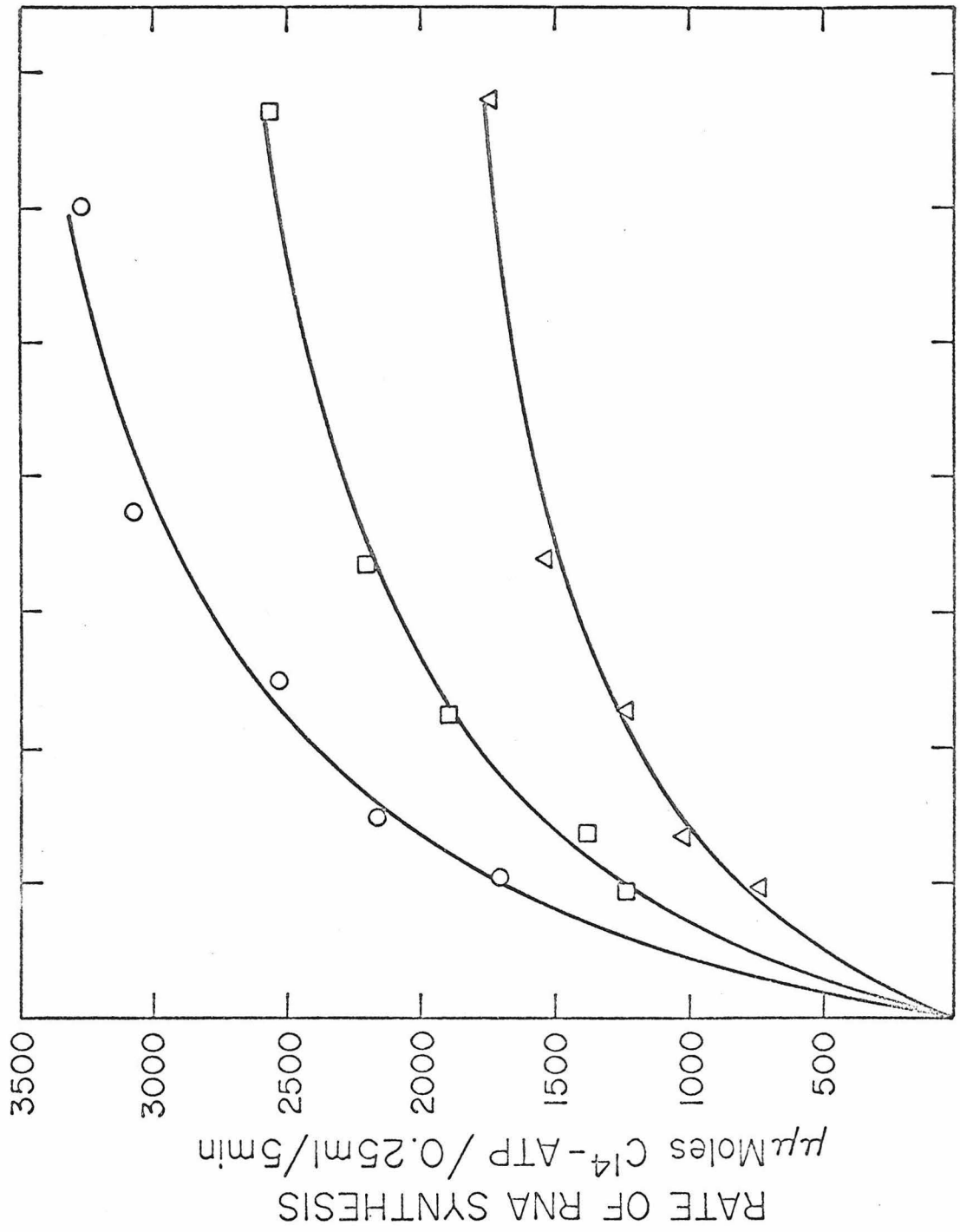
RATE OF RNA SYNTHESIS
μμMoles C14-ATP /0.25ml/5min
3500
3000
2500
2000
1500
1000
500
0
2
4
6
8
10
12
14
DNA CONCENTRATION, μg/0.25 ml

