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INTERACTION OF MODEL PROTEINS AND DNA

by

REGINA SANTELLA

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1976

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

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ABSTRACT

INTERACTION OF MODEL PROTEINS AND DNA

by REGINA SANTELLA

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Random copolymers of lysine and either tyrosine or phenylalanine or histidine were used as model proteins to study the role of these nonbasic amino acids in protein-DNA interaction. Complexes between the various copolypeptides and DNA were made by direct mixing and studied by absorption, circular dichroism (CD), and fluorescence spectroscopy and thermal denaturation.

The binding of all copolypeptides to DNA results in an increase in absorption from both DNA and the copolypeptide. The CD studies show that DNA undergoes a structural transition from B toward C form with polypeptide binding. The extent of this transition varies with the copolypeptide used and follows the order: poly(Lys^{48},His^{52}) pH 7.0 > polylysine > poly(Lys^{58},Phe^{42}) > poly(Lys^{48},His^{52}) pH 9.2 > poly(Lys^{50}, Tyr^{50}).

Thermal denaturation studies show that the nonbasic amino acids affect the thermal stability of protein-bound regions. Copolypeptide-DNA complexes show two melting bands: one at T_m due to the melting of free base pairs and another at T_m' due to the melting of protein-bound base pairs. Poly(Lys^{50},Tyr^{50})-bound base pairs melt at the same temperature (98-99°) as polylysine-bound base pairs (99-100°) under the same conditions while poly(Lys^{48},His^{52})-bound base pairs melt lower (94°). Thus, the presence of tyrosine does not affect the thermal

stability of protein-bound DNA while histidine decreases the stability compared to polylysine-bound DNA. Complexes with poly(Lys^{58},Phe^{42}) show three melting bands: one at $T_{m,I}$(47-48°) for free base pairs and two for protein-bound base pairs, $T_{m,II}$(73-75°) and $T_{m,III}$(89-90°). A mechanism is proposed which accounts for these results and involves intercalation of the phenylalanine chromophore into the DNA as the temperature is raised.

Fluorescence measurements of copolypeptide-DNA complexes show enhanced spectra over that of free DNA and free copolypeptide with a peak at 300nm and a shoulder at 360nm when excited at 250nm. The fluorescence intensity of the complexes with poly(Lys^{50},Tyr^{50}) and poly (Lys^{58},Phe^{42}) show further enhancement as the temperature is raised but not the complex with poly(Lys^{48},His^{52}). This indicates a partial stacking of the aromatic amino acid with the DNA bases at higher temperatures. In addition to the variable temperature fluorescence studies, the effect of increasing temperature on absorption and CD was also studied.

Fluorescence studies were extended to non-fluorescent polypeptide binding to DNA and showed that polylysine and polyarginine induce DNA fluorescence comparable to that seen with fluorescent copolypeptides. The fluorescence intensity was shown to depend upon the polypeptide used, the fraction of DNA bound, the length of the polypeptide, and the secondary structure of the bound protein.

Complexes of the various polypeptides with mononucleotides and single-stranded polynucleotides was also examined. There is preferential fluorescence enhancement of poly(Lys^{50},Tyr^{50}) and poly(Lys^{58},Phe^{42})

complexes with 10^{-3}M dAMP and dGMP but not with dTMP or dCMP. Polylysine-mononucleotide complexes show no fluorescence. This could indicate some role for the aromatic amino acids in protein-DNA interaction, possibly a stacking interaction with the purine nucleotides. Polypeptide-polynucleotide complexes show enhanced spectra over that of the free polypeptide and free polynucleotide in all polylysine, poly(Lys^{50},Tyr^{50}) and poly(Lys^{58},Phe^{42}) complexes. These results indicate that nucleic acid fluorescence can be induced by protein binding and provide an additional method for studying protein-nucleic acid interaction.

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I would like to thank Dr. Hsueh Jei Li for the guidance he has given me during the course of this research. I am grateful for many hours of helpful discussions and encouragement along the way.

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CHAPTER I

INTRODUCTION

There is a great deal of current interest in the processes of recognition between proteins and nucleic acids. Knowing these processes is crucial to the understanding of many biological functions including gene regulation at the chromosomal level and protein synthesis. For example, synthesis of mRNA in an operon is controlled by a highly specific interaction of a repressor protein and an operator region of DNA.[1,2] Many other proteins also interact with DNA, such as repair enzymes, DNA and RNA polymerases,[3] histones[4] and nonhistone proteins.[5]

Because of the complexity of macromolecules and varied types of forces (electrostatic, H-bonding, hydrophobic) involved in the interactions, there is a definite need to work with simplified model systems to study these interactions. Polylysine and polyarginine have been used extensively as models for histones in studying the effect of basic amino acid residues of a protein when it interacts with DNA.[6-10] The early studies showed an irreversible electrostatic interaction between polypeptides with positively charged lysine or arginine residues

and DNA with negatively charged phosphate.[6] These complexes showed a binding of one lysine or arginine per phosphate and remained soluble until this ratio approached one.[11] At high ionic strength polylysine binds preferentially the (A + T)-rich DNA,[7,8] while polyarginine the (G + C)-rich DNA.[6]

These early studies were made on complexes prepared by reconstitution using salt gradient dialysis. Both polypeptide and DNA were first mixed in a high salt solution where their electrostatic interactions were minimized and then the salt was gradually dialyzed out. Complexes prepared by this method, however, show strong light scattering which makes optical measurements less precise.

Other studies were made on complexes prepared by the method of direct mixing where the polypeptides were added directly to the DNA at low ionic strength.[12-15] The complexes were studied using thermal denaturation and circular dichroism. Thermal denaturation of polylysine-DNA complexes showed two melting bands, one at 47-49° due to the melting of free DNA regions and the other at 99-101° due to the melting of polylysine-bound regions.[13] The changes in the CD of DNA due to polylysine binding indicated a change in DNA conformation from a B toward a C conformation.[12] These changes in DNA structure are similar to those induced by high salt concentration[16,17] and have been interperted as due to dehydration in the vicinity of the DNA molecule.[12]

Similar studies were carried out on polyarginine-DNA complexes prepared by the method of direct mixing.[14,15] Thermal denaturation of polyarginine-DNA complexes showed three melting bands, T_m of free DNA at 48-50°, $T'_{m,I}$ at 91° and $T'_{m,II}$ at 99°.[14] It was suggested that the two melting bands at higher temperature were due to the binding of

polyarginine to the two opposite grooves of DNA. The induced CD changes in polyarginine-bound DNA are similar to those induced by polylysine binding.[15]

To study the effect of nonbasic amino acids when a protein interacts with DNA, Friedman and Ts'o[18] examined complexes between polytyrosine and DNA. However, polytyrosine is insoluble in aqueous solution at pH 7.0.

The solubility problems can be minimized by using copolymers of lysine and the amino acid to be studied. The presence of charged lysine residues increases the solubility of the copolypeptide and permits studies in aqueous solution at physiological pH. Such copolypeptides have been used as model proteins in fluorescence,[19] NMR[20] and CD[21] studies. For example, Cernosek et al[21] used the sequential polytripeptide $(Lys\text{-}Ala\text{-}Gly)_n$ as a model for histone H 1 and showed a CD spectrum very similar to that of histone H 1.

Sponar et al.[22] have used sequential copolymers of alanine, lysine and proline to study the changes in DNA induced by the binding of these copolypeptides. They showed that the CD of the complex depended upon the sequence of the copolypeptide used. These limited studies show that copolypeptides should serve as useful model proteins in protein-DNA interaction.

To obtain detailed information on the effect of each individual amino acid in a protein when it interacts with DNA, a systematic study was undertaken using random copolymers of lysine plus one type of nonbasic amino acid. Using these copolypeptides, the effect of each amino acid residue could be determined more precisely. Since the effect upon DNA structure and thermal stability by polylysine binding is known

complexes of DNA and the various copolymers can be studied and the effect of each individual amino acid in a protein when it interacts with DNA can be extrapolated.

As part of this study of model protein-DNA interaction, Pinkston and Li[23] used random copolymers containing varying amounts of lysine and alanine to study the effect of both the hydrophobic, aliphatic amino acid residues and the α-helical structure in protein-DNA interaction. Poly(Lys^{40},Ala^{60}) was reported to have an α-helical structure which becomes distorted when the copolymer binds to DNA. The presence of alanine residues was shown to reduce the capacity of polylysine to induce the conformational transition of DNA from a B toward a C form. The melting temperature of the bound base pairs was also found to be lower than that for polylysine binding, implying that the presence of a rigid α-helix in the copolypeptide prevents its lysine residues from fully interacting with the phosphates on DNA.

The results to be presented here represent another part of this study of model protein-DNA interaction. Random copolymers containing lysine and one other type of amino acid residue, including histidine and the aromatic residues tyrosine, phenylalanine and tryptophan, were used. The copolypeptides with aromatic amino acids are especially interesting since it has been suggested that they play an important role in protein-nucleic acid interaction.[24-28] In particular, intercalation of aromatic residues between the DNA base pairs could be an important factor in the recognition processes between a protein and DNA.

Numerous studies have been carried out on the interaction of aromatic amino acid residues and nucleic acids.[24-31] Helene et al[29]

studied the interaction of tyrosine and tyramine with nucleic acids and their derivatives. Quenching of tyrosine and tyramine fluorescence upon interaction with DNA and its derivatives has been observed in frozen aqueous solution. NMR studies at room temperature showed an upfield shift of ring protons in both the tyrosine and purine or pyrimidine rings after they were mixed. They interperted these results as due to the intercalation of aromatic amino acid residues into the DNA and formation of a stacked complex with the mononucleotides. Similar results were reported for tryptophan,[31,32] tryptamine,[33] and 5-hydroxy-tryptamine.[33]

Oligopeptides of the general structure (lys-X-lys) where X is an aromatic residue have also been studied by Helene and his colleagues. They showed that two types of complexes were formed when these oligopeptides were bound to single or double-stranded polynucleotides.[30] One type involved only electrostatic interaction between lysyl residues and phosphates without direct interaction of the aromatic amino acid with the bases. The second involved a stacking of the aromatic amino acid with the bases leading to quenching of the fluorescence. Stacked complexes were favored in single-stranded polynucleotide complexes. NMR data of these oligopeptides show upfield shifts of the tryptophan and tyrosine protons complexed with poly(A) supporting the stacked complex.[27,28] A change in the CD spectra is also seen resulting from the unstacking of the adenine bases when the complex is formed.[31]

Gabbay et al[24,25] studied the interaction of a large number of di- and tri-peptides with nucleic acids by NMR, viscosity, and CD. The NMR results also indicate a selective broadening and upfield chemical shift of the aromatic protons of the peptides when bound to DNA. A decrease in the specific viscosity of DNA was also observed when

peptides containing aromatic amino acids were bound. They suggested a model in which the aromatic residues were partially intercalated between the base pairs of DNA.The selective lowering of the specific viscosity of DNA by peptides containing aromatic amino acids was suggested to result from a tilting of adjacent base pairs and a decrease in the effective length of the helix.

These results all suggest the possibility of intercalation of aromatic amino acids into DNA. However, amino acids, di- and tripeptides are not the best model systems for studying protein-DNA interaction. In these systems the contribution of the charged groups at the ends ($-NH_3^+$ and $-COO^-$) to the interaction with DNA is expected to be large, while in a native protein of one to several hundred amino acid residues, the end effect is expected to be small. In addition, the presence of a greater number of lysine residues in the copolymers is expected to yield strong localized binding of the protein to DNA. Oligopeptides bind DNA in a reversible and nonlocalized fashion which is different from basic proteins.

Information on the effect of aromatic amino acids in protein-DNA interaction can therefore be obtained by using random copolymers containing lysine and one aromatic amino acid residue. These copolymers should bind DNA irreversibly because of their high lysine content and induce a second phase of DNA melting. By comparison to the melting of polylysine-bound DNA, the effect of each aromatic amino acid upon the thermal stability of protein-bound DNA can be determined. Similarly, by comparison to the changes in the structure of DNA induced by polylysine binding, the effect of the aromatic amino acid upon the structure of DNA

can be determined. Since the aromatic amino acids fluoresce, fluorescence spectroscopy can be used as an additional method for obtaining information about the interaction.

Previously, fluorescence studies on protein-DNA interaction have focused on the fluorescence from the aromatic amino acids because the bases in DNA fluoresce very weakly (quantum yield, $\Phi_f \leq 10^{-4}$) in aqueous solution at room temperature.[35] Almost all fluorescence work on nucleic acids has been done at low temperature using ethylene glycol glasses at 77°K. From these studies, fluorescence spectra and quantum yields of the mono- and polynucleotides were determined.[36-38] It is not easy however, to extrapolate this data to room temperature and aqueous solutions. Only recently have studies on DNA and its components at room temperature been reported.[39] Daniels has made use of a multiple scan instrument with signal accumulation techniques to study the fluorescence of monomers and DNA at room temperature.

The studies to be reported here indicate that the quantum yield of DNA fluorescence at room temperature can be greatly increased by polymer binding even if the polymer does not contain any aromatic amino acids. Because of these interesting results it was decided to study in more detail the nature of this enhancement. To do this, the fluorescence studies were extended to the interaction of the various copolypeptides and polylysine with mononucleotides and single-stranded polynucleotides.

From these studies the nature of the interaction of several amino acids (tyrosine, phenylalanine, and histidine) with DNA has been examined and the results are discussed.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Copolymer L-lysine-L-tyrosine hydrobromide (1:1), poly(Lys^{50},Tyr^{50}), with a molecular weight of 82,500, copolymer L-lysine-L-phenylalanine hydrobromide (1.4:1), poly(Lys^{58},Phe^{42}), with a molecular weight of 30,000, copolymer L-lysine-L-histidine hydrobromide (1:1.1), poly(Lys^{48}, His^{52}), with a molecular weight of 64,000 and oligomer L-lysine-L-tryptophan hydrobromide (1:1.1), oligo(Lys^{48},Trp^{52}),were purchased from Miles Laboratories. Poly L-lysine hydrobromide, polylysine, of varied molecular weights as well as polyL-arginine hydrochloride, polyarginine, with a molecular weight of 30,000 were also purchased from Miles Laboratories. Copolymer L-lysine-L-alanine hydrobromide of varied ratios (4.3:1), (1:1.5) and (1:4.3), poly(Lys^{81},Ala^{19}), poly(Lys^{40},Ala^{60}) and poly(Lys^{19},Ala^{81}) were synthesized in our laboratory by Margaret Pinkston.

Calf thymus DNA was purchased from Sigma Chemical Co. and purified by phenol extraction. Micrococcus luteus DNA was purchased

from Miles Laboratories and used without further purification. The sodium salt of poly(adenylic acid), poly(A), was purchased from Miles Laboratories. The sedimentation coefficient, supplied by the manufacturer is 12.35. The potassium salts of poly(guanylic acid), poly(G), $S_{20,w}$= 7.8, and poly(cytidylic acid), poly(C), $S_{20,w}$= 10.0 were purchased from P.L. Biochemicals.

The sodium salts of 2'-deoxyguanosine 5'-phosphate, dGMP, 2'-deoxyadenosine 5'-phosphate, dAMP, 2'-deoxycytidine 5'-phosphate, dCMP, and 2'-deoxythymidine 5'-phosphate, dTMP, were purchased from Sigma Chemical Co.

Trypsin and trypsin soy bean inhibitor were purchased from Sigma Chemical Co.

METHODS

Preparation of Polymer Solutions

Solutions of poly(Lys^{50},Tyr^{50}) and poly(Lys^{58},Phe^{42}) were prepared by dissolving and dialyzing the copolymers in 0.001M Tris (pH 6.8). The concentrations were determined by the absorbance at 277nm for poly(Lys^{50}, Tyr^{50}) using an extinction coefficient of 1.4×10^3 $M^{-1}cm^{-1}$ for the tyrosine residues[40] and at 257nm for poly(Lys^{58},Phe^{42}) using an extinction coefficient of 210 $M^{-1}cm^{-1}$ for the phenylalanine residues.[40] Solutions of oligo(Lys^{48},Trp^{52}) were prepared by dissolving the polymer in 2.5×10^{-4} M EDTA (pH 8.0). The concentration was determined by the absorbance at 279nm using an extinction coefficient of 5.5×10^3 $M^{-1}cm^{-1}$.[40]

Solution of poly(Lys^{48},His^{52}), polylysine, polyarginine, and poly (Lys^m,Ala^n) with m + n = 100%, were prepared by dissolving and dialyzing the polymers in 2.5×10^{-4} M EDTA. For the concentration determination

of these polymers, a sample was hydrolyzed in 6N HCl (100°,24hr) followed by ninhydrin determination[41], using lysine, arginine, or an appropriate mixture of lysine and the non-basic residues as the standard.

Preparation of Nucleotide Solutions

Solutions of calf thymus and Micrococcus luteus DNA were prepared by dissolving the DNA in either 0.001M Tris (pH 6.8) or 2.5×10^{-4} M EDTA (pH 8.0) and dialyzing against the appropriate buffer. The concentration of nucleotide in DNA was determined using the molar extinction coefficient of 6.5×10^3 $M^{-1}cm^{-1}$ at 260nm for calf thymus DNA and 7.0×10^3 $M^{-1}cm^{-1}$ for M. luteus DNA.

Solutions of the mono- and polynucleotides were also prepared as above with the exception that the mononucleotides were not dialyzed. The following molar extinction coefficients at the appropriate wavelengths were used to determine the concentration of nucleotide.[40]

	ϵ (10^3 $M^{-1}cm^{-1}$)	λ (nm)
dAMP	15.0	259
dCMP	9.0	271
dGMP	13.0	254
dTMP	9.6	267
poly(A)	9.4	259
poly(G)	9.9	254
poly(C)	6.2	271

Preparation of Complexes

Polypeptide- and copolypeptide-DNA complexes were made by the slow addition of appropriate amounts of 10^{-3} M polypeptide or copolypeptide to 10^{-4}M DNA. A similar procedure was used for the mono- and polynucleotides except that the nucleotide concentration varied from 10^{-5} to10^{-3}M. The input ratio, r, of polypeptide or copolypeptide to DNA or synthetic nucleotide is reported in amino acid residues per

nucleotide.

Trypsin Digestion of Complexes

Trypsin digestion of the complexes was accomplished by the addition of 0.01 mg/ml trypsin solution to the complexes to give a final concentration of 10μ g/ml of complex. The samples were incubated at room temperature for varied times and digestion was stopped by the addition of 0.02 ml of 1 mg/ml trypsin inhibitor to 5 ml of complex solution.

Preparation of Denatured DNA

Denatured calf thymus DNA was prepared by heating a sample in a sealed tube at 100^{o} for 10 min followed by rapid cooling on ice.

Instrumentation

Sample absorbances were measured on a Beckman Model S-25 spectrophotometer. Absorption spectra were taken on a Cary 17 spectrophotometer. CD spectra, taken on a Jasco spectropolarimeter, Model J-20, are reported as $\Delta\epsilon = \epsilon_L - \epsilon_R$ in $M^{-1}cm^{-1}$. For free DNA and the complexes, M represents moles of nucleotide per liter; for the free polypeptide or copolypeptide solutions, M represents moles of amino acid residues per liter. Fluorescence emission spectra were recorded in the ratio mode of a Perkin Elmer MPF 2A spectrofluorimeter.

Thermal denaturation measurements were made on a Gilford spectrophotometer, Model 2400-S with a constant heating rate of 2/3 ^{o}C per min. The hyperchromicity, h, is the percent increase in absorbance at 260nm and dh/dT is the derivative of the melting curve.

In order to obtain titration curves, some complexes were centrifuged in a Sorvall SS-34 rotor at 4° for 10 min at 10,000rpm. The supernatants were collected for concentration determination.

CHAPTER III

STUDIES ON THE INTERACTION BETWEEN POLY(L-LYSINE^{50},L-TYROSINE^{50}) AND DNA

Previous studies on the effect of tyrosine on protein-DNA interaction have suggested that the aromatic ring intercalates into the DNA.[24,25,29,30] These studies however, have used di- or oligopeptides which bind DNA in a reversible, non-localized fashion because of their weak binding affinity. In addition, the effect of the charged groups at the ends contributes greatly to the interaction. This is not the case in a native protein. Poly(Lys^{50},Tyr^{50}) is a better model for studying the role of tyrosine in protein-DNA interaction since it binds in a irreversible localized fashion to DNA.

Most of the previous studies on polypeptide-DNA interaction were carried out in 2.5 x 10^{-4}M EDTA, pH 8.0 (EDTA buffer). A stock solution of 10^{-2} M poly(Lys^{50},Tyr^{50}) was also prepared in EDTA buffer. However, when the stock solution was diluted with EDTA buffer to 10^{-3}M, the solution became cloudy. For some reason, as yet unknown, there is aggregation of this copolypeptide upon dilution in EDTA buffer. An alternate buffer, namely 0.001M Tris (ultrapure grade from Schwarz- Mann)

pH 6.8, (Tris buffer) was then used. A weighed sample of the copolypeptide was dissolved in Tris buffer and the absorption at 277nm determined. Beer's law is obeyed in Tris buffer and the molar extinction coefficient was determined to be 1.4×10^3 $M^{-1}cm^{-1}$ for tyrosine residues in the copolypeptide. This is in agreement with the value for free tyrosine.[40]

Titration of Calf Thymus DNA by Poly(Lys^{50},Tyr^{50})

Poly(Lys^{50},Tyr^{50})-calf thymus DNA complexes were made by adding varying amounts of 10^{-3} M copolypeptide to 5 ml of 10^{-4} M DNA. The complexes were then centrifuged and the absorbance at 260nm of the supernatants measured. Figure 1 shows the titration curve of calf thymus DNA by poly(Lys^{50},Tyr^{50}). The complexes are completely soluble and remain in the supernatant until the input ratio, r, reaches 2.0 amino acid residues per nucleotide. At this point, with one lysine and one tyrosine per nucleotide in the complex, precipitation occurs sharply. The residual absorbance in the supernatant after precipitation ($r > 2.1$) may be due to the tyrosine absorbance of the excess copolypeptide in solution. This result is in agreement with previous studies on polylysine,[11,13,42] polyarginine,[14] and protamine[43] in which precipitation occurs when the number of positively charged amino acid residues added is approximately equal to that of negatively charged phosphates in DNA.

Absorption Spectra of Poly(Lys^{50},Tyr^{50})-DNA Complexes

Figure 2 shows the absorption spectra of free poly(Lys^{50},Tyr^{50}), calf thymus DNA and a complex with r = 1.0. Both DNA and the copolypeptide absorb light at the same wavelength. ϵ_m, ϵ_f^D and ϵ_f^P represent

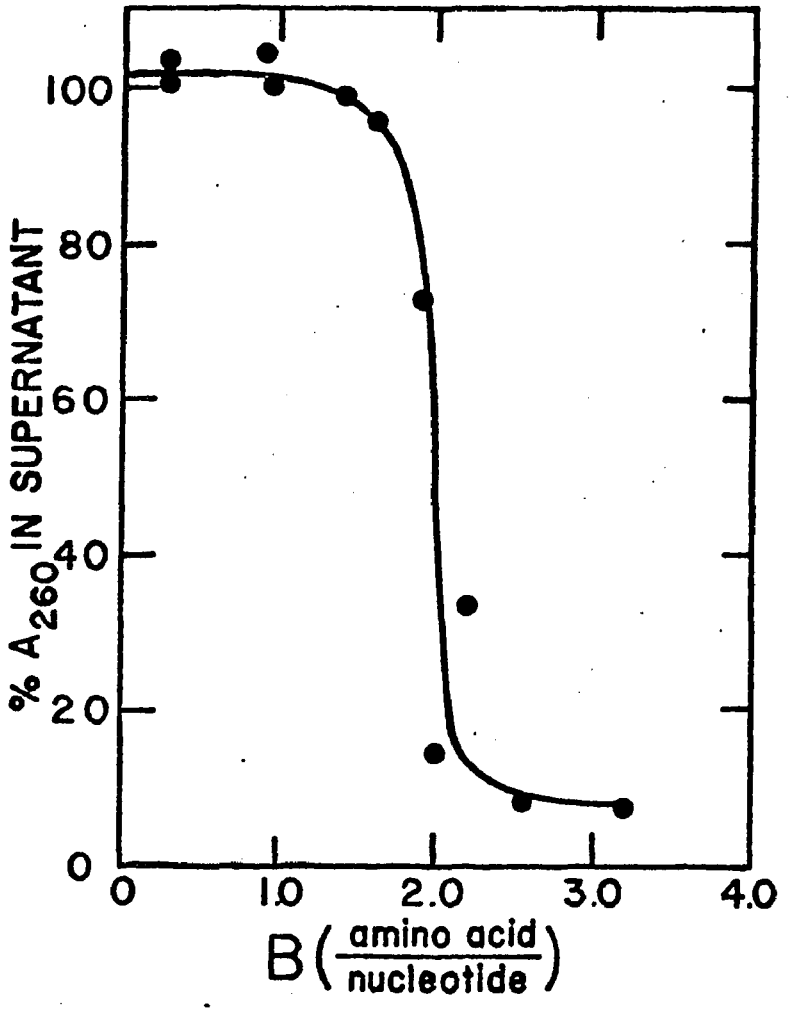


Figure 1. Titration curve of DNA by Poly(Lys^{50},Tyr^{50}).

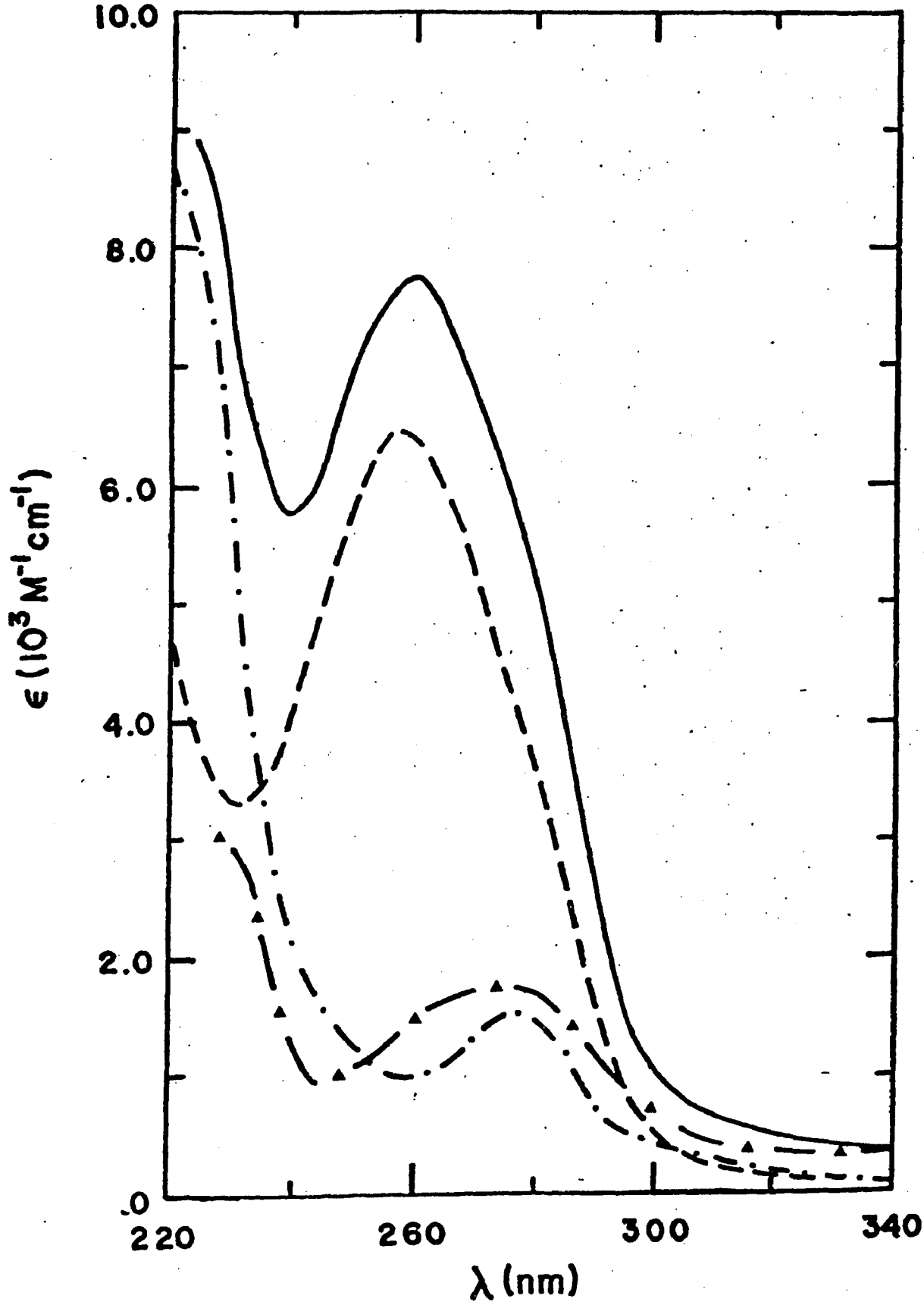


Figure 2. Absorption spectra of DNA (---), poly(Lys^{50},Tyr^{50})(-·-) and a complex with r = 1.0 (___). The absorbance gain after complex formation from Eq.(1) is shown. (-▲-).

respectively, the molar extinction coefficient of the complex, free DNA and tyrosine in the free copolypeptide. The increase in absorbance with complex formation can then be expressed as

$$\delta\epsilon = \epsilon_m - (\epsilon_f^D + \frac{r}{2}\epsilon_f^P) \quad (1)$$

where r/2 is the number of tyrosine residues per nucleotide since the copolypeptide has 50% tyrosine residues. This difference in absorption can be calculated and is shown in Figure 2. The difference spectrum has a broad band between 260 and 280nm. This indicates that the spectral changes resulting from complex formation are contributed by both the DNA and the tyrosine absorption.

Circular Dichroism Spectra of Poly(Lys^{50},Tyr^{50})-DNA Complexes

Figure 3 shows the CD spectra of free poly(Lys^{50},Tyr^{50}) at two pH's and their difference spectrum. At pH 6.8 there is a positive CD band at 228nm and a negative one at 215nm. Two small positive CD bands at 273 and 304nm are also observed at high concentration. The CD spectrum of free copolypeptide at pH 9.5 is very different from that at pH 6.8. At pH 9.5 there is a small positive band at 250nm and a large negative band at 222nm. As seen from the difference CD spectrum in Figure 3, deprotonation on tyrosine residues yields a reduction in CD near 230nm and an increase near 210nm.

When poly(Lys^{50},Tyr^{50}) is complexed with DNA several CD changes are induced (Figure 4). Above 240nm, the negative band at 245nm becomes more negative and the crossover at 256nm shifts to the red. A slight red shift is also observed for the positive band near 275nm.

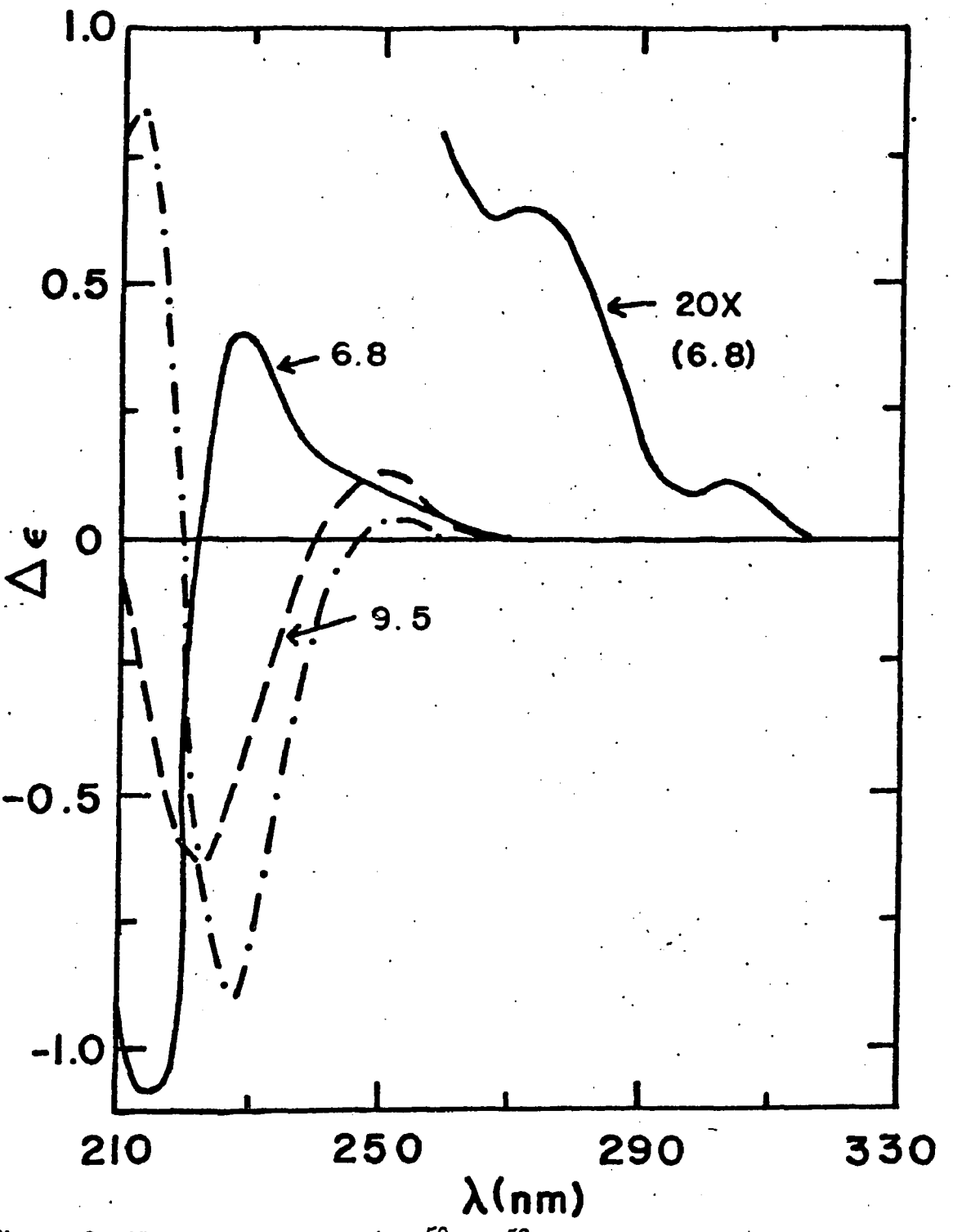


Figure 3. CD spectra of Poly(Lys^{50},Tyr^{50}) at pH 6.8 (–),pH 9.5 (---), and the difference(-.-). The CD spectrum above 270nm is a 20-fold magnification.

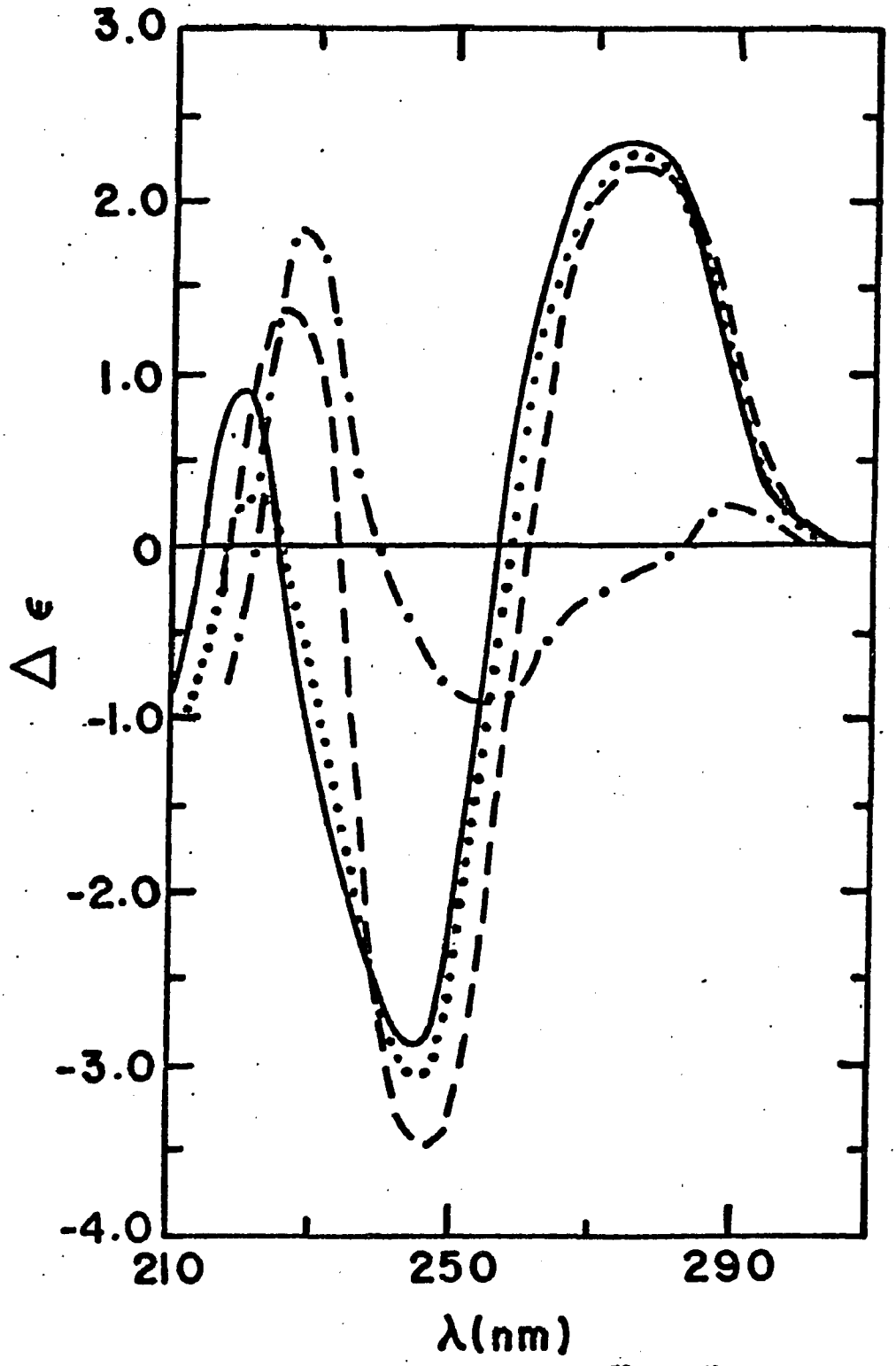


Figure 4. CD spectra of DNA and Poly(Lys^{50},Tyr^{50})-DNA complexes. r=0 (—); 0.31 (...); 1.55 (---); and the difference between r= 0 and r= 1.55 (-·-).

Below 240nm, there is a significant red shift. In order to examine the spectral changes due to copolypeptide binding, a difference CD spectrum was taken between DNA and a complex with r = 1.55. This difference CD spectrum is similar to that of polylysine-DNA complexes[12] with a negative band at about 255-260nm, except that the amplitude is much smaller in the copolypeptide complex. The difference spectrum above 270nm is small and varies slightly in complexes of varied r values. Such a variation could be due to a small contribution of tyrosine CD in this wavelength region.

The positive difference CD at 230nm in Figure 4 coincides with the CD of poly(Lys^{50},Tyr^{50}) in the free state at this wavelength (Figure 3). If it is assumed that this difference CD is solely contributed by the bound copolypeptide it will be equal to $r\Delta\epsilon_{t,b}$ where $\Delta\epsilon_{t,b}$ is the CD of bound copolypeptide and r is the input ratio of amino acid residues per nucleotide. Under this assumption it is estimated that $\Delta\epsilon_{t,b}$ of this complex (r = 1.55) at 230nm is 1.3, which is about three times the corresponding $\Delta\epsilon_{t,f}$ of free copolypeptide at pH 6.8 (Figure 3). Though peptide bonds in the copolypeptide also have some CD contribution around 230nm, the amplitude and the sensitive response of this CD to pH implies that the CD in this wavelength range could be contributed mainly by the tyrosine chromophores or the interaction among these chromophores with DNA.

