

Interaction Specificities of Some Small
Molecules and Proteins with DNA

By

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To my family, to whom I am so devoted.....

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TABLE OF CONTENTS

	Page
Acknowledgments	iii
List of Tables	v
List of Figures	vi
Abstract	x
INTRODUCTION	1
Early History	1
DNA--Structure	9
DNA--Stability	16
Hydrogen Bonding	16
Stacking of the Bases	19
Electronic Interactions of the Bases	20
Phosphate-Phosphate Interactions	22
Histones	22
Problem I	25
Problem II	28
Problem III	30
RESULTS AND DISCUSSION	36
Results and Discussion--I	36
Proton Magnetic Resonance Studies	37
Tm Studies of Helix-Coil Transition	39
Ultraviolet Absorption Studies	44
Circular Dichroism Studies	46
Viscometric Studies	56
Equilibrium Dialysis Studies	68
Results and Discussion--II	74
Results and Discussion--III	88
EXPERIMENTAL	105
Reactions	107
Viscosity Studies	114
Circular Dichroism Studies	116
Proton Magnetic Resonance Studies	116
Melting Temperature Studies	117
Equilibrium Dialysis Studies	117
BIBLIOGRAPHY	120
BIOGRAPHICAL SKETCH	126

LIST OF TABLES

	Page
Table 1. The Effect of Increasing Length of the Polynucleotide (Ap)nA on the UV Absorption Spectrum	20
Table 2. Amino Acid Composition of Histone Fractions in Mol-%. F ₁ : Very Lysine Rich; F _{2a} , F _{2b} : Slightly Lysine Rich; F ₃ , F ₄ : Arginine Rich	23
Table 3. Absorption Properties of N-Methyl-1,10-Phenanthroline Cations, I, in 0.01 M MES buffer, pH 6.2 (0.005 M Na ⁺)	45
Table 4. Summary of the Circular Dichroism and Viscometric Titration Studies of Salmon Sperm DNA by Cations I	48
Table 5. Summary of the Scatchard-Type Treatment of the Binding Studies of Cations I to Various Nucleic Acids	65
Table 6. The Effect of Sodium Ion on the Binding of Reporter <u>11</u> to Salmon Sperm DNA	75
Table 7. The Effect of Basic Proteins on the Binding of Reporter <u>11</u> to Salmon Sperm and Calf Thymus DNA	81
Table 8. Binding Data of Reporter <u>11</u> to Native and Reconstituted Chromatin and Free DNA	86

LIST OF FIGURES

	Page
Figure 1. Schematic Diagram of One Pair of Chromosomes as a Cell Goes Through the Division Process	2
Figure 2. Schematic Diagram of Griffith's Experiments Involving the "Transforming Principle."	5
Figure 3. The Principle Behind the Hershey-Chase Experiment	7
Figure 4. The Chemical Components of Deoxyribonucleic Acids	8
Figure 5. Schematic Representation of the Watson-Crick-Wilkins Double Helix of DNA	10
Figure 6. Watson-Crick-Wilkins Base-Pairs	9
Figure 7. Structure of a Section of a DNA Chain	12
Figure 8. Comparison of a Watson-Crick-Wilkins DNA model with Strands of Opposite Polarity and Similar Polarity	13
Figure 9. (a) Watson-Crick Base-Pair. (b) Hoogsteen Base-Pair. (c) Anti-Hoogsteen Base-Pair	17
Figure 10. The Watson-Crick Base-Pairs in Acidic and Basic Media.	18
Figure 11. The Anti and Syn Conformations of a Purine and Pyrimidine Nucleoside	20
Figure 12. Exciton Splitting of the Energy Levels	21
Figure 13. The Ten Different Intercalating Sites in DNA	26
Figure 14. N-Methyl-1,10-Phenanthroline Cation	27
Figure 15. The 2,4-Dinitroaniline Reporter Molecule	28

Figure 16.	The Effect of Increasing Peptide Concentration on the Viscosity of DNA	32
Figure 17.	A Schematic Diagram Showing the Possible Effect of Increasing Concentration of DNA on the Specific Viscosity	33
Figure 18.	The N-Methyl-1,10-Phenanthroline Cation .	36
Figure 19.	The Temperature-Dependent Partial Proton Magnetic Resonance Spectra of <u>3</u> and Salmon Sperm DNA- <u>3</u> Complex	38
Figure 20.	The Effect of Increasing Concentrations of Cations I on the T _m of the Helix-Coil Transition of Salmon Sperm DNA	41
Figure 21.	The Effect of Increasing Concentrations of Cations I on the T _m of the Helix-Coil Transition of Poly d(A-T)-Poly d(A-T) . . .	43
Figure 22.	The Effect of Increasing Concentration of <u>3</u> on the Observed Ellipticity, (θ) _{obs} , at 340 nm in the Presence of 5.60 x 10 ⁻⁴ M P/1 of Salmon Sperm DNA	49
Figure 23.	The Induced Circular Dichroism Spectra of Salmon Sperm DNA- <u>1</u> , <u>2</u> , <u>5</u> , and <u>7</u> Complexes at Saturation	51
Figure 24.	The Induced Circular Dichroism Spectra of Salmon Sperm DNA- <u>3</u> , <u>4</u> , and <u>6</u> Complexes at Saturation	52
Figure 25.	The Induced Circular Dichroism Spectra of Salmon Sperm DNA- <u>8</u> and <u>9</u> Complexes at Saturation	54
Figure 26.	The Cations, I, which Exhibit a Negative Induced CD Upon Binding to Salmon Sperm DNA	55
Figure 27.	Schematic Illustration of the Possible Intercalation Complexes of <u>5</u> to DNA with Long Axis of the Molecule Pointing at Opposite Chains	57
Figure 28.	Schematic Illustration of the Possible Intercalation Complexes of <u>5</u> to DNA with the Long Axis of the Molecule Pointing at Opposite Grooves	58

Figure 29.	The Effect of Increasing Concentrations of <u>1</u> on the Relative Specific Viscosity . . .	60
Figure 30.	The Effect of Increasing Concentrations of Cations <u>1</u> on the Specific Viscosity of Near Infinitely Dilute Solution of Salmon Sperm DNA	63
Figure 31.	Schematic Illustrations of the Possible Complexes of <u>1</u> to DNA Showing Lengthening (a) and (c) as Well as Bending of the Helix (b) at the Intercalation Site	67
Figure 32.	The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of Cation <u>1</u> with Poly d(A-T)-poly d(A-T), Salmon Sperm DNA, and <u>Micrococcus luteus</u> DNA . . .	70
Figure 33.	The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of Cation <u>6</u> with Poly d(A-T)-poly d(A-T), Salmon Sperm DNA, and <u>Micrococcus luteus</u> DNA	71
Figure 34.	Schematic Illustrations of the Complexes of <u>1</u> to DNA Showing the Possible Separation Distances Between Base-Pairs Required to Accommodate Unsubstituted (b) and Methyl Substituted N-Methyl-1,10-Phenanthrolium Cation (c).	73
Figure 35.	The 2,4-Dinitroaniline Reporter Molecule. . .	74
Figure 36.	The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of <u>11</u> with Salmon Sperm DNA at Varying Na^+ Concentrations	77
Figure 37.	The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of <u>11</u> with Salmon Sperm DNA, Salmon Sperm DNA + 16 $\mu\text{g/ml}$ of Poly-L-lysine, and Salmon Sperm DNA + 24 $\mu\text{g/ml}$ of Poly-L-lysine . .	80
Figure 38.	The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of <u>11</u> with Calf Thymus DNA, Calf Thymus DNA + 60 $\mu\text{g/ml}$ Histone II, and Calf Thymus DNA + 60 $\mu\text{g/ml}$ Histone IV .	84

Figure 39.	The Effect of Increasing Concentrations of Basic Proteins on the Relative Specific Viscosity of a 2.44×10^{-4} M P/1 Salmon Sperm DNA Solution	89
Figure 40.	The Effect of Increasing Concentrations of Basic Proteins on the Relative Specific Viscosity of a 2.84×10^{-4} M P/1 Calf Thymus DNA Solution	90
Figure 41.	The Effect of Increasing Concentration of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 and 30 $\mu\text{g/ml}$ Histone III	95
Figure 42.	The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 and 30 $\mu\text{g/ml}$ Histone III	96
Figure 43.	The Effect of Increasing Concentration of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 and 30 $\mu\text{g/ml}$ Histone III	97
Figure 44.	The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 and 30 $\mu\text{g/ml}$ Histone III.	98
Figure 45.	The Effect of Increasing Concentration of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 and 10 $\mu\text{g/ml}$ Poly-L-lysine	100
Figure 46.	The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 and 10 $\mu\text{g/ml}$ Poly-L-lysine	101
Figure 47.	The Effect of Increasing Concentrations of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 and 30 $\mu\text{g/ml}$ Histone III	102
Figure 48.	The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 and 30 $\mu\text{g/ml}$ Histone III	103

Abstract of Dissertation Presented to the
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INTERACTION SPECIFICITIES OF SOME SMALL
MOLECULES AND PROTEINS WITH DNA

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Major Department: Chemistry

The research in this dissertation deals with several aspects of the interactions of some reporter molecules and proteins with nucleic acids. In an attempt to define the 3-dimensional size of the 10 possible intercalation sites in DNA, a series of substituted N-methyl-1,10-phenanthroline cations were synthesized. The interactions between nucleic acids of different base-composition and the aromatic cations were studied by melting temperature (T_m), proton magnetic resonance (pmr), ultraviolet (uv) absorption, induced circular dichroism (CD), equilibrium dialysis, and viscometric techniques. The planar cations were found to intercalate between base-pairs of DNA as evidenced by (1) total

broadening of the pmr signals, (2) enhanced viscosity, (3) induced circular dichroism, and (4) dramatic stabilization of the DNA helix toward denaturation. In addition, selective interactions with DNA were observed as a function of the position and number of substituents on the N-methyl-1,10-phenanthrolium ring. For example, the more highly substituted systems exhibited (i) higher affinity, (ii) greater stabilization of the DNA helix, and (iii) higher viscosity upon binding to DNA. Selective binding to G-C sites (and/or a combined G-C/A-T site) by the more highly substituted aromatic cations was observed. The aromatic cations which contained methyl groups at the 3,8 positions of the N-methyl-1,10-phenanthrolium ring, instead of H-substituents, exhibited a negative CD upon binding to salmon sperm DNA, as opposed to a positive induced CD observed for the other cations. A model was proposed whereby the 3,8-dimethyl substituted cations cannot assume all possible geometries in the intercalating site due to steric restrictions. Furthermore, a second model was proposed (to help explain the viscosity data) which was based on the suppositions that the "thickness" of the aromatic ring of the N-methyl-1,10-phenanthrolium ring was not uniform and was dependent on the position of substitution of methyl groups. Therefore, in some cases, intercalation led not only to lengthening of the helix but also to slight bending of the helix at the point of intercalation.

The effect of sodium ion, poly-L-lysine and histones on the binding of a reporter molecule to DNA was studied by equilibrium dialysis technique. It was found that these systems not only lower the affinity of the reporter molecule to DNA, but also diminish the number of strong binding sites to DNA. The same effect on the binding of the reporter molecule to DNA was found in native, reconstituted, S and M chromatin. Since considerable evidence exists that the reporter molecule binds to DNA from the minor groove, the possibility that the basic proteins may also bind to this site cannot be ruled out. In addition, it was found that the reporter molecule binds more strongly to the open, exposed DNA in S chromatin than the packaged, compacted DNA in M chromatin.

Viscosity studies, involving various poly-L-lysine- and histone-DNA complexes, showed selective interactions of the basic proteins for DNA as evidenced by the different decreases in the relative specific viscosities. Exchange of Histone III and poly-L-lysine between DNA helices was monitored by a viscometric technique. It was found that at 10° , 0.02 M Na^{+} and pH 6.2, poly-L-lysine freely exchanged, but Histone III did not. Further evidence for the selective interactions of proteins for DNA was found when exchange studies showed that in 0.1 M Na^{+} , pH 6.2 and 10° , Histone III freely exchanged between calf thymus DNA helices but not between salmon sperm helices.

INTRODUCTION

Early History

Before 1900, little was known about the chemical composition of chromosomes. They had been observed in detail through microscopes in cells undergoing mitosis. Mitosis is separated into five distinct phases and is illustrated in Figure 1 for one pair of chromosomes. The chromosomes become visible and more condensed during prophase, when each consists of two strands joined by a centromere. The chromosomes line up at the cell's equatorial plane, and the centromere divides, allowing the strands to separate and form the daughter chromosomes during metaphase. The migration of the strands to opposite sides of the cell is called anaphase. In telophase, the migration of the strands is completed and the cell splits in two. During interphase, the rest phase between telophase and prophase, the division of the cell is completed and the chromosomes become lengthened and less condensed.

At this time, the composition and function of chromosomes was still unknown. An Augustinian monk, Gregor Mendel,¹ had already completed his work on the characteristics of the pea plant and had published that hereditary factors, both dominant and recessive forms, are passed from generation to generation. The problem was to identify Mendel's inheritance factors, now called genes, in the cell.