FIGURE 18

TABLE IX

Template Activity of DNA-Histone IV Complex

Lysine + Arginine / Nucleotide	V_{max} (μμmoles ATP/ 5 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 5 min/μg DNA)	%	% Free DNA regions*
0	4,094 ± 178	2.82 ± 0.36	1,451 ± 127	100	100
0.60	3,192 ± 120	3.18 ± 0.32	1,002 ± 69	69	60
0.80	2,192 ± 129	3.38 ± 0.53	649 ± 67	45	47

*Calculated from stoichiometry of histone IV lysine and arginine to DNA phosphates of 1.5.

‡Rate constants were calculated by direct fitting of experimental data of Figure 19 to Michaelis-Menten equation with proper weighting factors on IBM System/360 Model 50 computer.

CHAPTER VIII

Structure and Function of Nucleohistones

Physical characterizations of the DNA complexes with various purified histone fractions prepared as described in Chapter II are presented in Chapters III to VI. The template activity of nucleohistone IV in support of RNA synthesis is presented in Chapter VII. In this chapter, the author intends to discuss the implication of the experimental findings to the understanding of the molecular structure and function of nucleohistones and with other known facts to construct a molecular picture of nucleohistones to the limit of present knowledge.

Thermal denaturation of double helical DNA of high molecular weight may be divided into two kinds of processes: (1) The nucleation stage, i.e., denaturation first occurs at some labile loci of the long DNA strand and is followed by growth of denatured regions. (2) The merging together of the adjacent denatured loci and shortening of the intervening helical region. This picture is consistent with the electron microscopic observation of DNA partially denatured in the presence of formaldehyde (Beer and Thomas, 1961) which shows "puddles" of denatured loci. Recent theoretical calculation of the melting curves for DNA show that at the T_m of the transition the average length of helix plus adjacent coil section is approximately 600 base pairs (Crothers, 1968). Therefore, a distinctive transition to be visible in the melting profiles requires a homogeneous segment of DNA (either "naked" or complexed with histones) of several hundred

base pairs. A molecule of histone IV has only 27 cationic amino acid side chains and histone I about 65. A single molecule of histone, therefore, only occupies a segment of DNA of less than 50 base pairs. The biphasic nature of the melting profiles suggests that very long stretches of DNA are completely "naked," while other long stretches are complexed with several histone molecules situated side by side. This kind of cooperative binding of proteins to DNA is similar to that formed with polylysine, polyarginine and protamine as discussed in Part II of this Thesis.

From the melting profiles, it can be noted the DNA complexes with various histone species each has its characteristic transition temperature. This fact provides positive evidence that the complexes prepared in the present way have homogeneous and characteristic molecular structures. This provides a starting point for further study of the characteristic structure of nucleohistones of DNA complexed with various histone species. In the absence of such characteristic structure one deals with DNA-histone complexes of a variety of molecular conformations because of complexity of DNA-histone interaction. The author would like to emphasize, however, that the above statement is a one-way argument. A complex without a characteristic T_m need not have no characteristic structure. If histone molecules were not so cooperatively disposed on DNA, the various lengths of short DNA segments between complexed regions would also lead to heterogeneous melting.

DNA complexes with combinations of several histone species show only biphasic instead of multiphasic melting although T_m of nucleohistones I differs from those of nucleohistones IIb and IV by $6° \sim 8°C$. This would imply that mixtures of histones bind heterogeneously to DNA, and that there is no very long stretch completely complexed with many identical histone molecules. The sharpness of the transition is generally less than that of DNA complexed with a single histone species. This also indicates the heterogeneity of the complexed region. The biphasic nature of the melting profiles suggests that cooperativeness of binding operates even in the presence of mixture of histones.