Thermal Denaturation of Poly(Lys^{50},Tyr^{50})-DNA Complexes

Figure 5 shows the derivatives of the melting curves of poly(Lys^{50}, Tyr^{50})-DNA complexes. The binding of more copolypeptide to DNA raises Tm, the melting temperature of free base pairs, from 61 to 75°, but not

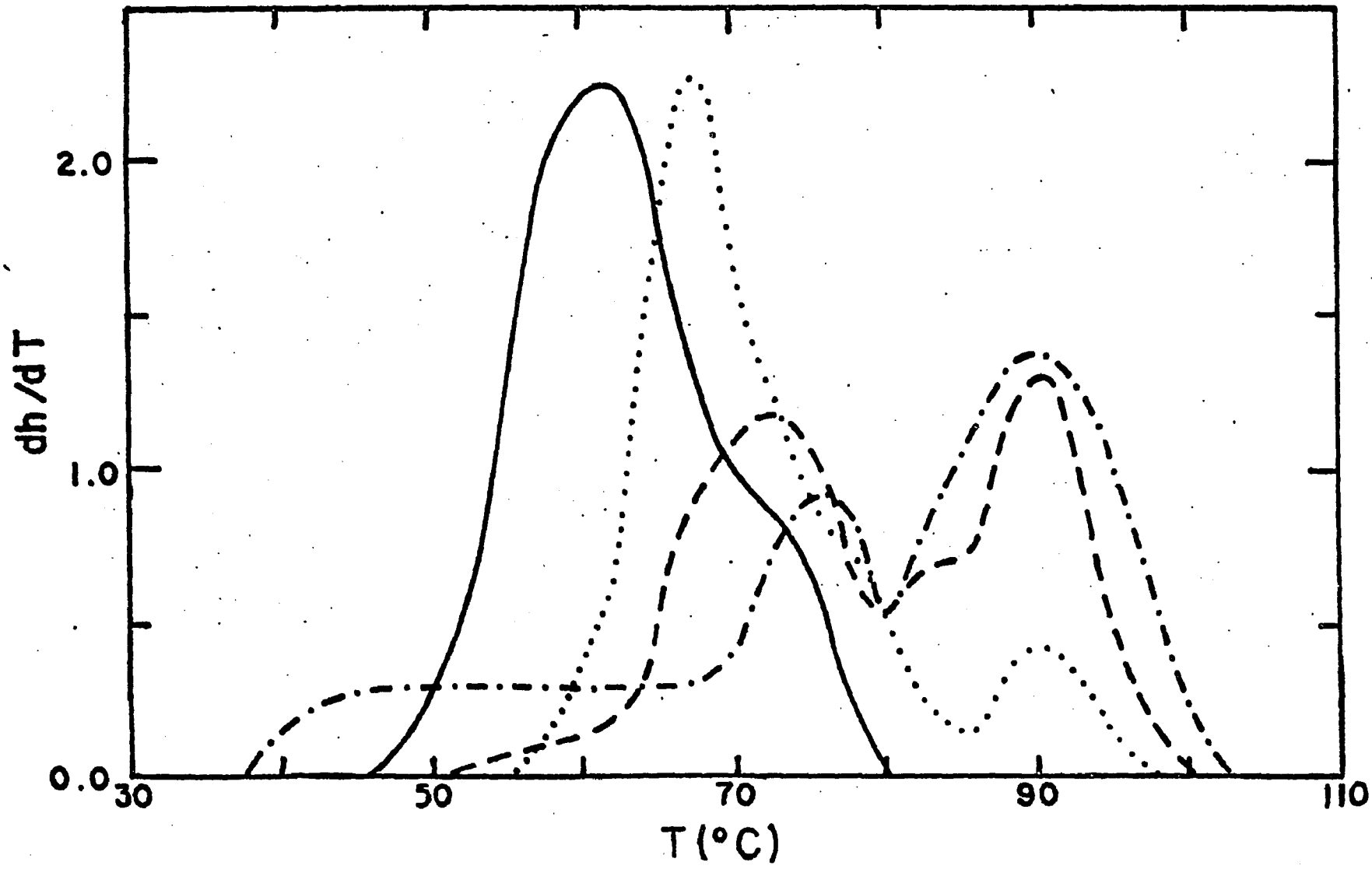


Figure 5. Derivative melting curves of Poly(Lys^{50},Tyr^{50})-DNA complexes. r= 0 (—); 0.36 (...); 1.08 (---); and 1.44 (-·-).

T_m', at 90°, the melting temperature of bound base pairs. A significant amount of melting occurs before Tm; this has not been observed with polylysine[13] or polyarginine[14] complexes.

Table I summarizes some of the melting results of poly(Lys^{50},Tyr^{50})-DNA complexes. As observed in polylysine-DNA[13], polyarginine-DNA[15] and protamine-DNA[43] complexes, h_{max} is reduced as more copolypeptide is bound to DNA. However, this decrease in h_{max} is reversed for complexes with high r values. This unexpected increase in h_{max} for complexes with high coverage is accompanied by the appearance of an early melting before Tm (Figure 5 and Table I) and an increase in A_{360}/A_{260} after melting. It is possible that some structural changes on the copolypeptide bound regions and aggregation of the complex might occur as the temperature is raised. For instance, for the complex with r = 1.44, about 30% of the total melting occurs before Tm and A_{360}/A_{260} is increased from 0.095 before melting to 0.146 after melting. Later in this chapter it will be shown that the former is due to structural changes on the copolypeptide-bound regions at higher temperature and the latter can be attributed to a slight increase in light scattering during the melting.

Since one of the main objectives of this study is to determine the effect of tyrosine residues on the structure and thermal stability of protein-bound regions, a comparison should be made to polylysine-DNA complexes. Previously, thermal denaturation studies on polylysine-DNA complexes were reported in 2.5×10^{-4} M EDTA. As mentioned earlier, this buffer cannot be used for poly(Lys^{50},Tyr^{50}). Two alternate methods were used to compare copolypeptide complexes with polylysine complexes: (a) polylysine-DNA complexes were prepared and studied under the same conditions as used for poly(Lys^{50},Tyr^{50}) namely, 0.001M Tris, pH 6.9,

Table I

Melting Characteristics of Poly(Lys^{50},Tyr^{50})-DNA Complexes

r amino acid / nucleotide	h_{max} (%)	A_{360}/A_{260}		T_m	T_m'	Fraction of Melting Area (%)		
		30°	110°			T_m	T_m'	residual
0	36.7	0.040	0.030	61.0	-	100.0	-	-
0.36	32.5	0.041	0.039	67.0	90.0	90.0	10.0	-
0.72	32.6	0.035	0.031	68.0	89.0	65.7	28.4	5.9
1.08	33.0	0.041	0.059	72.0	90.0	45.9	47.2	6.9
1.44	36.0	0.095	0.146	75.0	90.0	22.5	48.6	28.9

(b) poly(Lys^{50},Tyr^{50})-DNA complexes were prepared in Tris and dialyzed to EDTA buffer.

Thermal Denaturation and CD Studies of Polylysine-DNA Complexes in Tris Buffer

Polylysine-DNA complexes were prepared by direct mixing in Tris buffer and examined in the same buffer. The results are summarized in Table II. The CD results of the complexes in Tris buffer are similar to those in EDTA buffer.[12] As more base pairs are bound by polylysine, there are red shifts for both the peak at 275nm and the crossover at 256nm and a reduction in $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$.

The melting properties of polylysine-DNA complexes in Tris and EDTA buffer are similar to one another with respect to a reduction in h_{max} and a proportional increase in $A_{T_m'} / h_{max}$ for complexes with higher r values. However, the T_m' of the bound base pairs is shifted from 98° in EDTA buffer[12] to 92° in Tris buffer. In addition, the T_m of free base pairs, raised by one to three degrees in EDTA buffer when the r value of the complex is increased, increases by 10° in Tris buffer. This has also been found in poly(Lys^{50},Tyr^{50})-DNA complexes in Tris buffer (Figure 5 and Table I).

When polylysine-DNA complexes were dialyzed from Tris to EDTA buffer, the T_m of pure DNA and the complexes was lowered from 65-75° in Tris buffer down to 47-49° in EDTA buffer. The T_m' on the other hand was shifted from 92° in Tris buffer up to 98° in EDTA buffer. This indicates that prehaps there is a small amount of impurity in Tris, probably divalent cations. In the absence of polylysine, these divalent cations bind DNA [44] with a slight shift of T_m. When polylysine is

Table II

Melting and CD Characteristics of Polylysine-DNA Complexes Made in Tris Buffer

r	h_{max}(%)	A_{320}/A_{260}		T_m	T_m'	$A_{T_m'}/h_{max}$	λ_{max} (nm)	c (nm)	$\frac{278}{246}$
		30°	110°						
0	36.6	0.017	0.020	65.5	--	0	275	256.0	0.81
0.18	34.5	0.029	0.025	67.5	92.0	17.8	278	258.5	0.68
0.36	35.9	0.018	0.027	69.5	91.0	32.2	280	260.0	0.60
0.54	33.4	0.044	0.028	75.0	92.0	51.6	281	262.0	0.53
0.72	32.8	0.043	0.050	73.5	91.0	67.3	283	263.0	0.53
0.90	29.4	0.046	0.054	75.0	92.5	90.6	284	266.5	0.44

bound to DNA, these cations could possibly be replaced and move to the remaining free DNA regions and further stabilize them against melting. This could explain the increment of about 10° in the T_m of the complexes in Tris and its reduction after being transferred back to EDTA buffer because EDTA is capable of removing divalent cations from DNA.

When poly(Lys^{50},Tyr^{50})-DNA complexes were dialyzed from Tris to EDTA buffer there were similar changes in the T_m and T_m' values. The T_m of pure DNA and the complexes was lowered from 61-75° in Tris buffer to 47-49° in EDTA buffer. The T_m' was increased from 89-90° in Tris to 98-99° in EDTA. Since, when placed in the same buffer, the melting temperature of DNA base pairs bound by polylysine or poly(Lys^{50},Tyr^{50}) is the same, the presence of 50% tyrosine does not affect the thermal stability of the copolypeptide-bound regions.

Determination of β Value for Poly(Lys^{50},Tyr^{50})-DNA Complexes

Because of the contribution of the absorbance of tyrosine to the apparent hyperchromicity and the problem of structural changes and aggregation during melting, the melting data cannot be analyzed as accurately as has been done for other polypeptide-DNA complexes.[13,14,43] However, if it is assumed that the fraction of melting area under T_m (A_{T_m}/A_T) is proportional to the fraction of free base pairs in a complex, the following equation can then be used to determine F, the fraction of base pairs bound by the copolypeptide

$$F = 1 - \frac{A_{T_m}}{A_{Total}} = \frac{A_{T_m'}}{A_{Total}} \qquad (2)$$

The β value or ratio of amino acid residues per nucleotide in the copolypeptide-bound regions can then be determined from

$$r = \beta F \qquad (3)$$

Figure 6 shows the plot of Equation (3) which yields $\beta = 2.0$. Thus, in copolypeptide-bound regions, there are 2.0 amino acid residues per nucleotide or 1.0 lysine and 1.0 tyrosine per nucleotide.

Fluorescence Studies on Poly(Lys^{50},Tyr^{50})-DNA Complexes

The relative fluorescence emission spectra of poly(Lys^{50},Tyr^{50}), excited at 260 or 280nm,are shown in Figure 7. There are two emission peaks, 307 and 400nm. The peak at 307nm corresponds to that of free tyrosine which has a maximum emission at 307nm.[45] The peak at 400nm is probably due to bityrosine which can be formed from two adjacent tyrosines under basic conditions by a light induced reaction.[46,47] Because the quantum yield of bityrosine is so much greater than that of tyrosine, the presence of a few percent of this dimer can yield a strong emission band at 400nm.[46]

Figure 8 shows the relative fluorescence emission at 307nm of poly(Lys^{50},Tyr^{50}) excited at 280nm as a function of concentration. There is a linear relationship between fluorescence emission and concentration for the entire concentration range used for the fluorescence studies.

The fluorescence emission spectra of a poly(Lys^{50},Tyr^{50})-DNA complex ($r = 1.6$) excited at 260 or 280nm are shown in Figure 7. In both cases the emission spectra are normalized with respect to the emission of the free copolypeptide at 307nm. A significant amount of fluorescence (about 60%) is quenched at 307nm whether excitation is at 260 or 280nm. The quenching after complex formation does not result from the screening effect of the DNA absorption. This was shown by the following experiment. The fluorescence of a solution of 4×10^{-5} M copolypeptide and 5×10^{-5} M DNA was measured and compared to that of

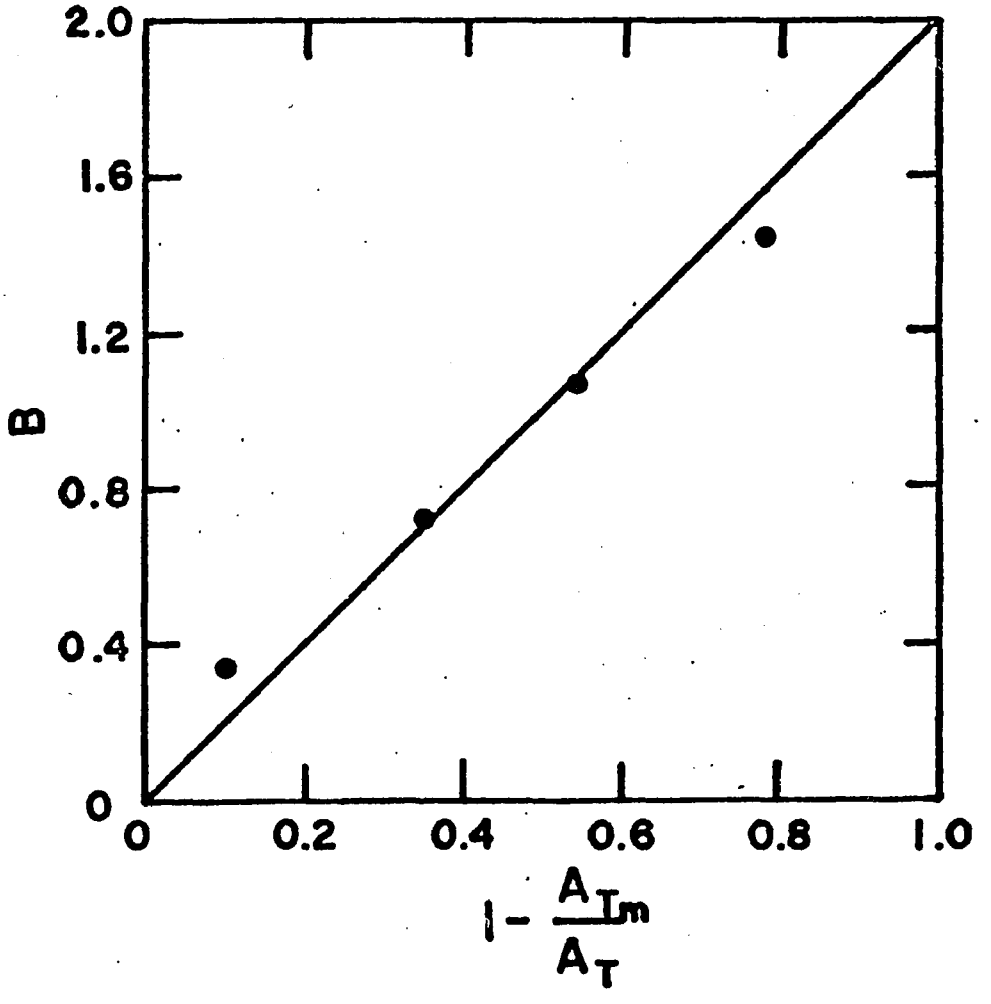


Figure 6. Plot of equation (3).

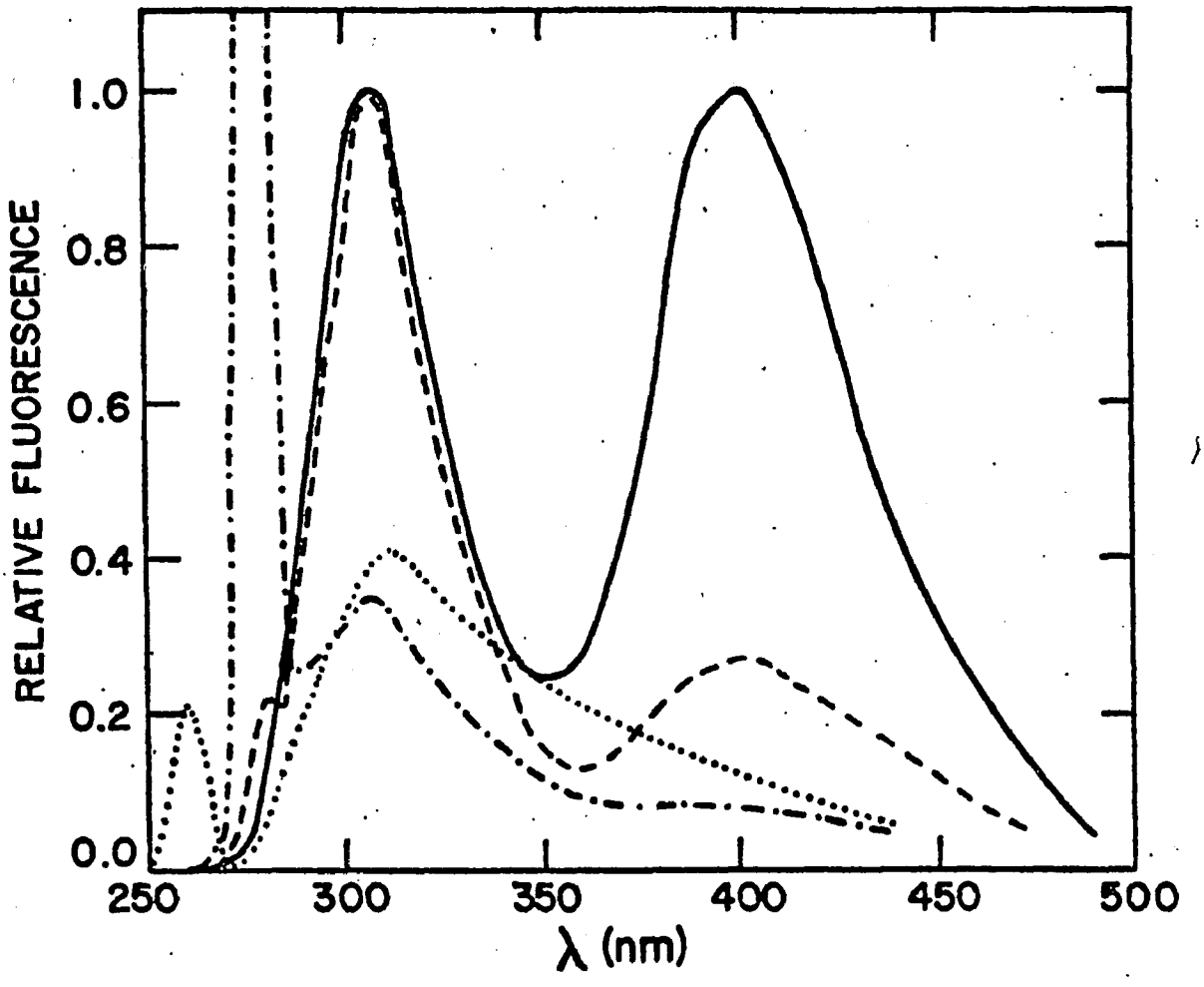


Figure 7. Fluorescence spectra of Poly(Lys^{50},Tyr^{50}) and its complex with DNA (r= 1.6). Free copolymer (__) and the complex (...) excited at 260nm, free copolymer (---) and the complex (-·-) excited at 280. Copolymer intensity set to 1.0 at 307nm.

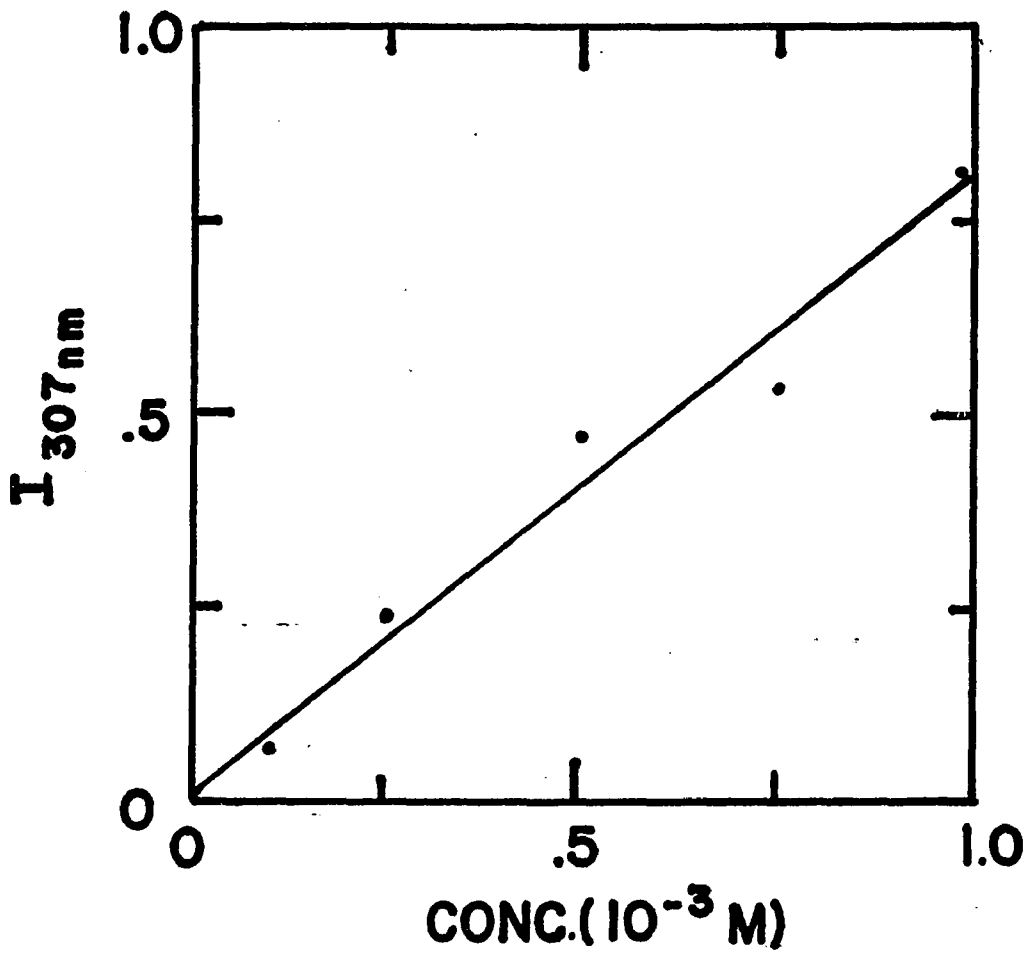


Figure 8. Fluorescence intensity of poly(Lys^{50},Tyr^{50}) at 307nm as a function of concentration. Excitation wavelength is 280nm.

a 4×10^{-5} M copolypeptide and 1×10^{-4} M DNA solution. Doubling the DNA concentration does not change the amount of bound tyrosine but does approximately double the absorbance at 280nm. In the more concentrated DNA solution the fluorescence intensity was reduced only by 7% compared to the 60% quenching observed when free copolypeptide is bound to DNA.

Figure 9 shows the relative fluorescence emission spectra of poly(Lys^{50},Tyr^{50}) and two complexes, one made with calf thymus DNA ($r = 1.02$) and the other with M.luteus DNA ($r = 0.97$) excited at 250nm. At this excitation wavelength, the emission spectrum of the complex is enhanced about twofold over that of free copolypeptide. However, since 280nm is the wavelength of maximum absorption for tyrosine, excitation at 250nm is expected to be much less efficient than at 280nm for tyrosine emission. For instance, the emission at 307nm of free copolypeptide excited at 250nm is only about one fourth of the intensity when excited at 280nm.

The discrepancy between the results observed with excitation at 250nm (enhancement) and at 280nm (quenching) can be explained as emission from two different fluorescent species, namely DNA-bound tyrosine and protein-bound DNA. The main fluorescent species is bound tyrosine when excited at 280nm, while it is protein-bound DNA when excited at 250nm. The latter is supported by fluorescence results of polylysine-DNA complexes to be presented in Chapter VI.

The change in fluorescence emission with variable excitation wavelength can be seen in Figure 10 for a complex with $r = 1.35$. With excitation at 260, 270 or 280nm the light scattering peak interferes with the emission peak near 300nm. For these wavelengths, a filter was

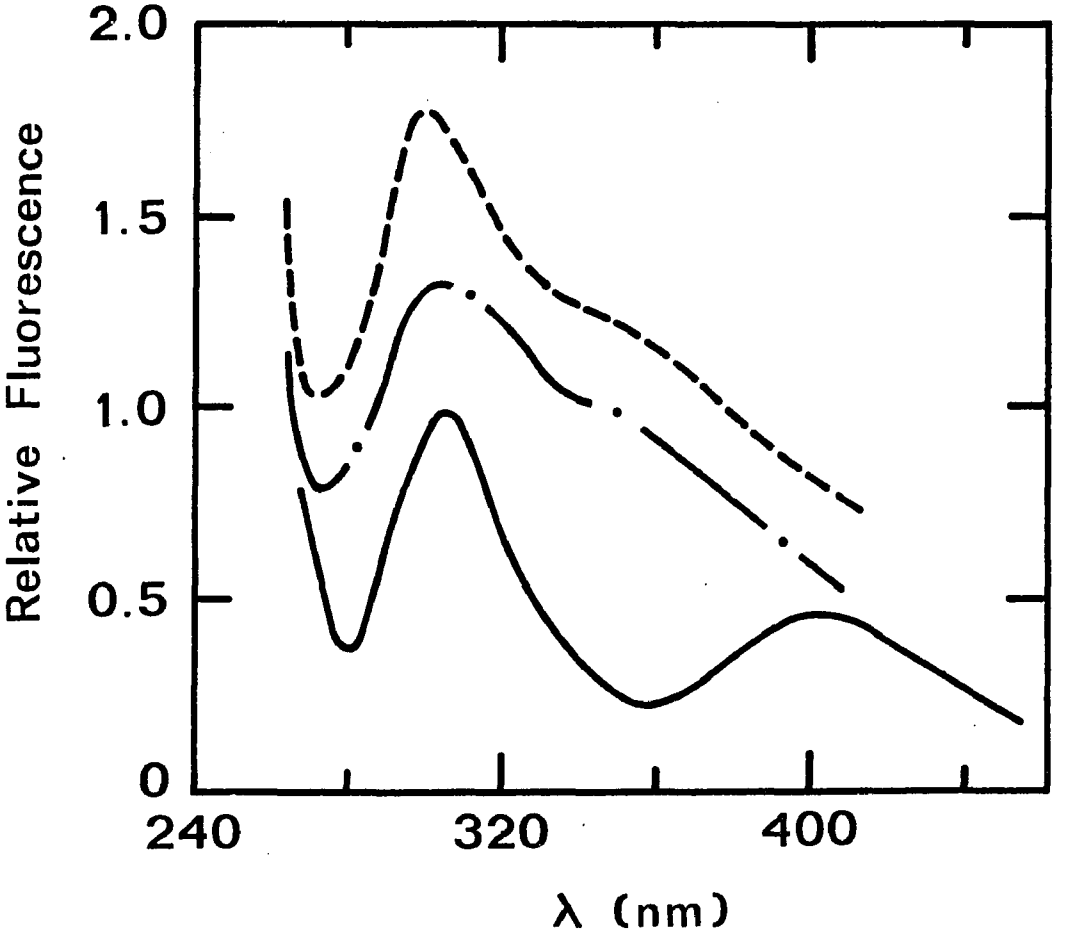


Figure 9. Fluorescence spectra of poly(Lys^{50},Tyr^{50}) and a complex with calf thymus DNA (r = 1.02) (---) and M.luteus DNA (r = 0.97) (...). Excitation wavelength is 250nm.

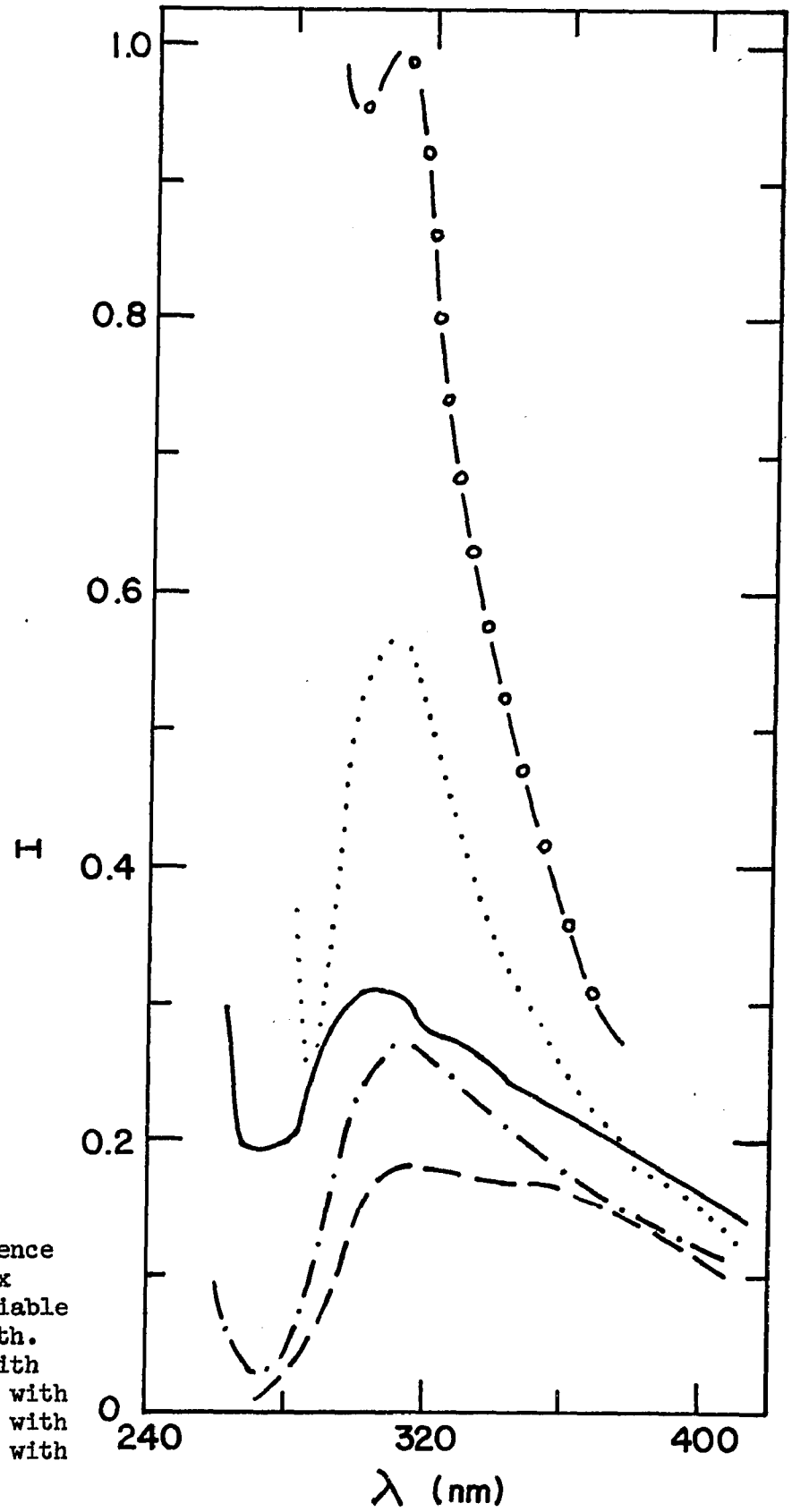


Figure 10. Fluorescence spectra of a complex (r = 1.35) with variable excitation wavelength. 250nm (__), 250nm with filter (---), 260nm with filter (-·-), 270nm with filter (...), 280nm with filter (-o-).

used to reduce the amount of emission at wavelengths below 290nm from reaching the photomultiplier. The filter was used for all excitation wavelengths except that, for excitation at 250nm, the emission spectrum was measured with or without the filter since the light scattering peak is far from the emission at 300nm. It can be seen from Figure 10 that the use of the filter slightly distorts the shape of the spectrum with excitation at 250nm since the filter does not have a sharp cutoff at 290nm. More importantly, it can be seen that the shape of the emission spectrum changes as the excitation wavelength is shifted to 280nm. There is a large increase in emission around 300-310nm which is probably due to an increase in emission from bound tyrosine as the excitation wavelength approaches its absorption maximum.

Variable Temperature Studies on Poly(Lys^{50},Tyr^{50})-DNA Complexes

One possibility for the early melting seen in the thermal denaturation studies is that the structure of copolypeptide-bound regions is temperature-dependent. Some structural fluctuation in copolypeptide-bound regions may occur before they are denatured at T_m'. Structural changes on pure DNA before its melting temperature have been observed by CD but not by absorbance.[48-50] Since the tyrosine chromophore in the complex is not as well protected as the bases in DNA, any structural fluctuation on copolypeptide-bound regions could possibly cause a detectable spectral change in tyrosine. To study this possibility in more detail, studies were carried out at variable temperatures using absorption, fluorescence, and CD.

Absorption Spectra of Poly(Lys^{50},Tyr^{50})-DNA Complexes at Variable Temperatures

Figure 11 shows the difference absorption spectra of calf thymus DNA, poly(Lys^{50},Tyr^{50}) or a complex with r = 1.3 taken at room and at a higher temperature. For copolypeptide alone, there is only a very small increase in absorbance near 230nm and 280nm from 22 to 80° For pure DNA, with a concentration equivalent to that of free base pairs in the complex there is a very small spectral change near 265nm at 50° which is greatly increased after 60° when the DNA is denatured. As expected, these difference spectra are similar to one another and to that of native DNA.

The difference spectra of the complex show several interesting bands. At 50 or 60°, there are peaks at 235 and 290nm and a broad band between them. The middle band appears as a peak when the temperature is 70° or higher and is due to partially or fully denatured free base pairs. As shown in Figure 5, although pure DNA is significantly denatured at 60°, the free base pairs in the complex are not denatured until about 70°. Therefore, the spectral changes at 50 or 60° can be attributed entirely to structural changes on the copolypeptide-bound regions in the complex.

Absorption Spectra of Poly(Lys^{50},Tyr^{50}) at Variable pH

Figure 12 shows the absorption spectra of poly(Lys^{50},Tyr^{50}) at pH 6.8 and 11.0 and their difference spectrum. The difference absorption spectrum due to deprotonation has a major peak at 245nm and a minor peak at 295nm. These peaks are very similar to those in the difference spectra of copolypeptide-DNA complex at higher temperature. This result implies that partial deprotonation may occur for bound

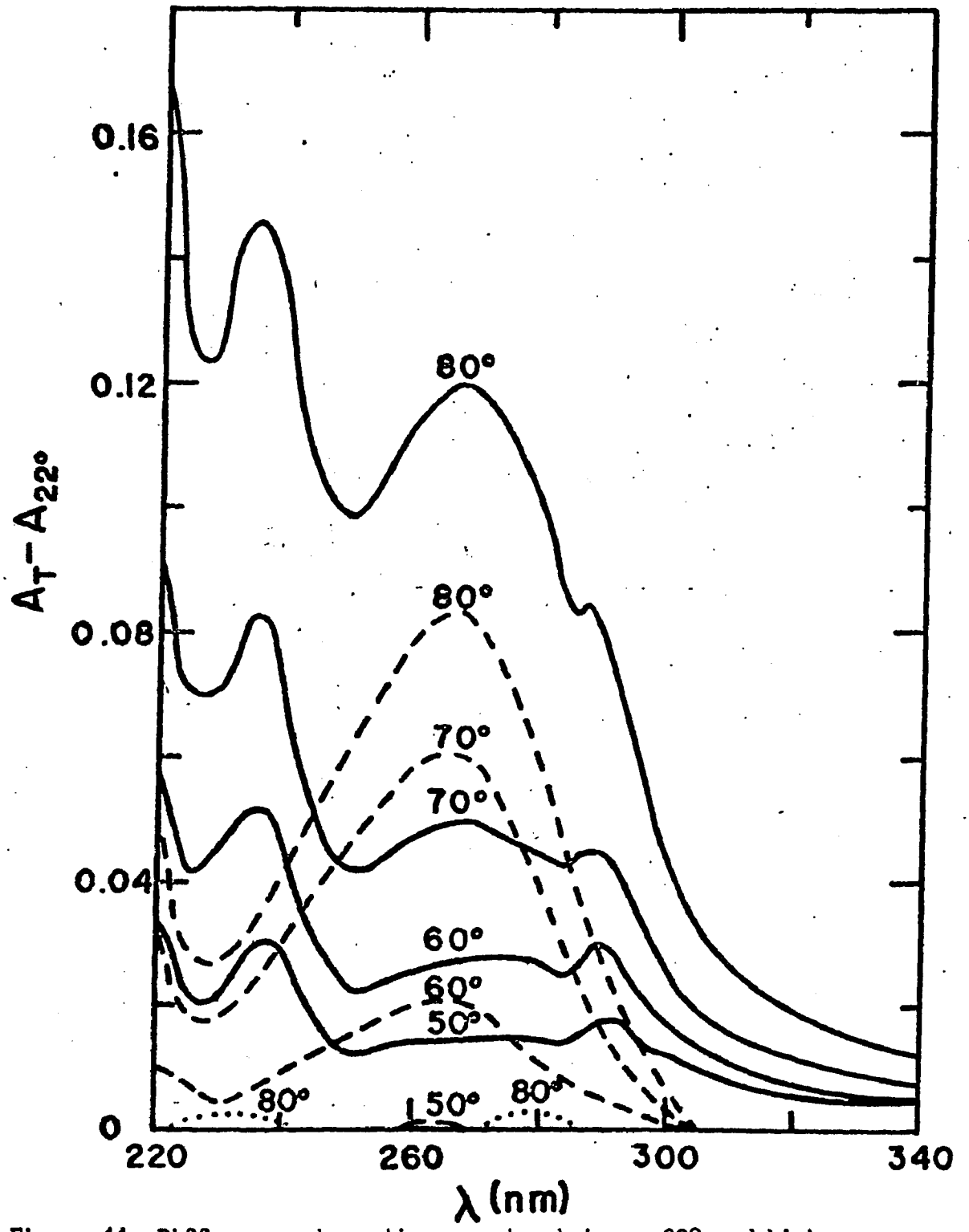


Figure 11. Difference absorption spectra between 22° and higher temperatures for DNA (---), free copolymer (...) and a complex with r= 1.3 (—).