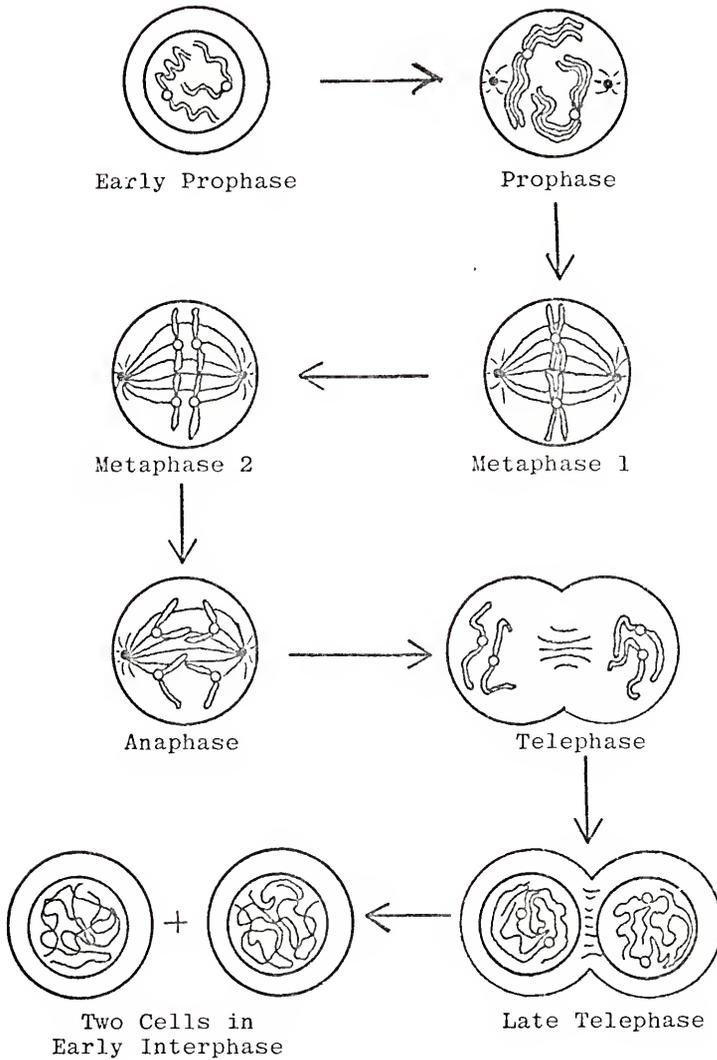


Figure 1. Schematic Diagram of One Pair of Chromosomes as a Cell Goes Through the Division Process.

Sutton,² in 1902, proposed that the behavior of chromosomes during mitosis could account for the distribution of genes to the daughter cells. In addition, the special form of cell division (i.e., meiosis) that takes place in the formation of germ cells (egg and sperm) when the chromosome number is halved, provides a neat explanation for the fact that only one gene of each pair is passed on from each parent. Proof of these speculative ideas was supplied by Morgan,³ who from 1909 to 1940 worked with Drosophila melanogaster, the fruit fly. When Morgan examined the fruit flies' chromosomes carefully, he noted that one out of the normal four pairs of chromosomes present in the body cells of fruit flies was different in the two sexes. In females this fourth pair consisted of two straight chromosomes (called X chromosomes); but, in males only, one of the pair was straight while the other was hook-shaped (Y chromosome). Here was the first clear evidence that the chromosomes carry inherited information--in this case, for sex.

Before World War II, Schlesinger,⁴ an Hungarian chemist working in London, found that bacteriophages (viruses which invade and infect only bacteria) consisted only of protein and nucleic acid. Speculation of the "inheritance factor" or gene was high and it was generally thought that proteins, rather than nucleic acid, held the key to life. This concept might seem reasonable, since nucleic acid consists of only four variables, the bases adenine, guanine, cytosine, and thymine, whereas proteins consist of at least twenty different amino acids.

In England, Griffith⁵ experimented with pneumococci bacteria. He found if either dead encapsulated bacteria or live nonencapsulated bacteria were injected into mice, the mice survived. However, if both dead encapsulated bacteria and live nonencapsulated bacteria were injected into mice, a significant number of mice contracted pneumonia and died. Two explanations for this phenomenon were suggested. The first is that the dead virulent bacteria came back to life, and the second is that the live harmless bacteria became virulent. Griffith suspected the second explanation was more likely and so a search began for the "transforming principle" which is passed from dead virulent bacteria to the live avirulent form, enabling transformation of the latter to the virulent strain. This "transforming principle" behaved like a gene.

Dawson⁶ showed that this transformation could occur in a test tube, if the two types of pneumococci were mixed. Further studies by Alloway⁷ showed that live avirulent non-encapsulated bacteria could be transformed to the virulent strain by placing the bacteria in a cell-free solution prepared from the juices of broken-up dead encapsulated bacteria. This showed that the "transforming principle" was chemical in nature (see Figure 2).

In 1944, Avery and co-workers⁸ treated Alloway's cell-free mixture with protein-digesting enzymes; however, the "transforming principle" was left untouched and active. A fibrous, thread-like material was isolated, which, when

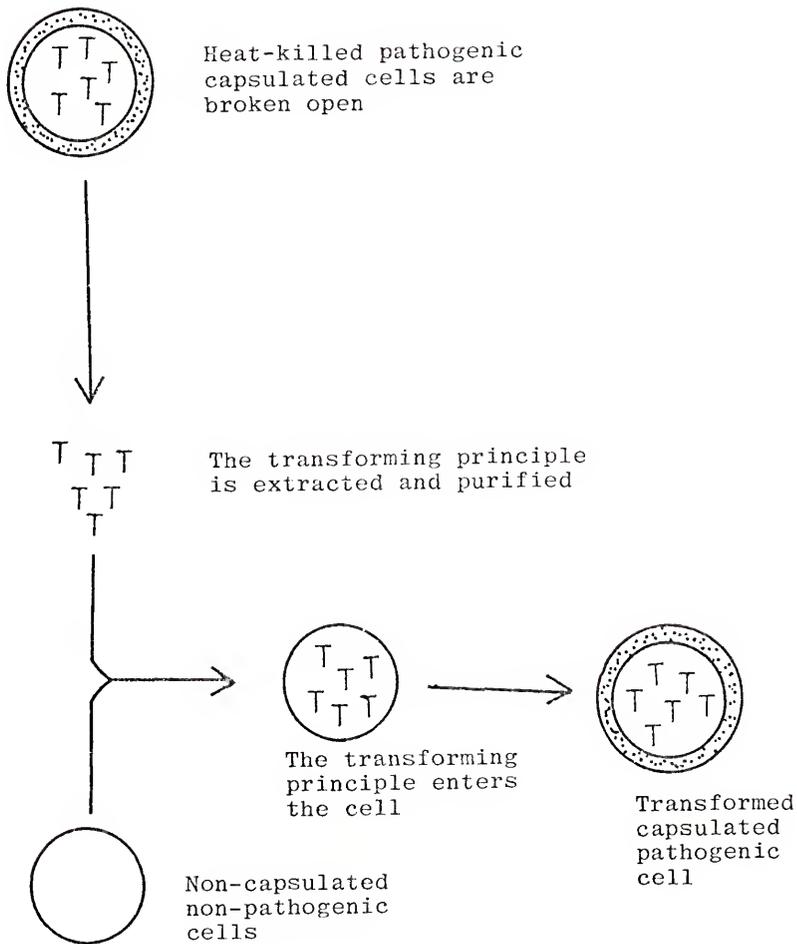


Figure 2. Schematic Diagram of Griffith's Experiments Involving the "Transforming Principle."

redissolved, yielded a potent "transforming" material. Chemical analysis of this substance showed it to be composed mainly of nucleic acid.

Hershey and Chase⁹ confirmed this finding in 1952 by the now famous Hershey-Chase experiment (see Figure 3). The protein component of T2 phages was labelled with radioactive sulphur and the DNA component with radioactive phosphorus. Bacteria were infected with the radioactively labelled T2 phages and, after a few minutes, the mixture was spun in a blender to shake the protein coat off from the cell. This mixture was placed in a high-speed centrifuge, where huge gravity forces were created, forcing all the bacteria to the bottom of the centrifuge tube. The bacteria formed a "pellet" that left behind a bacteria-free solution. The "pellet" contained most of the radioactive phosphorus and the bacteria-free solution contained most of the radioactive sulphur. If the blending stage was omitted, both phage DNA and protein remain attached to the bacteria.

Hershey and Chase explained that the only possible interpretation of this experiment is that the T2 bacteriophage acts like a tiny disposable protein syringe loaded with viral DNA. In order to infect a bacterium, it attaches itself by its tail to the wall of the bacterium and injects its DNA inside the cell. If then agitated in a blender, the empty phage syringes are shaken loose; the phage DNA stays with the bacteria while the protein syringe floats free in the solution. If no blender is used, the phage husks remain clinging to the outside of the bacteria.

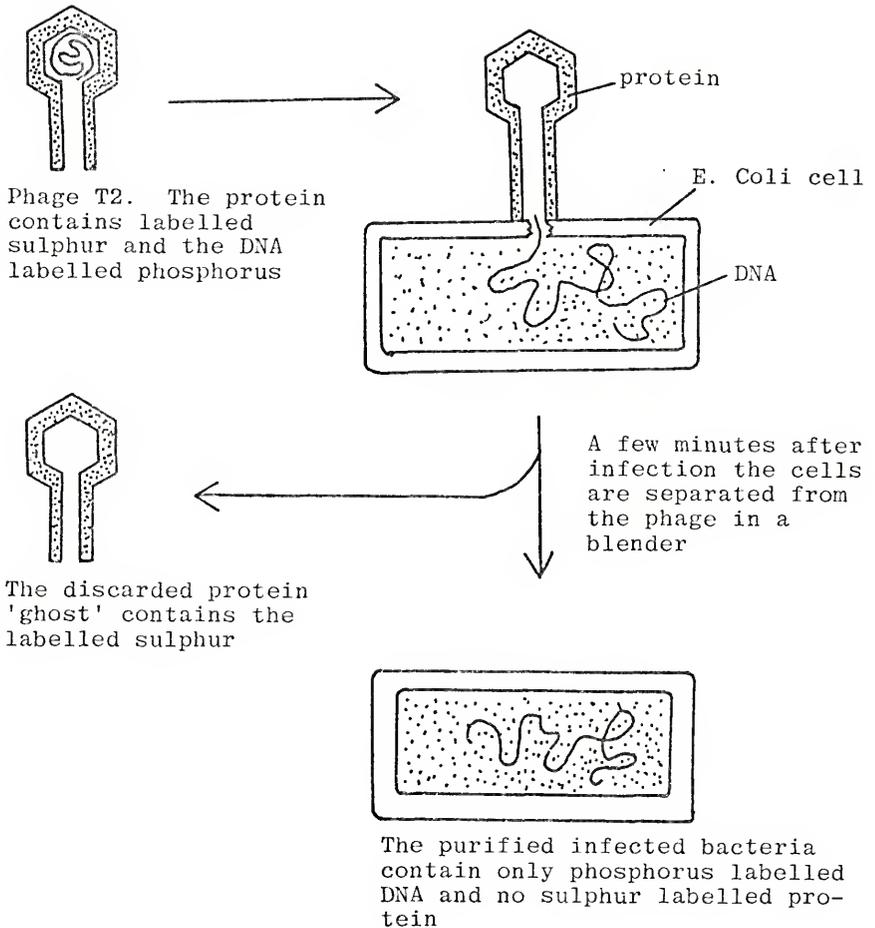


Figure 3. The Principle Behind the Hershey-Chase Experiment. Radioactively labelled phosphorus appeared in the bacteria and labelled sulphur in the detached "ghosts" of the infecting T2 phage.

Until 1952, the structure of nucleic acid was unknown; however, its chemical components were known (Figure 4).

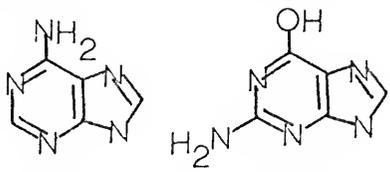
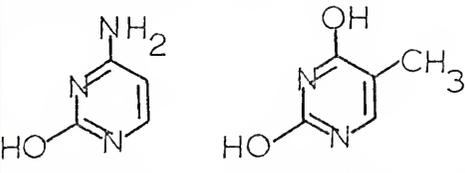
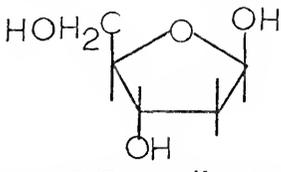
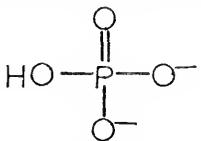
Purine Bases	 <p style="text-align: center;">Adenine Guanine</p>
Pyrimidine Bases	 <p style="text-align: center;">Cytosine Thymine</p>
Pentose Sugars	 <p style="text-align: center;">D-Deoxyribose</p>
Phosphate	

Figure 4. The Chemical Components of Deoxyribonucleic Acids.

On work involving DNA from numerous sources, Chargaff and Lipschitz¹⁰ noted that there were certain similarities for DNAs of various species. These observations, known as Chargaff's Rules, are that (1) the amount of adenine equals the amount of thymine; (2) the amount of guanine equals the amount of cytosine; and (3) for a particular species, the A-T/G-C ratio is a constant.

In 1953, Watson and Crick^{11,12} proposed a structural model for nucleic acid based largely on the chemical findings of Chargaff.

DNA--Structure

The Watson-Crick-Wilkins model of nucleic acid consists of two polynucleotide strands which fit together to form a right-handed double-stranded helix. The two strands are held together by specific hydrogen bonds formed between complementary bases and hydrophobic forces that favor a stacked base geometry (see Figure 5). Figure 6 illustrates the specific Watson-Crick-Wilkins base-pairing scheme which accounts for Chargaff's rules.

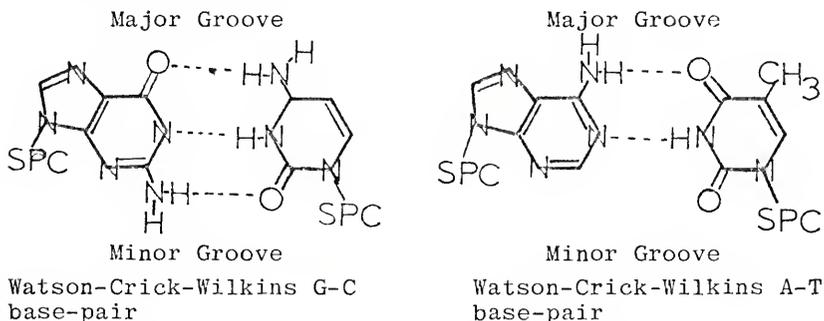


Figure 6. Watson-Crick-Wilkins Base-Pairs.