The general shape of UV absorption spectrum of DNA is not changed by complexing with histone (Chapter VI). DNA bases are the main UV absorbing chromophores in this region. It can therefore be concluded that there is no major interaction of histones with the bases to cause changes in absorption spectrum. The bases are stacked at the core of DNA double helical structure. The interaction of histone to DNA mainly occurs at the outside of this core and through the phosphate groups of DNA. Although no shift of UV absorption maximum has been found for the DNA-histone complex, less hypochromicity has been observed for nucleohistone than pure DNA (Bonner and Tuan, 1968). This is interpreted as due to a slight tilting of DNA bases in nucleohistone. In the present study, whether there is decreased hypochromicity in the reconstituted complexes has not been elaborately explored.

DNA complexes with poly-L-lysine and poly-L-arginine have very high T_m, 98°C, in comparison with the T_m of nucleohistones, 75° to 83°C. Protamine, which contains 70 mole % of its amino acid residues as arginine, has its T_m very close to that of polyarginine. At first look, the non-basic amino acid residues of histones would appear to have the effect of decreasing T_m of their DNA complexes. Since DNA-polylysine and DNA-polyarginine have similar T_m, it appears that the cationic side chains of lysine and arginine have similar effects on stabilizing the double helical structure of DNA. As is shown in Part II of this Thesis, DNA complexes with these homopolymers possess a stoichiometry of one lysine or arginine residue per nucleotide residue. Each consecutive DNA phosphate is bound to each consecutive cationic side chain of the polypeptides. The lower T_m of nucleohistone as compared with that of homopolymer complexes would suggest this is not the picture for nucleohistones. Molecular model building shows that the polyarginine chain can fit snugly into the small groove on the DNA molecule with each arginine side chain matched to each DNA phosphate (Wilkins, 1956). Lysine side chains are only slightly shorter than those of arginine and may also assume the same structure.

As discussed in Chapter V, the stoichiometric ratio for the charged residues is not unity for nucleohistones Ia and Ib. The ratio is 0.8 cationic side chain per nucleotide. If one assumes all of the lysine and arginine residues are bound to DNA, there must still be 20% of DNA phosphates which are free. Histone I contains 9 mole % of its amino acid as proline (Table I) which interferes with helix

formation, and this histone exhibits, therefore, a low content of α-helical structure as studied by optical rotatory dispersion (Bradbury et al., 1965; Jirgensons and Hnilica, 1965). In solution and solid film, infrared studies also show the presence of β-structure in histone I (Bradbury et al., 1967). Histone I molecules appear, therefore, to assume an extended chain conformation. Partial sequence studies on one pure component of this class of histone molecules show three-fourths of the 61 lysine and 22 proline residues and one-half of the 56 alanine residues to be in the C-terminal half of the molecule (Bustin et al., 1969). A most probable structure for nucleohistone I is as follows: The DNA binding part of the histone molecule is in extended chain conformation. The spacing of cationic side chains is longer than that of DNA phosphates, so that each consecutive charged side chain of histone I cannot match each consecutive phosphate. At least 20% of phosphate groups are skipped over and are thus free. This kind of molecular structure is consistent with the following observations: (1) low T_m (Table VI); (2) low binding coefficient for DNA (Akinrimisi, Bonner and Ts'o, 1965); (3) easy dissociation by salts (Ohlenbusch et al., 1967); (4) high percentage of free phosphates in native nucleohistone as studied by interaction with toluidine blue (Miura and Ohba, 1967); (5) easy extraction by acid, such as 5% perchloric acid (Johns, 1964).

The stoichiometric ratios of cationic side chains of histone to phosphate groups of DNA observed for nuclechistones IIb and IV are considerably higher than unity and are very distinct from that of