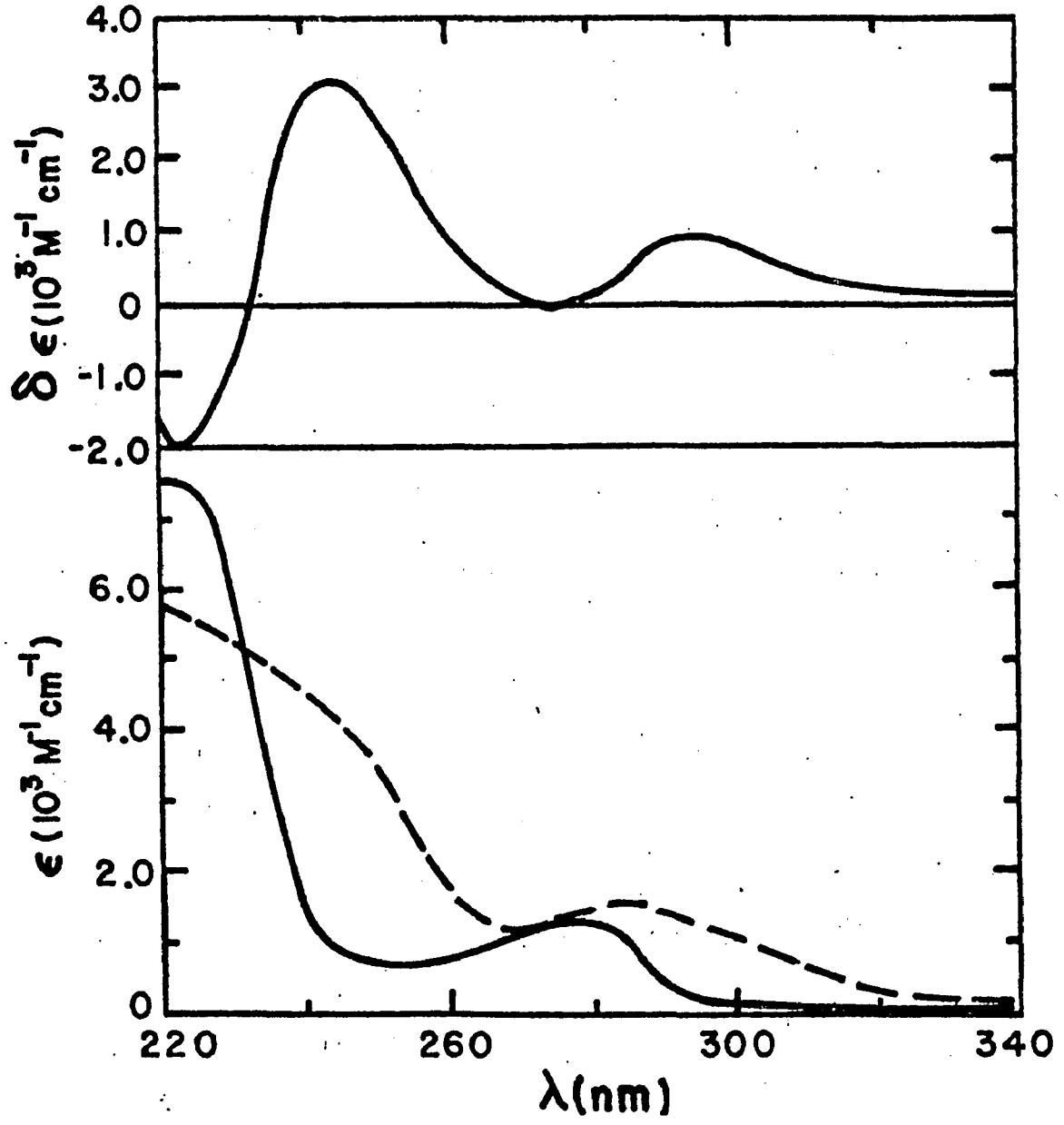


Figure 12. Absorption and difference absorption spectra of free Poly(Lys^{50},Tyr^{50}) at pH 6.8 (—); 11.0 (---); and the difference (—).

tyrosine at higher temperatures before denaturation.

Circular Dichroism Spectra of Poly(Lys^{50},Tyr^{50})-DNA Complexes at Variable Temperatures

The CD spectra of calf thymus DNA at variable temperatures are shown in Figure 13 and of a complex with r = 1.35 in Figure 14. For the complex, significant spectral changes have been observed at 275, 245 and 230nm. Figure 15 shows the temperature dependence of the CD spectra at these three wavelengths for the complex and pure DNA. For pure DNA, spectral changes at these wavelengths before its T_m (about 60°) are small compared with those which accompany melting after 60°. On the other hand, there are very significant spectral changes on the complex before its T_m (about 70°). These differences in CD between DNA and the complex indicate that significant structural changes have occurred on copolypeptide-bound regions in the complex even before the double helical DNA is denatured.

Fluorescence of Poly(Lys^{50},Tyr^{50})-DNA Complexes at Variable Temperatures

Emission at 307nm as a function of temperature is shown in Figure 16 for free poly(Lys^{50},Tyr^{50}) and a complex with r = 1.16. For the copolypeptide, whether it is excited at 250 or 280nm, there is a decrease in fluorescence with increasing temperature. For the complex however, the results with 250 or 280nm excitation are very different. The fluorescence almost doubles between 70 and 90° if excited at 250nm while it is slightly quenched if excited at 280nm. If we are looking mainly at tyrosine fluorescence with 280nm excitation, this decrease in fluorescence can be attributed to the partial deprotonation on the

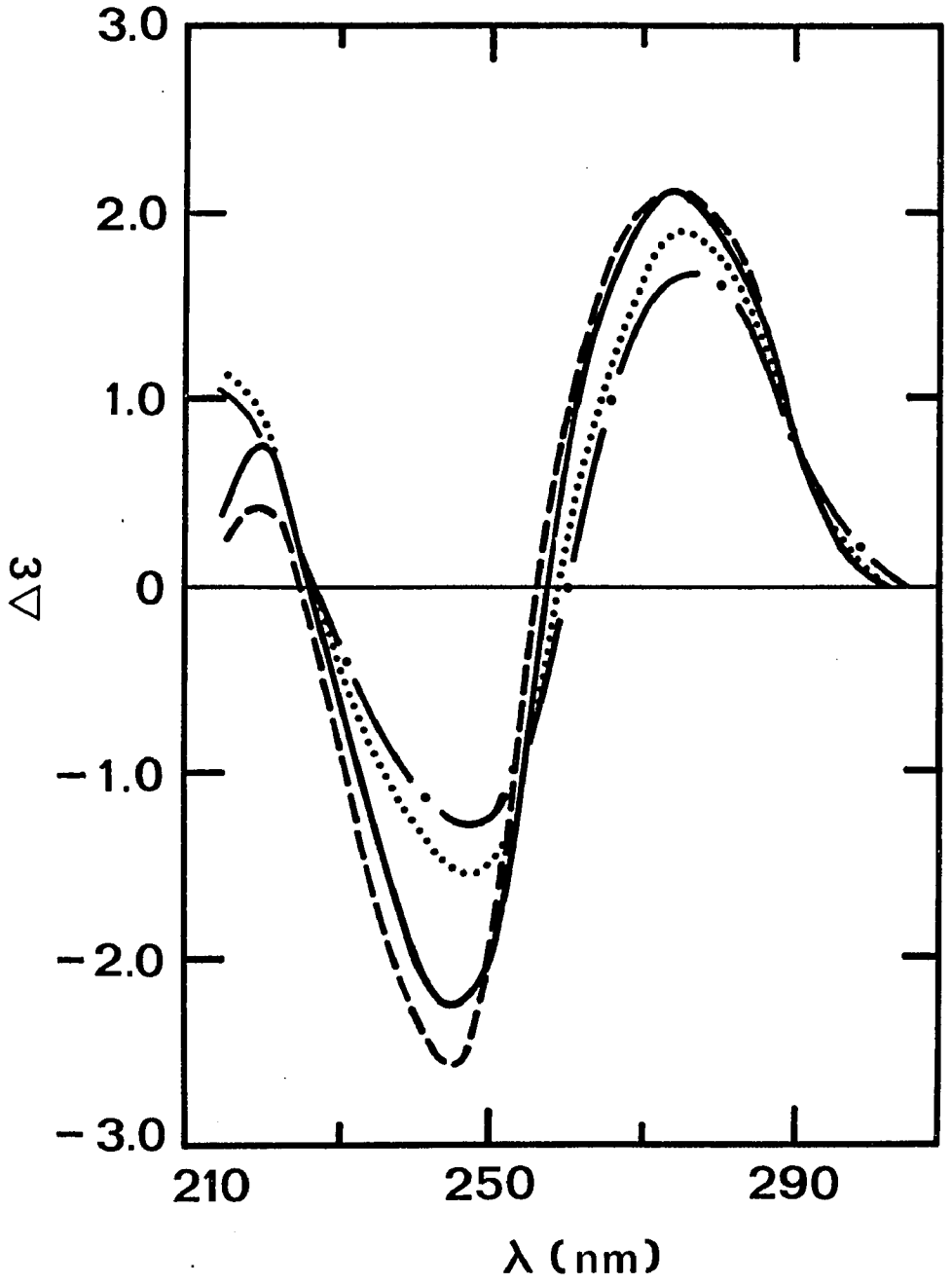


Figure 13. CD spectra of calf thymus DNA at various temperatures: 22° (__); 50° (---); 70° (...) and 80° (-.-).

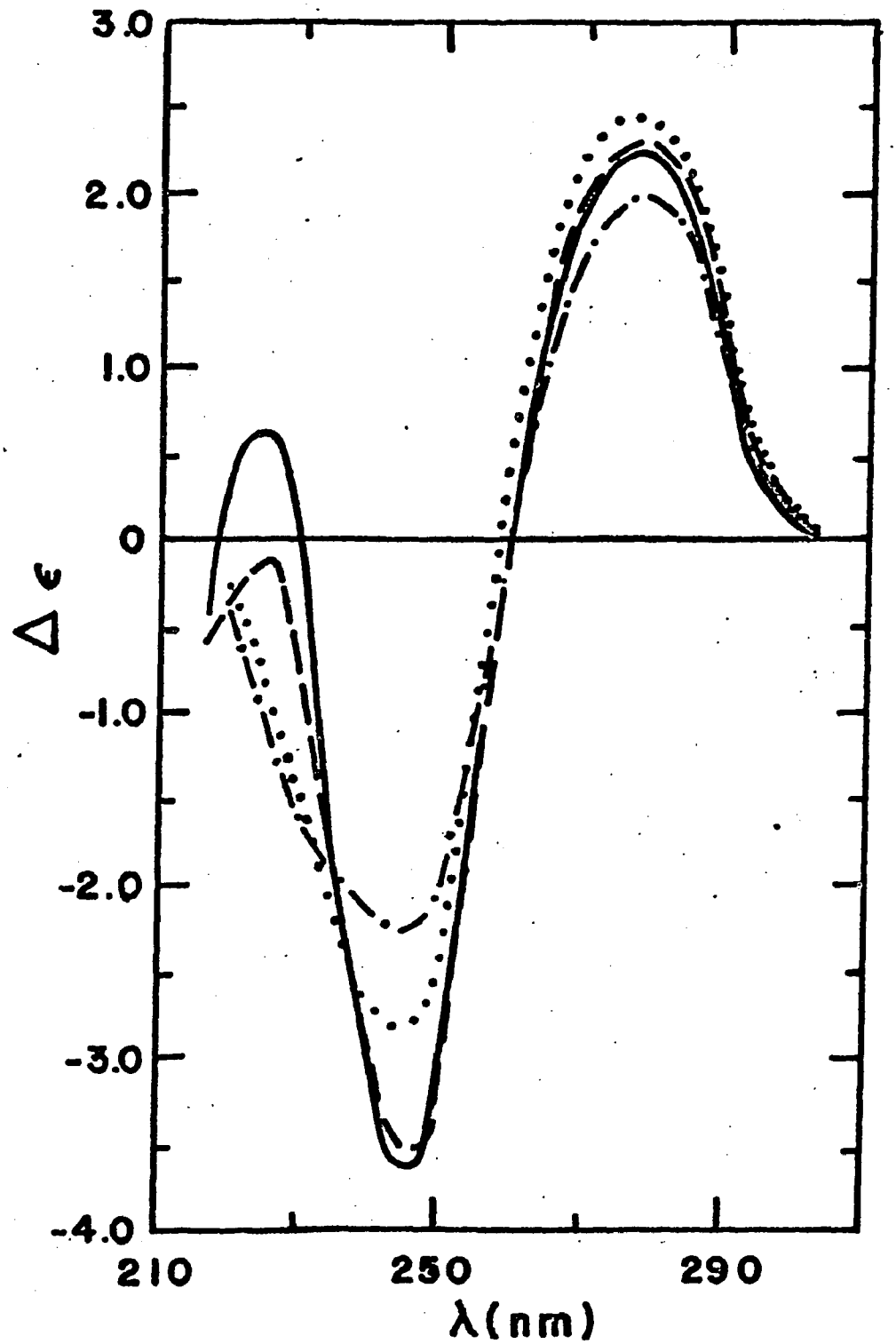


Figure 14. CD spectra of a Poly(Lys^{50},Tyr^{50})-DNA complex (r= 1.34) at various temperatures: 22°(—); 50° (---); 70° (...); and 80° (-·-).

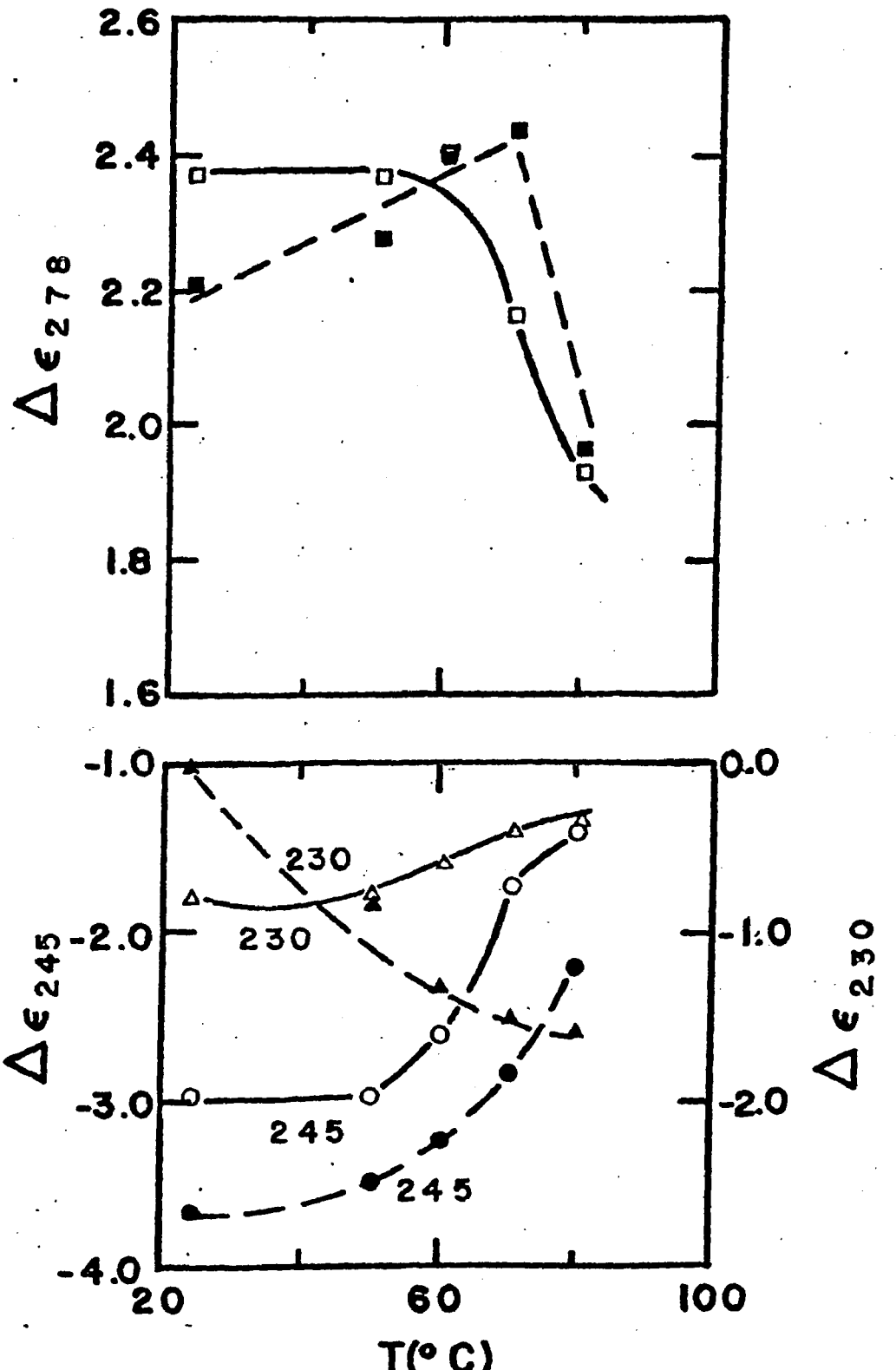


Figure 15. Temperature dependence of CD of DNA and Poly(Lys^{50}, Tyr^{50})-DNA complex (r = 1.34). DNA (○,△,□) and the complex (●,▲,■).

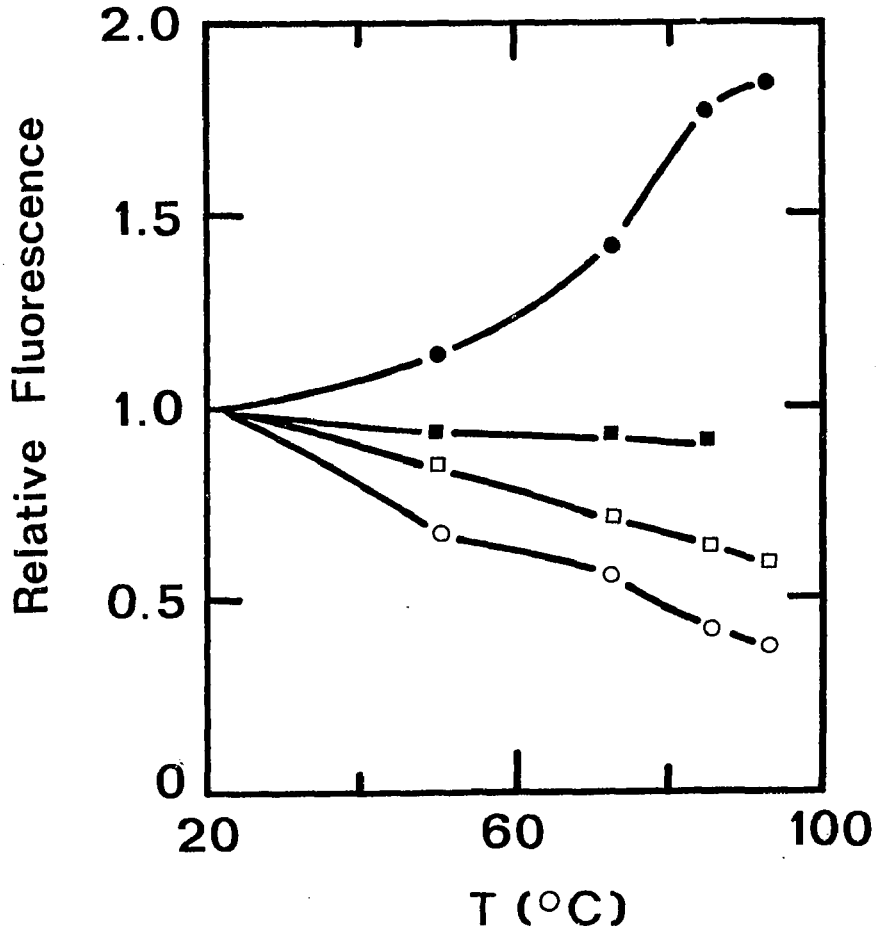


Figure 16. Temperature dependence of fluorescence at 300nm for poly(Lys^{50},Tyr^{50}) excited at 250nm (o) and 280nm (□) and a complex with r = 1.16 at 250nm (●) and at 280nm (■).

tyrosine since the fluorescence of free poly(Lys^{50},Tyr^{50}) decreases by 80% when the pH is raised from 6.8 to 9.5.

Discussion

This study was initally undertaken to determine whether the presence of non-basic amino acid residues in a protein will increase or decrease the thermal stability of protein-bound DNA and whether it will also change the DNA structure. The results show that there is no detectable effect of tyrosine on the thermal stability of polylysine-bound base pairs. In copolypeptide-bound regions, there is one tyrosine per nucleotide. If tyrosine has any significant effect on the thermal stability of a DNA base pair, a change in T_m' would be expected. This finding is different from reports on thermal stabilization of DNA by oligopeptides including tyrosine residues.[24,26] Thermal stabilization of DNA by these oligopeptides could possibly be due to the presence of $--NH_3^+$ at the N-terminal in tyrosylpeptides[24] and at both the N-terminal and lysine side chain in oligopeptides of lysine and tyrosine [26] rather than to the tyrosine chromophore itself. Reversible binding between DNA and an oligopeptide is however, quite different from an approximately irreversible binding between DNA and a copolypeptide with 50% of its residues carrying positive charges.

Figure 4 shows that the presence of tyrosine in poly(Lys^{50},Tyr^{50}) reduces the conformational effect on DNA resulting from polylysine binding (Table II and Reference 12). For instance, when 80% of the base pairs are bound by this copolypeptide, the peak is shifted from 275 to 278nm, the crossover from 256 to 260nm and $\Delta\epsilon_{278}/-\Delta\epsilon_{246}$ is reduced from 0.81 to 0.62. These CD effects on DNA are equivalent to those in a

polylysine-DNA complex with about 20 to 30% of its base pairs bound. Thus, the presence of 50% tyrosine residues in the copolypeptide reduces the structural effect on polylysine-bound DNA by a factor of three to four.

Another interesting result is the structural fluctuation in copolypeptide-bound regions before they are denatured. The similarity of the peaks at 235 and 290nm in the difference absorption spectrum of a complex at elevated temperatures (Figure 11) and those of free copolypeptide at neutral and high pH (Figure 12), implies that partial deprotonation may occur for bound tyrosine at higher temperatures. This suggestion of partial deprotonation on bound tyrosine is also supported by the CD results. As shown in Figures 14 and 15, a sharp decrease in CD at 230nm occurs before 60°. Deprotonation of the tyrosine in free copolypeptide also causes a large decrease in CD at this wavelength (Figure 3). These results suggest that a full or partial deprotonation could possibly occur on bound tyrosine residues at higher temperatures. This deprotonation might include hydrogen bonding between the -OH of tyrosine and the phosphates or other moieties of DNA.

The fluorescence quenching on tyrosine when the copolypeptide is bound to DNA is in agreement with previous results on the binding of tyrosine or tyrosine-containing oligopeptides to DNA.[29,34] This is true only when excited at 280nm, the absorption maximum of tyrosine. When the complexes were excited at 250nm, an enhancement in fluorescence was observed. However, it will be shown by data to be presented in Chapter VI, that this fluorescence comes mainly from DNA since polylysine binding to DNA also causes an enhanced fluorescence spectrum similar to that in Figure 9. The enhanced fluorescence at higher temperatures with 250nm

excitation depends upon the presence of tyrosine and is not seen in polylysine-DNA complexes (Figure 52). Prehaps a stacking interaction between the tyrosine chromophores and the DNA bases might occur as the temperature is increased. Additional evidence for this will be presented when the interaction of poly(Lys^{50},Tyr^{50}) with single-stranded synthetic polynucleotides is presented in Chapter VI.

CHAPTER IV

STUDIES ON THE INTERACTION BETWEEN POLY(L-LYSINE^{58},L-PHENYLALANINE^{42}) AND DNA

To continue the studies on the role of aromatic amino acids in protein-DNA interaction, the binding of a random copolymer containing 58% lysine and 42% phenylalanine to DNA was examined. Previous studies have suggested that phenylalanine, like tyrosine, can intercalate into DNA.[24,25,30,31] Once again, these reports are based on the use of oligopeptides. The results from the studies on poly(Lys^{50},Tyr^{50})-DNA complexes showed that random copolomers of lysine and an aromatic amino acid are useful model proteins and can provide additional information not available with oligopeptide studies.

Because of the low solubility of poly(Lys^{58},Phe^{42}) in EDTA buffer, a test of Beer's law was made on this copolypeptide in Tris or EDTA buffer. Figure 17 shows absorption at 257nm, the absorption maximum for phenylalanine, plotted as a function of copolypeptide concentration. A linear relationship is found in both Tris and EDTA buffer but the line goes through the origin in Tris buffer but not in EDTA. For this reason, Tris buffer was used for the studies on this copolypeptide. A sample of weighed copolypeptide was dissolved and the absorption at 257nm was

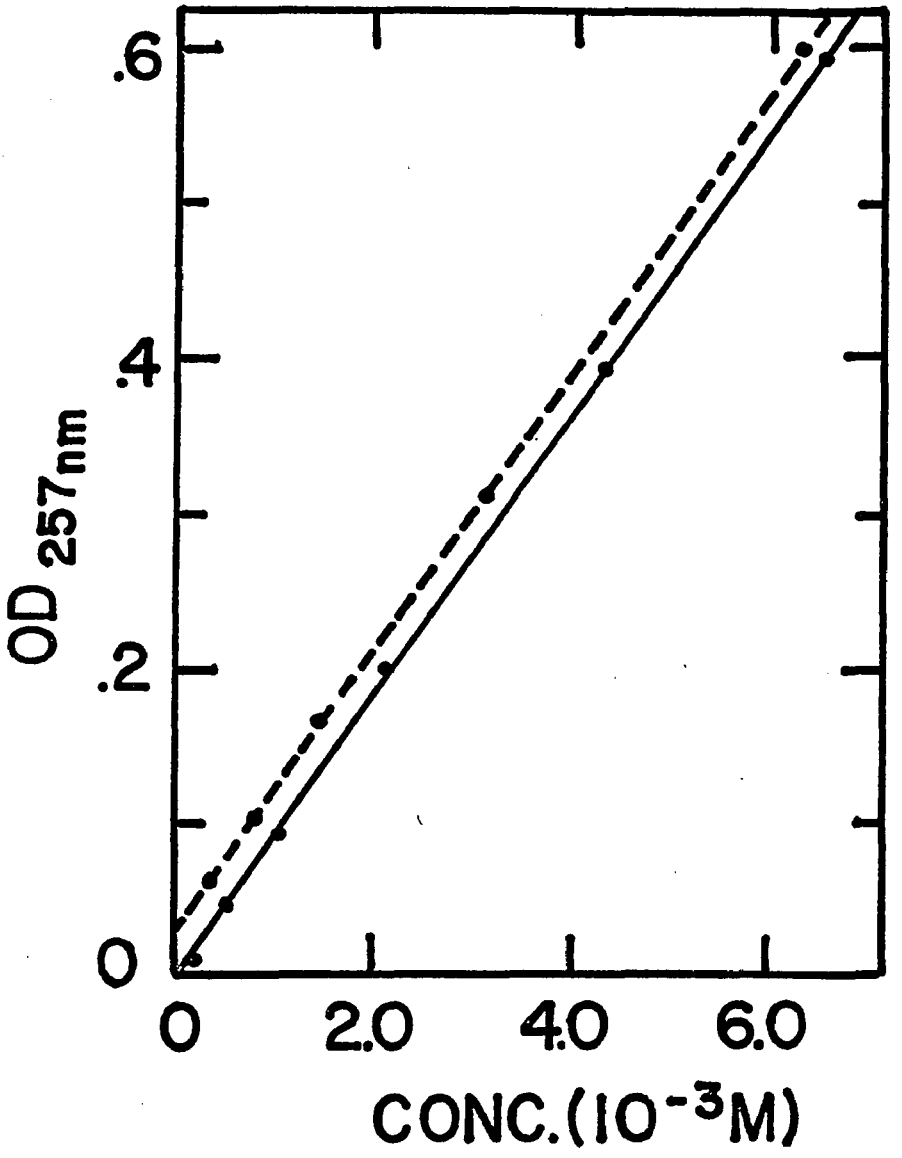


Figure 17. Absorption of poly(Lys^{58},Phe^{42}) at 257nm as a function of concentration in Tris (___) or EDTA (---) buffer.

measured. The extinction coefficient of phenylalanine in the copolypeptide was determined to be 210 $M^{-1}cm^{-1}$ which is close to the value of free phenylalanine, 195 $M^{-1}cm^{-1}$.[40]

Circular Dichroism Spectra of Poly(Lys^{58},Phe^{42})

Figure 18 shows the CD spectra of poly(Lys^{58},Phe^{42}) at varied concentrations. Near 260nm the CD is very weak with two negative peaks at 261 and 268nm. The CD below 250nm is larger with a negative peak at 233nm and a positive peak at 217nm. Similar results were reported by Peggion et al[51] on random-coiled copolymers of L-lysine and L-phenylalanine. As shown in Figure 18, the positive peak at 217nm is sensitive to concentration while the negative peaks at 233nm and near 260nm are not. The concentration dependence of the CD at 217nm could imply some intermolecular interaction between copolypeptide molecules, presumably through interaction among phenylalanine residues.

Titration of Calf Thymus DNA by Poly(Lys^{58},Phe^{42})

Complexes of poly(Lys^{58},Phe^{42}) and calf thymus DNA were made and centrifuged as described earlier for poly(Lys^{50},Tyr^{50}). Figure 19 shows the titration curve of calf thymus DNA by poly(Lys^{58},Phe^{42}). Precipitation occurs at r = 1.7 amino acid residues per nucleotide which is equivalent to 1.0 lysine and 0.7 phenylalanine per nucleotide. As with poly(Lys^{50}, Tyr^{50}), precipitation occurs when the number of positively charged amino acid residues added is approximately equal to the number of negatively charged phosphates on DNA.

Absorption Spectra of Poly(Lys^{58},Phe^{42})-DNA Complexes

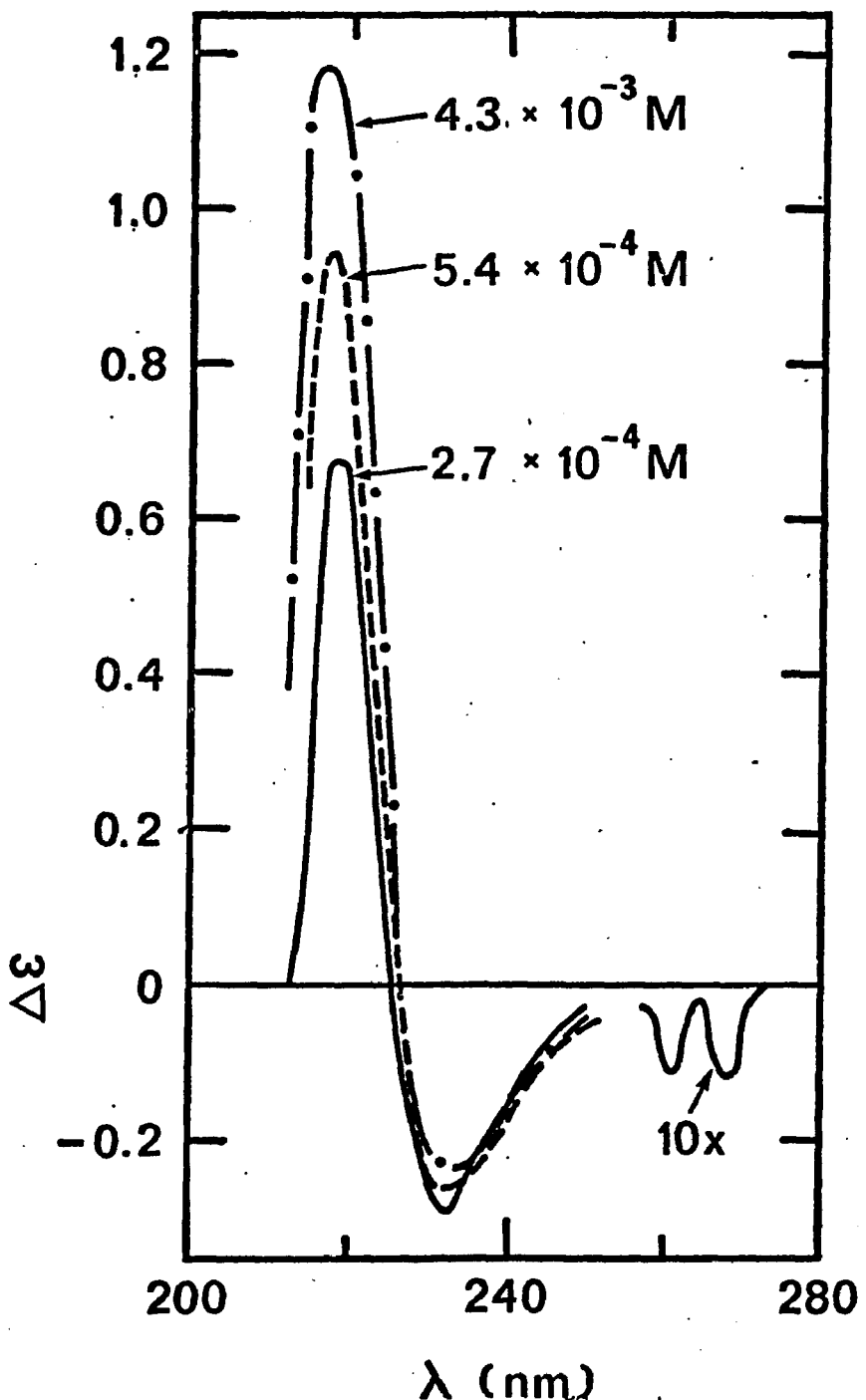


Figure 18. CD spectra of Poly(Lys^{58},Phe^{42}) in 0.001M Tris (pH 6.8). Concentrations are indicated.

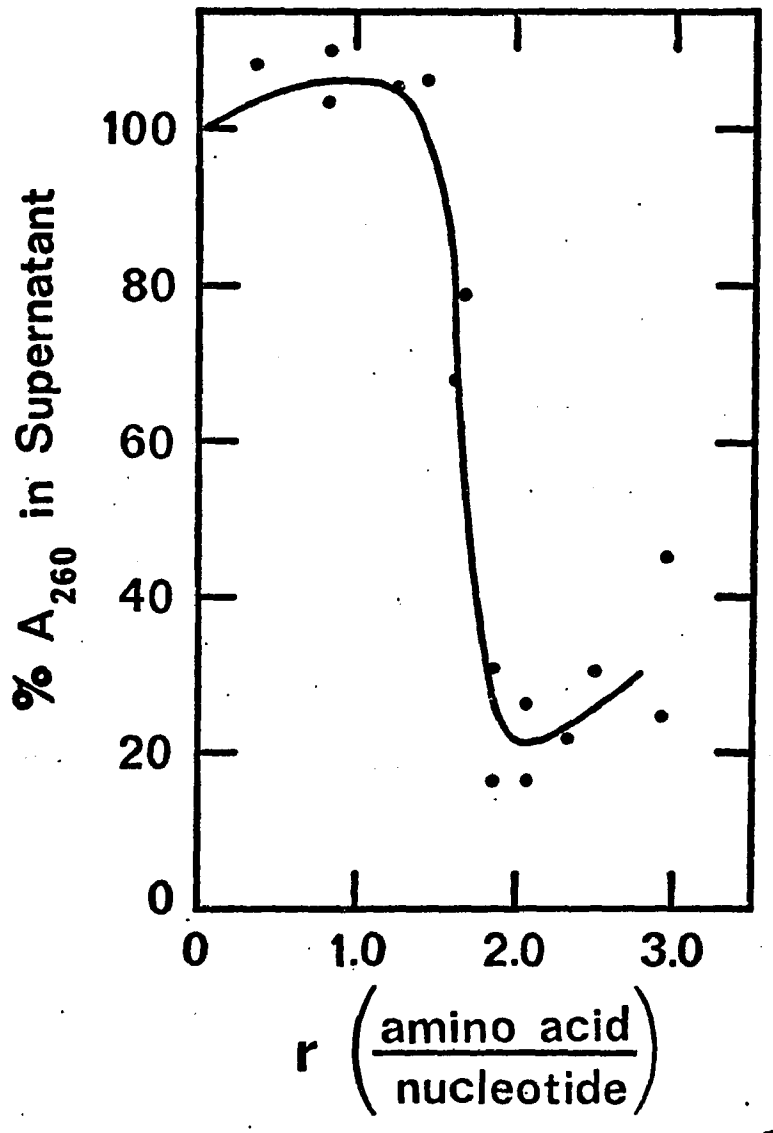


Figure 19. Titration curve of DNA by Poly(Lys^{58},Phe^{42}).

Poly(Lys^{58},Phe^{42}) has an absorption peak at 257nm and two subpeaks at 252 and 263nm,respectively (Figure 20), representing the absorbance from the phenylalanine residues.[45] The absorbance near 260nm is extremely small compared to that of DNA. Formation of a complex between DNA and this copolypeptide results in an enhancement of absorbance. If ϵ_m, ϵ_f^D and ϵ_f^P are defined as the molar extinction coefficients of the complex, free DNA and free copolypeptide respectively, the gained absorbance after binding can be calculated from

$$\delta\epsilon = \epsilon_m - (\epsilon_f^D + r\epsilon_f^P) \qquad (4)$$

This differs from Equation (1),used for poly(Lys^{50},Tyr^{50}), in that r is used instead of r/2. This is because ϵ_f^P for poly(Lys^{58},Phe^{42}) is expressed in terms of amino acid residues (lysine + phenylalanine). The gained absorbance shows a peak at 265nm and a greater increase below 230nm. The increased absorbance near 260nm is about ten times that of free copolypeptide and is too large to be accounted for solely by the phenlyalanine chromophore. The spectral changes after complex formation, as shown in Figure 20, can be contributed by both DNA and phenylalanine and indicate strong electrostatic interaction between the phenylalanine chromophore and the bases in DNA.

Circular Dichroism Spectra of Poly(Lys^{58},Phe^{42})-DNA Complexes

Figure 21 shows the CD spectra of complexes between poly(Lys^{58}, Phe^{42}) and calf thymus DNA. The CD changes induced by copolypeptide binding show a red shift for the crossover near 255nm, and a red shift and reduction of the positive band near 275nm. Similar changes are observed when M. luteus DNA with 70% guanine + cytosine is used

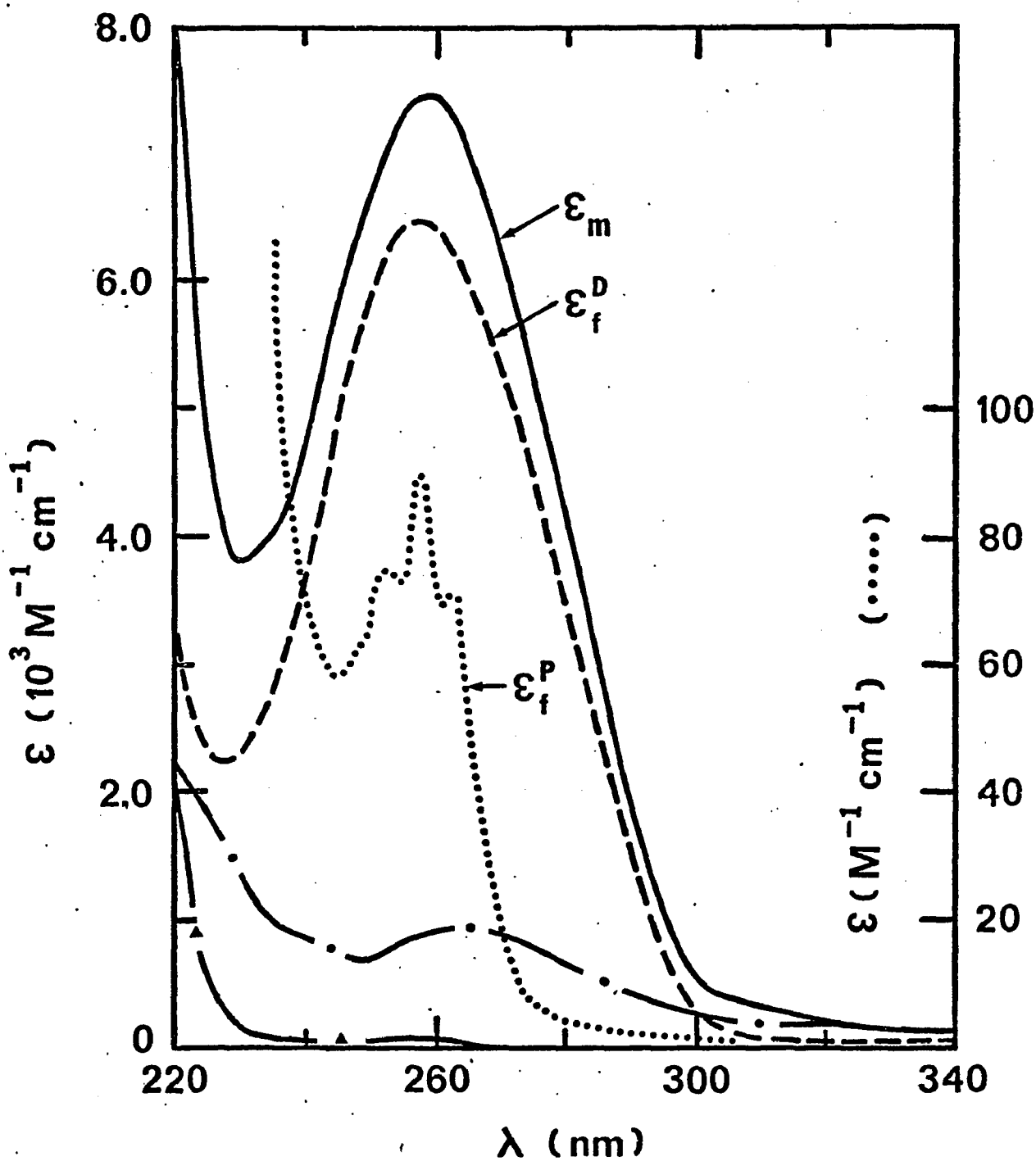


Figure 20. Absorption spectra of calf thymus DNA (---), poly(Lys^{58},Phe^{42}) in two scales (...for the scale to the right and ▲ to the left) and a complex (r = 1.13) (—). The absorbance gain after complex formation, equation (4) (-·-).