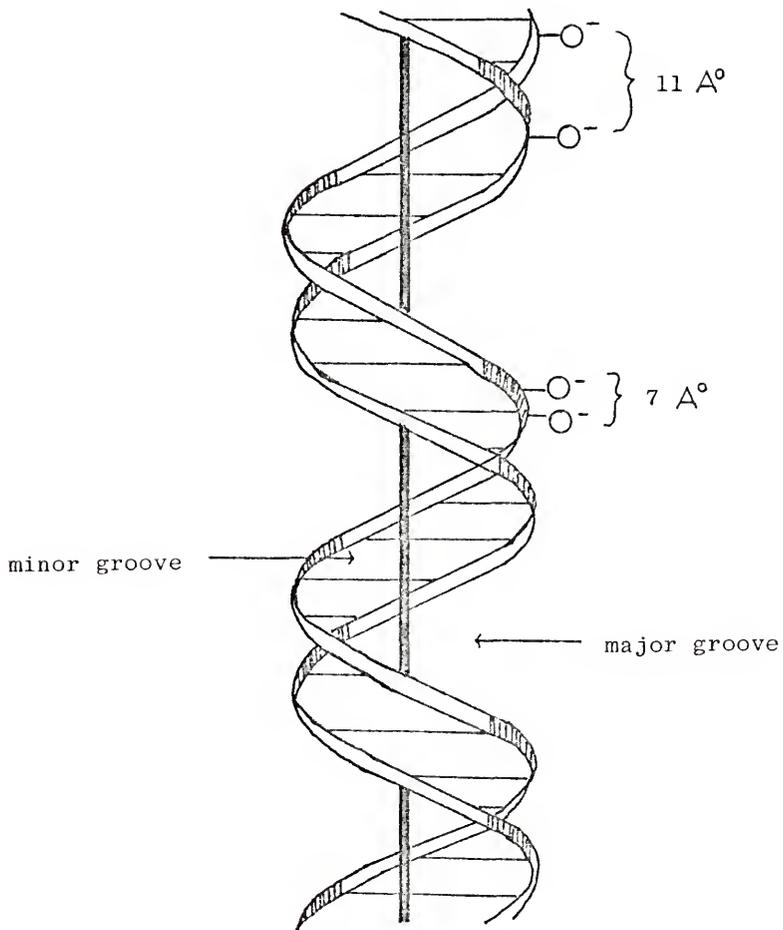


Figure 5. Schematic Representation of the Watson-Crick-Wilkins Double Helix of DNA. The outer helical strands represent the sugar-phosphate backbone, the horizontal lines represent the base-pairs, and the vertical line is the helix axis.

The individual DNA strands are composed of monomer nucleotide units which have been enzymatically joined to produce an alternating sugar-phosphate-sugar backbone, while the bases are stacked on top of one another (Figure 7). The D-deoxyribose sugar, which exists in the furanoside form, has two hydroxyl groups at the 3' and 5' positions. In order to obtain maximum symmetry, the two complementary strands are placed anti-parallel. In other words, one strand has its sugar-phosphate chain directed 3'→5' while the other chain is directed 5'→3' (Figure 8). This aspect of the double helix was proven correct by Josse and co-workers¹³ using nearest neighbor analysis.

The phosphate groups are formally phosphate diesters. At neutral pH, the phosphates exist as monoanions and, due to this charged character, the oxygen atoms are not equivalent in that one lies parallel to the helical axis (axial) and the other lies perpendicular to it (equatorial).

Langridge and co-workers,¹⁴ from x-ray diffraction data, found that the double helix makes one complete turn every 34 angstroms, which is known as the pitch. In addition, there are ten base-pairs in a pitch, or a translation of 3.4 angstroms between each base-pair. Since one turn of the double helix encompasses 360° of rotation, the model predicts that the average angle between successive base-pairs is 36°. The planes of the base-pairs are perpendicular to the helical axis.

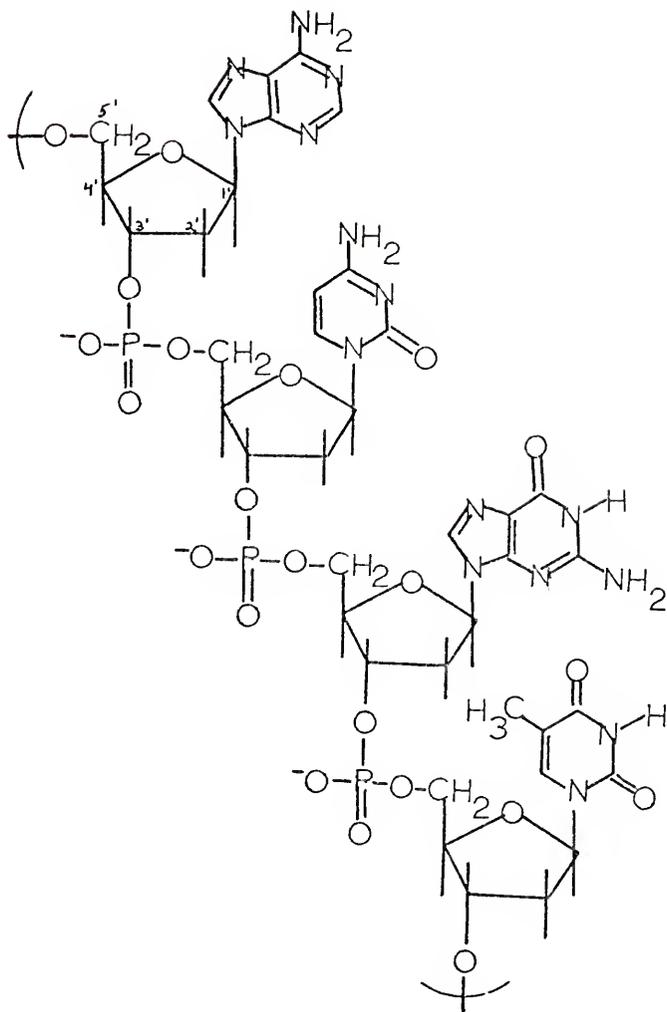


Figure 7. Structure of a Section of a DNA Chain.

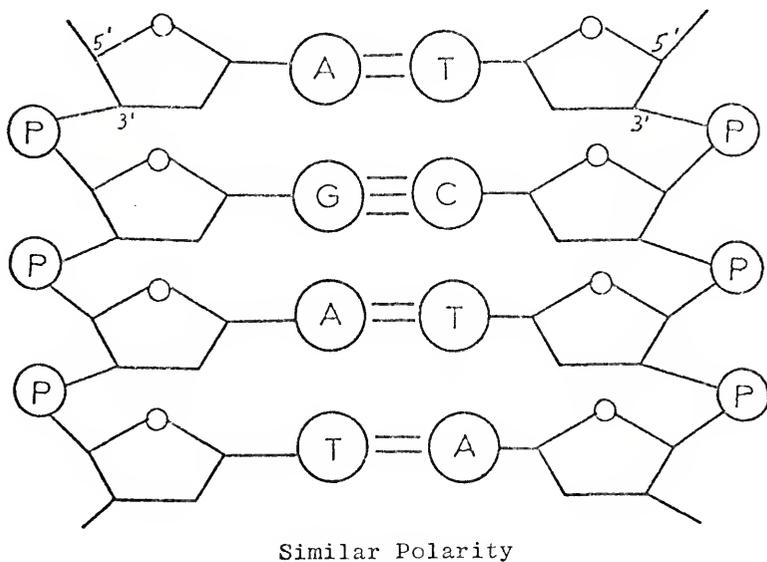
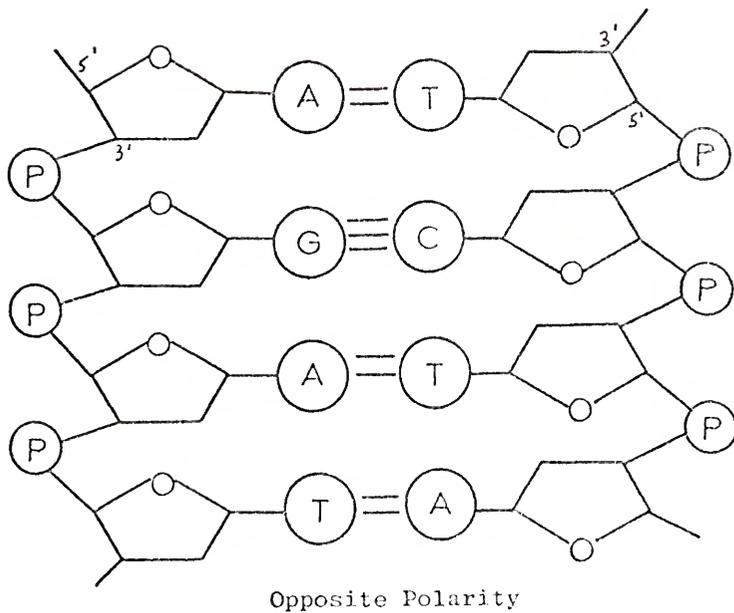


Figure 8. Comparison of a Watson-Crick-Wilkins DNA Model with Strands of Opposite Polarity and Similar Polarity.

As a consequence of the twist of the double helix, there exist two distinct grooves (i.e., the major and minor). Inspection of Figure 6 shows the positions of the major and minor grooves and, in addition, that (1) the third hydrogen bond of the G-C base-pair is located in the minor groove, and (2) the methyl group of thymine is located in the major groove. The significance of the two grooves with respect to interactions of DNA with small molecules and protein will be discussed later.

All x-ray work on DNA has been done with fibers. Unlike x-ray analysis of single crystals, fibers do not produce enough definable data points so that a definite arrangement of atoms can be made. The resolution is not good enough to construct a DNA molecule from the data alone. Usually, molecular models are built and fitted to the available data. In this way, models which are inconsistent with the data or are stereochemically unfeasible are ruled out. Eventually, this elimination of models yields at least one model which is consistent with the available x-ray data. Donohue^{15,16,17} has questioned this method of x-ray approach and has suggested that it not be used.

In the B form of DNA (where the base-pairs are perpendicular to the helical axis and the pitch is 34 angstroms with 10 base-pairs per turn), x-ray studies of the fiber cannot distinguish between a left- and a right-handed helix.¹⁸ It is only because the A form of DNA (where the base-pairs are at an angle of 15° from the perpendicular and the pitch is 28 angstroms with 11 base-pairs per turn) can be

established as a right-handed helix that it can be assumed the B form is also. There have been at least eight structures proposed for DNA, all varying in the angle of tilt of the base-pairs from the perpendicular and the pitch of the helix (A, B, C, P, P₂, J₁, J₂, and S).¹⁹

Different media from which the fibers of DNA are drawn for x-ray analysis yield slightly different structures for the same DNA molecule (i.e., Li⁺, K⁺, Na⁺, or Mg⁺⁺ salts).²⁰ Above 80% relative humidity, x-ray data suggest the B structure, but below 80% relative humidity the data suggest the A form. In addition to dependence on salt and humidity, it has been shown that the A-T/G-C ratio of the DNA will cause a change in structure,²¹ suggesting that the secondary structure of DNA is a function of the primary sequence. It is apparent that DNA structure is a function of many variables and extrapolation of fiber structures to solution may not be justified. The necessity of using DNA in its fiber form for the x-ray analysis and then using these results to postulate a structure for DNA in solution, assumes that its molecular structure does not change once it is solvated. In fact, Bram's studies,^{22,23} using a low angle x-ray scattering technique on DNA in solution, supports the contention of DNA structure in fiber form differing from DNA structure in solution.

Evidence supporting the concept that DNA exists as a right-handed helix in solution comes from Gabbay and co-workers.²⁴ This work involved the synthesis of two optically active enantiomeric reporter molecules (i.e.,

the DNP derivatives of L- and D-prolyl diammonium salts) and studying their binding affinities to various DNAs. It was found that one of the reporters (i.e., the D-enantiomer) was bound more than the other to all DNA systems, which was consistent with a right-handed DNA helix in solution.

DNA--Stability

The stability of the double helix in solution depends on a delicate balance between unfavorable interstrand phosphate anion repulsions and favorable base-pairing and stacking, and is sensitive to changes in pH, temperature, solvent, ionic strength, and the presence of counterions.

Hydrogen Bonding

Donohue²⁵ and Donohue and Trueblood²⁶ showed that there were 29 possible base-pairs which could be formed from the four bases in DNA. Figure 9 shows three possible hydrogen bonding schemes between adenine and thymine. The methyl groups are in much closer proximity to one another in the Hoogsteen type base-pairing than the Watson-Crick model.^{27,28} The anti-Hoogsteen base-pair was found when adenosine and 5-bromouridine were co-crystallized.^{29,30} The Watson-Crick model for A-T base-pairing has not been observed in crystals. This does not mean that in solution the Watson-Crick base-pairing does not exist.