nucleohistone I. One must be aware of the possibility of artifacts in the stoichiometric ratio caused by aggregation of histone molecules. In fact, such aggregation is the reason why reconstituted nucleohistone IV showed no stabilization of DNA against thermal denaturation as observed by earlier workers. In the present study, introduction of urea during the process of salt gradient dialysis diminishes this complication very much. The stoichiometric ratio of (lysine + arginine)/nucleotide for nucleohistone IV of 1.5 is still somewhat abnormally high. It indicates that some slight molecular aggregation is still present. But as compared with the stoichiometry of 1.2 observed for nucleohistone IIb, it suggests that in these nucleohistones the cationic side chains of histones are not completely bound to DNA phosphates. It may be that a considerable fraction of lysine and arginine residues which are not in the DNA binding part of the histone molecule are free. Such free basic groups have been observed in native nucleohistone by acid-base titration studies (Walker, 1965). In view of the following properties as compared to those of nucleohistones I (see references previously mentioned); (1) higher T_m; (2) higher binding coefficient; (3) more difficultly dissociated by salts; (4) essentially no extraction by 5% perchloric acid; (5) stoichiometric ratio higher than unity, a different molecular picture of the DNA-histone complexes is suggested. Longer segments of consecutive cationic side chains of histones IIb and IV appear to be bound to consecutive DNA phosphate groups. This would account for all of the above observations.

The complete amino acid sequence of calf thymus histone IV has already been worked out (DeLange et al., 1969). This is shown in Figure 19 with all of the cationic amino acid residues indicated by square boxes. The cationic residues can be grouped into two major and two minor clusters: A, residues #1 to #23; B, #35 to #45; C, #75 to #79; and D, #91 to #95. By assuming α-helical structure from residues #1 to #16 and #20 to #23, all of the cationic side chains in cluster A are in proper position to interact with DNA phosphates. The structure is shown in CPK model in Plate 1. Lysine and arginine side chains all assume extended configuration as determined by X-ray crystallography (Marsh and Donohue, 1967). Residues #8, #12, #16, #18 and #20 are all separated by equal distances, and the spacing between #3 and #8 is only slightly longer. The distance is compatible with spacing of DNA phosphates on the same strand. Residues #17, #19 and #23 are also separated by the same phosphate distance and can bind to the phosphates of opposite strands. By skipping two phosphate groups, residue #5 can also bind to a third phosphate. The histone molecule can sit at the large groove of DNA and assume the necessary curvature if only one or two hydrogen bonds of the long helix segment are broken. The bottom of the helix (faces the floor in Plate 1) is all composed of amino acids with short side chains, and can thus come into intimate contact with the DNA groove surface. The possible structure of cluster B is shown by CPK model in Plate 2. Residues #36 to #43 are in α-helical conformation. In this way, the cationic side chains of #35, #39, #43 and #45 are in proper position to react with DNA phosphate

FIGURE 19. Primary structure of calf thymus histone IV. Cationic amino acid residues are in square boxes. The dashed boxes indicate basic amino acid clusters. Residue #16 exists as free or acetylated in nature (DeLange et al., 1969).