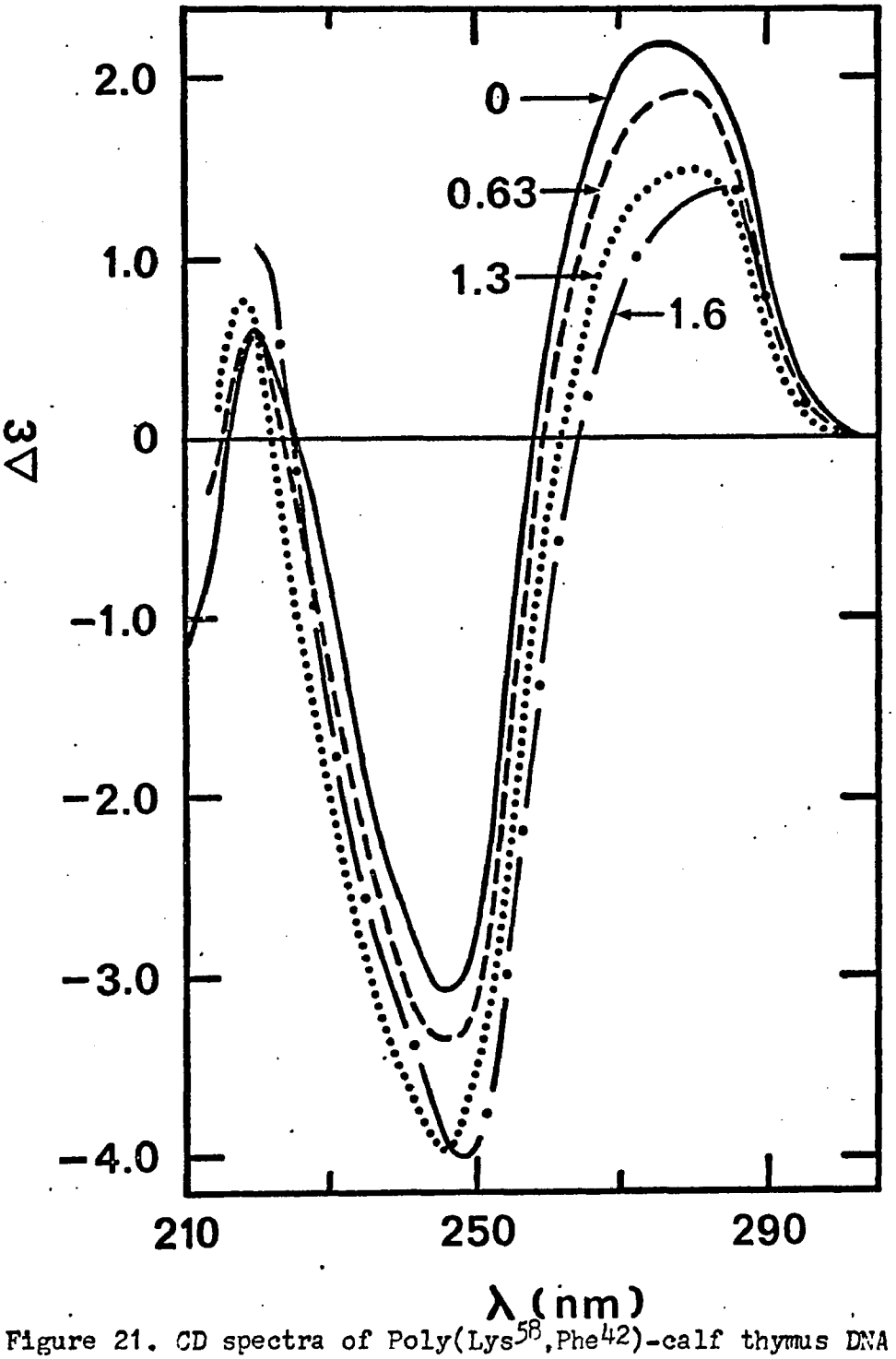


Figure 21. CD spectra of Poly(Lys^{58},Phe^{42})-calf thymus DNA complexes.

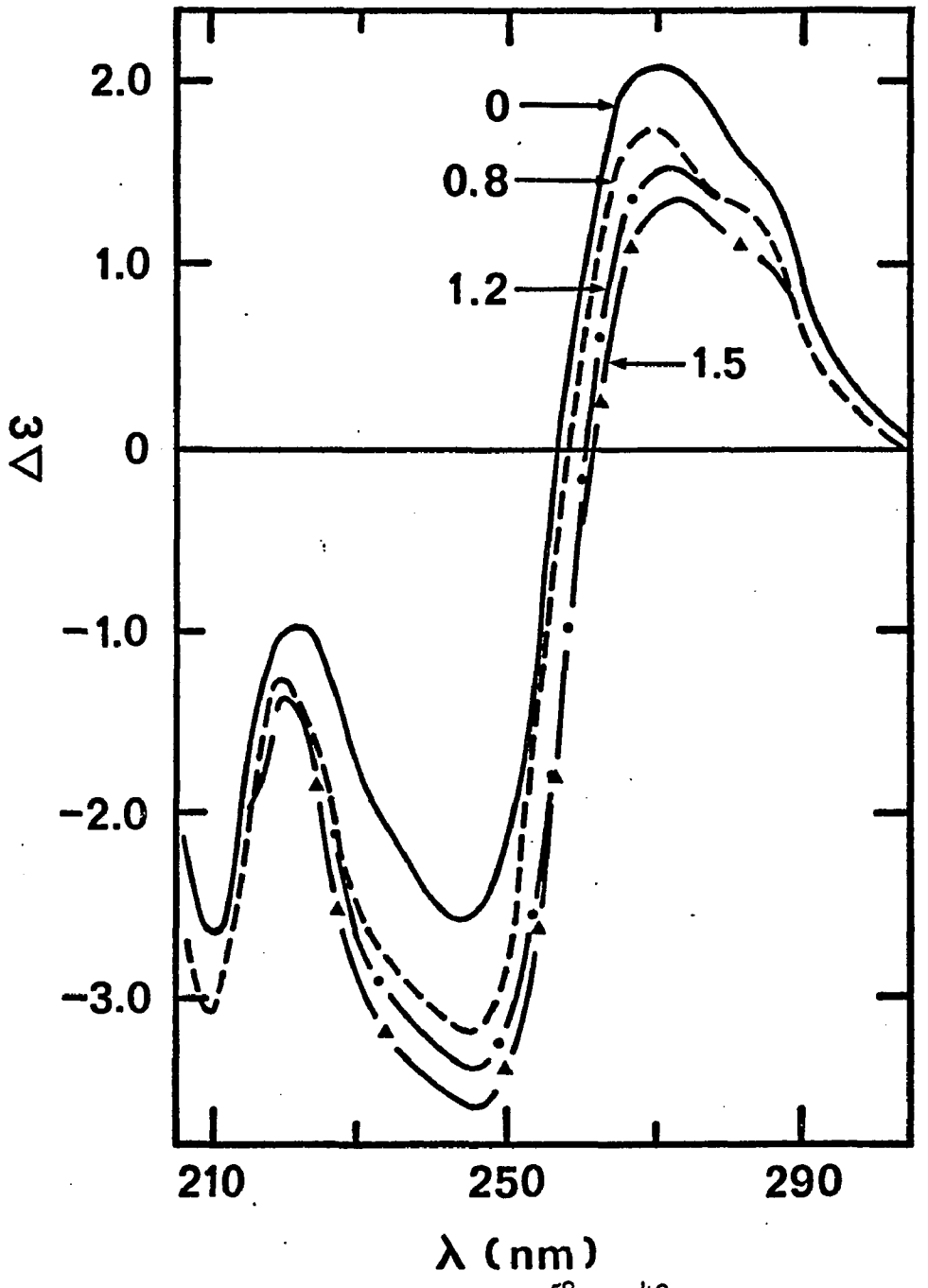


Figure 22. CD spectra of Poly(Lys^{58},Phe^{42})-M.luteus DNA complexes.

(Figure 22). Since the contribution from phenylalanine to the CD above 230nm is very small compared to that from DNA, the induced CD changes in these regions could be accounted for mainly by the DNA. For the CD near 220nm, although poly(Lys^{58},Phe^{42}) has a substantial contribution, the noise to signal ratios at these wavelengths are normally high. Thus small changes in these regions cannot be accurately followed.

The CD of the complexes, measured at 278 or 235nm, depends linearly on the input ratio, r, of copolypeptide to DNA. This implies that the measured CD can be decomposed into two components, $\Delta\epsilon^{D}_{f}$ of free base pairs and $\Delta\epsilon^{D}_{b}$ of copolypeptide-bound base pairs. The measured CD, $\Delta\epsilon_{m}$, of each complex is a sum of these two components.

$$\Delta\epsilon_{m} = (1-F)\Delta\epsilon^{D}_{f} + F\Delta\epsilon^{D}_{b} \qquad (5)$$

$\Delta\epsilon^{D}_{b}$ is then calculated for complexes with varied r values. The results for two complexes are shown in Figure 23 with a consistent $\Delta\epsilon^{D}_{b}$ having a positive maximum at 280nm, a crossover at 263nm and $\Delta\epsilon_{280} = 1.5$, about two-thirds that of pure DNA at the same wavelength. The CD changes on DNA due to binding follow the order of polylysine > poly(Lys^{58}, Phe^{42}) > poly(Lys^{50},Tyr^{50}).

Thermal Denaturation Studies on Poly(Lys^{58},Phe^{42})-DNA Complexes

For thermal denaturation studies, the complexes were dialyzed from Tris buffer to EDTA buffer. In EDTA buffer, in addition to T_{mI} at 47° for free base pairs, the binding of poly(Lys^{58},Phe^{42}) induces two characteristic melting bands, $T_{m,II}$ at 74° and $T_{m,III}$ at 90°. The area under the two higher melting bands is proportional to r, the input ratio of copolypeptide to DNA. (Figure 24)

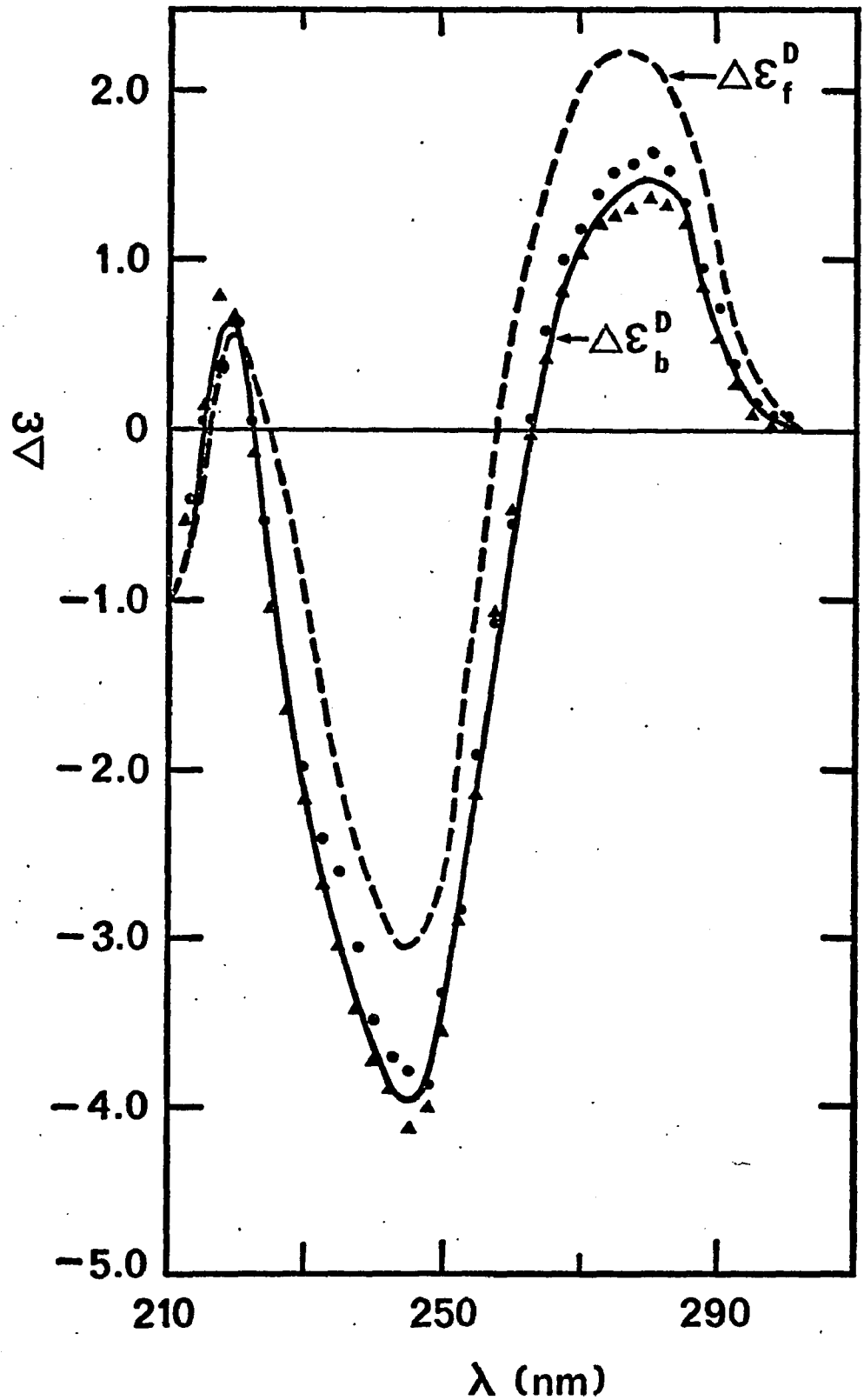


Figure 23. Calculated CD spectrum, $\Delta\epsilon_b^D$, for calf thymus DNA bound by poly(Lys^{58},Phe^{42}) from equation (5) (r = 0.66)(●) and (r = 1.32)(▲). $\Delta\epsilon_f^D$ of free calf thymus DNA is included.

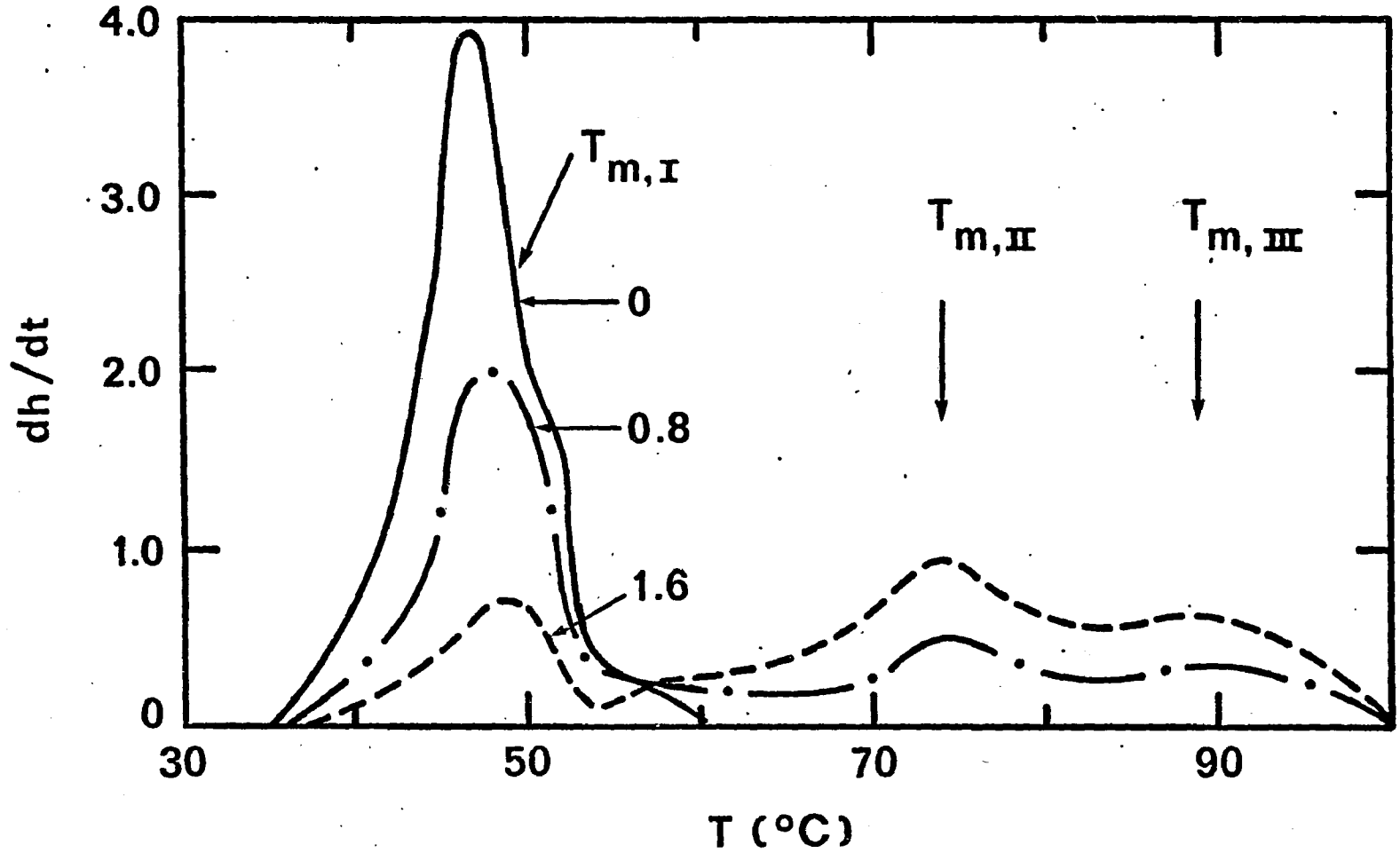


Figure 24. Derivative melting curves of Poly(Lys^{58},Phe^{42})-calf thymus DNA complexes in EDTA.

If M. luteus DNA (70% G + C) is used instead of calf thymus DNA (42% G + C) similar melting results are observed except that, each melting temperature is increased slightly, $T_{m,I}$ at 59°, $T_{m,II}$ at 79° and $T_{m,III}$ at 96°, due to the higher G + C content (Figure 25). The ratio of the area under melting band III to that under melting band II is also independent of the G + C content. This excludes the possibility that the two bands at higher temperature result from the melting of bound A·T and G·C pairs.

A full interpretation of melting band II and III will be given later, after further experiments are presented. If, however, it is assumed that $T_{m,III}$ corresponds to the melting of copolypeptide-bound DNA base pairs, it is seen that the presence of phenylalanine destabilizes the copolypeptide-bound regions. Under these conditions polylysine-bound DNA melts at 98°, as does poly(Lys^{50},Tyr^{50})-bound DNA, but $T_{m,III}$ for poly(Lys^{58},Phe^{42})-DNA is only 90°. This destabilization could be due to the effect of phenylalanine on full charge neutralization on the phosphates by lysine residues. To study this possibility a sample of DNA and two complexes (r = 0.92 and 1.30) were dialyzed into 0.005M and 0.01M NaCl (Figure 26). At higher ionic strength $T_{m,I}$ of free base pairs is raised significantly and $T_{m,III}$ is also raised from 90 to 95°. This result is in contrast to a lack of change in T_m' in polylysine-DNA and polyarginine-DNA complexes at higher ionic strength[52,14] and implies that there is no full charge neutralization on the phosphate lattice with copolypeptide binding.

In order to determine the β value or ratio of amino acid residues per nucleotide in copolypeptide-bound regions, equations 2 and 3 can be used as was done for poly(Lys^{50},Tyr^{50})-DNA complexes. A plot of Equation (3) is shown in Figure 27 and the slope β , is determined to

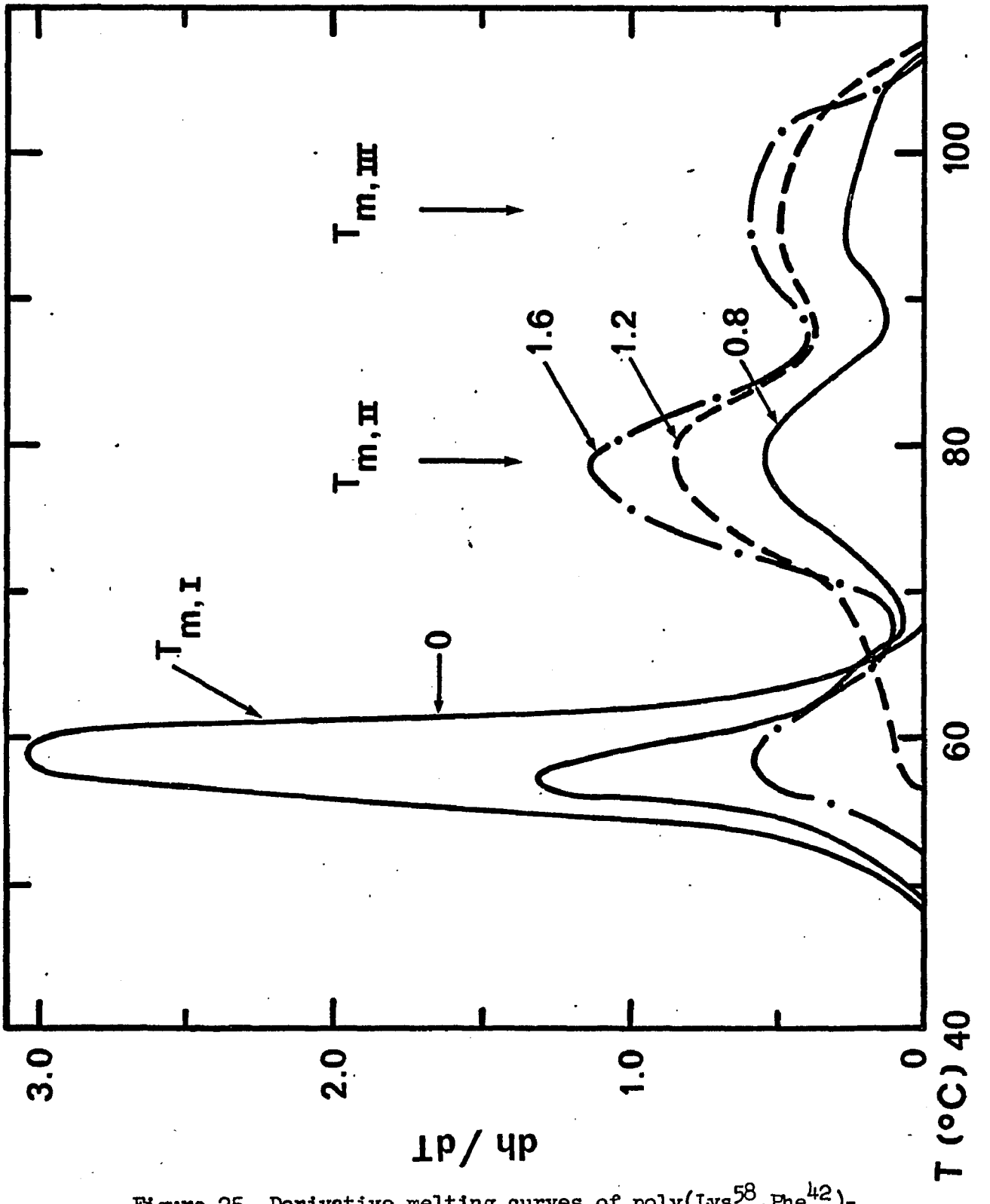


Figure 25. Derivative melting curves of poly(Lys^{58},Phe^{42})-M. luteus DNA complexes in EDTA buffer. r values are indicated.

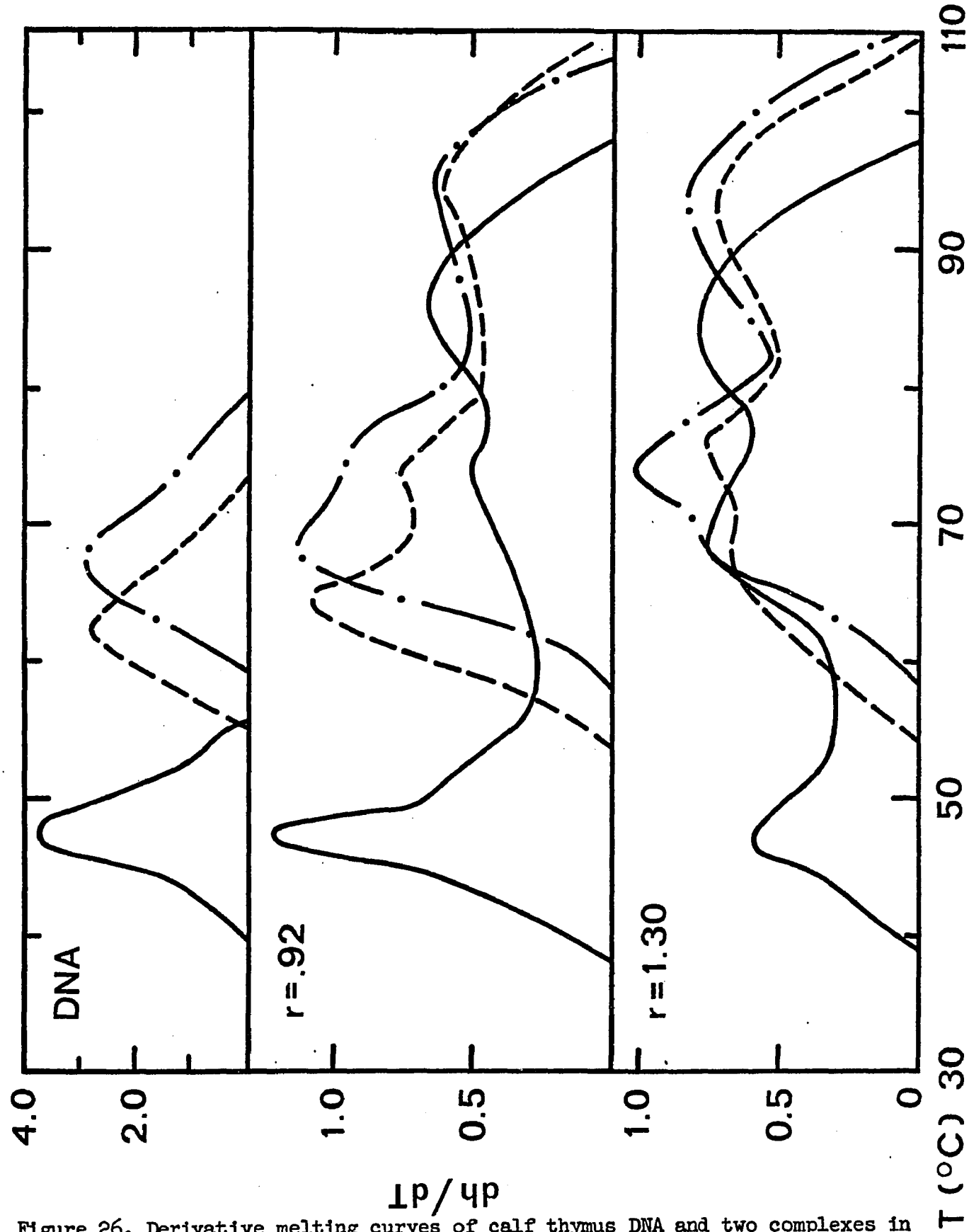


Figure 26. Derivative melting curves of calf thymus DNA and two complexes in 0.005M NaCl (---) and 0.01M NaCl (-·-).

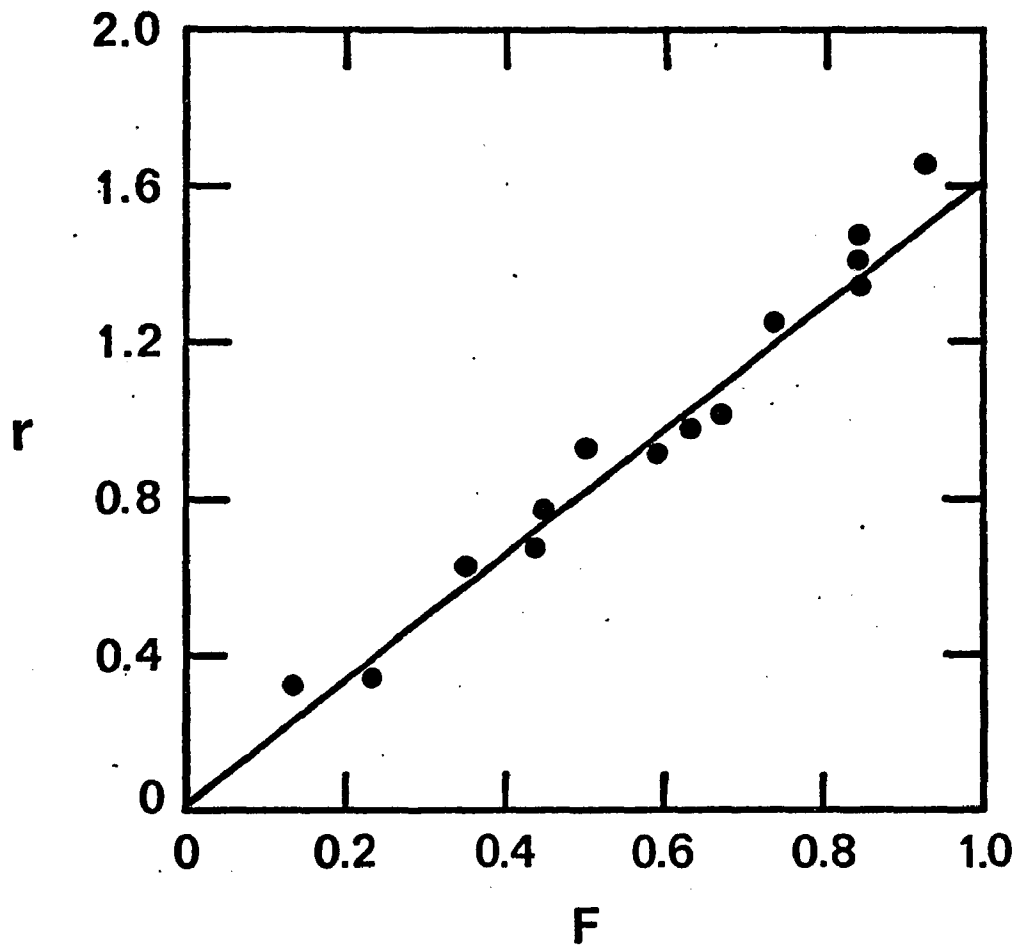


Figure 27. Linear plot of equation (3).

be 1.6 amino acid residues per nucleotide, equivalent to 0.93 lysine and 0.67 phenylalanine residues per nucleotide.

Fluorescence Studies on Poly(Lys^{58},Phe^{42})-DNA Complexes

The relative fluorescence emission spectrum of poly(Lys^{58},Phe^{42}) excited at 250nm is shown in Figure 28a. The emission maximum is at 280nm which is identical to that of phenylalanine.[45] Figure 29 shows the relative fluorescence at 280nm as a function of copolypeptide concentration. There is a linear relationship between fluorescence and concentration from 0 to 10^{-3}M, the concentration range for all the experiments.

When complexes are formed by binding poly(Lys^{58},Phe^{42}) to DNA from either calf thymus or M. luteus, a broad fluorescence spectrum with a peak at 300nm is obtained (Figure 28a). The spectra are similar to those seen with poly(Lys^{50},Tyr^{50}) binding to DNA (Figure 9). The fluorescence of DNA alone is small compared to that of the copolypeptide or complexes.

As shown in Figure 28b, the binding of poly(Lys^{58},Phe^{42}) to denatured calf thymus DNA or poly(A) shows spectral changes very similar to those induced by the binding of the copolypeptide to native DNA. Again, the fluorescence of denatured DNA or poly(A) alone is very small. These results imply that the induced fluorescence could possibly originate from a strong electronic interaction of the phenylalanine chromophore with nucleic acids, most likely a stacking interaction with the bases.

Figure 30 shows the emission spectrum of a calf thymus complex ($r = 1.3$) at variable excitation wavelength. The use of a filter again distorts the shape of the spectrum, but, unlike the results with a

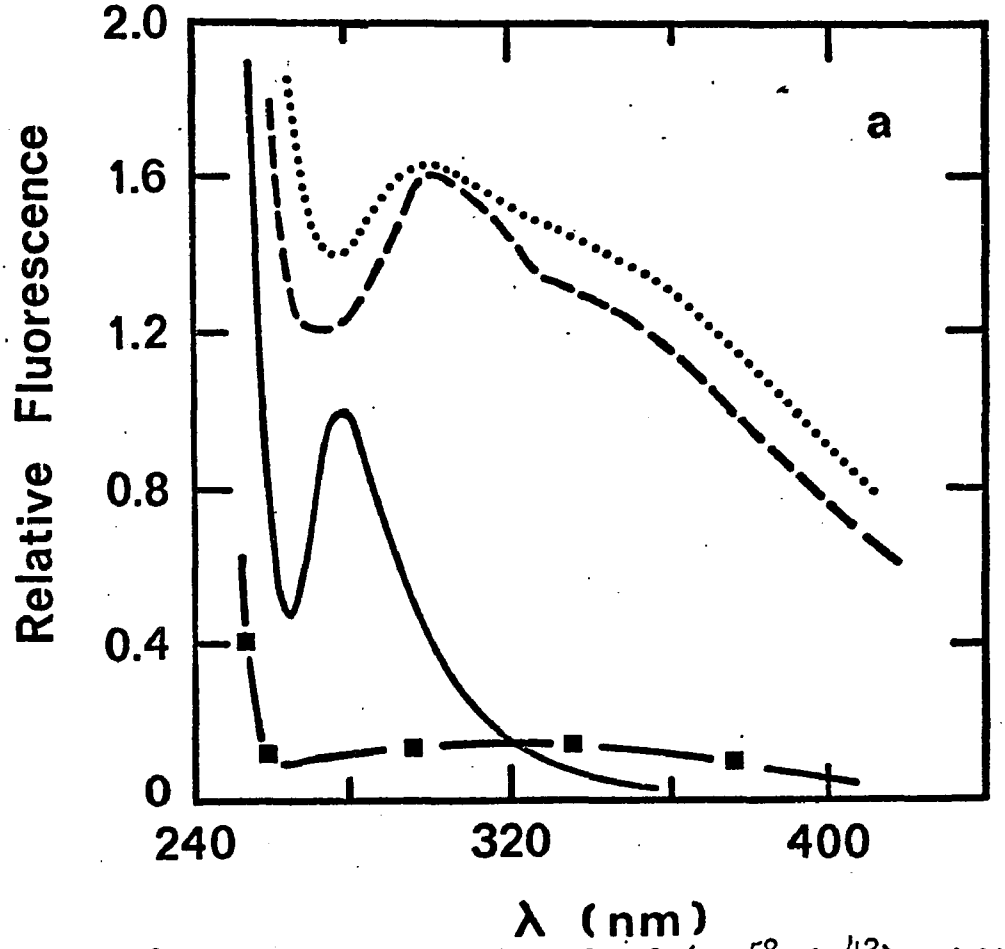


Figure 28a. Fluorescence spectra of Poly(Lys^{58},Phe^{42}) and its complex with calf thymus DNA (r = 1.35)(...) and with M. luteus DNA (r = 1.25)(---) and calf thymus DNA alone(■). Excitation is at 250nm.

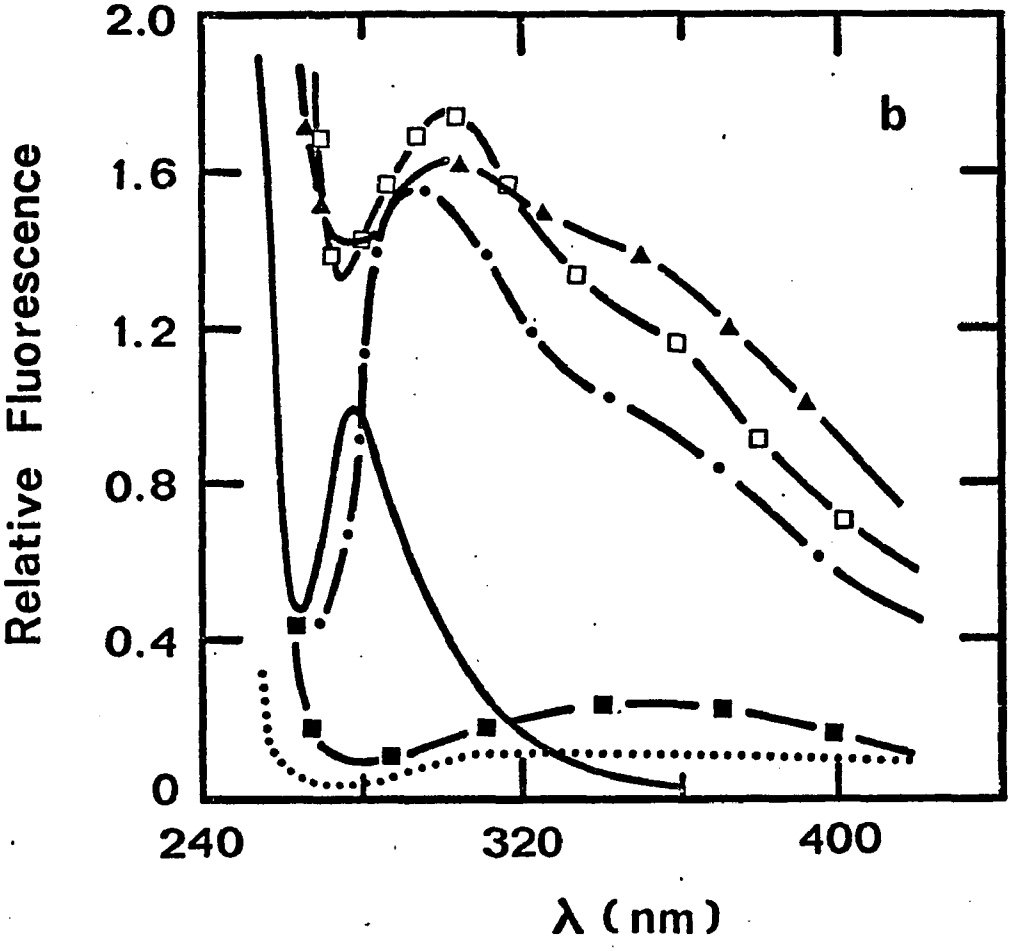


Figure 28b. Fluorescence spectra of Poly(Lys^{58},Phe 42)(—) and its complex with calf thymus DNA (▲)(r = 1.35), with denatured DNA ( )(r = 1.35) and with poly(A)(●) (r = 0.75); denatured DNA alone (■) and poly(A) alone (···). Excitation wavelength is 250nm.