X-ray analysis of single crystals of guanine and cytosine has yielded only the Watson-Crick hydrogen bonding scheme, presumably due to the fact that three hydrogen bonds stabilize

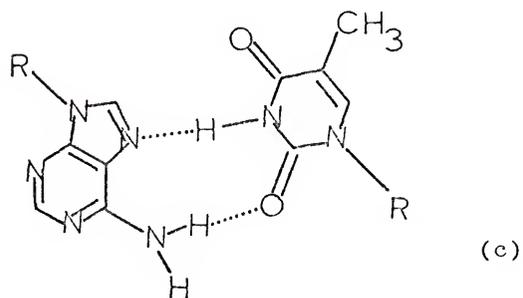
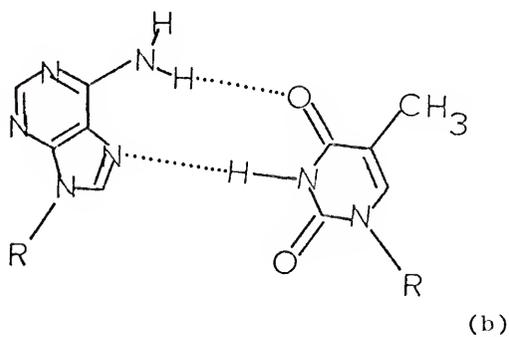
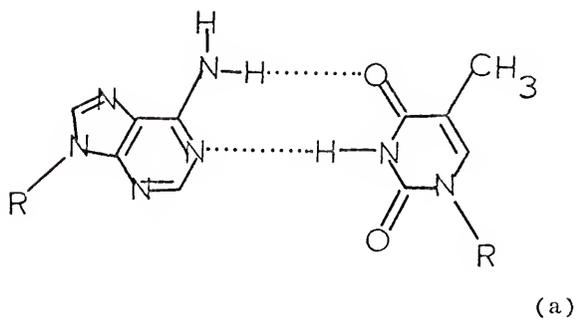


Figure 9. (a) Watson-Crick Base-Pair.
 (b) Hoogsteen Base-Pair.
 (c) Anti-Hoogsteen Base-Pair.

Stacking of the Bases

The bases of nucleic acid are essentially non-polar molecules and in aqueous solution they have been shown to associate by vertical stacking of one base on top of another. The process is considered to be entropy driven in that the associated bases are less hydrated than the individual bases themselves, and, as a result, a release of water molecules from the hydration shells around the bases occurs.

Studies done with different dinucleoside phosphates³³ have shown that some have a higher tendency to stack than others. For instance, ApA and UpU show a dramatic difference in their ability to stack. At -70°C , both systems have an ordered, stacked structure. As the temperature is increased, UpU loses its structure readily, whereas ApA does not. It was proposed that as the temperature increases, the oscillation of the bases increases, until the amplitude becomes large enough to allow for solvation of the bases which disrupts the stacking.³⁴

Chan and Nelson,³⁵ using nuclear magnetic resonance, showed that the stacking conformation of ApA is anti-anti. The phenomenon of base stacking requires the anti-conformation. Figure 11 shows adenine and thymine in their syn- and anti-conformers.

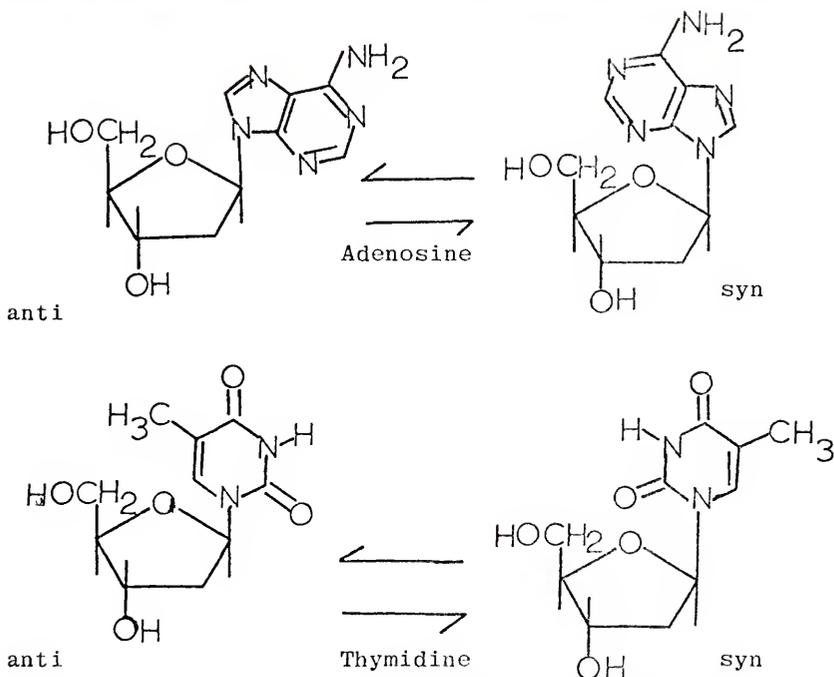


Figure 11. The Anti and Syn Conformations of a Purine and Pyrimidine Nucleoside.

Electronic Interactions of the Bases

The electronic interaction among bases in nucleic acids is well known. The effect of increasing number of nucleotide units in a polynucleotide chain on the uv spectrum is seen in Table 1.

TABLE 1

The Effect of Increasing Length of the Polynucleotide (Ap)_nA on the UV Absorption Spectrum

<u>n</u>	<u>λ^{max}</u>	<u>ε^{max/monomer}</u>
0	260	15,000
1	257	13,600
2	257	12,600
3	257	11,300
4	257	11,300
5	257	10,800
poly A 300	256	9,000

As the number of nucleotide units is increased, there is a progressive decrease in the extinction coefficient of the adenine bases and a blue shift in the wavelength maximum.³⁶ Tinoco,^{37,38,39} employing Davydov's exciton theory, stated that for molecular crystals, light is not absorbed by a single molecule, but excitation is distributed over many molecules. The excited energy levels of the bases are considered to be split. The lower energy level has the electronic vectors of the base anti-parallel, whereas the higher energy level has the electronic vectors parallel (Figure 12). The absorption of a photon results in the

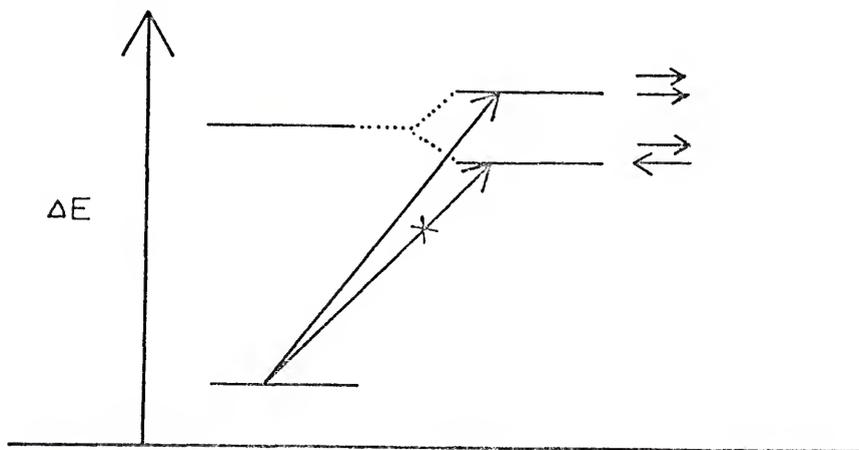


Figure 12. Exciton Splitting of the Energy Levels.

transition of a ground state electron into the excited levels; however, the transition to the lower energy excited level is

forbidden by selection rules, and, as a result, the energy required for absorption of light is increased, causing a blue shift in the absorption spectrum.

The hypochromism results from an intensity interchange between transition moments of card stack arrangements of bases so that the lower energy transition becomes hypochromic (260 nm) while the higher energy transitions (220 nm and below) become hyperchromic.

When DNA is melted into the destructured random coils, the stacking of the transition moments of the neighboring bases is decreased, resulting in an increase in the optical density of the solution (hyperchromicity).

Phosphate-Phosphate Interactions

At neutral pH, the phosphate groups along the backbone of the DNA double helix are charged, and, as a result, the association of the two strands is not a favorable process. This charge repulsion between strands is a primary disruptive force. Cations shield the anionic charge of the phosphate groups (reducing the Coulombic repulsion between strands) and effectively stabilize the double helix.

Histones

In eukaryotic organisms (i.e., organisms with cells that contain a nucleus), there are at least five histone fractions associated with DNA. These histones are divided into three broad classifications: very lysine rich, slightly lysine rich, and arginine rich. The amino acid compositions of the

five fractions are given in Table 2. The fraction numbers refer to the histone elution sequence in column chromatography.

TABLE 2

Amino Acid Composition of Histone Fractions in Mol-%.
 F₁: Very Lysine Rich; F_{2a}, F_{2b}: Slightly Lysine Rich;
 F₃, F₄: Arginine Rich⁴⁰

Amino acid	Fraction				
	FI	FIIa	FIIb	FIII	FIV
Alanine	23.5	10.5	10.5	12.5	6.9
Arginine	2.5	11.5	7.5	13.0	13.7
Aspartic acid	2.8	6.0	5.5	5.0	4.9
Glutamic acid	6.0	8.5	9.0	11.0	5.9
Glycine	6.8	12.5	7.0	6.5	16.7
Histidine	0.5	2.0	2.5	2.1	2.0
Isoleucine	1.6	4.5	5.0	5.0	6.0
Leucine	4.4	10.5	6.0	8.5	7.0
Lysine	26.3	10.5	14.5	9.0	11.0
Methionine	--	--	0.7	0.7	2.0
Phenylalanine	0.8	1.6	2.0	2.5	2.0
Proline	7.9	3.0	4.5	4.5	0.9
Serine	6.2	3.1	9.0	7.0	2.0
Threonine	5.5	5.6	6.5	7.0	7.0
Tyrosine	0.5	3.0	3.1	2.0	3.9
Valine	5.0	7.0	6.8	6.0	8.8

It is well known that the amino acid sequence of the histones is the same from species to species.⁴¹ If this is true, it is reasonable to assume that histones serve a general function in the nucleus and not a specific one.

One function attributed to the histones is a "repressing effect" on DNA.⁴² The sequence of DNA contains information which codes for various enzymes and proteins. However, certain information coded in DNA is useless for a particular cell (i.e., the brain cell would have no need of the sequence in DNA coding for hemoglobin). Thus, certain parts of DNA are said to be repressed.

Two separate studies^{43,44} involving radioactive labelling and protein digestion by enzymes have shown that about half of the DNA in chromatin is covered by protein. This would leave the other half of the DNA exposed for informational transfer.

Huang and Bonner⁴⁵ found that histone-DNA complexes are unable to act as primers for RNA synthesis by RNA polymerase. Allfrey et al.⁴⁶ found similar results and, when the cell nuclei were treated with trypsin (resulting in hydrolysis of the histones), the nuclei continued to synthesize RNA at a higher rate than untreated nuclei. Addition of more histone resulted in the inhibition of the RNA synthesis.

Another role in which histones may be involved is the structural packaging of DNA.⁴⁷ In the cycle of mitosis, during interphase, the chromosomes are spread out and cannot be seen through a microscope. At this point, it is thought that DNA and histone synthesis occurs.⁴⁸ In early prophase, the chromosomes are compacted and condensed.

The amino acid composition of histones contains a high percentage of lysine or arginine. These amino acids have positive charges which interact electrostatically with the phosphate anions of DNA. This electrostatic binding is non-specific, although there is some evidence which indicates that poly-L-lysine prefers A-T sites and that poly-L-arginine prefers G-C sites.⁴⁹

Intercalation (the insertion of planar aromatic ring (i.e., proflavine⁵⁰ or ethidium bromide⁵¹) between the base-pairs of DNA), which may be very specific, may play an important role in histones recognizing certain sequences in DNA.^{52,53} Since histones do have some aromatic residues (phenylalanine and tyrosine), then possibly these residues might make histones more specific for certain DNA sequences than others.

Problem I

As mentioned previously, intercalation may play an important role in the recognition process of proteins for DNA. Brown⁵⁴ proposed a "bookmark" hypothesis whereby the aromatic amino acid residue may serve to anchor and prevent slippage of the protein along the DNA helix. Helene and co-workers,^{55,56} who studied the interactions of tryptamine, serotonin, and tyramine to nucleic acids and their components, found that the aromatic residues of the above systems are bound to DNA via an intercalation mechanism, and, therefore, they also suggested an "anchoring" role for the aromatic amino acids. Work by Gabbay and co-workers^{57,58,59} on the interactions of 70 different di-, tri-, and tetrapeptides, as well as di-, tri-, and tetrapeptide amides to DNA of various base compositions, supports the above conclusions. In addition, the results of pmr, viscosity, CD, T_m, and equilibrium dialysis suggest that not only site-specific intercalation of the aromatic residue of the peptides, but also a dependence on the primary structure

is involved. On this basis, a "selective bookmark" hypothesis was proposed, whereby the "bookmarks" (i.e., the aromatic residues of the proteins) could recognize the "pages of the book" (i.e., the intercalating sites).

As a consequence of the right-handed Watson-Crick-Wilkins double helix of DNA with A-T and G-C base-pairs, there are ten distinctly different intercalating sites illustrated in Figure 13.⁶⁰ Each site may provide a different environment

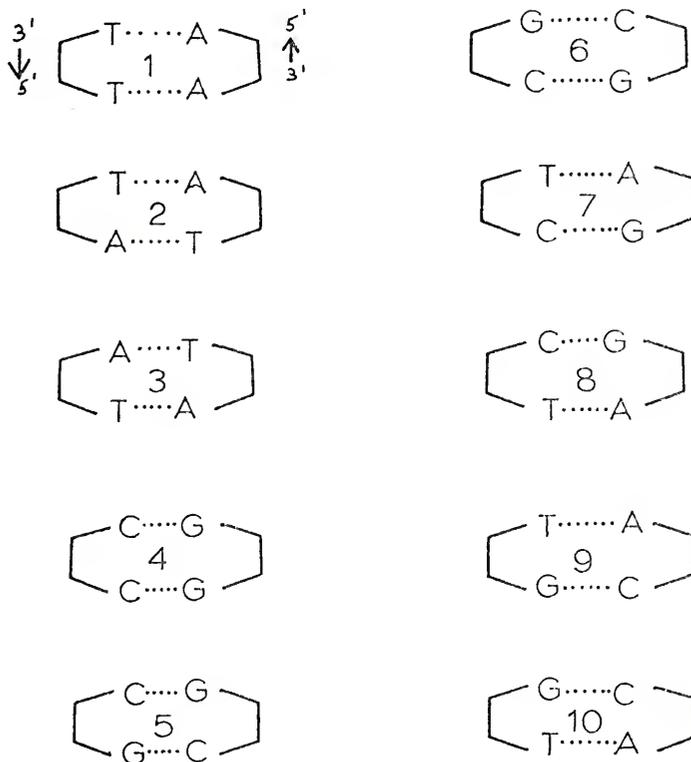
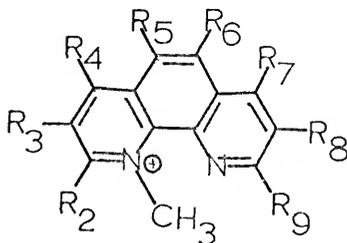


Figure 13. The Ten Different Intercalating Sites in DNA.