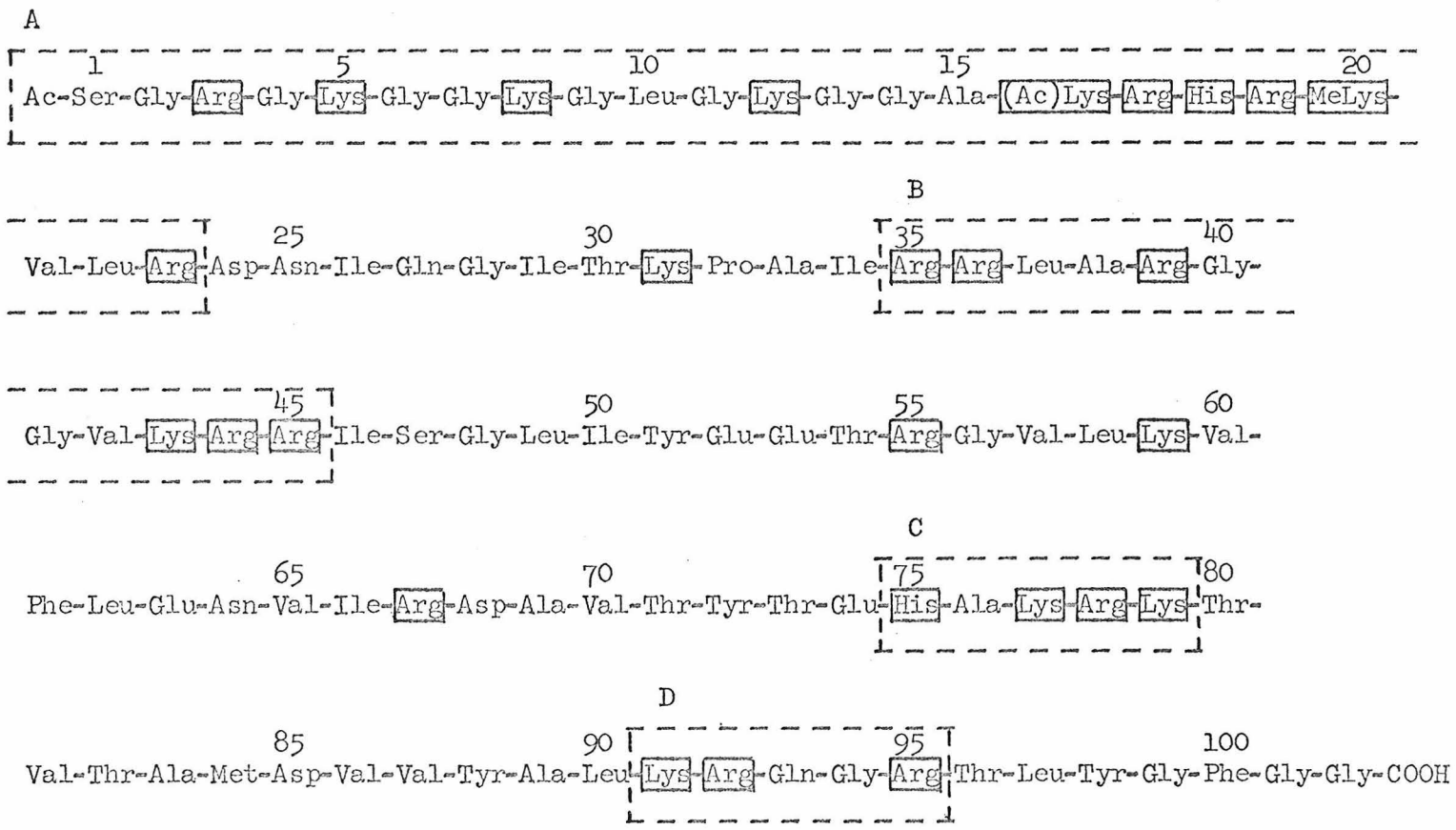
A
1 5 10 15 20
Ac-Ser-Gly-Arg-Gly-Lys-Gly-Gly-Lys-Gly-Leu-Gly-Lys-Gly-Gly-Ala-(Ac)Lys-Arg-His-Arg-MeLys-
B
25 30 35 40
Val-Leu-Arg-Asp-Asn-Ile-Gln-Gly-Ile-Thr-Lys-Pro-Ala-Ile-Arg-Arg-Leu-Ala-Arg-Gly-
45 50 55 60
Gly-Val-Lys-Arg-Arg-Ile-Ser-Gly-Leu-Ile-Tyr-Glu-Glu-Thr-Arg-Gly-Val-Leu-Lys-Val-
C
65 70 75 80
Phe-Leu-Glu-Asn-Val-Ile-Arg-Asp-Ala-Val-Thr-Tyr-Thr-Glu-His-Ala-Lys-Arg-Lys-Thr-
D
85 90 95 100
Val-Thr-Ala-Met-Asp-Val-Val-Tyr-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-COOH