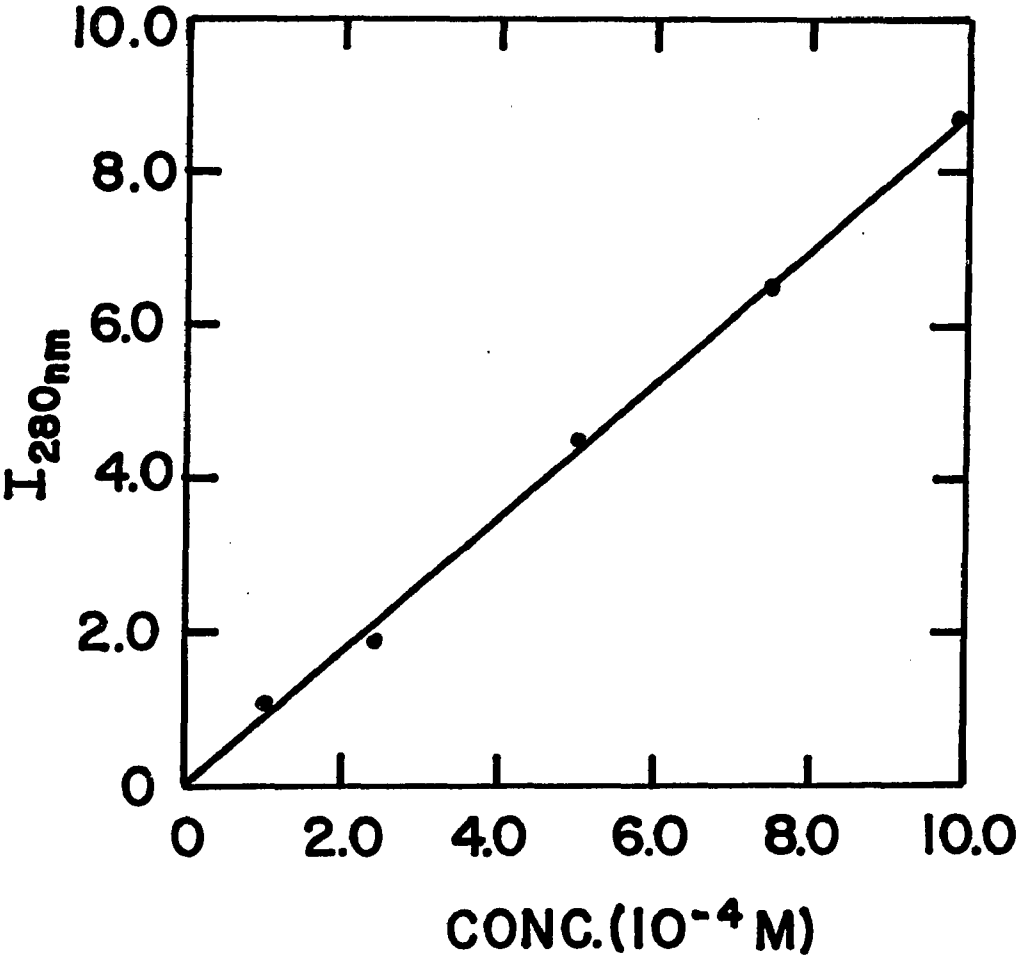


Figure 29. Fluorescence of poly(Lys^{58},Phe^{42}) at 280nm as a function of concentration. Excitation wavelength is 250nm.

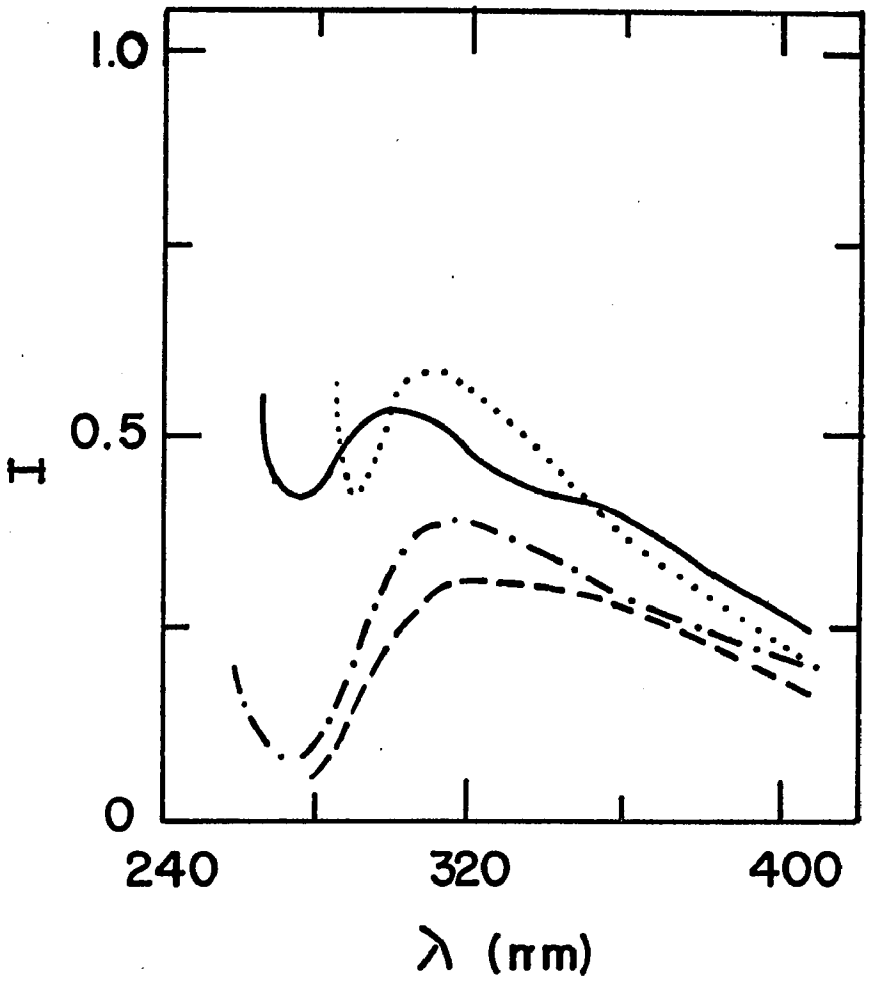


Figure 30. Fluorescence spectra of a calf thymus complex (r = 1.3) with variable excitation wavelength. 250nm (__), 250nm with filter (---), 260nm with filter (-·-), 270nm with filter (...).

poly(Lys^{50},Tyr^{50}) complex (Figure 10), there is no change in the general shape of the spectrum with increasing excitation wavelength. This finding supports the earlier interpertation that the emission spectrum of a poly(Lys^{50},Tyr^{50})-DNA complex, with excitation at 280nm, is significantly contributed by tyrosine residues.

Absorption Spectra of Poly(Lys^{58},Phe^{42})-DNA Complexes at Variable Temperatures

Figure 31 shows the change in absorbance for M. luteus DNA and a complex ($r = 1.04$) in EDTA buffer as a function of temperature. For pure DNA, the difference absorption spectra after partial (60°) and full denaturation (75°) are similar to each other with a peak at 272nm and a trough at 225-230nm. The amplitude near 225nm is about one-half that at 270nm. For the complex, however, the shape of the difference spectrum depends upon temperature. After 75°, the amplitude at 225nm becomes greater than the amplitude at 270nm. Similar temperature dependence of the difference absorption spectra has also been observed for complexes with calf thymus DNA. The spectral changes at $T_{m,II}$ (75°) for the complex are similar to those observed when the copolypeptide is bound to native DNA at room temperature. No such spectral changes at higher temperatures have been observed when denatured DNA was used for the initial complexes. These results imply that the spectral changes near $T_{m,II}$ are related to the double-helical structure of native DNA in the complex and that further interaction between phenylalanine and DNA occurs at this temperature.

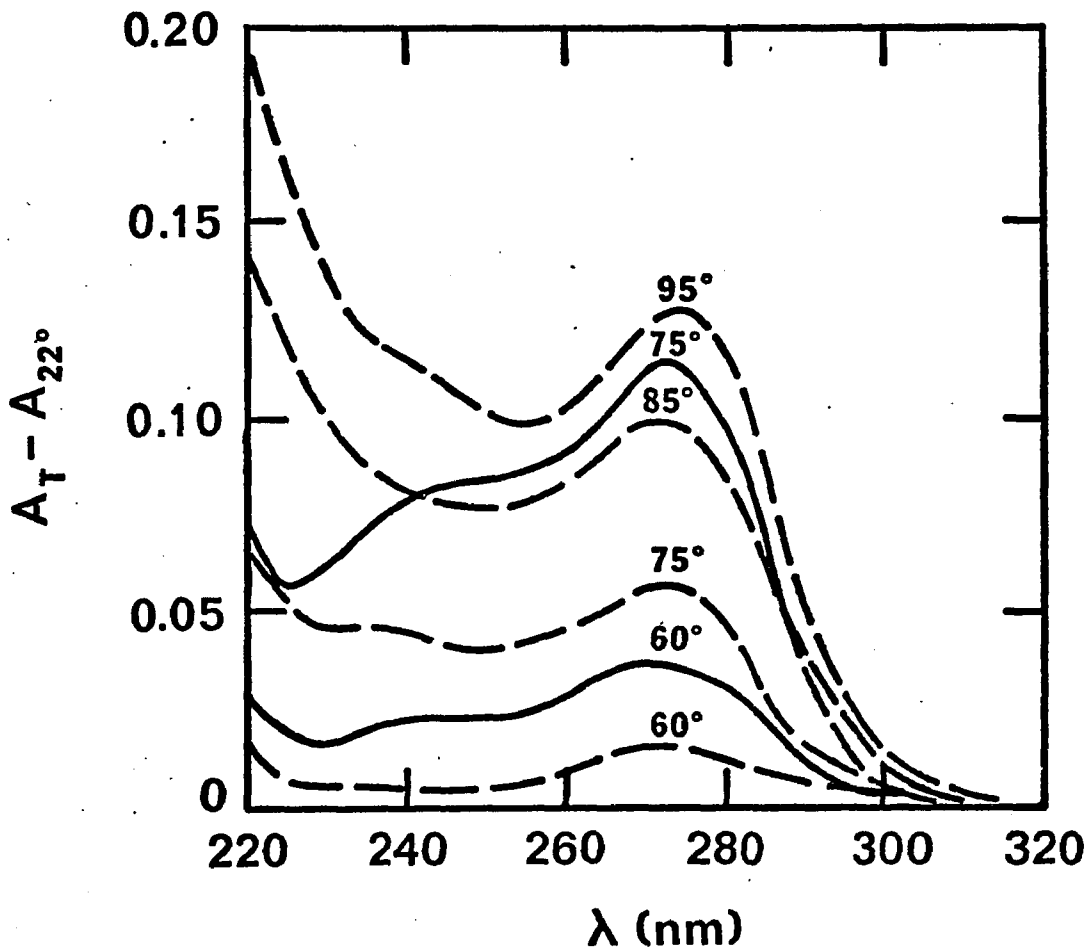


Figure 31. Difference absorption spectra between 22° and higher temperatures for M. luteus DNA (—) and a complex with r = 1.0 (---).

Circular Dichroism Spectra of Poly(Lys^{58},Phe^{42})-DNA Complexes at Variable Temperatures

CD spectra for M. luteus DNA (Figure 32) and a poly(Lys^{58},Phe^{42})-DNA complex (r = 0.95, Figure 33) were taken at various temperatures. Significant changes occur in both the positive and negative bands. $\Delta\epsilon_{278}$ for the positive band and $\Delta\epsilon_{245}$ for the negative band, in both DNA and the complex, as a function of temperature, are shown in Figure 34 in order to compare the temperature effects. For pure DNA, there is a decrease of $\Delta\epsilon_{278}$ and an increase of $\Delta\epsilon_{245}$ when the DNA is denatured at 60°. For the complex, major spectral changes occur in the $T_{m,II}$ melting region (after 60°). Although there are large spectral differences between pure DNA and the complex at room temperature (Figure 21) these differences are greatly reduced at 95° when pure DNA is fully denatured and the complex is close to complete denaturation.

Fluorescence of Poly(Lys^{58},Phe^{42})-DNA Complexes at Variable Temperatures

When poly(Lys^{58},Phe^{42})-DNA complexes are heated the enhanced fluorescence over that of free poly(Lys^{58},Phe^{42}) is further increased. For an M. luteus DNA complex (Figure 35), the broad fluorescent band at room temperature develops into two peaks at 310 and 360nm. For a calf thymus DNA complex (Figure 36) there is also an increase in fluorescence at higher temperatures. A plot of the relative fluorescence intensity as a function of temperature (Figure 37), measured either at 300 or 360nm, shows a sharp increase at 75° for the complex with M. luteus DNA and at 65° for the complex with calf thymus DNA which are in the temperature range of melting band II. For the calf thymus complex the fluorescence intensity levels off at 80°, the temperature at which

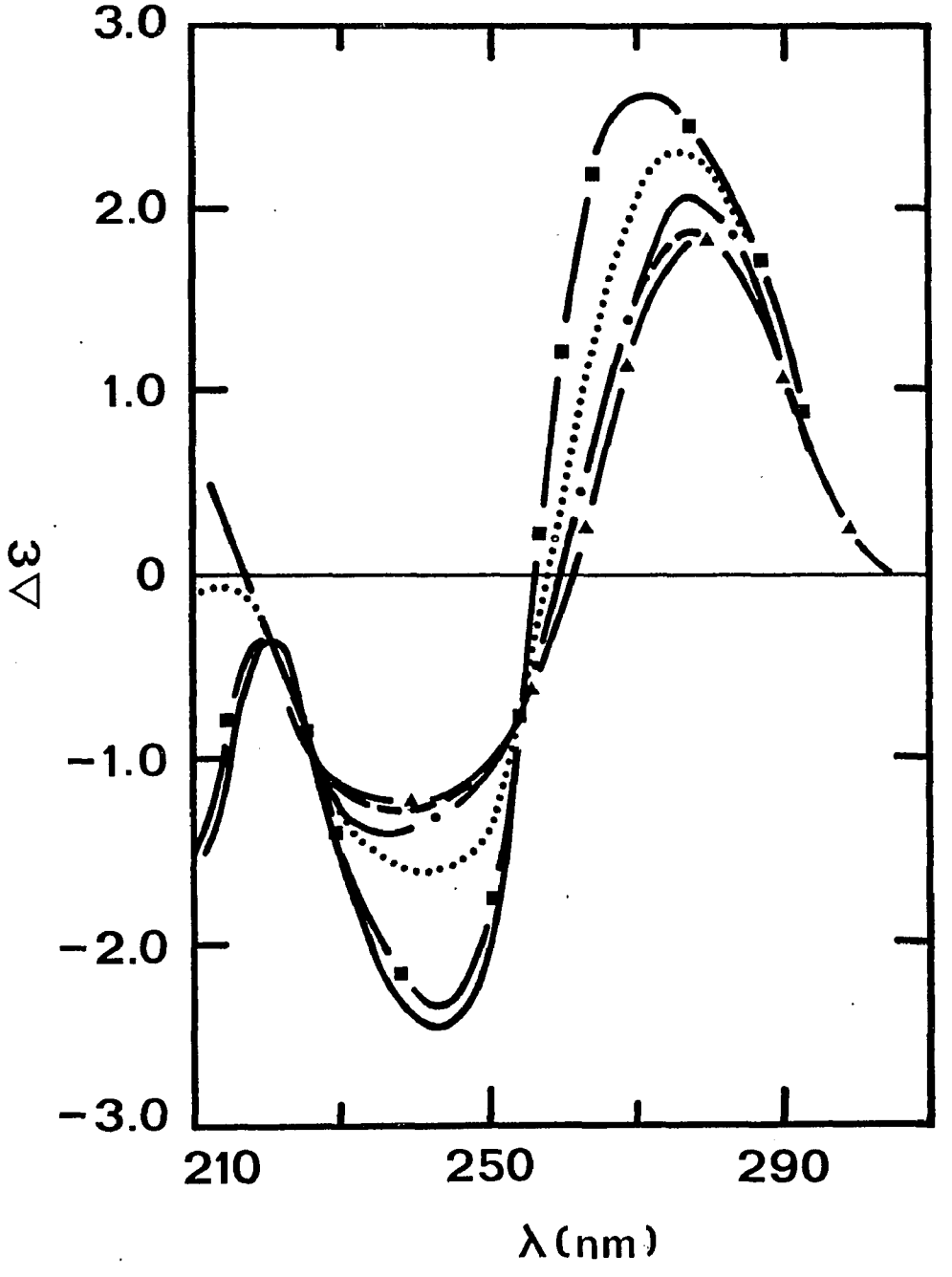


Figure 32. CD spectra of M. luteus DNA at various temperatures. 25° (—); 50° (-▪-); 60° (...); 75° (-·-); 85° (---); 95° (-▲-).

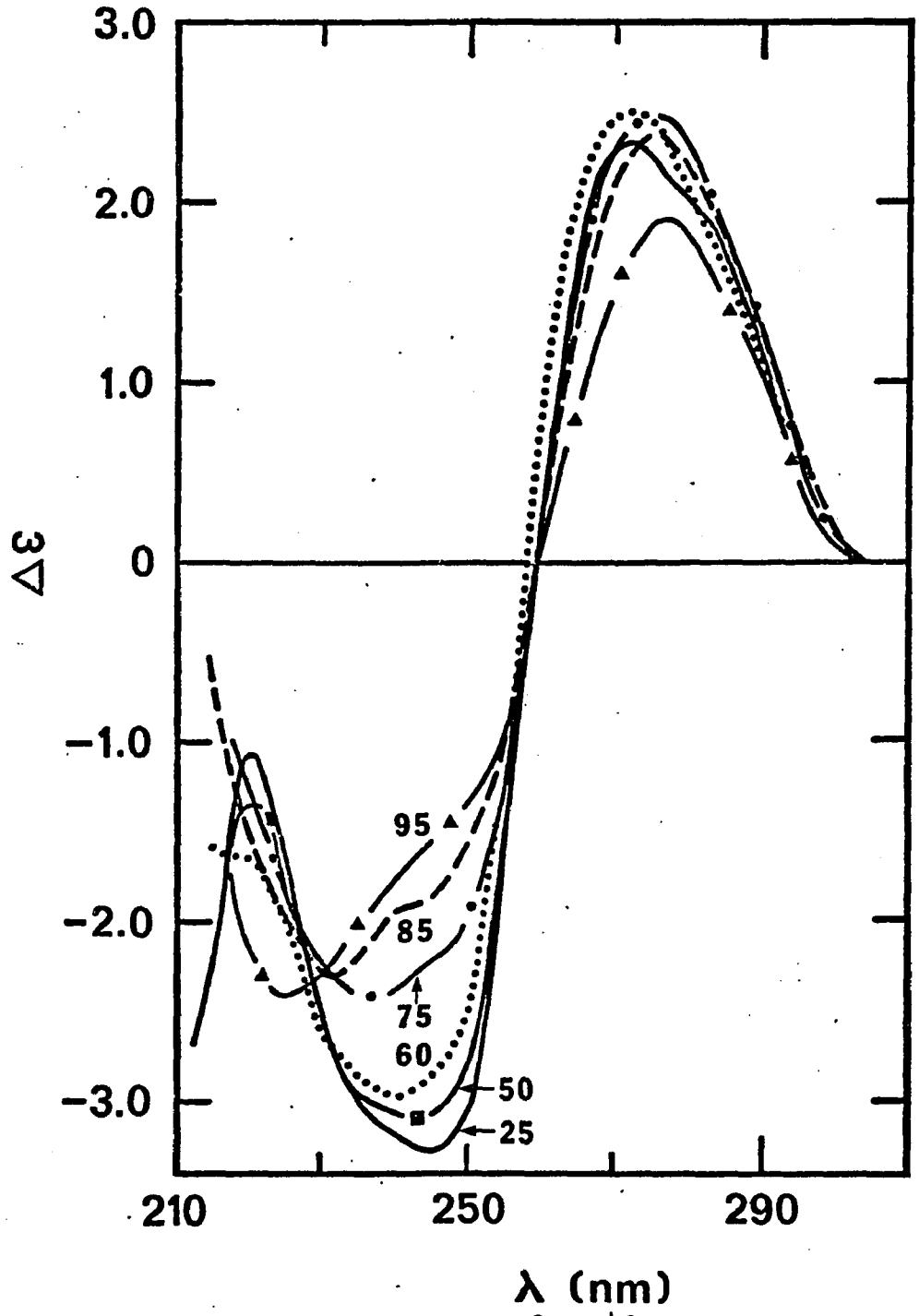


Figure 33. CD spectra of poly(Lys^{58},Phe^{42})-M.luteus DNA complex (r = 0.95) at various temperatures.

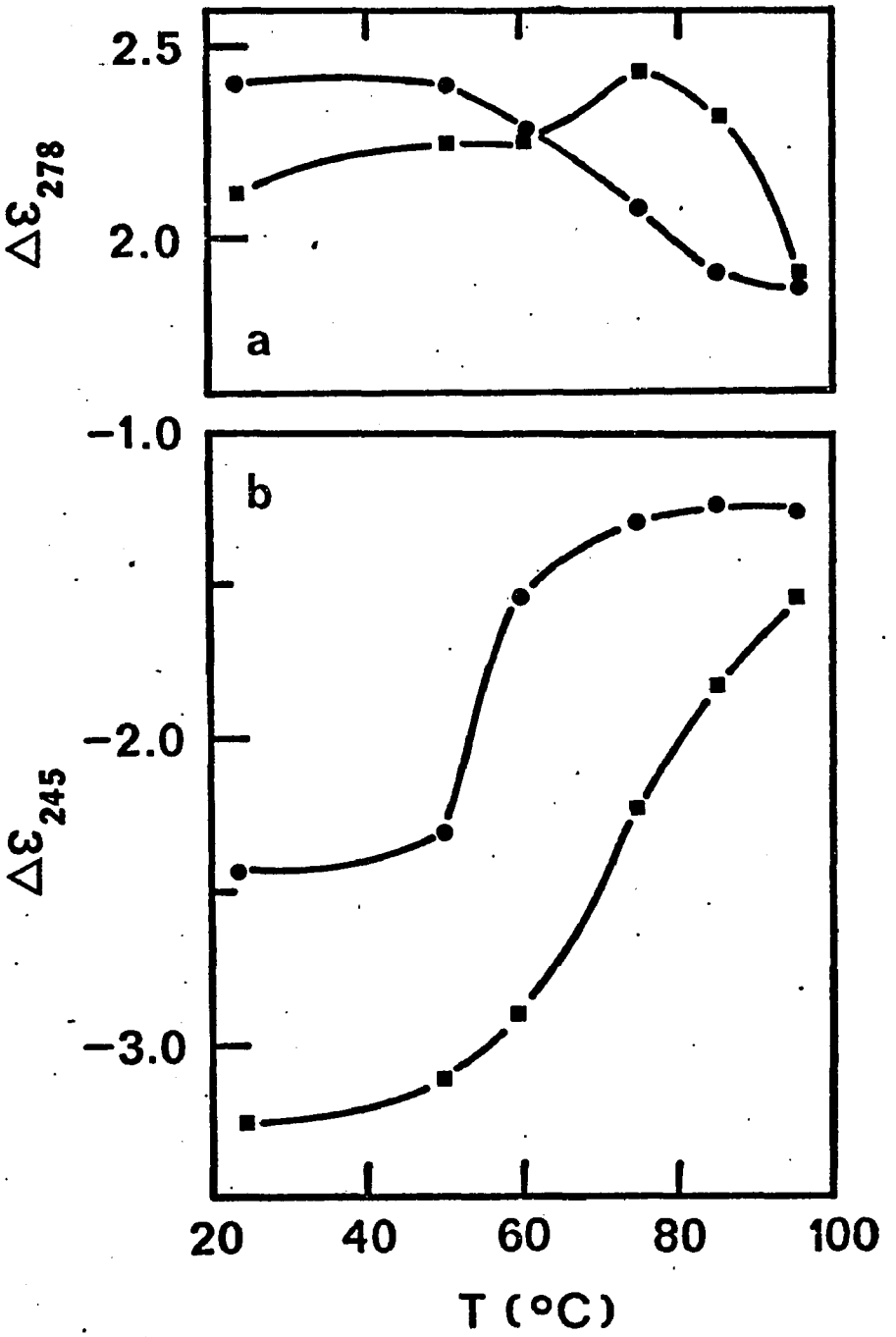


Figure 34. Temperature dependence of CD of DNA and poly (Lys^{50},Phe^{50})-M.luteus DNA complex (r =0.95) DNA (●) and the complex (■).

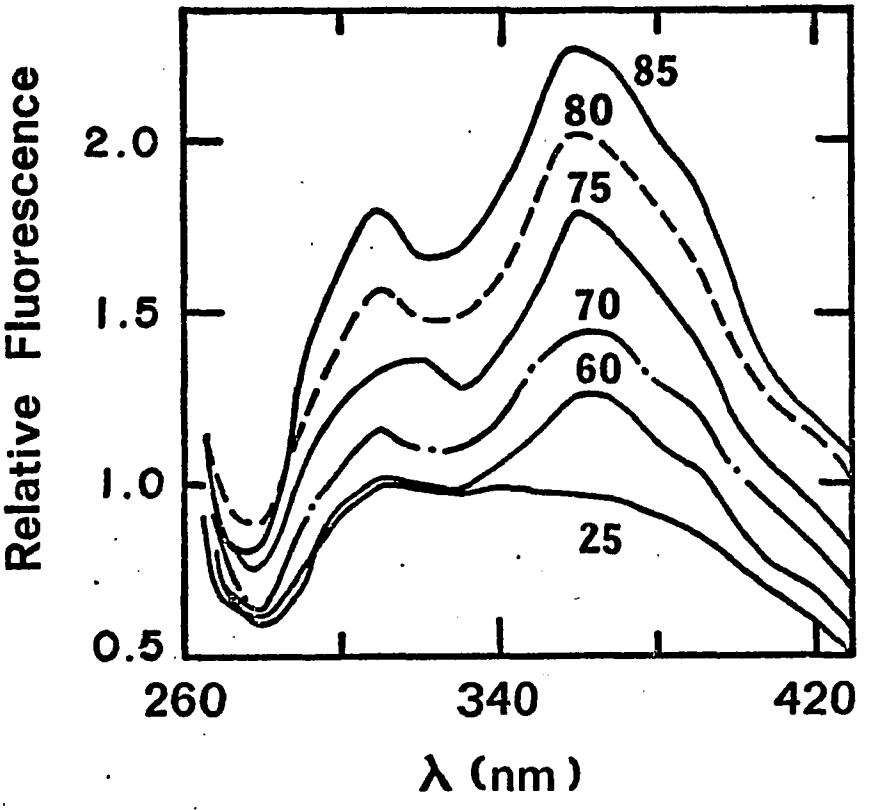


Figure 35. Temperature dependence of fluorescence spectra of poly(Lys^{58},Phe^{42})-M.luteus DNA complex (r = 1.04) in EDTA buffer. Excitation wavelength is 250nm.

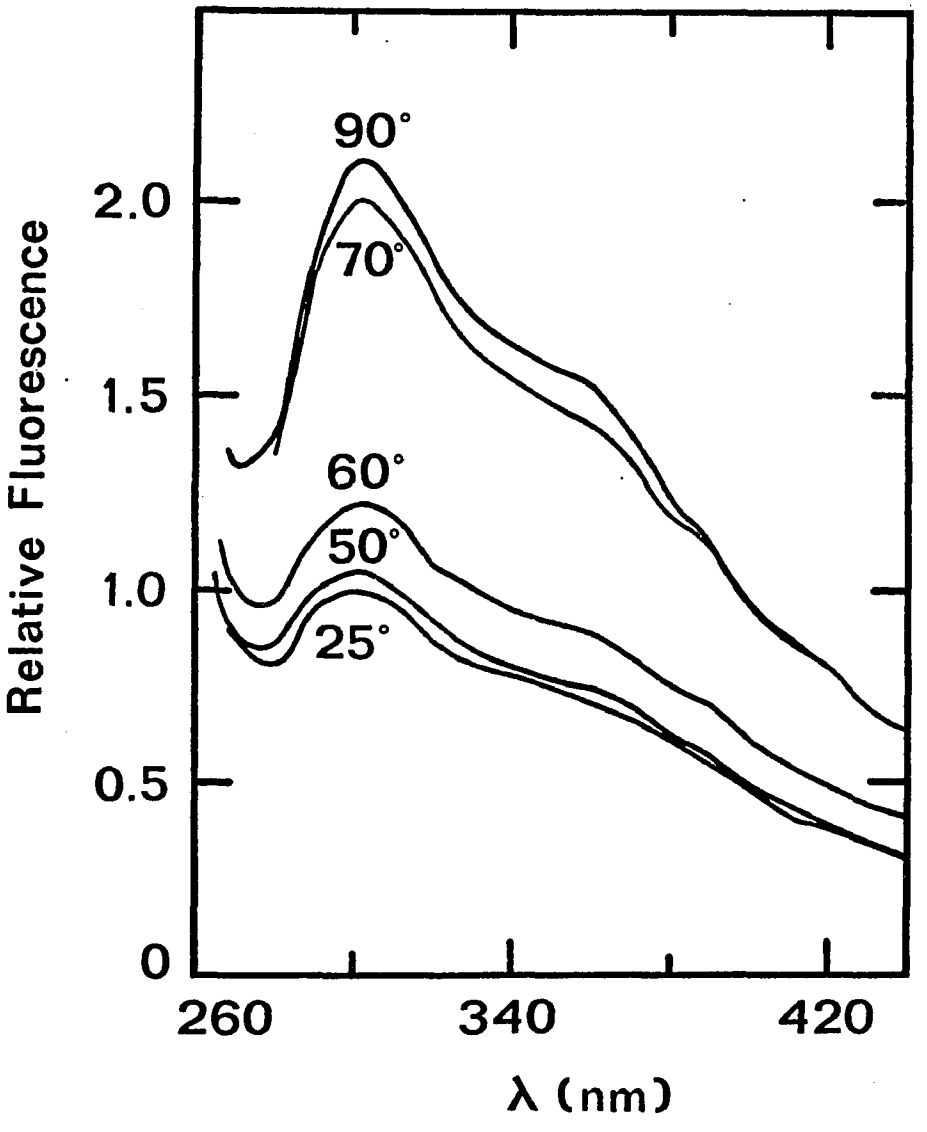


Figure 36. Temperature dependence of fluorescence spectra of a poly(Lys^{58},Phe^{42})-calf thymus DNA complex (r = 1.58) in EDTA buffer. Excitation wavelength is 250nm.

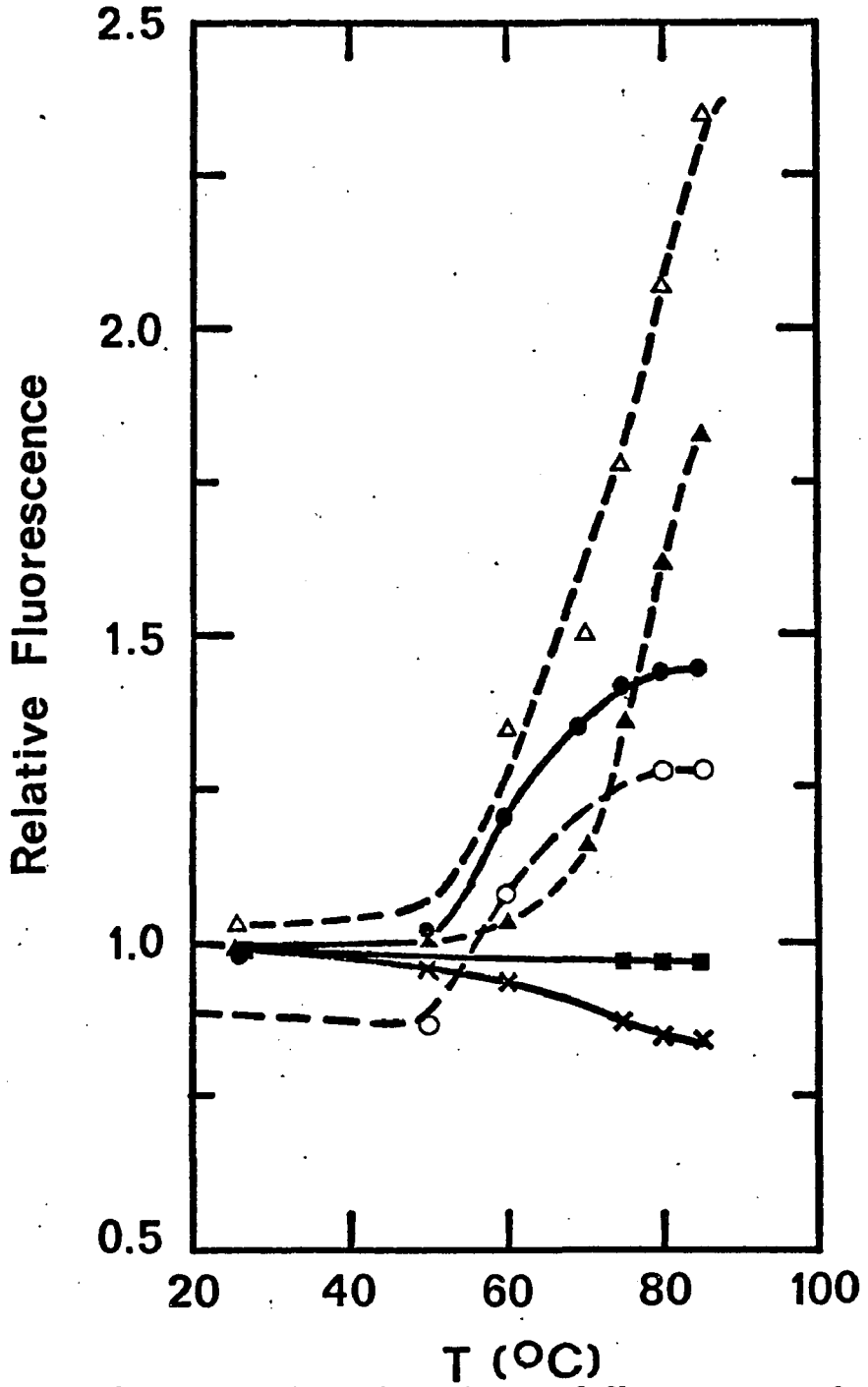


Figure 37. Temperature dependence of fluorescence of poly (Lys^{58},Phe^{42}) complexes with calf thymus DNA(r = 1.0) at 300nm(-●-)and at 360nm(-○-); with M.luteus DNA (r = 1.0) at 300nm(--▲--) and 360nm(--△--);with denatured calf thymus DNA at 300nm(-■-)(r = 1.35) and with poly(A) at 300nm(-x-)(r = 0.75).

melting band II is completed (Figure 24). For the M. luteus DNA complex the leveling off of fluorescence intensity is not apparent in Figure 37 since the highest temperature reached, 85°, is still in the range of melting band II of this complex.

Although the binding of poly(Lys^{58},Phe^{42}) to denatured DNA or poly (A) results in fluorescence enhancement (Figure 28b) similar to that observed for native DNA, the complex with denatured DNA shows no further fluorescence enhancement up to 85°. The complex with poly(A) shows a slight reduction in its intensity at higher temperatures (Figure 37). Parallel to these findings in fluorescence, no substantial hyperchromicity in absorbance at 260nm has been observed from 25 to 100° for complexes with denatured calf thymus DNA.

Discussion

In poly(Lys^{58},Phe^{42}) there is an approximately random distribution of phenylalanine and lysine residues along the copolymer as indicated by the specifications from Miles Laboratories. Therefore, only one melting band corresponding to the denaturation of the protein-bound regions would be expected. This has been observed in many other systems such as polylysine[12], protamine[43], poly(Lys^{40},Ala^{60})[23] and poly(Lys^{50}, Tyr^{50}) complexes. However, the melting curve of poly(Lys^{58},Phe^{42})-DNA complexes has three melting bands (Figure 24). From this and other experimental data the following mechanism can be proposed to describe the interaction of poly(Lys^{58},Phe^{42}) with DNA.

$$P + D \longrightarrow (PD)_{out} \xrightarrow{T_{m,II}} (PD)_{in} \xrightarrow{T_{m,III}} (PD)_{den} \qquad (6)$$

$(PD)_{out}$ is the complex formed at room temperature; $(PD)_{in}$ is the

intercalated complex at $T_{m,II}$ and $(PD)_{den}$ is the denatured complex at $T_{m,III}$. Melting band III corresponds to the ordinary denaturation of copolypeptide-bound regions but melting band II does not. This mechanism is supported by experimental data, particularly the results from fluorescence. When the polylysine fluorescence studies are presented later in Chapter VI, it will be seen that fluorescence enhancement at room temperature comes mainly from bound DNA rather than from the phenylalanine chromophore. However, the increased fluorescence at higher temperatures depends upon the presence of aromatic residues (phe or tyr, Figure 52). At $T_{m,II}$, the elevated temperature can cause enough unstacking of the DNA bases to allow for the intercalation of phenylalanine. This intercalation or stacking of the phenylalanine with the DNA bases causes a further enhanced fluorescence from DNA and phenylalanine. Also in support of this mechanism are the results of copolypeptide binding to poly(A) and denatured DNA. With these single stranded polynucleotides, a stacked complex can be formed at room temperature with an enhancement of fluorescence (Figure 28b). When these complexes are heated no further enhancement is observed (Figure 37). Interaction of copolypeptides with single stranded polynucleotides will be discussed further in Chapter VI.

A second mechanism could also be suggested:

$$P + D \longrightarrow PD \xrightarrow{T_{m,II}} PD_{den} \xrightarrow{T_{m,III}} P + D_{den} \qquad (7)$$

Here a native complex, PD is formed at room temperature; it is denatured at $T_{m,II}$ with the phenylalanine chromophore stacked with the denatured DNA; finally, the stacked complex is destroyed at $T_{m,III}$. This mechanism is supported by some of the experimental results. However, if the mechanism of Equation (7) were correct, one would expect a melting

band at $T_{m,III}$ for a complex of poly(Lys^{58},Phe^{42}) with denatured DNA. No such melting band has been observed when the absorbance change was monitored at 260nm. Therefore, the mechanism of Equation (7) is less likely than the mechanism of Equation (6).

Attempted Studies on Oligo(Lys^{48},Trp^{52})-DNA Interaction

In order to complete the study on the effect of aromatic amino acids on protein-DNA interaction, a copolymer containing 48% lysine and 52% tryptophan was purchased from Miles Laboratories. However, upon arrival, it was found to have a low molecular weight of 1,650. This corresponds to approximately 8 amino acid residues per chain, compared to about 400 for poly(Lys^{50},Tyr^{50}). Since no larger size was available an attempt was made to study the effect of tryptophan using this oligomer. The concentration was determined using the reported[40] extinction coefficient at 279nm of 5.5×10^3 $M^{-1}cm^{-1}$ for free tryptophan and complexes were made in EDTA. The melting curves showed only one melting band which shifted to higher temperature with increasing r value.

r	T_m
0	46
0.8	56
1.6	62

This increase in T_m could be due to stabilization by the lysine residues. No T_m' band is seen since the binding of such a small peptide is reversible.