(steric, as well as electronic) for the intercalating molecule. Inspection of the A-T and G-C base-pairs show (1) the presence of the third hydrogen bond of the G-C base pair in the minor groove, and (2) the methyl group of thymine of the A-T base-pair in the major groove. Theoretically, molecules might be designed to be more selective for some sites rather than others.

In order to test this concept, a series of substituted aromatic cations, I (see Figure 14), can be synthesized and



I

<u>Reporter</u>	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉
<u>1</u>	H	H	H	H	H	H	H	H
<u>2</u>	CH ₃	H	H	H	H	H	H	CH ₃
<u>3</u>	H	H	H	CH ₃	CH ₃	H	H	H
<u>4</u>	CH ₃	H	H	CH ₃	CH ₃	H	H	CH ₃
<u>5</u>	H	CH ₃	H	H	H	H	CH ₃	H
<u>6</u>	H	CH ₃	H	CH ₃	CH ₃	H	CH ₃	H
<u>7</u>	H	H	CH ₃	H	H	CH ₃	H	H
<u>8</u>	H	H	H	NO ₂	H	H	H	H
<u>9</u>	H	CH ₃	H	NO ₂	H	H	CH ₃	H
<u>10</u>	H	H	C ₆ H ₅	H	H	C ₆ H ₅	H	H

Figure 14. N-Methyl-1,10-Phenanthroline Cation.

their interaction specificities with nucleic acids of various base composition examined. The interactions of these compounds with DNA can be studied by T_m , nmr, viscosity, circular dichroism, and equilibrium dialysis techniques.

It is reasonable to predict that with increasing substitution of ring methyl groups, the compounds may exhibit more specificity for G-C binding sites rather than A-T binding sites because of the aforementioned methyl group of thymine in the major groove (i.e., increasing substitution of ring methyl groups on the 1,10-phenanthroline ring would increase steric interactions between these ring methyl groups and the methyl group of thymine).

Problem II

The structure of the reporter molecule, 11, is shown in Figure 15. This molecule has been well studied and is

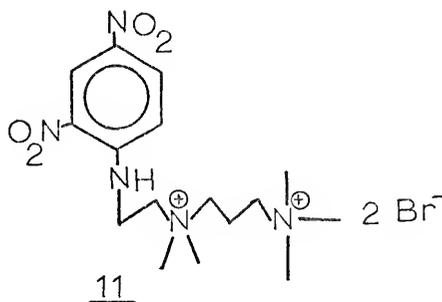


Figure 15. The 2,4-Dinitroaniline Reporter Molecule.

known to intercalate between base-pairs of DNA with the diammonium side chain lying exclusively in the minor groove of the helix.^{61,62,63} X-ray studies by Rosenberg et al.⁶⁴ showed that Na^+ lies in the minor groove of a Watson-Crick-Wilkens H-bonded dinucleoside phosphate. It would seem logical that if the diammonium side chain of reporter, 11, and Na^+ both lie exclusively in the minor groove, then the two must compete for the same binding sites. The effect of increasing sodium ion concentration on the binding of reporter molecule, 11, to DNA can be examined by equilibrium techniques (discussed below).

Simpson,⁶⁵ who studied the interaction of 11 with rabbit liver DNA and chromatin, found that the maximum number of binding sites is the same. It was concluded that proteins in the chromatin do not compete for the same DNA binding sites as the reporter molecule, 11, and, therefore, they interact with DNA in the major groove. On the other hand, the binding of actinomycin D, which is believed to bind to the minor groove of DNA,⁶⁶ is significantly reduced in chromatin as compared to free DNA.^{67,68} More binding studies of reporter molecule, 11, to DNA and DNA-protein systems are necessary to resolve this apparent contradiction.

Many researchers use reconstituted chromatin in their studies of chromatin. Reconstituted chromatin is chromatin which has been "reassembled" from its components (i.e., DNA,

histone proteins, and non-histone proteins). It is generally assumed that such "reassembled" chromatin (i.e., reconstituted chromatin) assumes the same properties and structures as native chromatin. If this assumption is correct, then the binding of reporter molecule, 11, to native and reconstituted chromatin could be tested by various techniques and similar binding specificities should be observed.

It is generally thought that doubling of the chromosomes and, consequently, the synthesis of new DNA is accomplished in the middle of interphase (i.e., the S phase of a cell).⁶⁹ It is at this phase where DNA is most dispersed throughout the nucleus and exposed for replication and transcription. During cell division (i.e., the M phase) the chromosomes are compacted and very dense. It is reasonable to assume that reporter molecule, 11, should bind more strongly to the open, exposed DNA (i.e., DNA in the S phase) than to packaged, condensed DNA (i.e., DNA in the M phase). In order to test this concept, the binding of 11 to S and M chromatin can be studied. The interaction specificities of reporter molecule, 11, to the various DNA and nucleoprotein complexes can be examined by binding studies using equilibrium dialysis, viscometric and spectroscopic techniques.

Problem III

One of the proposed functions of histones (previously discussed) is the unique and possibly selective packaging and unpackaging of DNA in the cycle of cell division. This

would be a general function of histones in eukaryotic cells and would be independent of nucleic acid sequence. However, the suggested "repressing" effect of histones would require specificity (i.e., the histones would have to discriminate between sequences in DNA).

Increasing peptide concentration on the viscosity of a DNA solution has been shown by Sanford⁷⁰ to decrease the viscosity of the solution (see Figure 16⁷¹). Since histones would have to be much more efficient than dipeptides in packaging DNA, then one would expect much more dramatic results in a graph of relative specific viscosity,

$\eta_{sp}^{complex} / \eta_{sp}^{DNA}$, versus increasing concentration of histone.

One can also get an idea of the specificity of histones using this technique by using DNAs of different sequences. Salmon sperm and calf thymus DNA are of the same A-T/G-C content, but of different sequence. If a histone fraction is more effective in decreasing the viscosity of one type of DNA solution over another, then this would suggest that the histone can distinguish between the two DNAs (i.e., between the different sequences of the DNAs).

Interpretation of these experiments may not be straightforward because of the possibility of exchange of histone between DNA strands. In other words, the proteins may not remain bound to their "original" sites on DNA and might undergo rearrangement and become bound to new sites. At least two works^{72,73} have shown that the exchange of

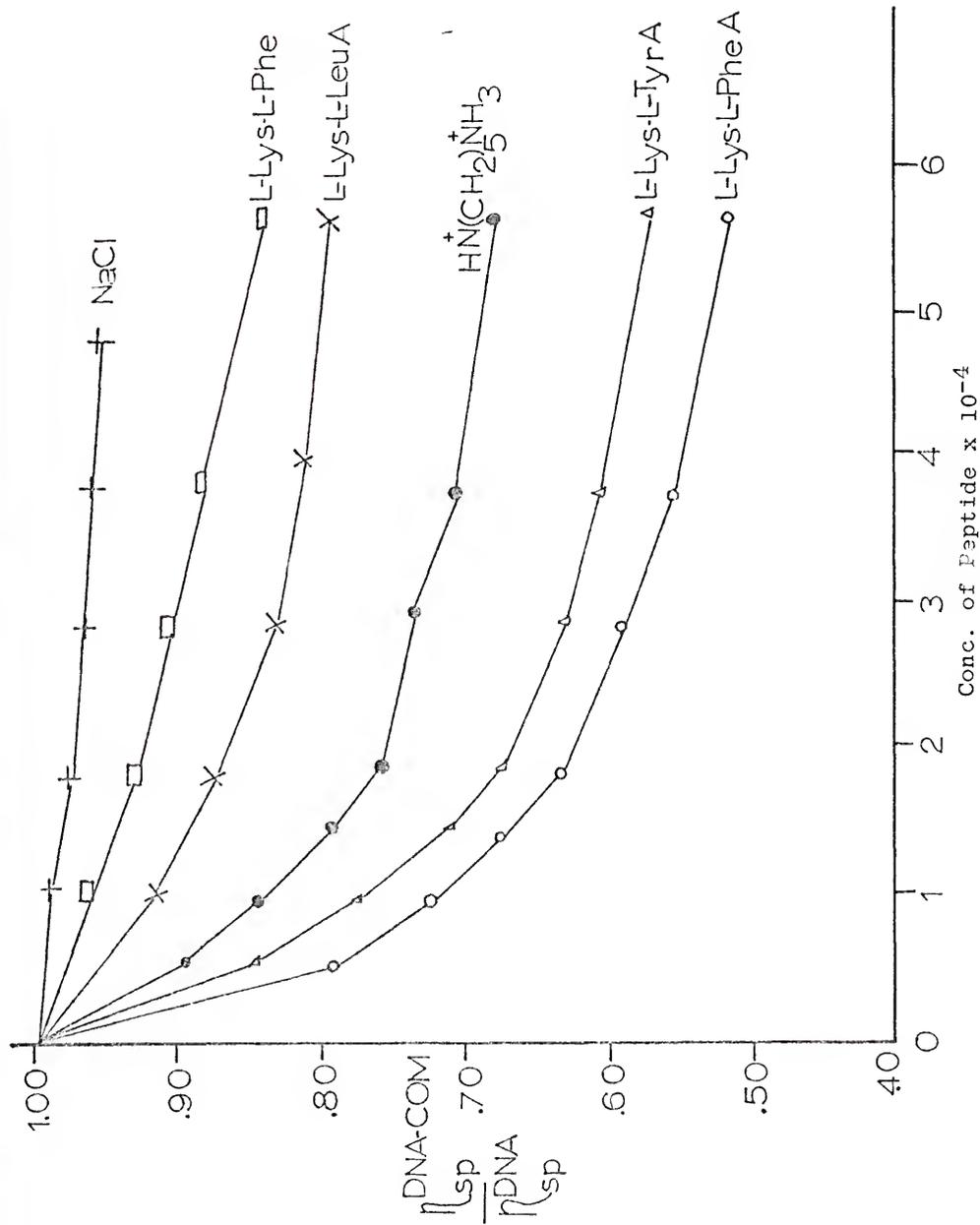


Figure 16. The Effect of Increasing Peptide Concentration on the Viscosity of DNA.

(Taken from Karl J. Sanford's Ph.D. Dissertation, University of Florida, 1972.)

nucleoproteins is dependent on salt concentration and also on the salt used. These exchange studies involved radioactive labelling.

It is possible to study the exchange of protein by viscosity. A graph of specific viscosity, η_{sp} , versus increasing concentration of DNA is shown in Figure 17 as a solid line.

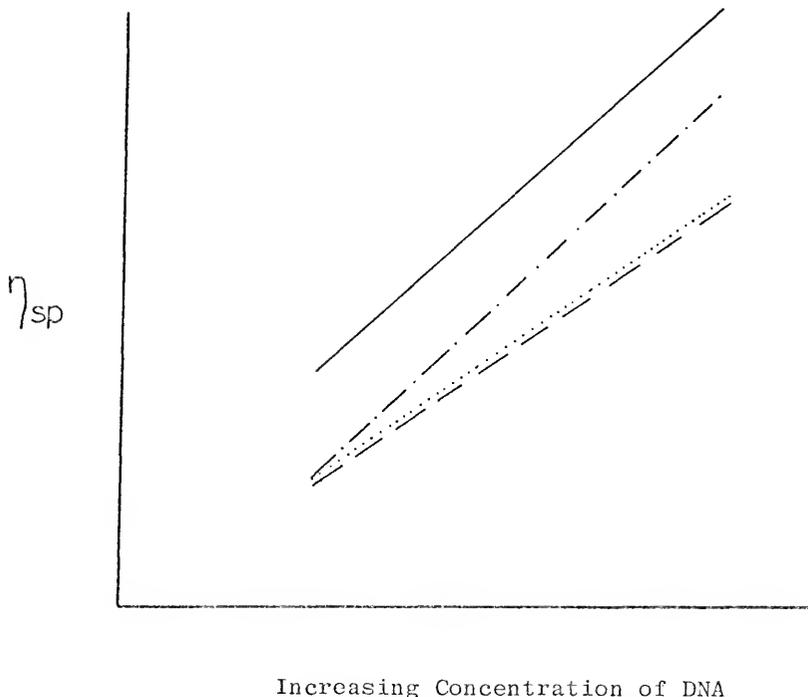


Figure 17. A Schematic Diagram Showing the Possible Effect of Increasing Concentration of DNA on the Specific Viscosity. DNA alone (—), DNA·Histone (·····), DNA·Histone if exchange (---), and DNA·Histone if none or little exchange (-·-·-).