FIGURE 19

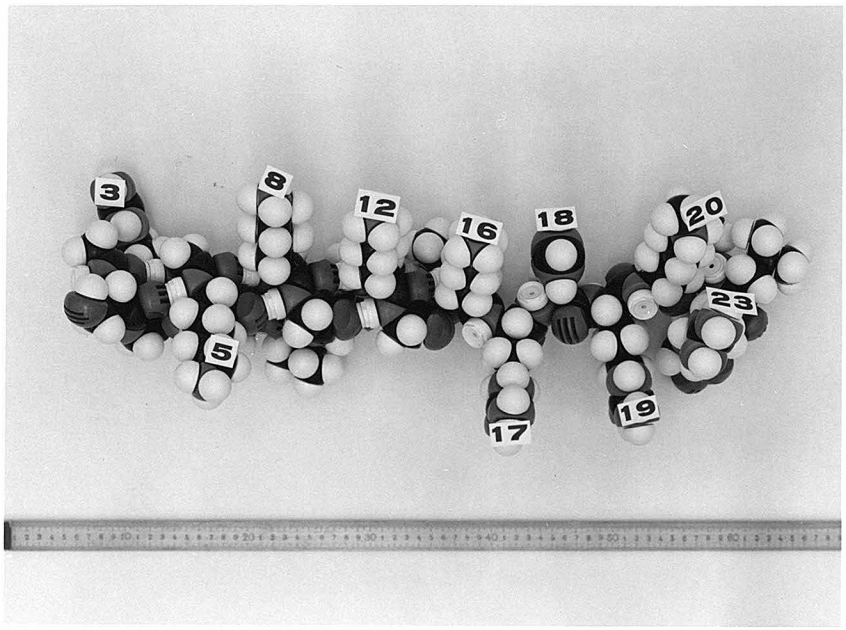

PLATE 1. CPK model showing the proposed structure of the binding part of the histone molecule from N-terminal end to residue #23 of calf thymus histone IV. Residue #1 to #16 and #20 to #23 are in right-handed α-helix conformation. All the cationic side chains (which carry the number labels) are in proper position to react with DNA phosphates provided the histone molecule lies parallel to the DNA grooves. Residue #16 is not acetylated.

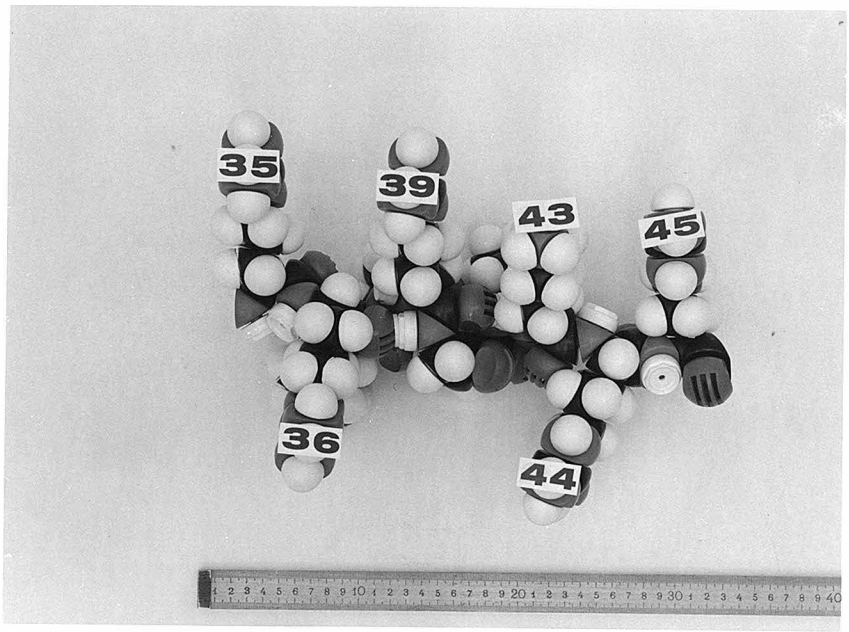

PLATE 2. CPK model showing the proposed structure of the binding part of calf thymus histone IV from residue #35 to #45. Residues #36 to #43 are in right-handed α-helix conformation. All the cationic side chains are in proper position to react with DNA phosphates, if the histone molecule lies parallel to DNA grooves.

groups on the same strand, and #36, #44 to phosphate groups of the opposite strand, skipping one phosphate in the middle.