The fluorescence spectrum of oligo(Lys^{48},Trp^{52}) excited at 250nm resembles that of free tryptophan with a peak at 350nm.[45] Complexing with DNA results in quenching of 50% of the tryptophan fluorescence. This result is in agreement with previous studies using oligomer binding to DNA but does not agree with the results obtained with poly(Lys^{50},Tyr^{50}) or poly(Lys^{58},Phe^{42}). This difference can be attributed to the different type of binding seen with small oligomers. Because this study is primarily concerned with polymer binding to DNA, it was decided not to pursue the work on oligo(Lys^{48},Trp^{52}).

CHAPTER V

STUDIES ON THE INTERACTION OF POLY(L-LYSINE^{48},L-HISTIDINE^{52}) AND DNA

A random copolymer of 48% lysine and 52% histidine was used to study the effect of histidine in a protein when it interacts with DNA. Histidine contains a five member ring and a weakly basic imidazolium group with a pK of 6.0.[52] Ideally, complexes would be made at two pH's, one at which histidine is mainly protonated and a second where it is mainly deprotonated, and the difference in DNA binding studied. However, in low ionic strength solution, DNA cannot be taken below pH 6.0 without substantial denaturation. Therefore, complexes were made at two pH's near the pK, 6.0 and 7.0 and at one above, 9.2.

Titration of Calf Thymus DNA by Poly(Lys^{48},His^{52}) at pH 6.0 and 7.0

Complexes of poly(Lys^{48},His^{52}) and calf thymus DNA were made in EDTA buffer at two pH's and centrifuged in a manner described earlier for poly(Lys^{50},Tyr^{50}) complexes. Figure 38 shows the titration curve of calf thymus DNA by poly(Lys^{48},His^{52}) at pH 6.0 and 7.0. At pH 6.0, precipitation occurs at r = 1.10 amino acid residues per nucleotide or 0.53 lysine and 0.57 histidine per nucleotide. At pH 7.0, precipitation

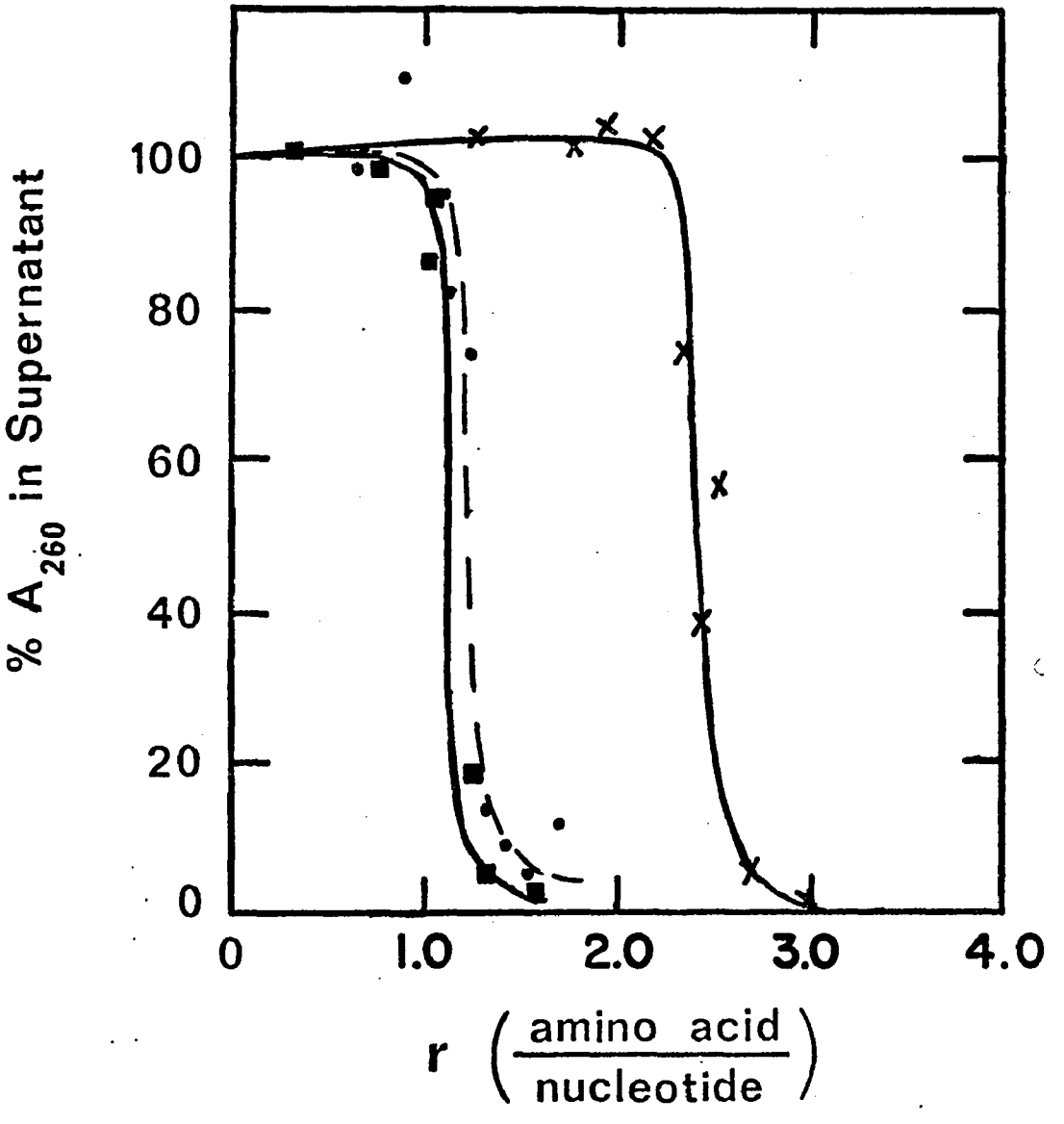


Figure 38. Titration curve of DNA by poly(Lys^{48},His^{52}) in EDTA buffer pH 6.0 (■), 7.0 (●) and 9.2 (X).

occurs at 1.23 amino acid residues per nucleotide or 0.59 lysine and 0.64 histidine per nucleotide. If precipitation of the complexes occurs when the negative charges on the phosphates are equal to the positive charges on the copolypeptide, it would appear that most of the histidines are positively charged at pH 6.0 and slightly less at pH 7.0.

Absorption Spectra of Poly(Lys^{48},His^{52})-DNA Complexes

Poly(Lys^{48},His^{52}) has very little absorption between 220 and 240nm in comparison to DNA and no absorption above 240nm. Formation of a complex between this copolypeptide and DNA results in an enhancement of absorbance as shown in Figure 39 for a complex with r = 0.66 at pH 7.0. Equation (4) can be used to calculate the increase in absorbance after copolypeptide binding. The result is shown in Figure 39. The gained absorbance has a peak at 270nm and a greater increase below 230nm. Since poly(Lys^{48},His^{52}) has no absorbance near 270nm, the increase in absorbance at this wavelength must come solely from DNA.

Circular Dichroism Spectra of Poly(Lys^{48},His^{52})-DNA Complexes

The CD spectra of free poly(Lys^{48},His^{52}) at different pH's are shown in Figure 40. At pH 7.0,there is a negative peak at 236nm and a larger positive peak at 222nm indicating that this copolypeptide is exclusively in a random coil conformation. As the pH is raised, there is a large decrease in the positive peak near 220nm. $\Delta\epsilon_{220}$ as a function of pH is shown in the inset of Figure 40 indicating a linear relationship between $\Delta\epsilon_{220}$ and pH. There is an increase and blue shift of the negative peak near 240nm possibly indicating formation of a more ordered structure at higher pH.

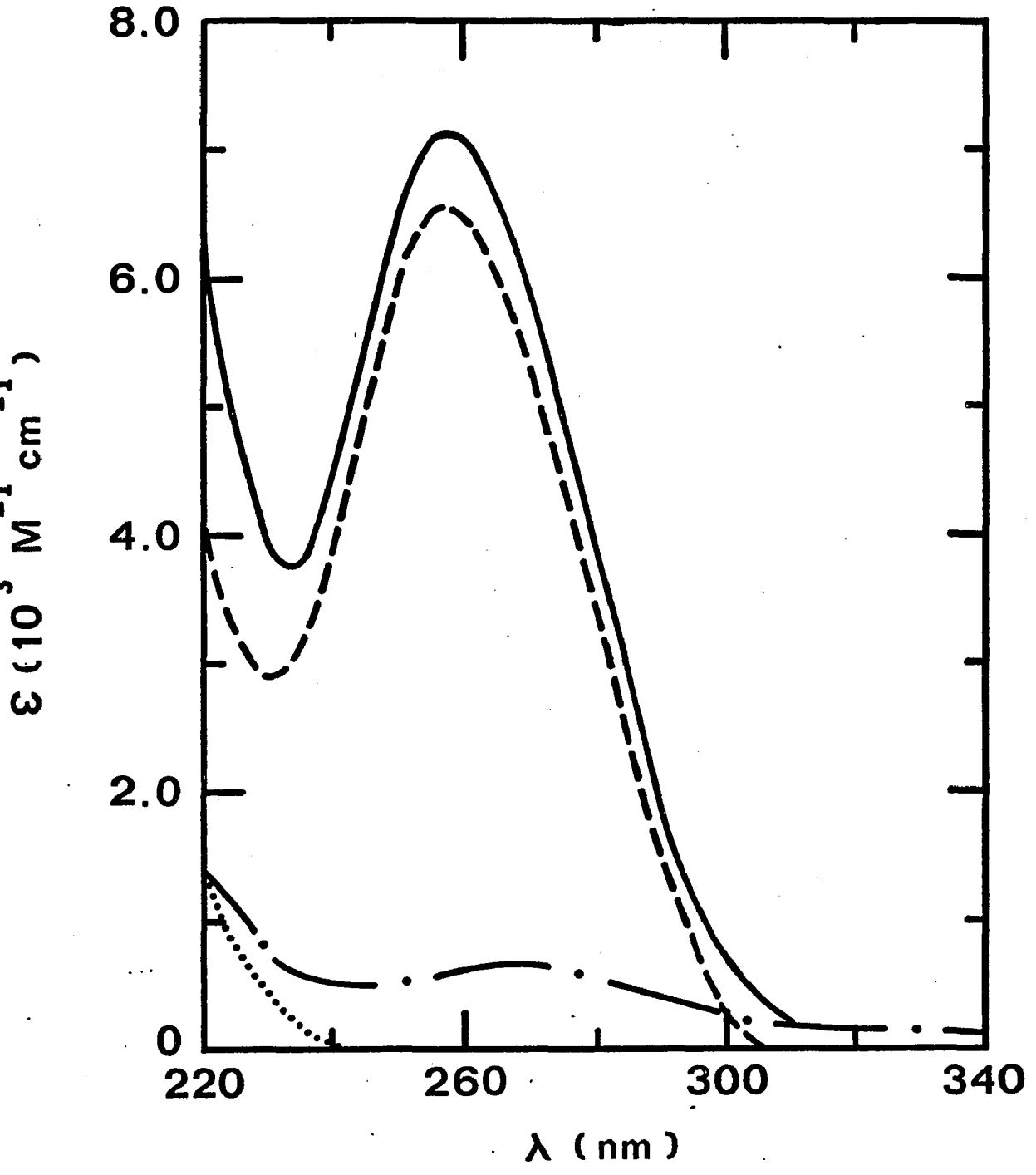


Figure 39. Absorption spctrum of DNA (---), poly(Lys^{48},His^{52}) and a complex (r = 0.60)(——). The absorbance gain after complex formation, calculated from Eq. (4) (·--·). pH 7.0

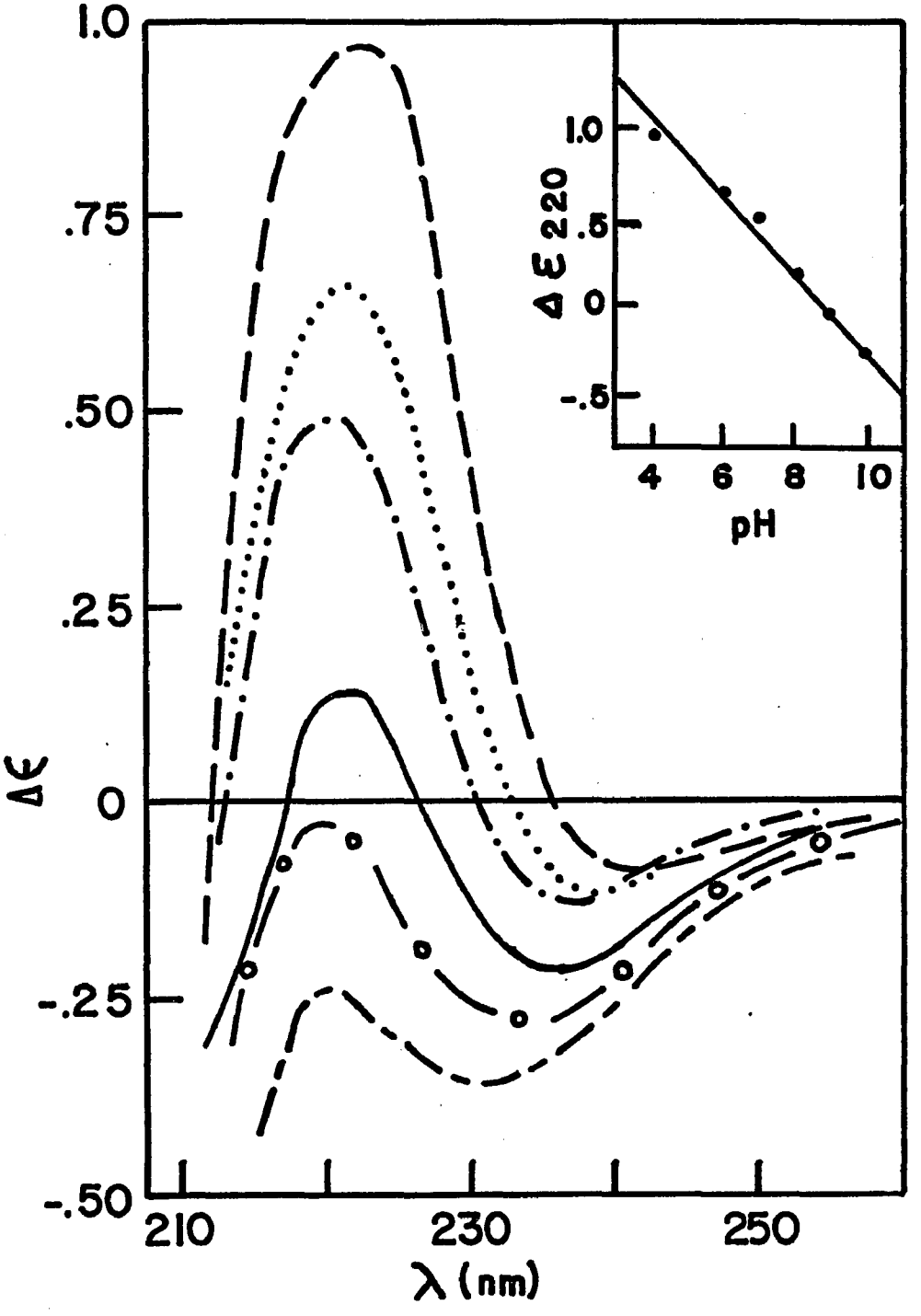


Figure 40. CD spectra of poly(Lys^{48},His^{52}) at variable pH. pH 4.0 (---), 6.0 (...), 7.0 (-·-), 8.1 (___), 9.1 (-o-) and 9.9 (_ • _). Inset $\Delta\epsilon_{220}$ as a function of pH.

The binding of this copolypeptide to DNA at pH 7.0 induces several CD changes (Figure 41).There is a red shift of the crossover near 255nm and a red shift and reduction of the positive band near 275nm. The negative band at 245nm becomes more negative and the positive band at 220nm increases. The measured CD can be decomposed into that due to the free and bound base pairs. Equation (5) can be used to calculate $\Delta\epsilon^{D}_{b}$ for the bound base pairs. The results are shown in Figure 41 with a positive maximum at 287nm, a crossover at 275nm and $\Delta\epsilon_{250}$ value of -5.25, more negative than that of polylysine. The CD changes on DNA due to binding follow the order of poly(Lys^{48},His^{52})pH 7.0 > polylysine > poly(Lys^{58},Phe^{42})> poly(Lys^{50},Tyr^{50}).

Thermal Denaturation Studies on Poly(Lys^{48},His^{52})-DNA Complexes

Figure 42 shows derivative melting curves of poly(Lys^{48},His^{52})-DNA complexes at pH 7.0. The T_m of free base pairs increases from 46 to 50° as the r value of the complex increases. The T_m' of the bound base pairs remains constant at 94°. If M. luteus DNA is used, similar results are obtained except that each melting temperature is raised, T_m 59-63° and T_m' to 100°. The increase in T_m with higher r value could be due to the presence of some cations associated with the free copolypeptide. When the copolypeptide is bound to DNA these ions become free to stabilize DNA. Evidence for this is the decrease in T_m to 46-47° when the complexes, made in EDTA, were dialyzed to EDTA.

Since the T_m' of poly(Lys^{48},His^{52})-bound regions (94°) is lower than that of polylysine-bound regions (98°), an attempt was made to see if there is full charge neutralization on the protein-bound regions.

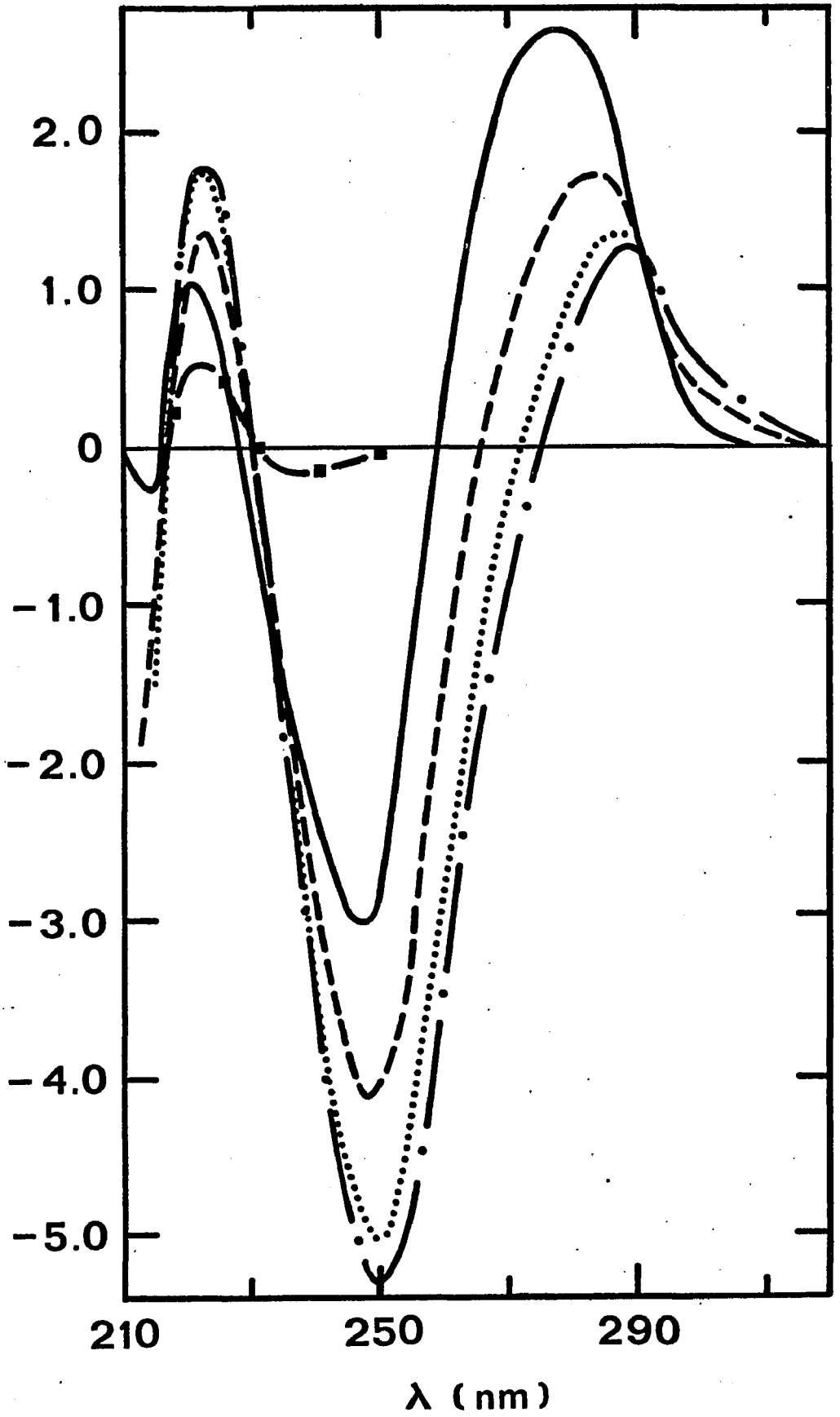


Figure 41. CD spectra of poly(Lys^{48},His^{52}) (-■-), DNA (——) and two complexes(r = 0.56)(---),(r = 0.94)(...). Δε per P (--·).

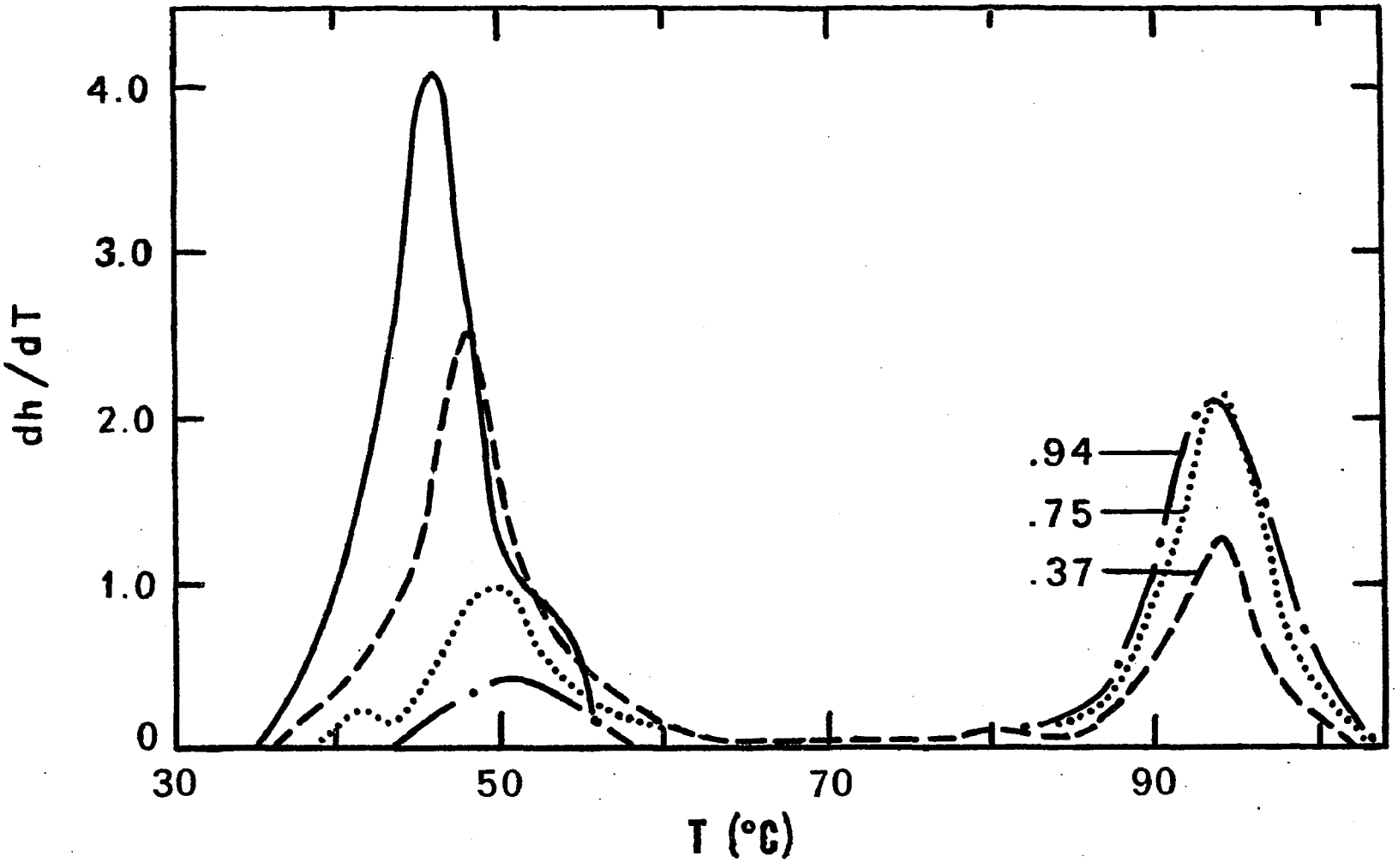


Figure 42. Melting curves of poly(Lys^{48},His^{52})-DNA complexes made at pH 7.0. The r value is indicated.

Figure 43 shows the melting curves of a complex (r = 0.60) in 0, 0.01 and 0.06M NaCl. With higher salt concentrations, the T_m of free base pairs increases from 48 to 80° while the T_m' at 94° remains unshifted. This indicates that in copolypeptide-bound regions the phosphates are nearly fully neutralized. The lowered T_m' would then be due to some destabilization on DNA by histidine.

To determine the β value or amino acid residues per nucleotide in copolypeptide-bound regions, equations (2) and (3) can be used. Plots of equation (3) for complexes made at pH 6.0 and 7.0 are shown in Figure 44. At pH 6.0, β is 1.07 amino acid residues per nucleotide, equivalent to 0.51 lysine and 0.56 histidine per nucleotide. At pH 7.0, β is 1.14 amino acid residues per nucleotide or 0.55 lysine and 0.59 histidine per nucleotide. This data also seems to indicate that the histidine residues carry a positive charge at pH 6.0 while at pH 7.0 slightly less are protonated.

Fluorescence of Poly(Lys^{48},His^{52})-DNA Complexes at pH 7.0

Since neither poly(Lys^{48},His^{52}) nor DNA has any measurable fluorescence at room temperature it was not expected that poly(Lys^{48}, His^{52})-DNA complexes would fluoresce. However, when measured, the complexes were found to fluoresce with approximately the same intensity as poly(Lys^{50},Tyr^{50})- and poly(Lys^{58},Phe^{42})-DNA complexes. Figure 45 shows the emission spectra of poly(Lys^{48},His^{52}), DNA and two complexes (r = 0.66 and 0.88) all excited at 250nm. Free copolypeptide or DNA show no significant fluorescence but the two complexes show a peak at 300nm and a shoulder at 360nm. Since the intensity of fluorescence increases with higher r value, a plot was made of intensity at 300nm as

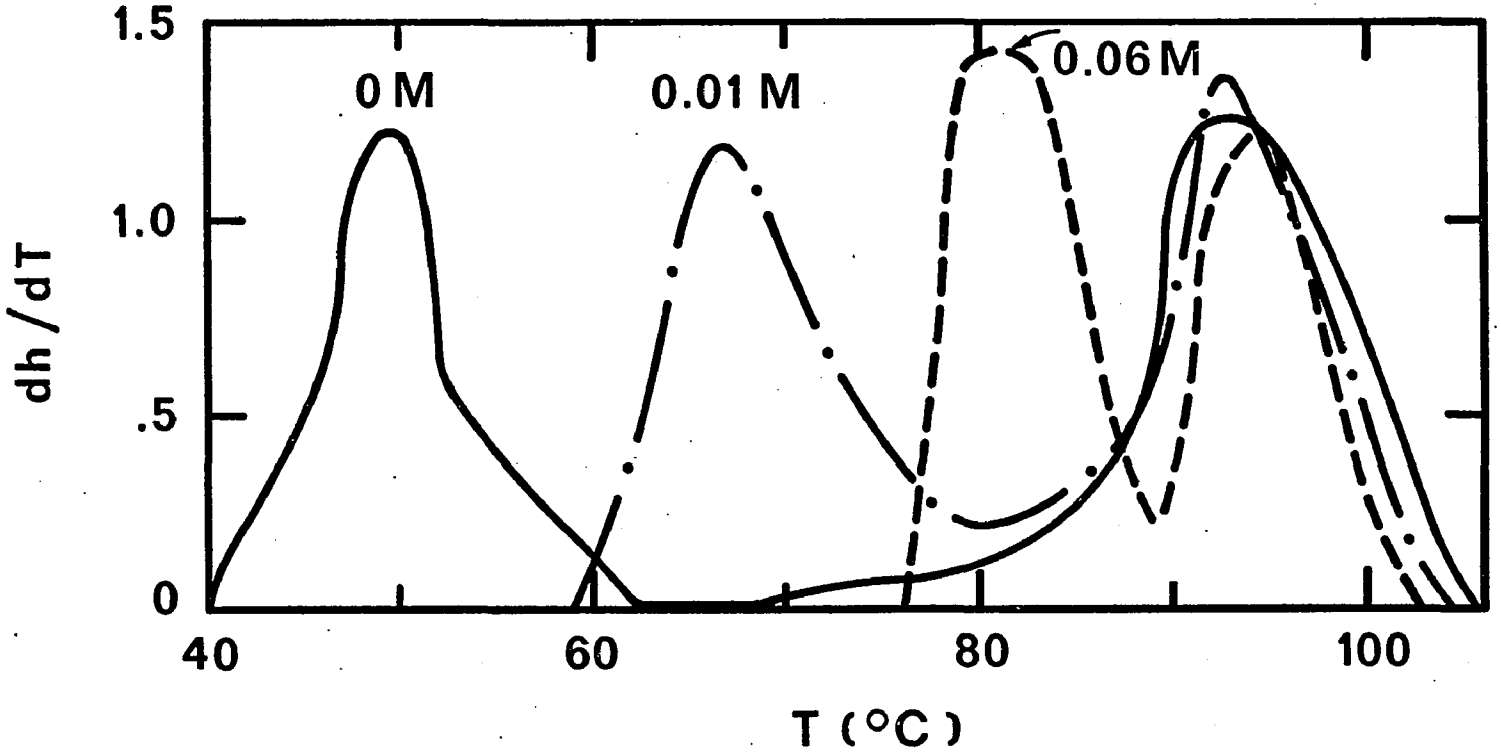


Figure 43. Derivative melting curves of a poly(Lys^{48},His^{52})-calf thymus DNA complex in in 0M NaCl (___), 0.01M NaCl (-·-·) and 0.06M NaCl (---).

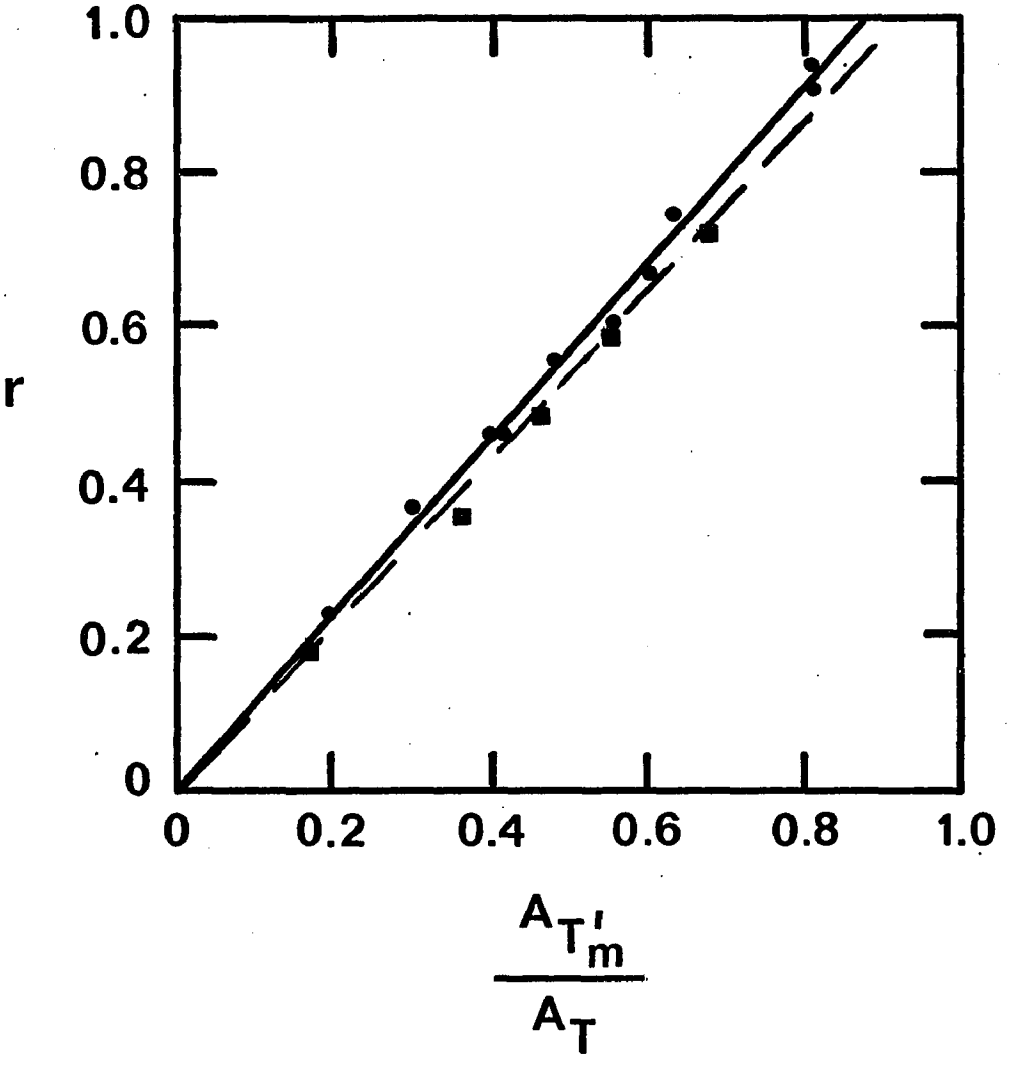


Figure 4. Linear plot of Equation (3) at pH 6.0 (■) and 7.0 (●).

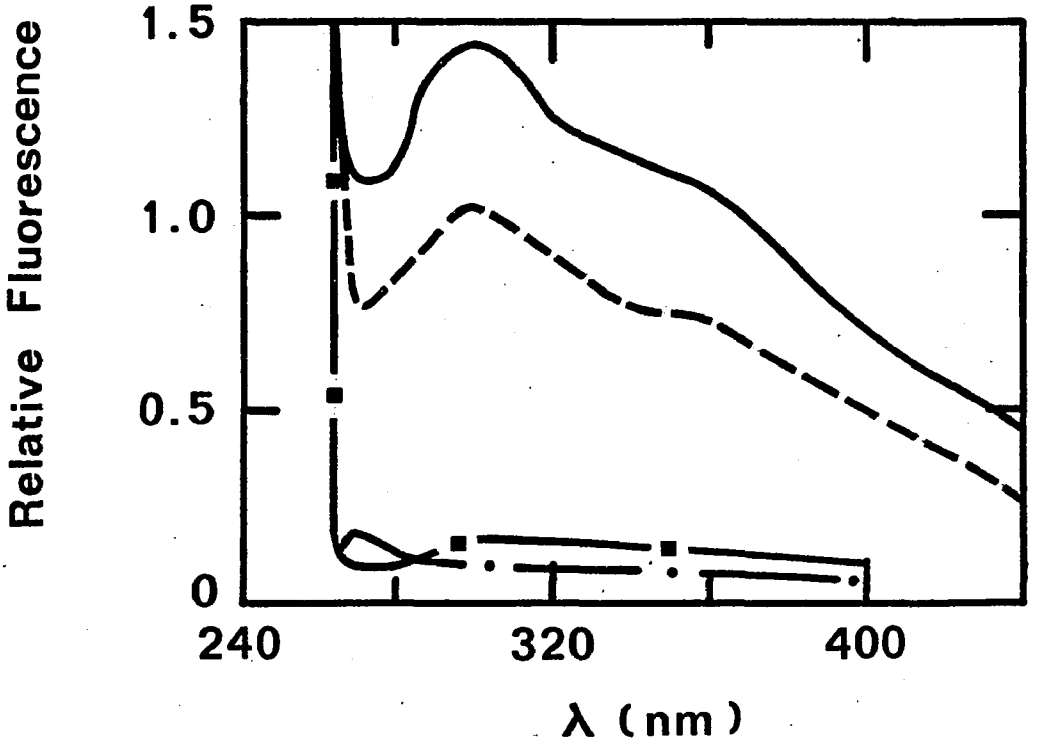


Figure 45. Fluorescence spectra of DNA (-■-), poly(Lys^{48},His^{52}) (-·-) and two complexes (r = 0.66)(---) and (r = 0.88) (——). Excitation wavelength is 250nm. The intensity is relative to poly(Lys^{58},Phe^{42})(1.2×10^{-4} M) at 280nm.

a function of r value. Figure 46 shows there is a linear relationship between fluorescence intensity and the r value of the complexes or fraction of DNA bound.

pH Dependence of Poly(Lys^{48},His^{52})-DNA Complexes

The above results on precipitation of DNA and determination of β value seem to indicate that most of the histidine residues carry a positive charge at pH 6.0 in the complex. If this is true it should be possible to raise the pH of the copolypeptide solution sufficiently to remove the charges from the histidine residues. Complexes with DNA could then be made and the effects on β value and structure of the complexes would then be studied. pH 9.2 was chosen since this is near the upper limit of pH in which DNA is not denatured at room temperature. It is also below the pK of positively charged lysine residues at pH 10.7.[40]

Solutions of calf thymus DNA and poly(Lys^{48},His^{52}) were adjusted to pH 9.2 by the addition of dilute NaOH. A titration curve of calf thymus DNA at pH 9.2 was determined from complexes prepared at this pH. Figure 38 shows that precipitation occurs at an r value of 2.42 amino acid residues per nucleotide or 1.16 lysine and 1.25 histidine per nucleotide. This is in contrast to the result at pH 6.0 where precipitation occurred at 1.10 amino acid residues per nucleotide and are consistent with the histidine residues being protonated at pH 6.0 and deprotonated at 9.2.