Since it is expected that a histone will decrease the viscosity of a given DNA solution, then the dotted line in Figure 17 might represent the graph of a η_{sp} versus increasing concentration

of DNA for a complex of DNA-histone (constant histone concentration). It is possible to make up a given concentration of DNA and histone in different ways. For instance, the complex can be made (1) directly, or (2) by adding half the DNA to the histone and then adding the other half of the DNA. In the latter case, if there is an immediate exchange of histone between DNA strands, then the viscosity of the first and second solutions would be the same. However, if the histone remains bound to the first half-portion of DNA and does not exchange, then the viscosity of the second solution must be greater than the first (see Figure 17). The effect of temperature, ionic strength, and base-sequence specificity on the equilibration of protein can also be monitored by the above technique.

It would be important to show that histones can indeed discriminate between sequences of DNA, if their role as selective repressors is to be fully accepted by the scientific community. It would also be important to show that the effect of histones on the packaging of DNA can be specific (i.e., one histone fraction being more efficient in packaging one DNA over another).

The results of these experiments cannot be related to the in vivo role of proteins because, in the present experiments, only systems of two components are being considered for simplicity. In a multi-component system (i.e., in vivo) there are many other factors which must be considered (i.e.,

non-histone proteins, enzymes, c-RNA, etc.).⁷⁴ However, it is important that the interactions of two component systems be fully understood before implications can be made for multi-component systems.

RESULTS AND DISCUSSION

Results and Discussion--I

Several investigators^{75,76,77} have shown that planar aromatic systems (i.e., proflavine, acridine orange, ethidium bromide, and reporter type molecules) intercalate between base-pairs of DNA as evidenced by (1) total broadening of the pmr signals, (2) enhanced viscosity, (3) induced circular dichroism, and (4) increased melting temperature (T_m) of the DNA helix. With one exception (i.e., 10), the N-methyl-1,10-phenanthrolium cations, I, (Figure 18)

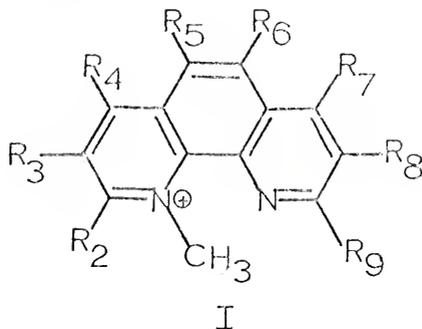


Figure 18. The N-Methyl-1,10-Phenanthrolium Cation.

were found to intercalate by the above criteria. In addition, selective interactions with DNA are observed as a function of

the position and number of methyl-substituents on the N-methyl-1,10-phenanthroline ring, I.

Proton Magnetic Resonance Studies

It is well known that, if the rate of molecular tumbling of molecules in solutions is lower than the typical Larmor frequencies, ω_0 (of the order of 10^8 - 10^9 radian sec^{-1} for protons in the conventional magnetic field), then T_2 , the transverse relaxation time, is considerably diminished, leading to substantial line broadening of the pmr signal.⁷⁸ This situation is obtained if the proton is contained in a rigid macromolecule (e.g., DNA),⁷⁹ or if the proton is contained in a slowly tumbling small molecule bound to a macromolecule. For instance, it has been shown by Gabbay and DePaolis⁸⁰ that the pmr signals of the aromatic protons of a molecule which intercalates between base-pairs of DNA are extensively broadened and indistinguishable from base line noise. The temperature-dependent pmr spectra of all the N-methyl-1,10-phenanthroline cations (except 10) in the presence and absence of DNA (sonicated) were taken at 37, 55, and 90°C (by C. Stuart Baxter). Attempts to examine the DNA-10 complex failed, due to an insolubility problem. In all cases, the pmr signals were indistinguishable from base line noise in the DNA-I complex at lower temperatures. This effect for the interaction of salmon sperm DNA with 5,6-dimethyl-N-methyl-1,10-phenanthroline chloride, 3, is illustrated in Figure 19. It should be noted that at the higher

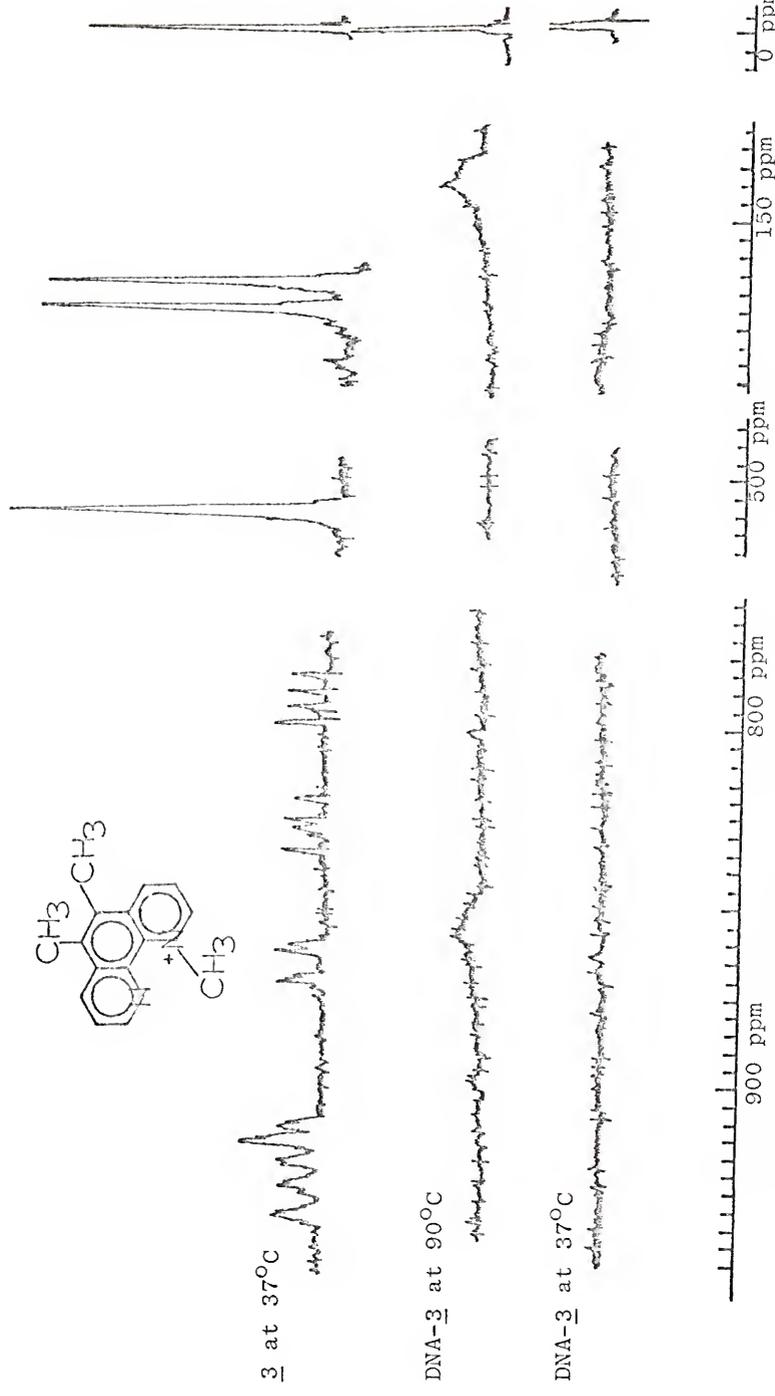


Figure 19. The Temperature-Dependent Partial Proton Magnetic Resonance Spectra of $\underline{3}$ and Salmon Sperm DNA- $\underline{3}$ Complex. Sonicated low molecular weight salmon sperm DNA was used at 0.15 mole of P/1 in D_2O in 10⁻⁴ sodium phosphate buffer (pH 7.0± 0.2). The concentration of $\underline{3}$ was 0.02 M.

temperatures, broad pmr signals and large upfield chemical shifts are observed for the CH_3 group protons of 3.

In summary, the pmr results indicate a common mode of binding of the cations, 1-9, to DNA. The total line broadening of the pmr signals of the cations is consistent with an intercalation mode of binding, since restricted rotation of the ring of I of the nucleic acid bound reporter molecule leads to incomplete averaging of the magnetic environment and substantial line broadening.

T_m Studies of Helix-Coil Transition

The effect of increasing concentrations of the phenanthroline cations, 1-7, on the T_m of the helix-coil transition of salmon sperm DNA and poly d(A-T)-poly d(A-T) is shown in Figures 20 and 21. Several interesting observations may be made. (1) A large increase in the T_m of the helix-coil transition of nucleic acids is observed for all phenanthroline cations, even at very low concentrations (i.e., 1×10^{-5} M and at a ratio of 4.2 base-pairs per reporter molecule). (2) The greater the methyl substitution of the N-methyl-1,10-phenanthroline ring, the higher the stabilization of the DNA helix. (3) The degree of stabilization is dependent on the position of methyl substitution. The following order is observed:

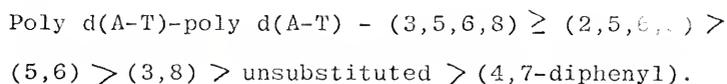


Figure 20. The Effect of Increasing Concentrations of Cations I on the T_m of the Helix-Coil Transition of Salmon Sperm DNA. The study was conducted in 0.01 M 2-(N-morpholino)ethane sulfonic acid buffer, pH 6.2 (0.005 M Na^+) using 8.40×10^{-5} M P/1 of salmon sperm DNA. The T_m in the absence of I is found to be 60.8° for salmon sperm DNA.

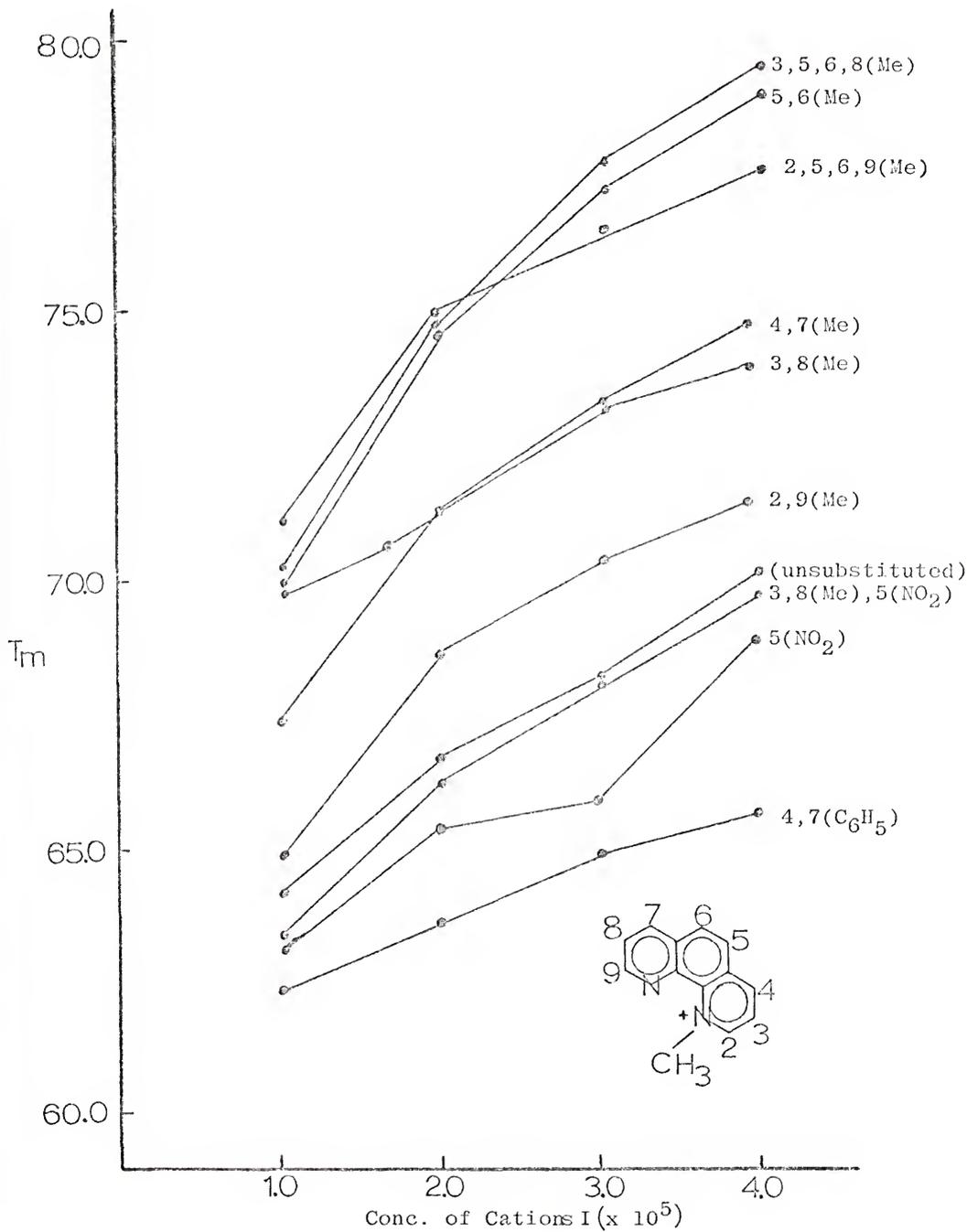
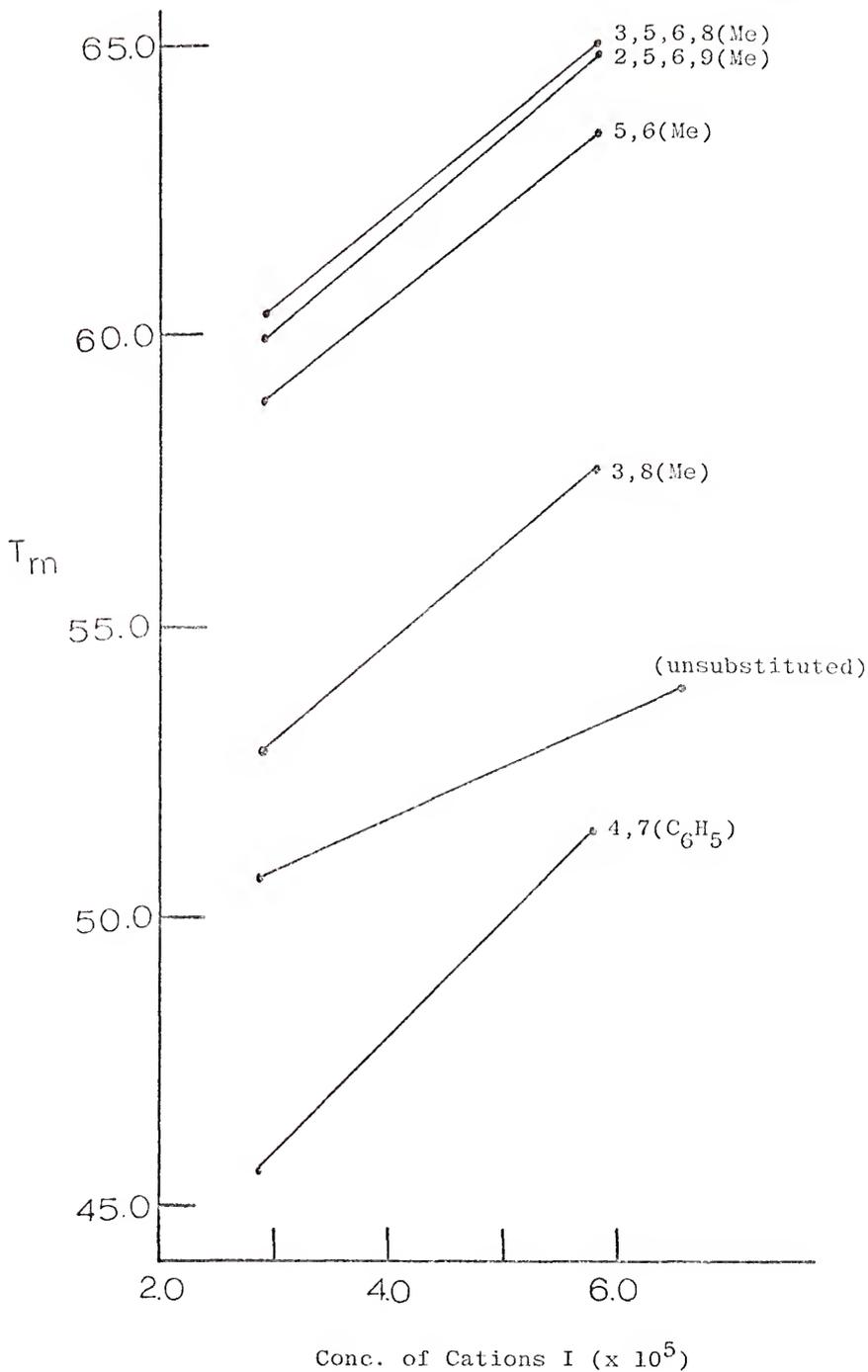