The experimental evidence for the above outlined structure is still very weak. Conformational analyses of histones of all classes except histone I in solution show a marked increase in optical rotatory dispersion parameters with increasing salt concentration and/or binding to DNA (Bradbury et al., 1965; Jirgensons and Hnilica, 1965; Tuan, 1967). It is possible that the proposed helical regions in clusters A and B are the main source of this salt-sensitive conformational change. The screening effect of salt and DNA on histone cations would facilitate the formation of α-helical structure in these regions. This is, however, but a working hypothesis for designing further experimentation.

As discussed in Chapter VII, the controversy about the biological effect of histone IV binding to DNA has been settled. DNA-histone IV complex is inactive in priming RNA synthesis. Since all of the cationic proteins, such as histones Ib, IIb, IV, protamines, polylysine, and polyarginine, which form stable complexes with DNA and stabilize the DNA double helical structure are all shown to block the template activity of DNA in support of RNA synthesis, it is highly suggestive that all histone species normally complexed with DNA in chromatin have the function of repression of gene activity. The chromatin isolated from cell nuclei shows reduced capacity for transcription by RNA polymerase, the DNA regions complexed with histones are the inactive regions, and the active portion may be those regions

which are free from histone or complexed with nonhistone proteins. Although the notion becomes clear all the histone fractions participate in repression of DNA template activity, it is still evident that some histone species may play additional structural roles in the chromatin.

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APPENDIX

Template Activity of Calf Thymus Chromatin

The statistical method of data analysis presented in Part II of this Thesis for the determination of template activity of DNA-polypeptide complexes can also be used for assessing the template activity of chromatin. In this kind of analysis, all the experimental points of the template saturation curves can be fully used to determine the quantity of interest. Especially when many chromatin preparations are to be compared for their template activities, a range of variation of the determined value will give some idea about the statistical significance of differences of two values. The use of the initial slope of the saturation curve $[\lim_{A\to 0}\left(\frac{dv}{dA}\right)]$ as a measure of template activity can also eliminate the effect of change of K.

The purified chromatin as isolated by the procedure described in Part I of this Thesis was sheared by Virtis homogenizer at 25 volts for 90 sec. The template activity of the supernatant after centrifugation at 12,000 g for 30 min (sheared chromatin) has been determined. The procedure for assay of RNA polymerase activity is similar to that described in Part II. Figure 1 shows the template saturation curves and Table I the derived rate constants. The template activity is 26% that of pure DNA. K is essentially identical for DNA and chromatin.

FIGURE 1. Template saturation curves of calf thymus chromatin. Sheared calf thymus chromatin and DNA were incubated for RNA synthesis. The reaction mixture contained 10 μmoles of Tris buffer, pH 8.0, 1 μmole of magnesium acetate, 0.25 μmole of $MnCl_2$, 3 μmoles of β-mercaptoethanol, 0.1 μmole each of C^{14}-ATP (1 μc/μmole), GTP, CTP and UTP, and indicated amount of the template in a total volume of 0.25 ml. Reaction was initiated by addition of 30 μg of E. coli RNA polymerase (f_4 fraction) and incubated at 37°C for 10 min. Reaction was stopped by 10% TCA. After filtration and washing through membrane filters, radioactivity was counted by Beckman LS-200B liquid scintillation system. Computer fitted curves are shown.