The derivative melting curves for complexes made at pH 9.2 indicate that the T_m of free base pairs is increased to 49-52° while the T_m' is decreased to 89-91°. As a control, polylysine-DNA complexes

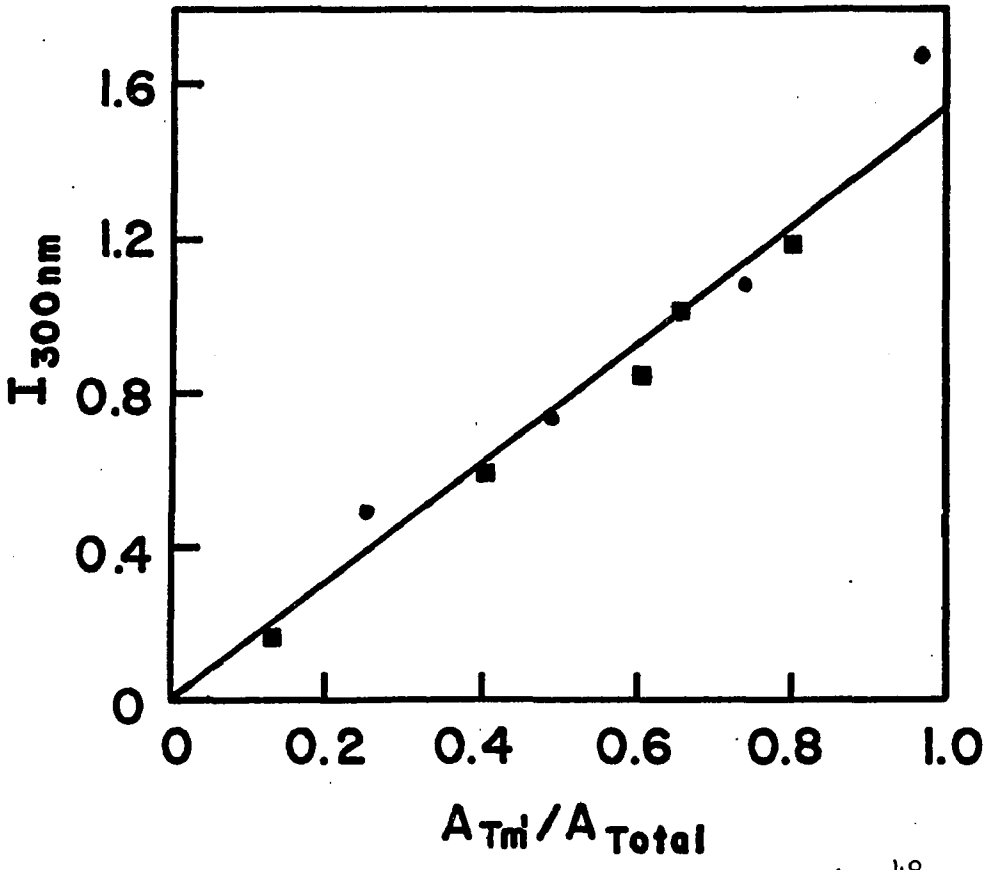


Figure 46. Fluorescence intensity at 300nm for poly(Lys^{48}, His^{52})-DNA complexes made at pH 7.0 (■) and 9.2 (●).

were made at pH 9.2 and thermally denatured. They showed the same increase in T_m but did not show any change in T_m' at this pH. When poly (Lys^{48},His^{52}) complexes were made at pH 9.2, adjusted to pH 7.0 and then melted at this pH, the T_m remained the same (49-50°) but the T_m' returned to the value found in the complexes made at pH 7.0 (94°). The changes in T_m can be attributed to stabilization by ions added in adjusting the pH. The lower T_m' could be due to deprotonation on histidine residues and also possibly a slight deprotonation on lysine residues at this pH.

The β value can be determined at pH 9.2 as was done at pH 7.0. Figure 47 shows a plot of equation (3) and a β value of 2.04 amino acid residues per nucleotide or 0.98 lysine and 1.06 histidine per nucleotide. At pH 9.2 the histidine residues have been deprotonated and the binding of the copolypeptide to DNA depends solely on the positively charged lysine. There is approximately one lysine per nucleotide in the bound region compared to 0.51 lysine at pH 6.0.

Figure 48 shows the CD spectra of poly(Lys^{48},His^{52})-DNA complexes made at pH 9.2. The red shift and reduction of the positive band near 275nm and the red shift of the crossover are not as great as at pH 7.0 (Figure 41). The measured CD can again be decomposed into that due to free and bound base pairs. $\Delta\epsilon_b^D$, calculated from equation (5), is shown in Figure 48. To compare the CD changes induced by complex formation at pH 7.0 and 9.2, $\Delta\epsilon_b^D$ for complexes prepared at these pH's are shown in Figure 49. At pH 7.0 there is a much greater red shift and reduction of the positive band near 275nm and a much greater red shift of the crossover near 255nm. At pH 9.2 there are over two amino acid residues per nucleotide compared to one amino acid at pH 7.0.

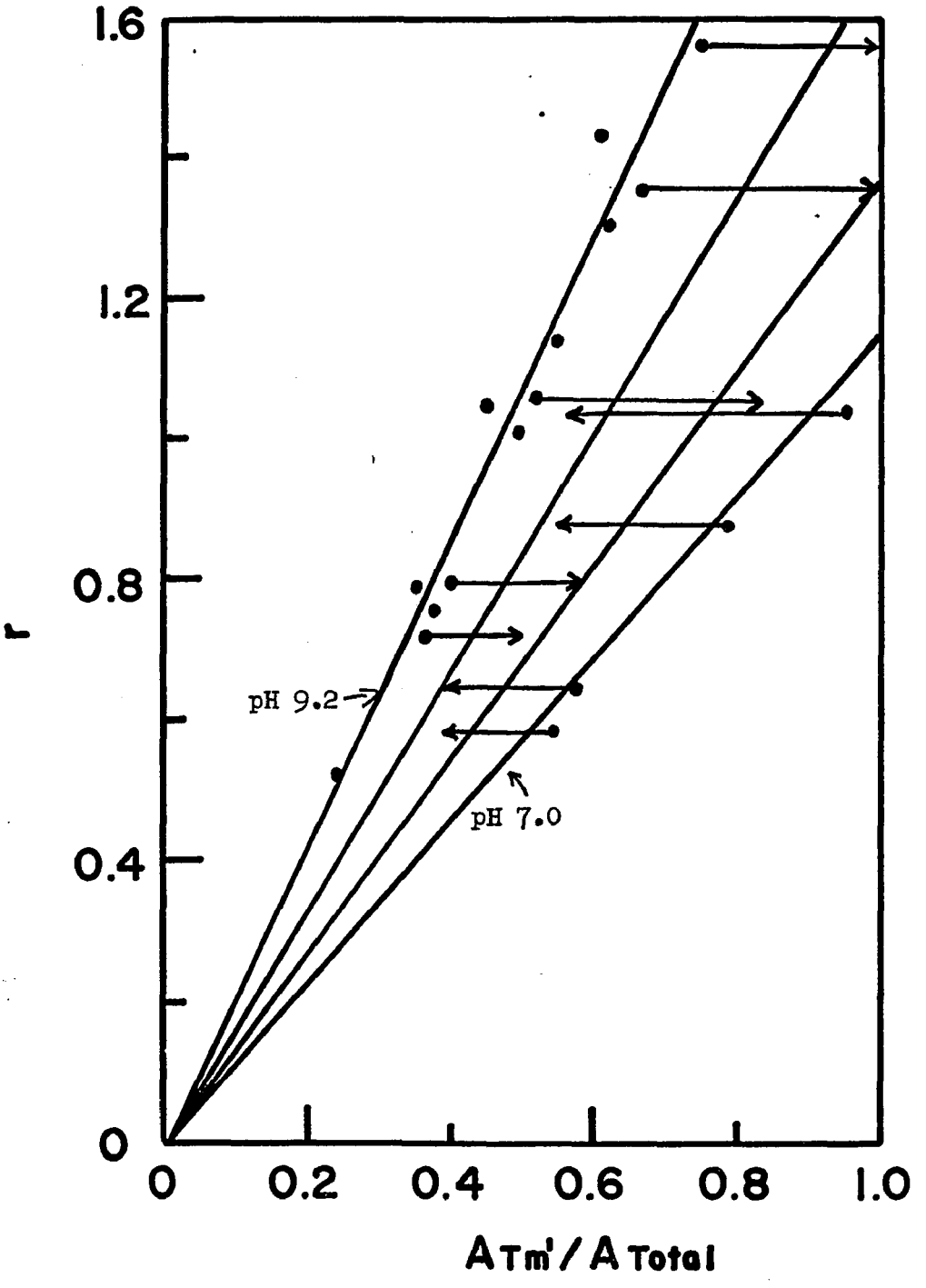
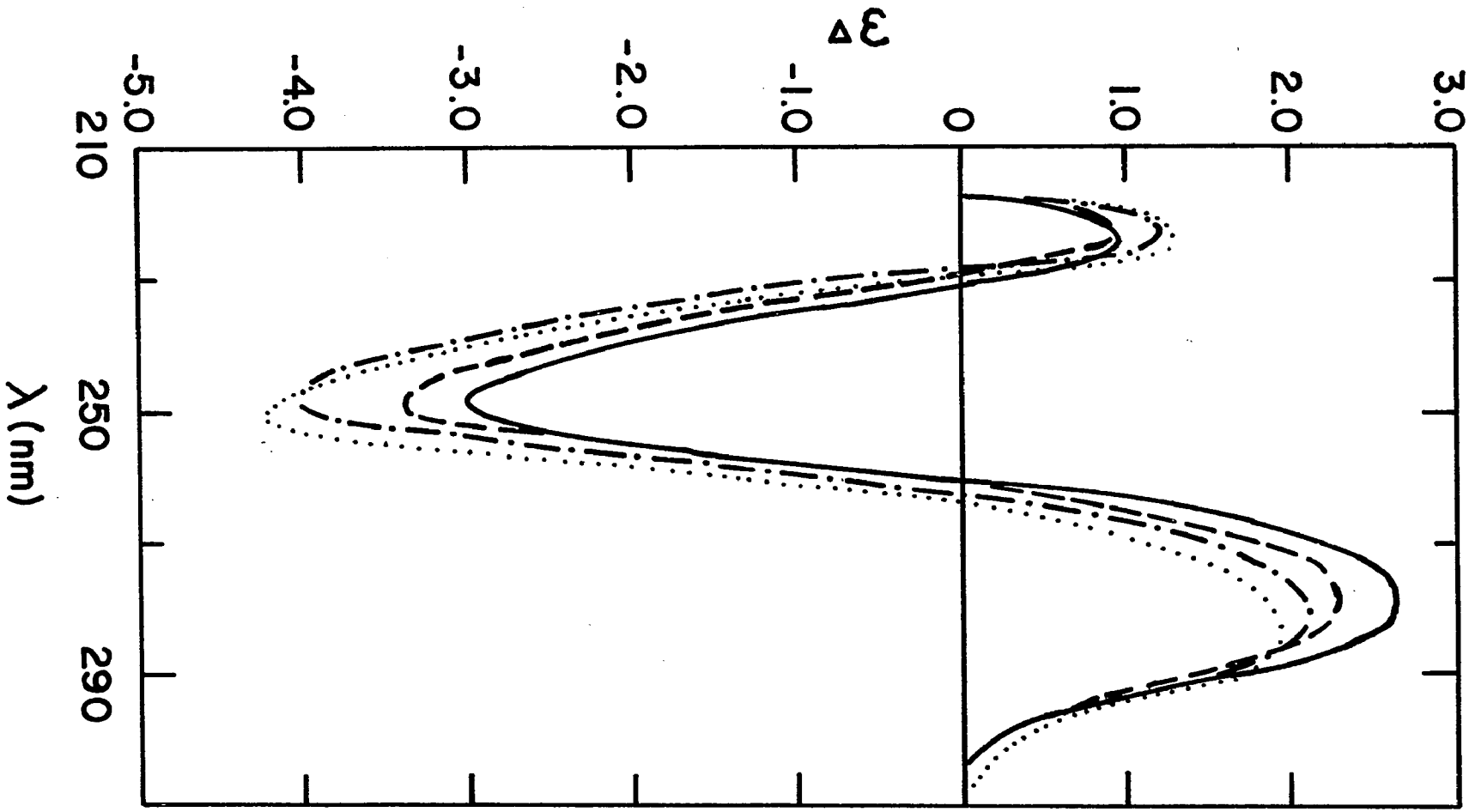


Figure 47. Plot of equation (3) for complexes made at pH 9.2 (•), made at 9.2 and adjusted to 7.0 (• ⟶) and made at pH 7.0 and adjusted to 9.2 (⟵ •)

Figure 48. CD spectra of DNA (___)and two complexes (r = 0.78)(---) and (r = 1.34)(-·-) at pH 9.2. $\Delta\epsilon_b^D$ for DNA bound at pH 9.2 (...).



Perhaps, with one amino acid per nucleotide at pH 7.0, the copolypeptide winds much more tightly around the DNA and causes a greater structural distortion in DNA than at pH 9.2.

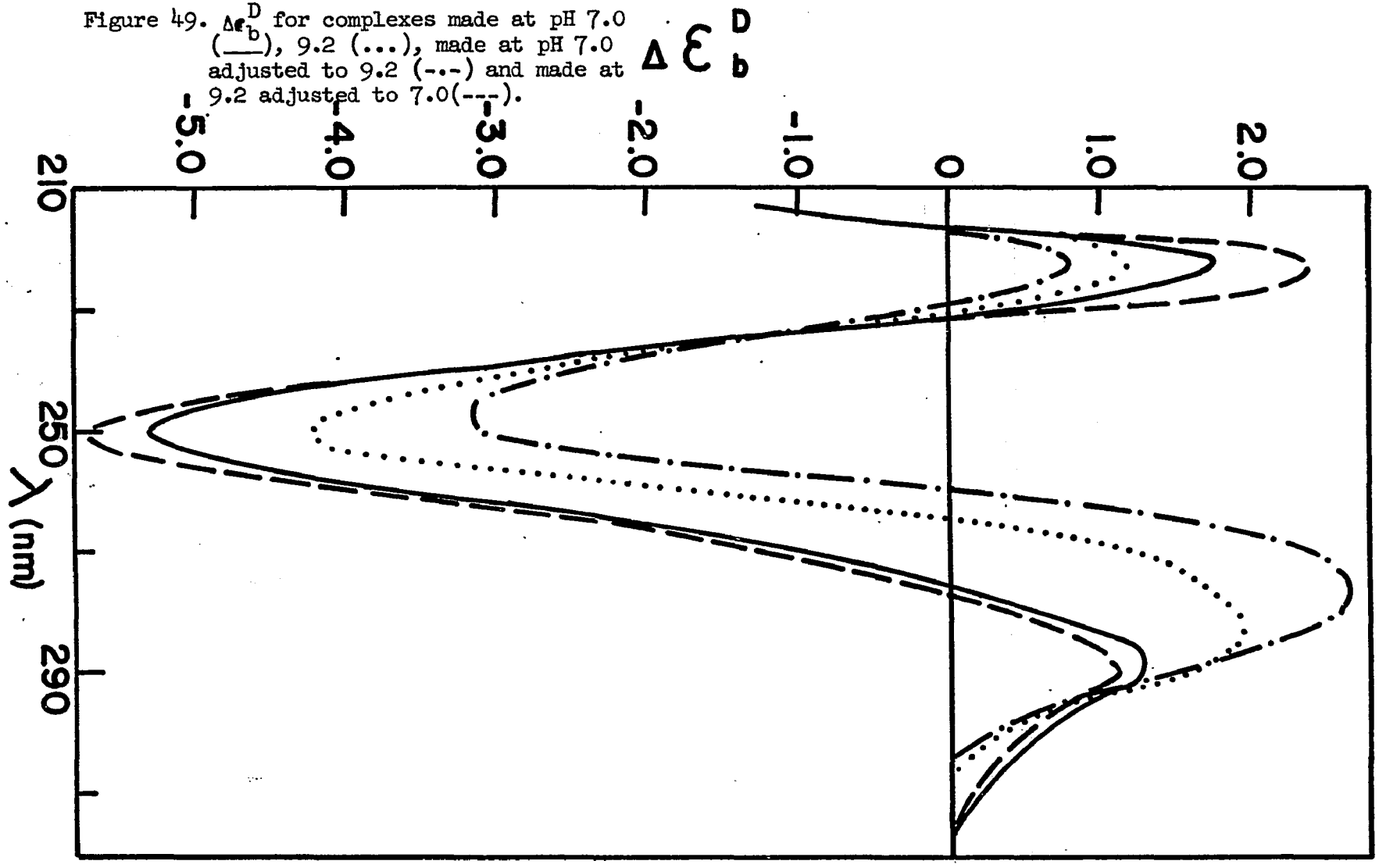
Fluorescence of the complexes, made at pH 9.2, were also determined. As shown in Figure 46, there is no difference in intensity at the same fraction of DNA bound for complexes prepared at pH 7.0 or 9.2.

After thermal denaturation and CD measurements were taken on the complexes made at pH 9.2, the pH was adjusted to 7.0 and both thermal denaturation and CD were taken again. Based upon thermal denaturation results, the β value of 2.04 at pH 9.2 decreases to approximately 1.3 amino acid residues per nucleotide at pH 7.0 (Figure 47). A decrease in pH would lead to partial protonation on histidine residues which can then bind the phosphates more effectively. If the copolypeptide was completely dissociated from and rebound to DNA, the β value would be expected to approach the 1.14 value obtained for complexes made at pH 7.0. The higher β value, 1.3 rather than 1.14, could suggest some rearrangement of the copolypeptide moleclue on DNA when the pH was lowered. For instance, some regions of the copolypeptide, such as near the ends, could become loose and rebind the DNA with histidine residues directly bound to the phosphates through ionic interaction.

The CD of the complexes made at pH 9.2 and adjusted to 7.0 resemble those of complexes made at 7.0 as seen from the $\Delta\epsilon_b^D$ calculated from these complexes (Figure 49). $\Delta\epsilon_b^D$ shows a large red shift and reduction of the peak at 275nm and a red shift and reduction of the crossover near 255nm from that of pure DNA, consistent with the lowered β value.

It is possible to reverse the above process by making the complexes

Figure 49. $\Delta\epsilon_b^D$ for complexes made at pH 7.0 (___), 9.2 (...), made at pH 7.0 adjusted to 9.2 (-.-) and made at 9.2 adjusted to 7.0(---).



at pH 7.0 and then adjusting the pH to 9.2. Under these conditions the fraction of DNA bound is decreased as determined by thermal denaturation. This in turn leads to an increase in β value as shown in Figure 47. The new β value, 1.7 amino acid residues per nucleotide, lies between the values for complexes made at pH 7.0 and 9.2. When the pH of the complex is raised the histidines loose their positive charges and can no longer bind to the phosphates through ionic bonding. In order to maximize ionic interaction, the copolypeptide in the complex could rearrange in such a way that the same copolypeptide molecule covers a smaller length of DNA such that those phosphates originally bound by histidine, can be bound ionically by lysine residues.

The CD spectra of complexes made at pH 7.0 and adjusted to pH 9.2 show only slight structural changes from that of free DNA. This can be seen from $\Delta\epsilon^{D}_{b}$ calculated for these complexes and shown in Figure 49. Structural changes on DNA are even less than those induced by complex formation at pH 9.2.

Discussion

The results of this study indicate that the presence of histidine residues in the copolypeptide decreases slightly the thermal stability of protein-bound regions. This is different from the results seen with poly(Lys^{50},Tyr^{50}) in which no significant destabilization is detectable. Prehaps electrostatic interaction between a phosphate and a protonated histidine is not as strong as that between a phosphate and a protonated lysine.

As shown in Figure 41, the presence of histidine in poly(Lys^{48},His^{52})

increases the conformational effect on DNA. There is a greater red shift and reduction of the positive peak near 275nm and a greater red shift of the crossover near 255nm than in polylysine complexes. Presumably, the side chain of histidine is more effective than that of lysine in inducing structural distortion in DNA. This effect could be due to the additional tightness of winding of the poly(Lys^{48},His^{52}) backbone along the groove of DNA to obtain maximum ionic bonding between histidine residues and phosphates. The fact that the complexes formed at pH 9.2, with approximately two amino acid residues per nucleotide in the bound region, have a much smaller structural change in DNA than at pH 7.0 is in agreement with this viewpoint.

The titration and thermal denaturation studies at variable pH suggest that the histidine residues in the complex are mostly protonated at pH 6.0 and deprotonated at 9.2 with resulting changes in the precipitation point and β value.

The induction of fluorescence in DNA by the binding of poly(Lys^{48}, His^{52}) is interesting. As mentioned in the Introduction (Chapter I), the only previous report of DNA fluorescence at room temperature required the use of a multiple channel spectrofluorometer. Because of the low intensity of DNA fluorescence, no measurements could be taken with the normal instruments. As seen in Figure 45 however, the binding of poly(Lys^{48},His^{52}), a non-fluorescent copolypeptide, results in the induction of DNA fluorescence of an amplitude similar to that observed in poly(Lys^{50},Tyr^{50})- and poly(Lys^{58},Phe^{42})-DNA complexes. Thus the binding of these copolypeptides to DNA sufficiently reduces the rate of nonradiatve energy dissipation,normally occurringat room temperature, to allow DNA fluorescence to be measured.

CHAPTER VI

FLUORESCENCE STUDIES ON THE INTERACTION OF POLYPEPTIDES WITH DNA AND ITS' DERIVATIVES

Induction of DNA Fluorescence by Polylysine and Polyarginine

At room temperature both DNA and polylysine show no detectable fluorescence (Figure 50). When they are complexed however, a strong fluorescence spectrum with a peak at 300nm and a shoulder at 360nm is induced. As shown in this figure, the more DNA bound by polylysine (higher r value), the greater the fluorescence intensity.

Similar to the effect of polylysine, polyarginine binding to DNA also induces DNA fluorescence (Figure 50). Polyarginine alone has no significant fluorescence.

To determine whether the size of the polylysine chain affects the intensity of fluorescence emission, polylysines of different chain length were used. For the same r value, or fraction of DNA base pairs bound, the fluorescence intensity of a complex with a polylysine of 33 residues is only about 50% of that with a polylysine of 166 residues. Thus the longer polylysine chain seems to be capable of inducing a greater amount of fluorescence for the same amount of DNA bound.

Figure 51 shows a plot of the relative fluorescence intensity at

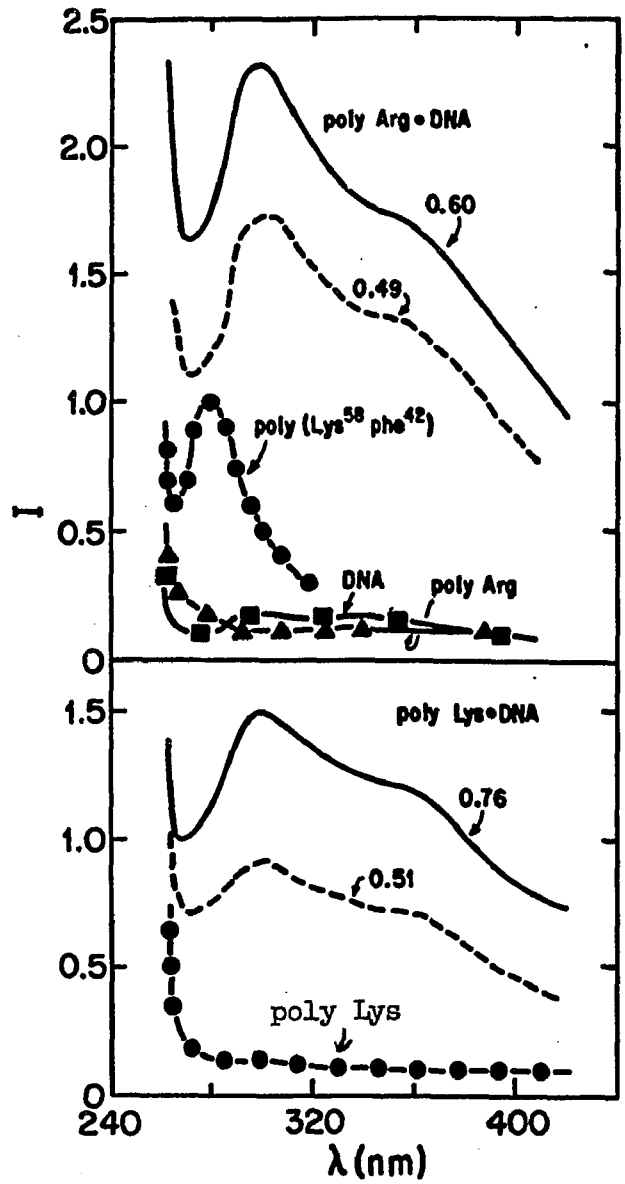


Figure 50. Fluorescence spectra of DNA, polyarginine polylysine and complexes with polyarginine and polylysine. The r values are indicated. Excitation wavelength is 250nm. Intensity relative to poly(Lys^{58},Phe^{42}) (1.2 x 10^{-4}M) emission at 280nm.

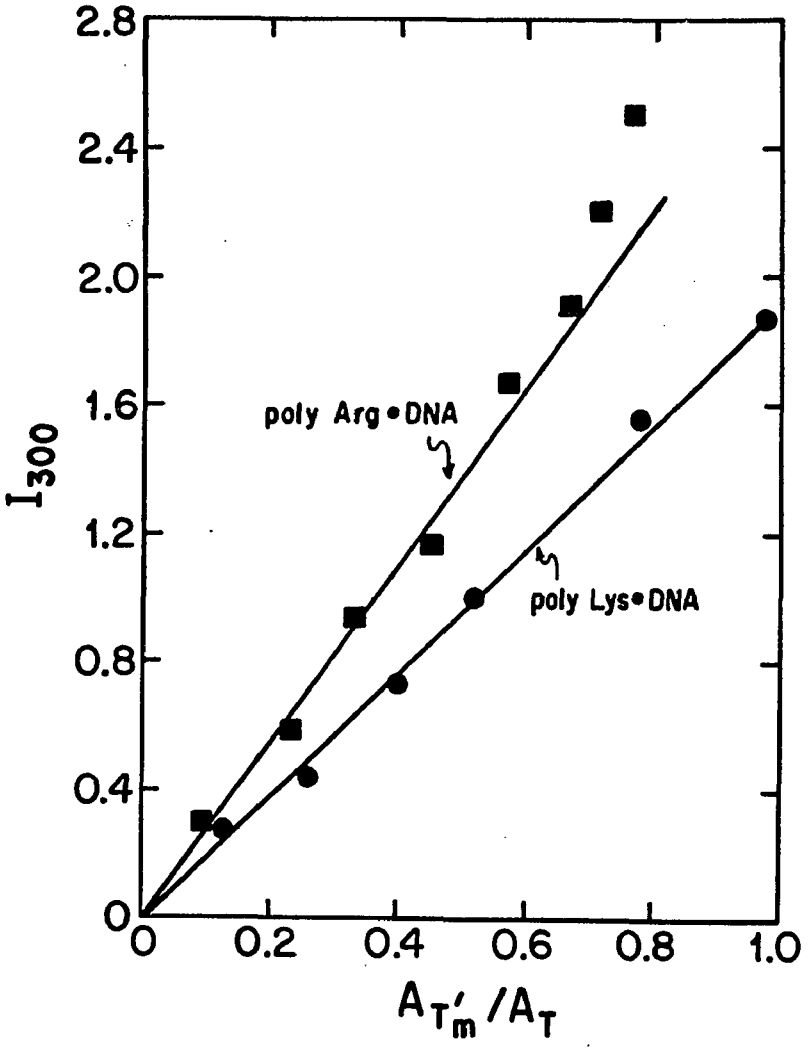


Figure 51. Fluorescence intensity at 300nm as a function of the fraction of DNA bound by polylysine or polyarginine. Excitation wavelength is 250nm. Polylysine of 166 residues length. Intensity relative to poly(Lys^{58},Phe^{42}) (1.2×10^{-4}M) emission at 280nm.

300nm as a function of the fraction of DNA bases bound by polylysine and polyarginine. There is a linear relationship between the fluorescence intensity and the fraction of bases bound by polylysine. For polyarginine complexes, the linear relationship is obeyed reasonably well until a higher r value in the complex is reached. The difference in fluorescence intensity indicates a difference in quantum yield of base pairs bound by different proteins. Although the chain length of a protein may have a significant effect on the quantum yield of induced fluorescence, the above difference cannot be due to this factor since the two polypeptides are of approximately equal chain length. In addition, poly(Lys^{48},His^{52}) induces even weaker fluorescence (Figure 45) although its molecular weight is about two times that of polylysine and polyarginine.

Complex Fluorescence as a Function of Temperature

Complexes of DNA with polylysine, polyarginine or other copolymers, in which the fraction of DNA base pairs bound was approximately the same, were heated in EDTA buffer and the fluorescence measured. Figure 52 shows the change in fluorescence intensity at 300nm as a function of temperature for these complexes. For the polylysine complex, there is no change in fluorescence as the temperature is raised. The readings at 100° were taken on samples heated at 100° in a sealed tube, then returned to the spectrofluorometer which was maintained at 85°. The poly(Lys^{48}, His^{52}) complex showed a slight decrease in fluorescence upon heating while the polyarginine complex showed a slight increase. Only complexes with poly(Lys^{50},Tyr^{50}) or poly(Lys^{58},Phe^{42}) show a significant increase in fluorescence at higher temperature. This has been attributed to a stacking interaction between the tyrosine or phenylalanine chromophore

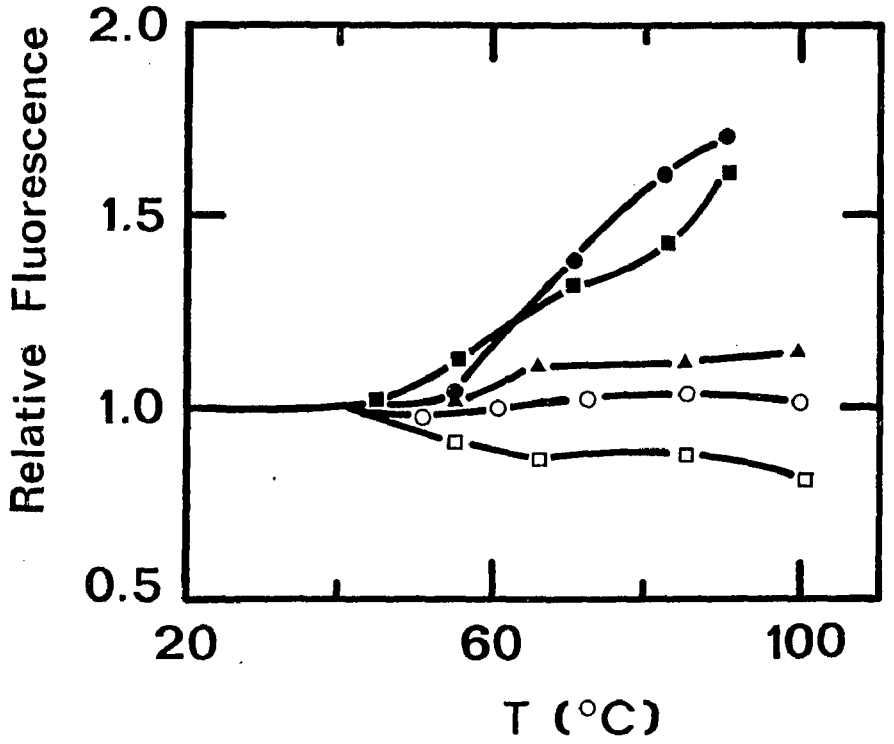


Figure 52. Temperature dependence of fluorescence intensity at 300nm of polypeptide-DNA complexes in EDTA buffer. A complex with poly(Lys^{58},Phe^{42})(r = 1.35)(●),with poly(Lys^{50},Tyr^{50})(r = 1.27)(■), with polyarginine (r = 0.60)(▲), with polylysine (r = 0.75)(○) and with poly(Lys^{48}, His^{52})(r = 0.75)(□). Excitation wavelength is 250nm.

with the DNA bases as the DNA is denatured.

Effect of Trypsin Digestion on Fluorescence of Complexes

Trypsin catalyzes the hydrolysis of peptide bonds to lysine and arginine residues.[53] A previous report from this laboratory discussed the use of trypsin in studying the mode of binding of various proteins to DNA.[54] Trypsin digestion of polylysine and polyarginine complexes were followed by thermal denaturation and CD. It was found that the T_m' band of polylysine-DNA disappears completely after $1\frac{1}{2}$ hr of trypsin digestion. A plot of the decrease in the area under the melting band at T_m' is shown in Figure 53. The original two melting bands at T_m and T_m' are replaced by one broad melting band with its peak falling between these two temperatures. This is due to the digestion of polylysine into short oligolysine fragments which, because of their small size, are not able to bind DNA irreversibly and induce an independent phase of melting. In contrast, when polyarginine-DNA complexes are digested, the area under T_m' is reduced by only 50% even after 5 hr (Figure 53). A broad melting band between T_m and T_m' is again seen due to the binding of the oligoarginine formed by the partial digestion of polyarginine.

Similar results were observed if the CD spectra of the complexes were followed. Thus, the CD spectrum of polylysine-DNA is almost completely restored to that of pure DNA after digestion for $1\frac{1}{2}$ hr. In contrast, the CD spectrum of a polyarginine-DNA complex is restored only one quarter of the way back to that of unbound DNA. These results were explained as probably due to the binding of polyarginine in the major groove of DNA in such a way that the main portion of the amide groups are buried and not accessible to trypsin. Polylysine, on the other hand

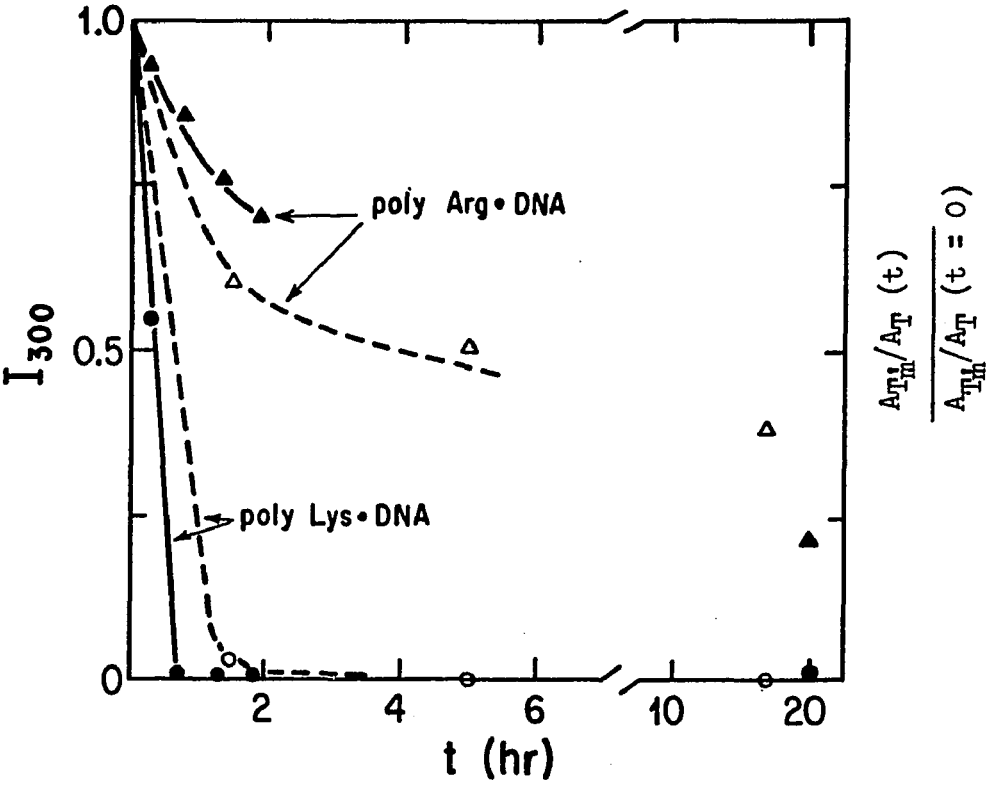


Figure 53. Effect of trypsin digestion on fluorescence at 300nm and area under melting band at T_m' for polylysine and polyarginine complexes with calf thymus DNA. Fluorescence results for polylys complex (r = 0.78)(●) and polyarg (r = 0.81)(▲); relative area under melting band at T_m' for polylys complex (r = 0.60)(○) and for polyarg complex (r = 0.60)(△).

is suggested to bind the minor groove of DNA in such a way that the peptide bonds are exposed to the trypsin.[54]

Because the binding of polylysine and polyarginine results in the induction of DNA fluorescence it was thought that fluorescence might be a useful third method for studying trypsin digestion of protein-DNA complexes. The fluorescence spectra of a polylysine-DNA complex (r = 0.78) and a polyarginine-DNA complex (r = 0.81) treated with trypsin, are shown in Figure 54a and b respectively. After 40 min, the fluorescence spectrum due to polylysine completely disappears while, the polyarginine-DNA complex still shows significant fluorescence even after 20 hr of trypsin digestion. A plot of fluorescence intensity at 300nm as a function of time of trypsin digestion is shown in Figure 53. Also plotted in this graph is the change in area under the melting band at T_m' for polylysine and polyarginine complexes. It can be seen that the fluorescence data is in agreement with the thermal denaturation.

Trypsin digestion was also used to study copolypeptide binding to DNA. Figure 55 shows the fluorescence spectra of a poly(Lys^{58},Phe^{42})-DNA complex (r = 1.39) after trypsin digestion. There is a decrease in the complex fluorescence but even after 20 hr some fluorescence remains. The fluorescence spectra for other DNA-copolypeptide complexes are not shown. Instead,Figure 56 summarizes the results by showing the decrease in intensity of emission at 300nm as a function of time of trypsin digestion for all complexes. Although the r value of the complexes varies, the fraction of DNA base pairs bound is approximately the same in all cases. After 20 hr of digestion none of the complexes shows complete digestion as seen with polylysine. Poly(Lys^{50},Tyr^{50}) is even more protected than polyarginine while poly(Lys^{58},Phe^{42}) and poly(Lys^{48},

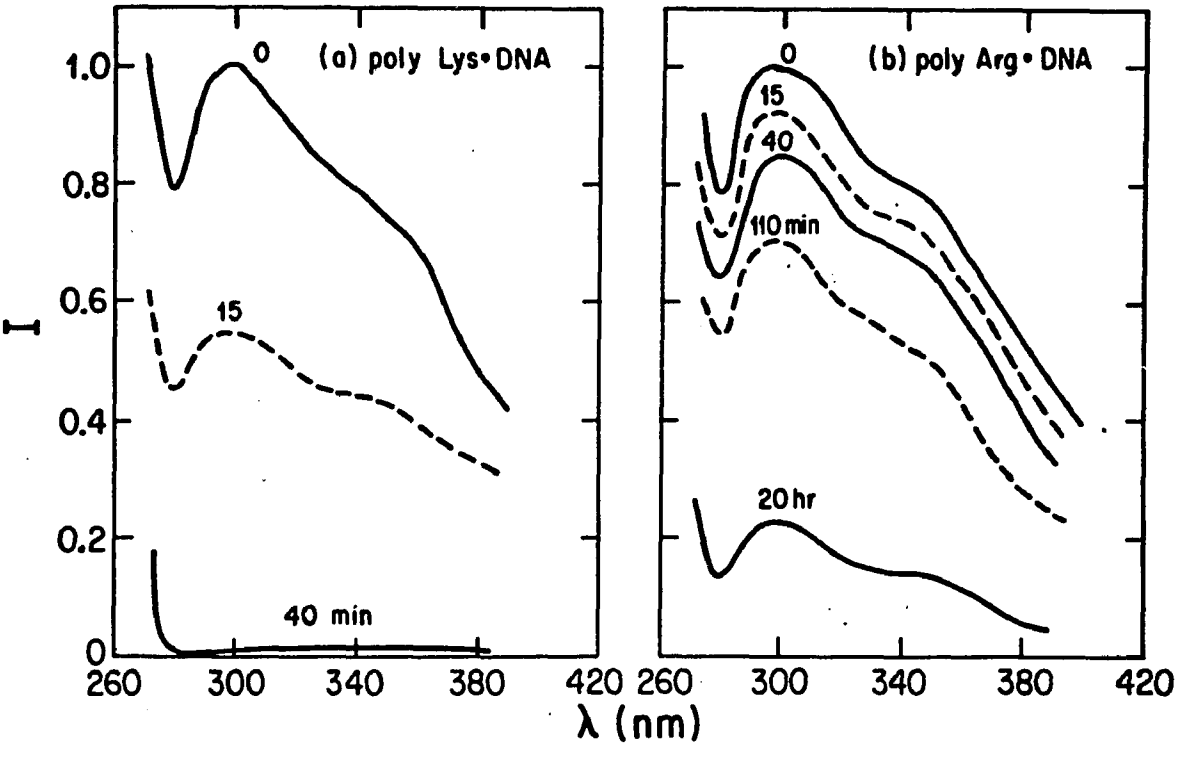


Figure 54. Fluorescence spectra of a polylysine-DNA complex (r = 0.78) and a polyarginine-DNA complex (r = 0.81) with trypsin digestion. Excitation wavelength is 250nm. Length of trypsin digestion is indicated.