Figure 21. The Effect of Increasing Concentrations of Cations I on the T_m of the Helix-Coil Transition of Poly d(A-T)-Poly d(A-T). The study was conducted in 0.01 M MES buffer, pH 6.2 (0.005 M Na^+) using 1.14×10^{-4} M P/I of poly d(A-T)-poly d(A-T). The T_m in the absence of I is found to be 41.9° for poly d(A-T)-poly d(A-T).



Salmon Sperm DNA - (3,5,6,8) \geq (5,6) \geq (2,5,6,9) $>$
 (3,8) $>$ (4,7) $>$ (2,9) $>$ unsubstituted $>$ (4,7-diphenyl).

(4) It is noted that the cation, 10, 4,7-diphenyl-N-methyl-1,10-phenanthroline chloride is the least effective in stabilizing the DNA helices to heat denaturation (Figures 20 and 21). The binding mode of 10 to DNA is different than the cations 1-9. The latter systems bind strongly to DNA via an intercalation mechanism, whereas 10 is not expected to intercalate between base-pairs of DNA due to the presence of the bulky 4,7-diphenyl groups which are twisted out of plane of the N-methyl-1,10-phenanthroline ring. A similar effect (i.e., steric hindrance to intercalation) has been observed with other types of substituted aromatic systems.⁸¹ (5) Tetramethyl ammonium ion at the same concentration as that used for cations I, has no significant effect on the T_m of the helix-coil transition. Clearly, therefore, the cations I, with the exception of 10, exhibit a high affinity to helical nucleic acids and stabilize the latter to heat denaturation.

Ultraviolet Absorption Studies

The interactions of the cations with native salmon sperm DNA were studied by uv absorption. Due to the overlapping uv absorption of DNA and λ^{\max} of I, the effect of binding to nucleic acids on the oscillator strength of the electronic transition of I could not be determined. The aromatic cations exhibit a λ^{\max} in

the 280 nm region ($\epsilon = 35,000$), with the lowest energy 0-0 band occurring above 300 nm with an extinction coefficient, $\epsilon = 6,500$ (Table 3). In all cases (except 10), it is

TABLE 3

Absorption Properties of N-Methyl-1,10-Phenanthroline Cations, I, in 0.01 M MES buffer, pH 6.2 (0.005 M Na⁺)

Compd.	λ^{\max}	ϵ^{\max}	λ^{\max}	ϵ^{\max}	$\lambda^{\text{shoulder}}_{0-0}$	$\epsilon^{\text{shoulder}}_{0-0}$
<u>1</u>	271	35,500	218	38,600	300	6,810
<u>2</u>	282	31,600	220	36,300	305	7,350
<u>3</u>	283	30,900	223	29,300	310	6,250
<u>4</u>	292	34,700	229	32,800	318	7,150
<u>5</u>	274	33,000	226	33,800	317	7,000
<u>6</u>	288	38,300	223	35,400	320	6,410
<u>7</u>	273	35,800	228	shoulder	302	5,140
<u>8</u>	272	27,700	242	18,500	328	5,150
<u>9</u>	282	22,800	220	25,300	372	4,750
<u>10</u>	284	43,900	217	37,100	319	11,600

observed that the 0-0 absorption band is shifted to the red upon binding to DNA. However, these effects cannot be quantitated, due to the overlap of nearby vibrational bands in the absorption spectra of I. Gabbay⁸² has shown that the absorption maximum is affected by the proximity of a charged environment (i.e., the surface of a DNA

molecule), thus this red shift is in accord with an intercalation model for the mode of binding of these cations to DNA. In addition, changes in the solvent environment also cause shifts in the absorption spectrum of the chromophore of the reporter molecule.

Circular Dichroism Studies

Studies of the circular dichroism induced in the electronic transition of I upon binding to salmon sperm DNA is also hampered by overlapping CD signal from the nucleic acid in the region below 300 nm. However, induced CD in the lowest energy 0-0 absorption band of I in the presence of DNA is noted in the region of 320-400 nm. For example, the addition of N-methyl-1,10-phenanthroline chloride, 1, to salmon sperm DNA leads to a positive CD band (between 320-360 nm) which increases with increasing concentration of 1 and finally levels off at a base-pair to cation ratio of 2.49. With the exception of 10, similar saturation effects are noted for all the cations, I. (Compound 10 does not exhibit an induced CD in the DNA complex, presumably because it cannot intercalate between base-pairs of DNA, due to the presence of the bulky, and out of plane, 4,7-diphenyl substituents). The above results strongly suggest that the induced CD observed in the absorption band of I in the DNA complex arises from an intercalation mode of binding. Moreover, the CD titration studies indicate that once all intercalation sites of DNA are filled by the cation I, further excess of the latter would not lead to

further changes in the CD spectrum (supporting evidence from viscometric titration data are given below).

The results of the CD titration studies with cations 1-9 are given in Table 4, and a typical CD titration for cation 3 with salmon sperm DNA is shown in Figure 22. It is noted that the induced CD signal in the absorption band of 1-9 upon binding to DNA reaches a saturation limit that varies from a minimum of 1.87 base-pair/cation (in the case of 7, 4,7-dimethyl-N-methyl-1,10-phenanthroline cation) to a high of 2.51 base-pair/cation (in the case of 5, 3,8-dimethyl-N-methyl-1,10-phenanthroline cation). Under the conditions of these experiments (i.e., the concentration of DNA was 5.60×10^{-4} M in P/1 in a low ionic strength buffer, 0.005 M Na^+ , MES buffer, pH 6.2), the cations I have a high binding affinity to DNA, where the apparent binding constant, K_a , is found to be greater than 1×10^4 (see equilibrium dialysis studies). Therefore, it can be easily shown by calculation that in the CD titration experiment (Figure 22) the cations I (1-9) are at least 80% bound to DNA prior to saturation of the strong binding sites.

For this reason, the values listed in Table 4 of base-pair to total cation concentration ratio at saturation of the CD signal, in fact, represent an approximation of the maximum number of strong binding sites for the intercalating cations. It should be emphasized, however, that the CD saturation value of base-pair/cation ratio will always be somewhat lower than that obtained by the Scatchard-type treatment (see

TABLE 4

Summary of the Circular Dichroism and Viscometric Titration Studies of Salmon Sperm DNA by Cations I^a

Compound	CD Studies ^b (B.P./Cation) ^d _{sat}	Viscometric Studies ^c (B.P./Cation) ^d _{sat}
<u>1</u>	2.49	2.32
<u>2</u>	2.26	2.07
<u>3</u>	2.04	1.99
<u>4</u>	2.06	2.26
<u>5</u>	2.51	2.32
<u>6</u>	2.27	2.04
<u>7</u>	1.87	1.94
<u>8</u>	2.30	2.50
<u>9</u>	2.80	1.80

^aAll studies were carried out in 0.01 M MES buffer, pH 6.2 (0.005 M in Na⁺). ^bCD titration studies employed a concentration of 5.6×10^{-4} M DNA P/1 using a 5 cm path length cells at 25°C. ^cViscometric titration studies employed a concentration of 5.6×10^{-4} M DNA P/1 and were carried out at 37°. ^d(Base-Pair/Cation)_{sat} is the calculated value of the ratio of base-pairs of DNA to total cation I present in solution, at saturation. It should be noted that these values do not represent the maximum number of binding sites for cations I to DNA since at saturation free cations in solution are also present.

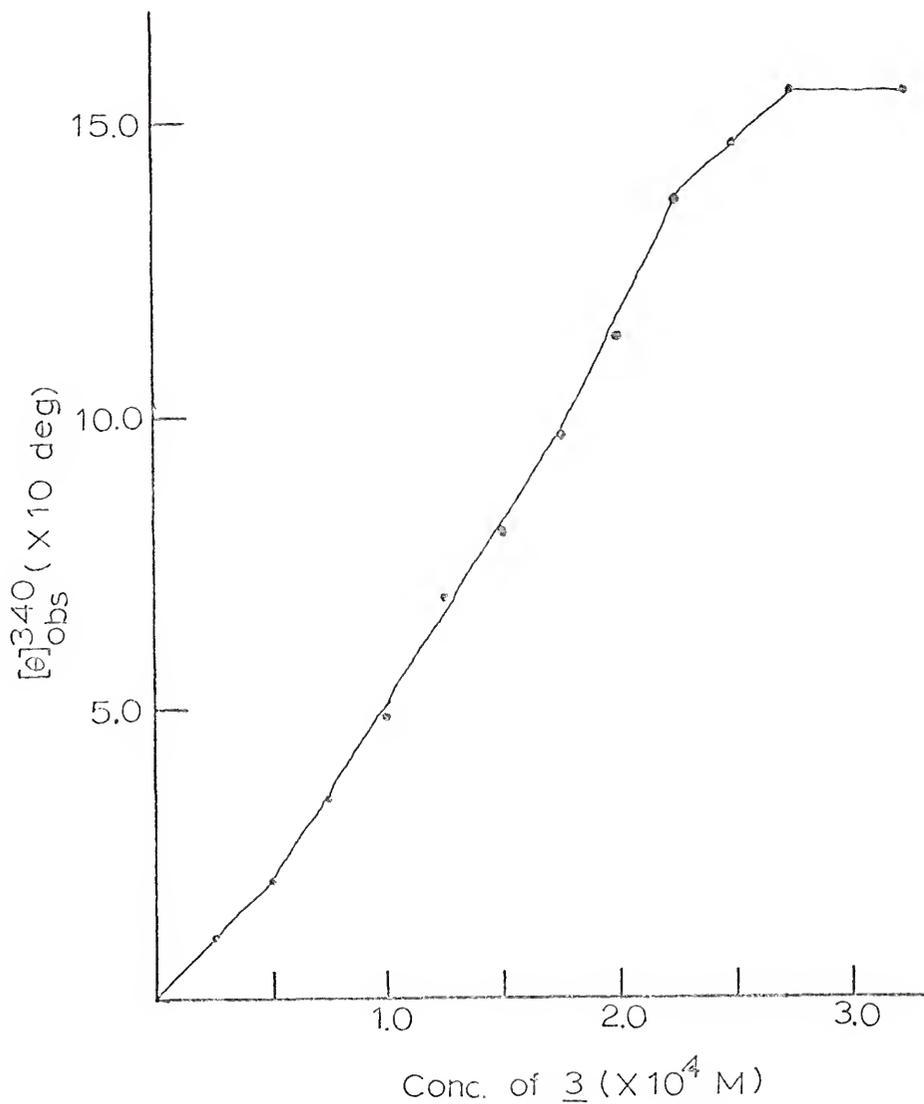


Figure 22. The Effect of Increasing Concentration of 3 on the Observed Ellipticity, $(\theta)_{\text{obs}}$, at 340 nm in the Presence of 5.60×10^{-4} M P/l of Salmon Sperm DNA.

equilibrium dialysis studies), since the latter method determines the maximum number of DNA strong binding sites per small molecule (i.e., the minimum number of base-pairs per binding site). This criterion, 100% binding of I to DNA at saturation, is not obtained by either the CD or the viscometric titration studies described in this work. Nevertheless, viscometric titration studies of DNA intercalating sites by cations I (using the same DNA concentration as the CD studies) show almost identical base-pair/cation ratios at saturation as the CD results cited above (see Table 4). It is, therefore, concluded (with a high degree of certainty) that the induced CD observed for the DNA-1-9 complexes arises from an intercalation mode of binding. The results of the pmr and Tm studies (cited earlier), and the viscosity studies (below), are consistent with this interpretation.