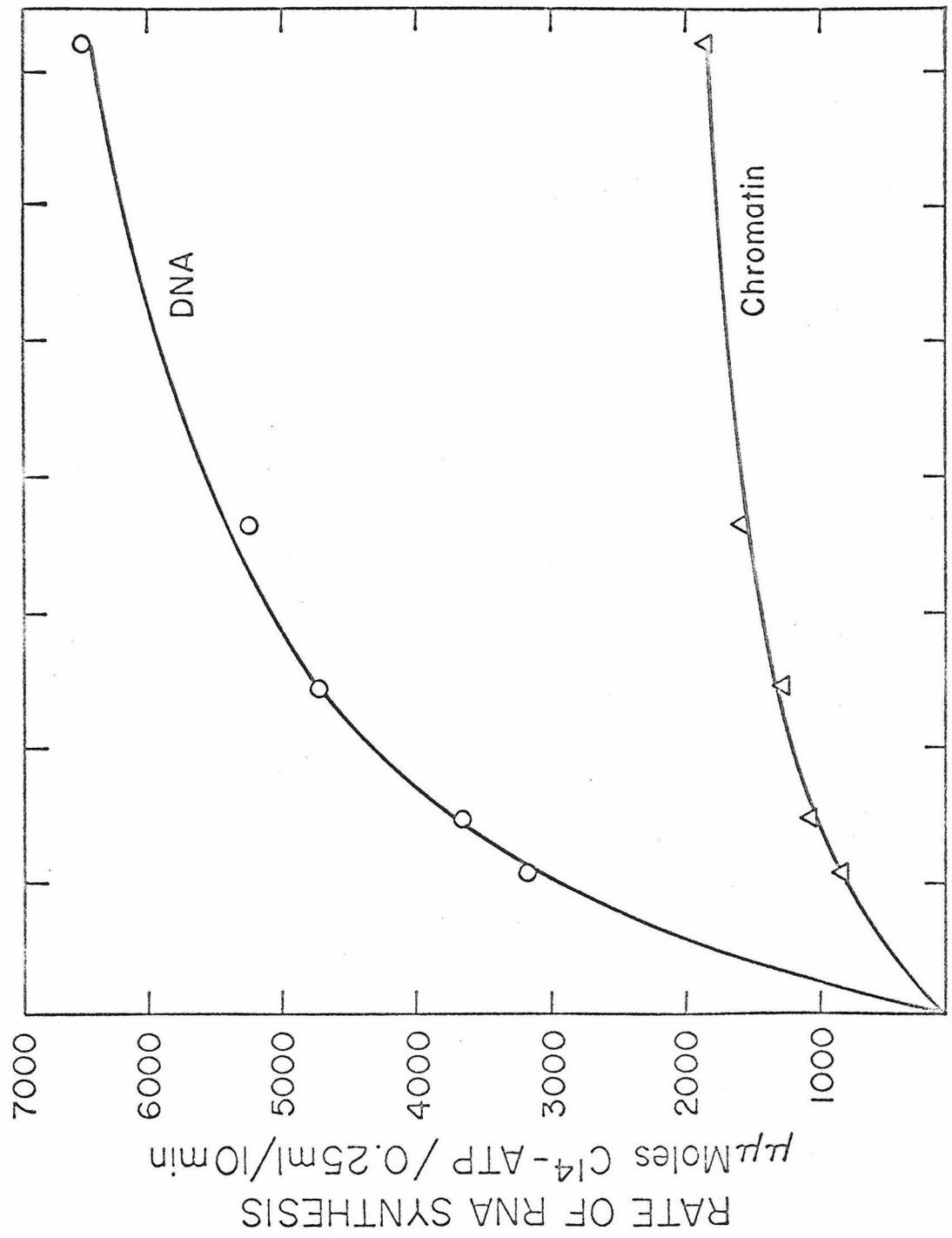
RATE OF RNA SYNTHESIS
μμMoles C14-ATP /0.25ml/10min
7000
6000
5000
4000
3000
2000
1000
0
2
4
6
8
10
12
14
DNA CONCENTRATION, μg/0.25 ml
DNA
Chromatin

FIGURE 1

TABLE I

Template Activity of Calf Thymus Chromatin

Template	V_{max} (μμmoles ATP/ 10 min/0.25 ml)	K (μg DNA/ 0.25 ml)	V_{max}/K (μμmoles ATP/ 10 min/μg DNA)	%
DNA	7,944 ± 250	3.34 ± 0.29	2,380 ± 139	100
Sheared chromatin	2,275 ± 72	3.72 ± 0.31	612 ± 34	26

*Purified calf thymus chromatin was sheared with Virtis homogenizer at 25 volts for 90 sec and centrifuged at 12,000 g for 20 min. Template activity of the supernatant determined.