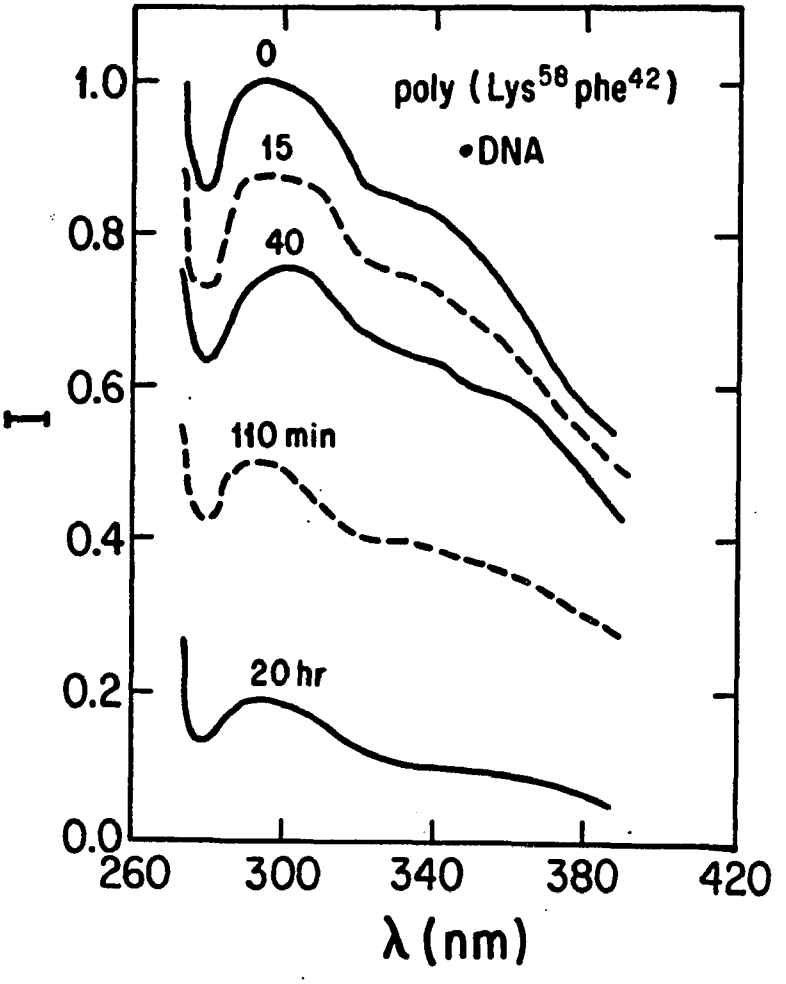


Figure 55. Fluorescence spectra of a poly(Lys^{58},Phe^{42})-DNA complex (r = 1.39) with trypsin digestion. Excitation wavelength is 250nm. Length of trypsin digestion is indicated.

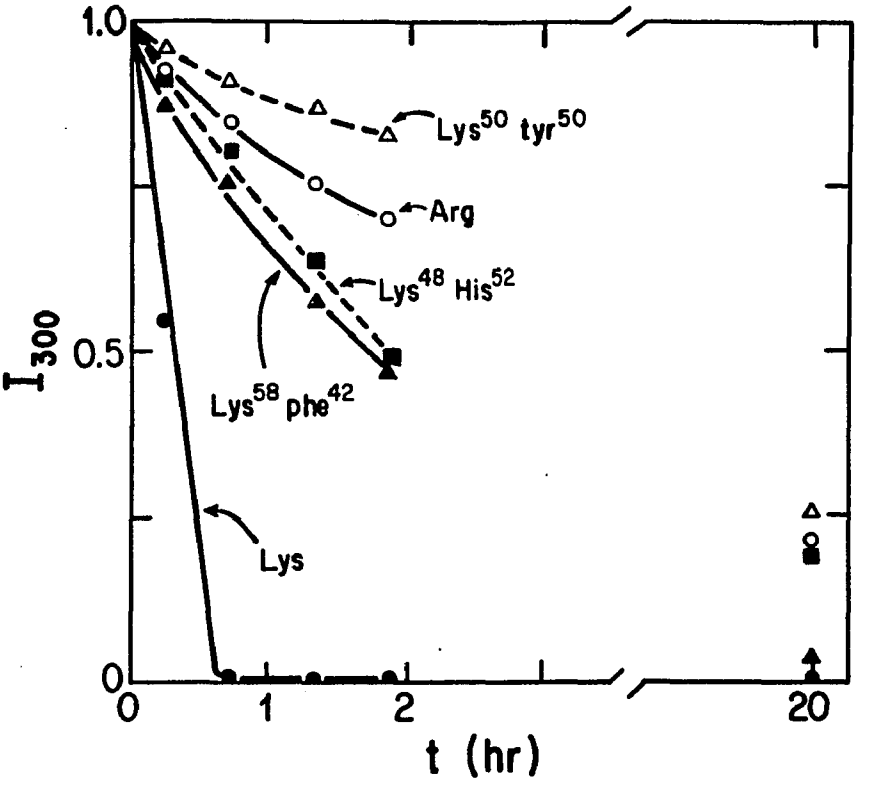


Figure 56. Fluorescence intensity at 300nm of polypeptide-DNA complexes as a function of time of trypsin digestion. The r value for the complex with polylys is 0.78, with polyarg,0.81, with poly(Lys^{50},Tyr^{50}), 1.30, with poly(Lys^{58},Phe^{42}), 1.39 and with poly(Lys^{48}, His^{52}), 1.50.

His^{52}) show intermediate protection between polylysine and polyarginine.

DNA Fluorescence Induced by Binding of Copolypeptides Containing Varying Amounts of Lysine and Alanine

Several copolypeptides containing varying amounts of lysine and alanine had been synthesized in this laboratory in order to study the effect of α-helical content and hydrophobic residues in a protein when it interacts with DNA. These studies showed that as the percentage of alanine in the copolymers increases the amount of α-helix also increases. Thus poly(Lys^{19},Ala^{81}) is 100% α-helix, while poly(Lys^{81},Ala^{19}) is a random coil.[55] The presence of a rigid α-helix was shown to prohibit some lysine residues from direct interaction with the phosphates on DNA. The β value for the complexes also changed greatly, 1.1 for poly(Lys^{81}, Ala^{19}), 2.5 for polyLys^{40},Ala^{60}) and 3.6 for poly(Lys^{19},Ala^{81}).[55] These results suggested that the copolymers might also differ in their ability to induce DNA fluorescence. Figure 57 shows the relative fluorescence intensity at 300nm of copolymer-DNA complexes as a function of the fraction of DNA bound. The slope is greater when the percent of lysine in the copolymer is higher. Thus at the same A_{T_m}/A_T, poly(Lys^{19},Ala^{81}) induces only one-fourth as much fluorescence as polylysine. Increasing amounts of α-helix may prevent a tight binding of the copolymer to DNA and show a lower efficiency in induced fluorescence.

Fluorescence Studies of Complexes between Polypeptides and Mono- or Polynucleotides

The results presented previously show the possibility of a stacking interaction between the tyrosine or phenylalanine chromophore

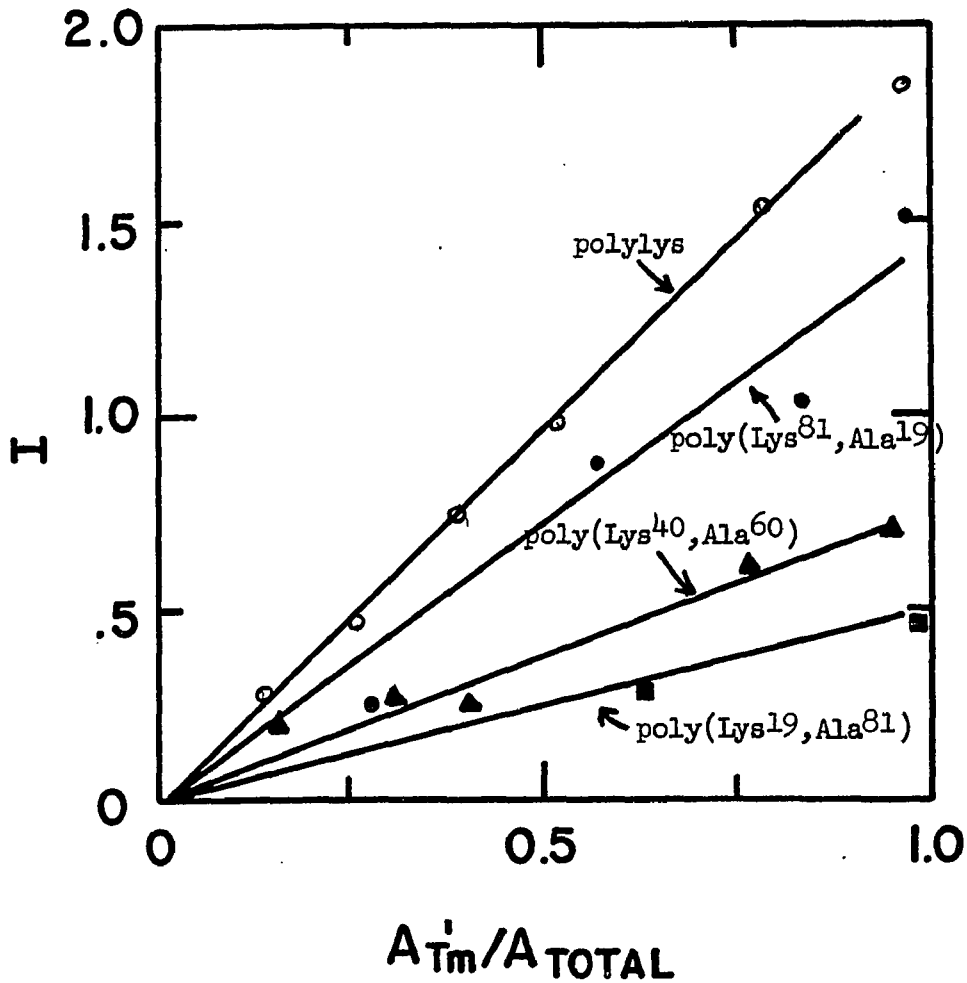


Figure 57. Fluorescence intensity at 300nm as a function of the fraction of DNA bound by polylysine and copolypeptides containing varying amounts of lysine and alanine. Excitation wavelength 250nm.

with the DNA bases. In addition, the binding of poly(Lys^{58},Phe^{42}) to poly(A) (Figure 28) with a fluorescence spectrum similar to that of binding to DNA, suggests an interaction with single-stranded RNA. Because of these results, it was decided to study in more detail the interaction of the various polypeptides with mono- and polynucleotides. If a stacking interaction is responsible for some of the fluorescence results it is possible that a stacked complex can be formed at high concentrations of mononucleotides. With polynucleotides, where the bases are held together by the phosphate backbone, stacking interaction with aromatic residues in the copolypeptide could occur more easily.

Complexes were made with the four deoxymononucleotides and three polynucleotides. Since the hydroxy group in the ribose moiety could possibly affect the type of interaction, complexes with both the ribose- and deoxyribose-mononucleotides were studied and the results were found to be identical.

Fluorescence of Polypeptide-Mononucleotide Complexes

Complexes were made between polylysine, poly(Lys^{50},Tyr^{50}) or poly (Lys^{58},Phe^{42}) and the four deoxyribonucleotides, dAMP, dTMP, dGMP and dCMP. The concentrations of mononucleotides range from 0 to 10^{-3} M. Figure 58 shows fluorescence spectra of free poly(Lys^{50},Tyr^{50}) and the 10^{-3} M mononucleotide complexes. The complexes with dTMP and dCMP show quenching of the tyrosine fluorescence, probably due to self-absorption, since the light scattering peak near 250nm disappears with the high absorption of the sample. Using 10^{-3} M dAMP a broad fluorescence band appears with a peak near 295nm and a shoulder at 360nm. At 10^{-3} M dGMP, an even stronger fluorescence spectrum is induced with a peak near 310nm

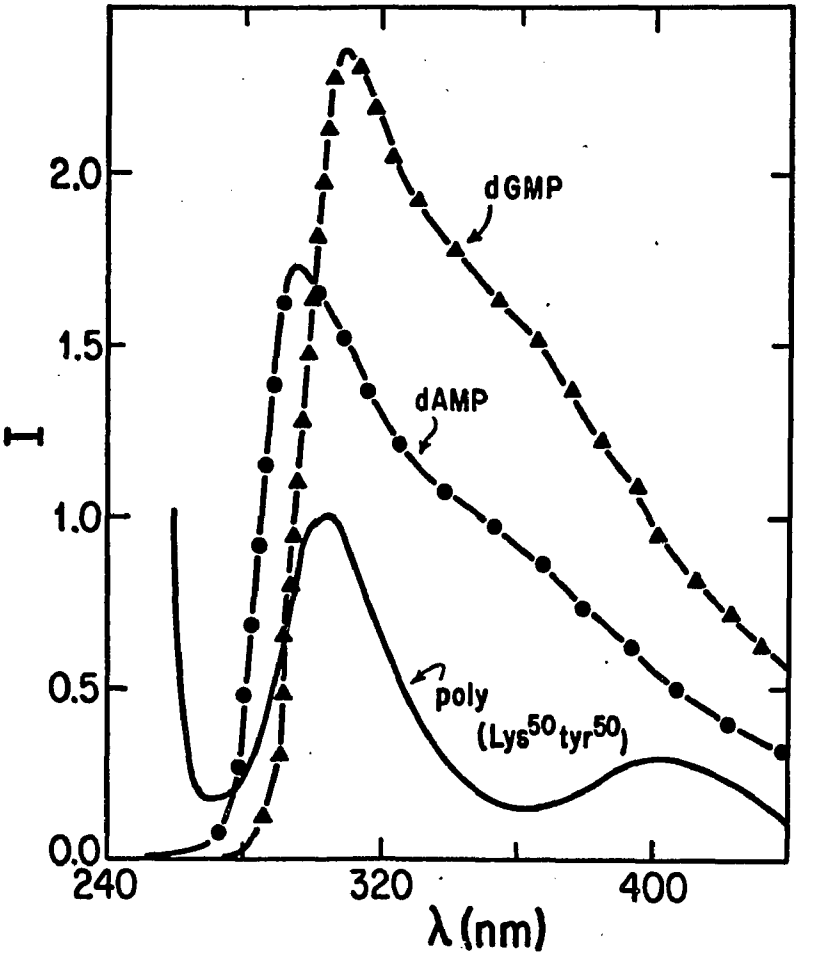


Figure 58. Fluorescence spectra of poly(Lys^{50},Tyr^{50}) and complexes with dAMP and dGMP excited at 250nm. Mononucleotide concentration is 10-3M and poly(Lys^{50},Tyr^{50}) is 1.38×10^{-4}.

and a shoulder at 360nm. Free mononucleotides have no significant fluorescence. The increase in fluorescence with dAMP and dGMP is even more impressive when the amount of quenching by self-absorption is concidered. 10^{-3} M solutions of mononucleotides have absorbances ranging from 9.0 to 15.0 which should be high enough to absorb any fluorescence produced. The strong fluorescence seen here indicates that the quantum yield of the complexes must be quite high.

The fluorescence spectra of free poly(Lys^{58},Phe^{42}) and the 10^{-3} M mononucleotide complexes are shown in Figure 59. Once again the dAMP complex shows enhancement with a peak at 300nm and a shoulder at 360nm. The dGMP complex shows stronger fluorescence with a peak at 310nm and a shoulder at 360nm. The general shape of the spectra are different from those seen with poly(Lys^{50},Tyr^{50}) and may indicate that the spectra depend upon the chromophore used (tyr or phe).

At low concentrations of mononucleotides (5×10^{-5} M) the spectra resemble those of free poly(Lys^{50},Tyr^{50}) or poly(Lys^{58},Phe^{42}) with a peak at 307 or 280nm, respectively, except of a reduced intensity due to the screening effect of the mononucleotides present. At 1 and 2×10^{-4} M mononucleotide, the shape of the spectra changes with the peak of emission shifting to 295nm for dAMP complexes with poly(Lys^{50},Tyr^{50}) and 300nm with poly(Lys^{58},Phe^{42}) and 310nm for all other complexes. The intensity of the complexes is still below that of the free copolypeptide. Table III summarizes the intensity and wavelength of emission maximum for poly(Lys^{50},Tyr^{50}), poly(Lys^{58},Phe^{42}) and polylysine complexes. Using polylysine, no significant amount of fluorescence can be induced no matter which mononucleotide is used. These results suggest that the presence of aromatic tyrosine or phenylalanine in the copoly-

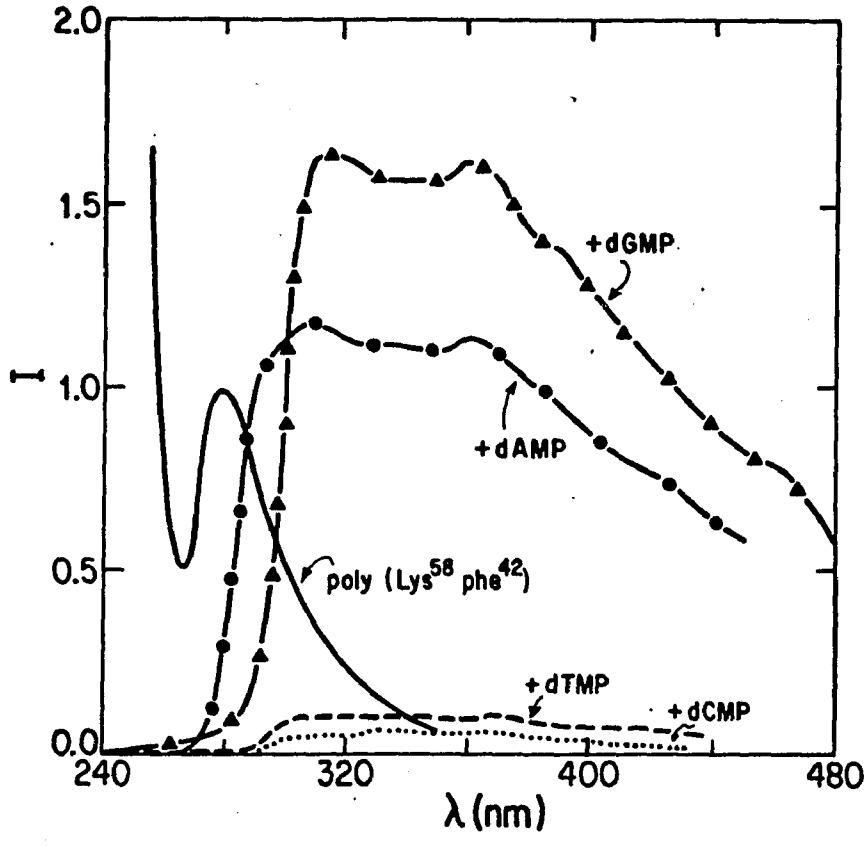


Figure 59. Fluorescence spectra of poly(Lys^{58},Phe^{42}) and complexes with dAMP,dGMP,dTMP and dCMP excited at 250nm. Mononucleotide concentration is 10^{-3}M and poly(Lys^{58},Phe^{42}) is 1.47×10^{-4}M.

TABLE III

Relative Fluorescence Intensity of Polymer-Mononucleotide Complexes

Polymer	Nucleotide	0	5×10^{-5}	10^{-4}	2×10^{-4}	10^{-3}
poly(Lys^{50},Tyr^{50})	dAMP 295nm	0.78	0.20	0.12	0.09	1.68
1.38×10^{-4}	dGMP 310nm	0.93	0.25	0.13	0.19	2.35
	dTMP 310nm	0.93	0.27	0.19	0.14	0.02
	dCMP 310nm	0.93	0.26	0.16	0.11	0.04
poly(Lys^{58},Phe^{42})	dAMP 300nm	0.45	0.18	0.16	0.20	1.45
1.47×10^{-4}	dGMP 310nm	0.27	0.12	0.18	0.32	1.63
	dTMP 310nm	0.27	0.15	0.13	0.13	0.10
	dCMP 310nm	0.27	0.15	0.10	0.10	0.04
polylysine	dAMP 300nm	0.01	0.02	0.03	0.05	0.03
7.4×10^{-5}	dGMP 310nm	0.01	0.03	0.03	0.03	0.02
	dTMP 310nm	0.01	0.01	0.02	0.01	0.01
	dCMP 310nm	0.01	0.02	0.02	0.01	0.01

peptide could possibly induce a stacking interaction with mononucleotides and fluorescence.

Fluorescence of Polypeptide-Polynucleotide Complexes

Complexes were made between polylysine, poly(Lys^{50},Tyr^{50}) or poly (Lys^{58},Phe^{42}) and poly(A), poly(C) and poly(G). The concentration of polynucleotide varied from 0 to 10^{-4} M. Figure 60 shows the fluorescence spectra of free polynucleotide, poly(Lys^{50},Tyr^{50}) and the copolypeptide-polynucleotide complexes, all excited at 250nm. The free polynucleotides have significant fluorescence compared to the free copolypeptide. Poly(G) shows the strongest fluorescence with a peak at 345nm. Poly(C) and poly(A) both show broad bands between 300 and 400nm. The copolypeptide - poly(G) complex (r = 0.83) shows an enhanced fluorescence over that of the free copolypeptide and free poly(G) with a peak at 335nm. The complex with poly(A) (r = 0.59) shows a peak at 300nm with a shoulder at 360nm. The strongest fluorescence is seen with the poly(C) complex (r = 0.70) having a peak at 310nm and an intensity 3.3 times that of free poly(Lys^{50},Tyr^{50}) and a shoulder at 360nm.

The fluorescence spectra of free poly(Lys^{58},Phe^{42}), poly(G) and complexes of poly(G) with polylysine (r = 0.60) and poly(Lys^{58},Phe^{42}) (r = 0.89) are shown in Figure 61a. The spectra of the complexes resemble that of poly(G) alone except that the peak is blue shifted from 345nm to 335nm for the poly(Lys^{58},Phe^{42}) complex and to 300nm for the polylysine complex.

Similar spectra for poly(C) complexes are plotted in Figure 61b. The complexes with polylysine and poly(Lys^{58},Phe^{42}) both show a peak at 310nm with a shoulder at 360nm.

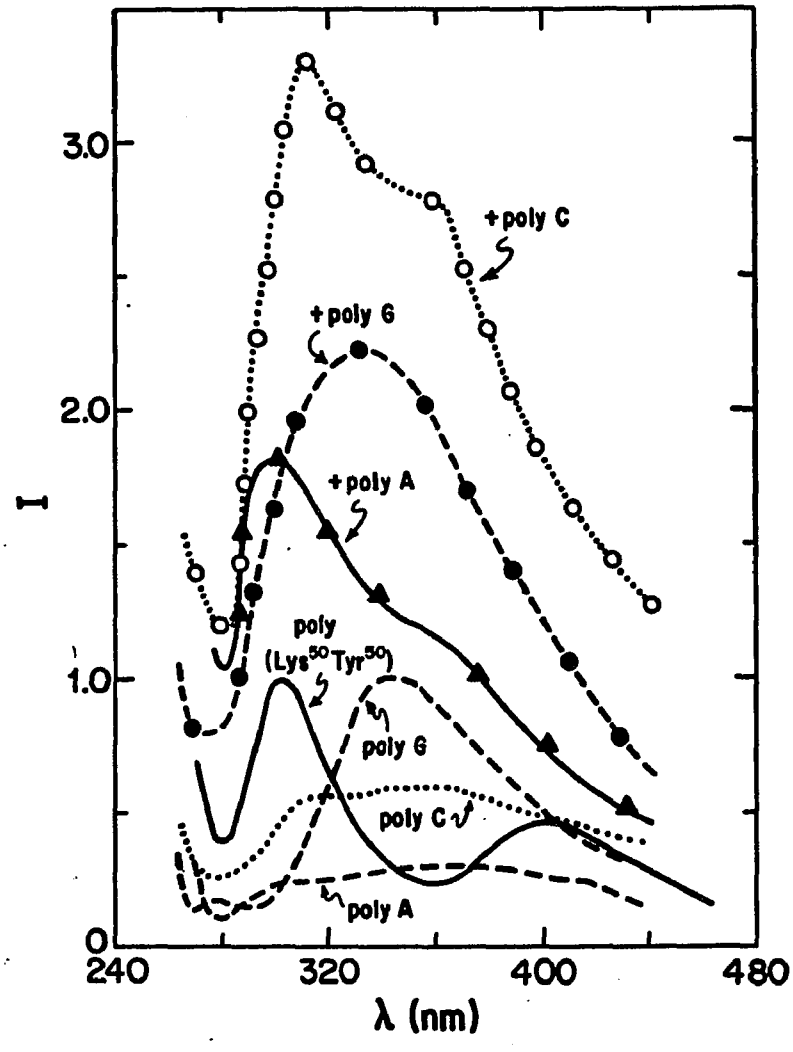


Figure 60. Fluorescence spectra of free poly(Lys^{50},Tyr^{50}), polynucleotides and copolypeptide-polynucleotide complexes excited at 250nm. Polynucleotide concentrations 10^{-4}M, poly(Lys^{50},Tyr^{50}) concentration 9.1 x 10^{-5}M. Poly(C) complex has an r value of 0.70, poly(G) of 0.83 and poly(A) of 0.59.

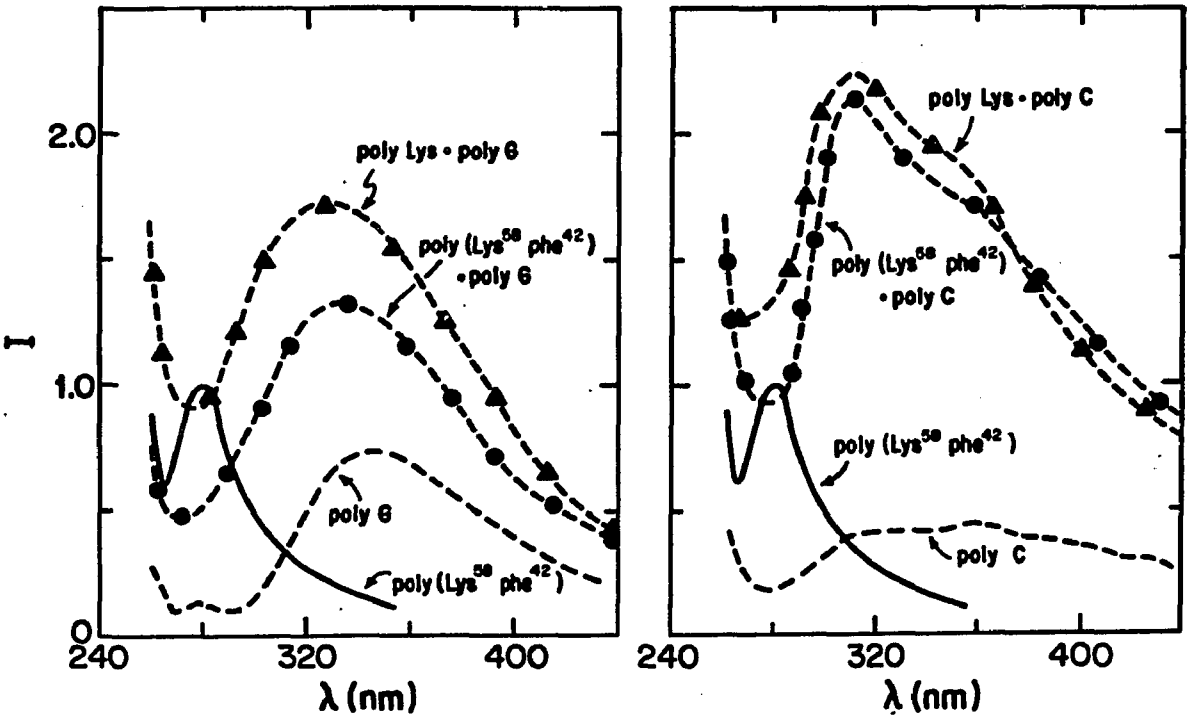


Figure 61. Fluorescence spectra of free poly(Lys^{58},Phe^{42}), poly(C), poly(G) and complexes with polylysine and poly(Lys^{58},Phe^{42}).excited at 250nm. Polynucleotide concentrations are 10^{-4}M, poly(Lys^{58}, Phe^{42}) concentration is 9.2 x 10^{-4}M and polylysine is 6.9 x 10^{-5}M. The r values for the complexes are polylys·poly(G) 0.60, polylys·poly(C) 0.52,poly (Lys^{58},Phe^{42})·poly(G) 0.89 and poly(Lys^{58},Phe^{42})· poly(C) 0.75.

Fluorescence intensities of the polynucleotide complexes relative to that of the free copolypeptide at its peak intensity are given in Table IV. The polylysine data are given relative to the intensity of free poly(Lys^{58},Phe^{42}) at 280nm. No enhancement is seen at 10^{-5} M polynucleotide and in some cases a slight quenching of the free copolypeptide is seen. However, at 5×10^{-5} M polynucleotide the fluorescence spectrum is enhanced with a shift in the peak position. No further increase in fluorescence was observed at a higher concentration of polynucleotide (10^{-4} M) since the polypeptide has only 5×10^{-5} M lysine residues which are fully bound by 5×10^{-5} M polynucleotide.

Discussion

The induction of fluorescence in complexes with nonfluorescent polypeptides, polylysine and polyarginine (Figure 50),suggests that the enhanced spectra seen with the binding of the various polypeptides to DNA comes mainly from DNA. Such an induced fluorescence has also been observed with poly(Lys^{48},His^{52})-DNA complexes (Figure 44) and with poly(Lys^{50},Tyr^{50}) and poly(Lys^{58},Phe^{42}) complexes. The intensity of this fluorescence depends upon the fraction of DNA bound (Figure 51) and the length of binding polypeptide. For instance, short oligolysines cannot induce fluorescence as shown in trypsin-treated polylysine-DNA complexes. (Figure 53).

Until now, fluorescence of DNA at room temperature has been measurable only with a multiple-scan spectrofluorimeter. The quantum yield is extremely low, 2×10^{-5}.[39] The fluorescence results presented here indicate that the binding of DNA by the various polypeptides enhances the quantum yield of DNA fluorescence. Close contact of

TABLE IV

Relative Fluorescence Intensity of Polymer-Polynucleotide Complexes

Polymer	Polynucleotide	0	10^{-5}	5×10^{-5}	10^{-4}
poly(Lys^{50},Tyr^{50})	poly(A) 300nm	0.95	0.64	1.91	1.82
9.1×10^{-5}	poly(G) 335nm	0.35	0.33	2.31	2.20
	poly(C) 310nm	0.93	0.95	3.54	3.31
poly(Lys^{58},Phe^{42})	poly(A) 300nm	0.45	0.51	1.54	1.60
9.2×10^{-5}	poly(G) 335nm	0.12	0.10	1.21	1.31
	poly(C) 310nm	0.27	0.29	2.23	2.16
polylysine*	poly(A) 300nm	0	0.21	1.53	2.61
6.9×10^{-5}	poly(G) 330nm	0	0.45	0.92	1.72
	poly(C) 310nm	0	0.47	3.00	2.24

* Intensity of polylysine complexes is given relative to the emission of poly(Lys^{58},Phe^{42})(9.2×10^{-5}M) at 280nm.

polypeptides with DNA seems to be important in the induced fluorescence as shown in Figure 57. Copolypeptides containing high percentages of alanine show very little fluorescence compared to that of DNA bound by polylysine. The former contain α-helical structure which may not wind in the groove of DNA as tightly as polylysine.

The results of polypeptide-mononucleotide complexes have some interesting implications. Fluorescence enhancement is seen only with very high concentrations of mononucleotides (10^{-3} M) and only with the purine mononucleotides, dAMP and dGMP but not with the pyrimidine mononucleotides, dTMP and dCMP. This is true for poly(Lys^{50},Tyr^{50}) and poly (Lys^{58},Phe^{42}). For polylysine no fluorescence has been observed for all nucleotide concentrations studied.(Table III). The fact that enhanced fluorescence of dAMP and dGMP complexes is seen only with copolypeptides containing aromatic amino acid residues indicates that the interaction is likely to be a stacking interaction of the nucleotide bases with the aromatic ring held together by the peptide backbone of the copolypeptide.

The preferential enhancement with the purine nucleotides could be due to a greater overlap of the aromatic ring with the larger rings of the purine bases compared to the pyrimidine bases. This result is not totally unexpected since a greater K_{app} for purines than for pyrimidines has been shown for complexes with tryptamine using NMR at much higher concentrations of mononucleotides (0.04M) and higher concentrations of tryptamine(0.1-0.3M).[56] The greater sensitivity of fluorescence measurements shows that the preferential stacking with the purine bases can be detected at much lower concentrations.

Figures 58 and 59 show that the shape of the induced spectra depends upon the copolypeptide used. For poly(Lys^{50},Tyr^{50}) complexes with dAMP and dGMP, the shoulder at 360nm is of much lower intensity than the main peak while, for poly(Lys^{58},Phe^{42}) complexes, it is of almost equal intensity. This also implies that the fluorescence probably results from the interaction between the particular amino acid and the mononucleotide and not just a stacking interaction among mononucleotides. This is further confirmed by the lack of fluorescence in polylysine complexes.

In contrast, the induction of polynucleotide fluorescence seems to be independent of the polypeptide used (with or without aromatic residues). For instance, polylysine-polynucleotide complexes show fluorescence spectra similar to those with poly(Lys^{50},Tyr^{50}) and poly (Lys^{58},Phe^{42}), with respect to both shape and amplitude (Table IV).

The induction of nucleic acid fluorescence by protein binding, demonstrated here, provides a new direction for research in understanding the excited states of nucleic acids and the mechanisms of quenching. It also provides an additional method for studying protein-nucleic acid interactions such as repressor-DNA, polymerase-DNA, mRNA-ribosome and others.

CHAPTER VII

DISCUSSION

The results presented here confirm the general usefulness of random copolymers of lysine with the nonbasic amino acids in studying protein-DNA interaction. The objective, to study interaction between DNA and copolypeptides containing lysine and either histidine or tyrosine or phenylalanine, can be divided into three areas: a) to determine whether the presence of nonbasic residues in a protein would increase or decrease thermal stability of protein-bound regions in a nucleoprotein; b) what are the structural changes in DNA induced by binding of these copolypeptides; c) whether aromatic residues intercalate into DNA.

Thermal denaturation of copolypeptide-DNA complexes shows different thermal stabilities of the protein-bound regions depending upon the nonbasic residues in the model protein. In poly(Lys^{50},Tyr^{50})-DNA complexes the presence of 50% tyrosine does not affect the thermal stability of the copolypeptide-bound regions. On the other hand, poly (Lys^{48},His^{52})-DNA complexes have a melting temperature of copolypeptide-bound regions lower than that of polylysine-bound base pairs. In the

poly(Lys^{58},Phe^{42})-DNA complexes there is a similar reduction in this temperature. In the case of poly(Lys^{48},His^{52}), the electrostatic interaction between a phosphate and a histidine may not be as strong as between a phosphate and a lysine. With poly(Lys^{58},Phe^{42}), intercalation of the phenylalanine chromphore in DNA could reduce the thermal stability of DNA.

Three different types of CD spectra have been reported for DNA in three conformations A, B, and C form.[57,58] When a protein is bound to B form DNA, the conformation of DNA can be unchanged,[59,60] distorted toward C form,[12,15,21,61] distorted toward A form[23,62] or toward another structure exhibiting a huge negative CD near 270nm.[63,64,65] All the CD changes in DNA induced by the copolypeptides studied here show a B→C transition. Polylysine binding to DNA induces a B→C transition[12] and its spectrum is used a a reference. Chang et al[12] reported that the $\Delta\epsilon_b$ of polylysine-bound DNA base pairs was close to that of pure DNA in 6.0M NaCl which is between that of B and C form DNA. They explain this result as partly due to dehydration of DNA by polylysine-binding. Some water molecules originally tightly bound on DNA can be replaced by polylysine. Other factors however, have to be considered. For example, if a free protein has a stable structure it might tend to keep its most stable structure such that, when it binds to DNA,the latter would be slightly deformed. The structural effect on DNA may also be due to the presence of some particular amino acids in the protein. The latter effects have been studied with respect to tyrosine, phenylalanine and histidine and each shows different effects from that of lysine.

As shown in Figure 4, the presence of tyrosine in poly(Lys^{50},Tyr^{50})

reduces the conformational effect on DNA by polylysine binding by a factor of three to four. In contrast, the effect on the CD of DNA induced by the binding of poly(Lys^{58},Phe^{42})(Figure 21) is comparable to that of polylysine. This could result from different modes of binding of these two copolypeptides to DNA. Tyrosine with its -OH group possibly forms hydrogen bonds with DNA while phenylalanine, without an -OH, favors stacking. The different effects on CD in these two complexes could also be due to different extents of dehydration in the grooves of DNA bound by these two copolypeptides.

The CD changes induced by poly(Lys^{48},His^{52}) were found to be dependent upon the pH at which the complexes were formed. At pH 7.0, the effect on the CD of DNA is greater that that induced by polylysine while at pH 9.2 this effect is smaller. Perhaps at pH 7.0, with one amino acid per nucleotide in the bound region, the copolypeptide bacbone winds much more tightly along the groove of DNA to allow maximum ionic interaction between the protein and DNA and also replaces much more water molecules (dehydration).

Because it is possible to calculate the CD of protein-bound base pairs, the effect on the CD of DNA by the binding of the various model proteins can be compared. Based upon this study the following order has been observed: poly(Lys^{48},His^{52})pH 7.0 > polylysine > poly(Lys^{58},Phe^{42}) > poly(Lys^{48},His^{52})pH 9.2 > poly(Lys^{50},Tyr^{50}).

Perhaps the most striking discovery in this research is the observation of DNA fluorescence in protein-DNA complexes (Figures 28,44 48,49). Several factors which influence the intensity of the induced fluorescence were examined. The intensity increases linearly with the

fraction of DNA bound (Figures 46,51). The size of the bound protein also has some effect on the intensity as shown with polylysine of variable chain lengths; the longer the polylysine the greater the induced fluorescence. In agreement with this observation is a decrease of fluorescence intensity of polypeptide-DNA complexes after trypsin digestion. The fluorescence data on complexes prepared from copolymers containing various amounts of lysine and alanine (Figure 57) indicates that the formation of α-helical structure in model proteins, which reduces their effect on the CD of DNA,[55] also reduces the fluorescence intensity. Thus the induced fluorescence also depends upon the secondary structure of the bound protein.

In addition to the fluorescence studies on double-stranded DNA, interaction of the various polypeptides with mono- and polynucleotides also results in fluorescence enhancement. This effect does not depend upon the presence of aromatic amino acids since polylysine-polynucleotide complexes show enhanced fluorescence comparable to that with poly(Lys^{50}, Tyr^{50}) and poly(Lys^{58},Phe^{42}).

Single-stranded poly(A) was shown to have its bases partially stacked at room temperature.[66,67] Davidson and Fasman[68,69] have studied poly(A)-polylysine complexes by ORD and UV spectroscopy. They showed that the UV absorption maxima shifted from 250 to 262nm with polylysine binding. The ORD spectra were also red shifted and decreased. They proposed that these results indicate a conformational change in the partially stacked bases but the exact nature of the rearrangement is not known. The present research shows a strong fluorescence enhancement of poly(A) after polylysine binding.

Fluorescence enhancement on mononucleotides in the presence of poly(Lys^{50},Tyr^{50}) and poly(Lys^{58},Phe^{42}), but not polylysine, suggests a role for the aromatic amino acid residues. The preferential enhancement with dAMP and dGMP further implies a role of stacking between aromatic chromophores and purine bases. Perhaps purine bases can accomodate a stacking interaction with aromatic amino acid chromophores better than pyrimidine bases.

The origin of nucleic acid fluorescence, when complexed with model proteins, is still not clear. The present research provides sufficient data to indicate that it is a genuine fluorescence from nucleic acids and is a general property of nucleic acid-protein complexes.

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