The induced circular dichroism in the absorption band of the cations 1-9 upon binding to salmon sperm DNA is shown in Figures 23, 24, and 25. The spectra were obtained under conditions of saturation of the intercalating sites (i.e., further addition of cations I to the DNA solution do not change the observed spectra). It should be noted that significant differences in the induced CD of DNA-1-9 complexes are observed. In addition, two interesting observations can be made. (1) The cations which contain methyl groups at the 5,6 positions on the N-methyl-1,10-phenanthroline ring (i.e., 3, 4, and 6) exhibit an induced CD upon binding to

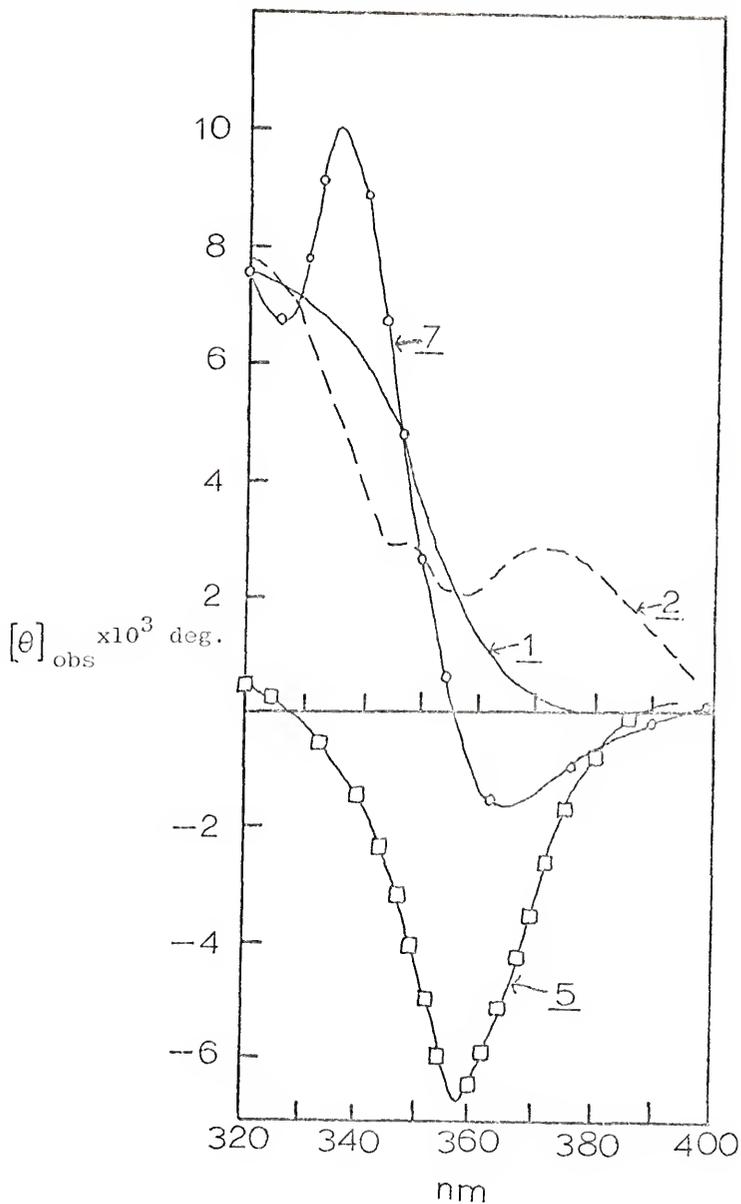


Figure 23. The Induced Circular Dichroism Spectra of Salmon Sperm DNA-1, 2, 5, and 7 Complexes at Saturation. The studies were performed in 0.01 M MES, pH 6.2 and 0.005 M Na^+ with 5.60×10^{-4} M P/1 salmon sperm DNA.

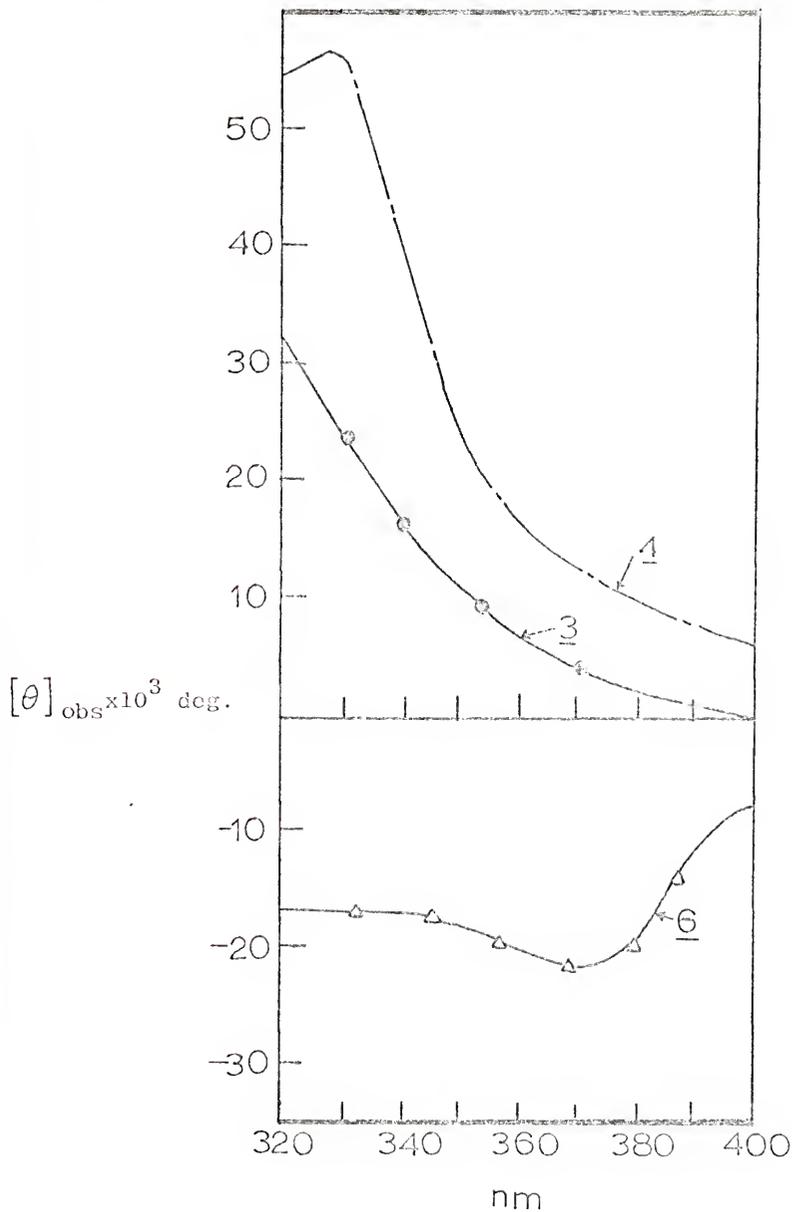
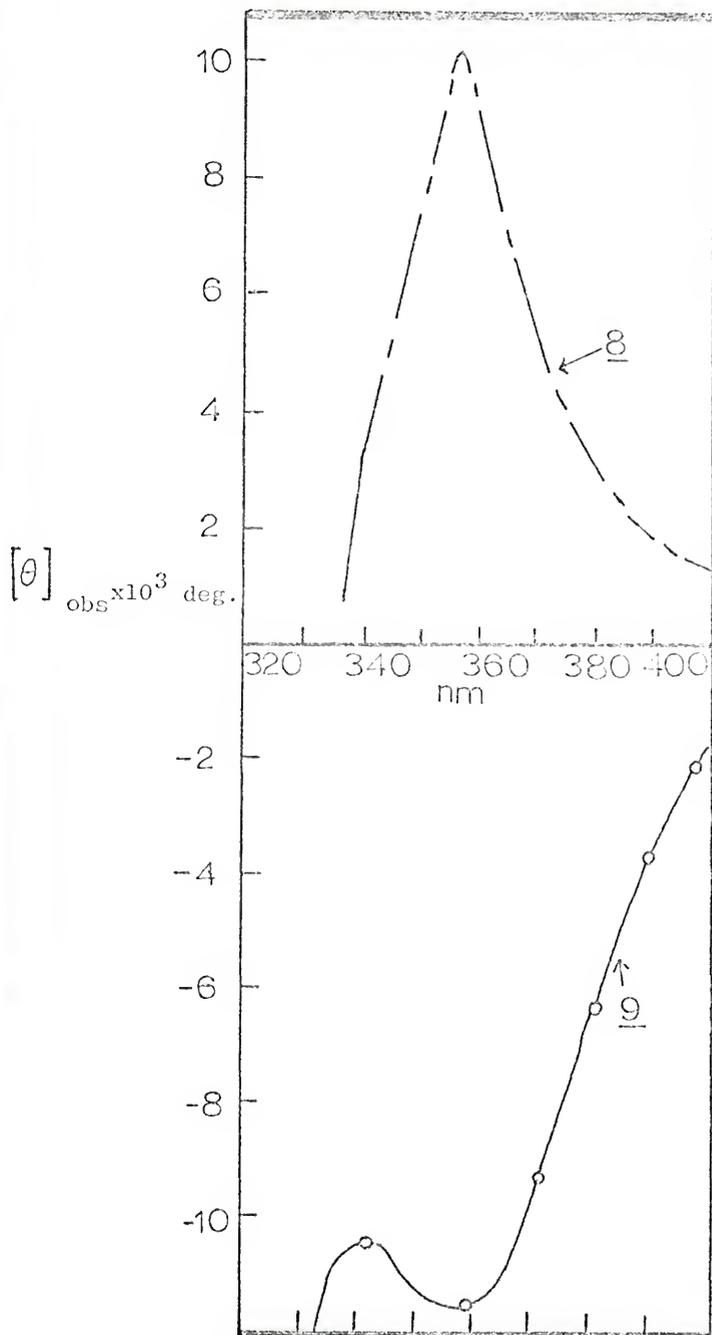


Figure 24. The Induced Circular Dichroism Spectra of Salmon Sperm DNA-3, 4, and 6 Complexes at Saturation. The studies were performed in 0.01 M MES, pH 6.2 and 0.005 M Na^+ with 5.60×10^{-4} M P/1 salmon sperm DNA.

Figure 25. The Induced Circular Dichroism Spectra of Salmon Sperm DNA-8 and 9 Complexes at Saturation. The studies were performed in 0.01 M MES, pH 6.2 and 0.005 M Na⁺ with 5.60×10^{-4} M P/1 salmon sperm DNA.



salmon sperm DNA which is several magnitudes greater than the unsubstituted and dimethyl-substituted cations, 1, 2, 5, 7, 8, and 9. One possible explanation for this effect is the fact that the absorption maximum, λ^{\max} , of 3, 4, and 6 (as the free cations) is red shifted by 10-16 nm, as compared to 1, 2, 5, 7, 8, and 9 (Table 3). Therefore, the higher induced CD in the 320-400 nm region (Figure 6) observed for the DNA 3, 4, and 6 complexes, may simply be due to the fact that λ^{\max} of these systems (283-292 nm) is nearer to the region in which the CD spectra are being observed. (2) The cations which contain methyl groups at the 3,8 positions (see Figure 26) of the N-methyl-1,10-phenanthroline ring (5, 6, and 9) exhibit a negative

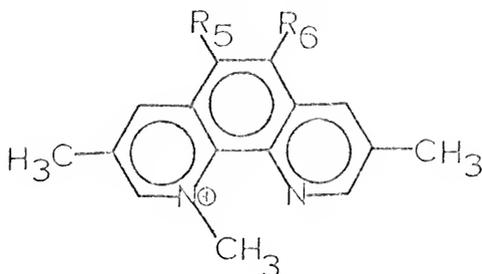


Figure 26. The Cations, I, which Exhibit a Negative Induced CD Upon Binding to Salmon Sperm DNA.

5, R₅=R₆=H; 6, R₅=R₆=CH₃; 9, R₅=NO₂

induced CD upon binding to salmon sperm DNA, as opposed to positive induced CD observed for the other cations. One possible explanation is that the 3,8-dimethylated cations, 5, 6, and 9, cannot assume all possible geometries in the intercalating site due to steric restrictions. For example, the distance between CH₃ groups at the 3 and 8 positions of I is approximately 11.2 angstroms (including the Van der Waals radius of the methyl groups); therefore, unfavorable steric interactions with the deoxyribofuranoside rings (on opposite chains) may be expected to occur for certain geometries. Molecular framework model studies indicate that an intercalation geometry for the 3,8-dimethylated cations, I, whereby the long axis of the molecule is approximately parallel with respect to the H-bonds of the base-pairs, is highly unfavorable, due to steric interactions with the sugar rings. A more reasonable intercalation geometry for the above systems is one in which the methyl groups at the 3 and 8 positions are pointing into opposite grooves (see Figures 27 and 28). The oppositely induced CD observed for the DNA-5, 6, and 9 complexes, as compared to the other cations (1-4, 7, and 8), can arise (on the basis of present theories)^{83,84} from different intercalation geometries.

Viscometric Studies

Planar molecules such as acridine orange, ethidium bromide, and proflavine intercalate between base-pairs in DNA and are accompanied by an increase in the viscosity

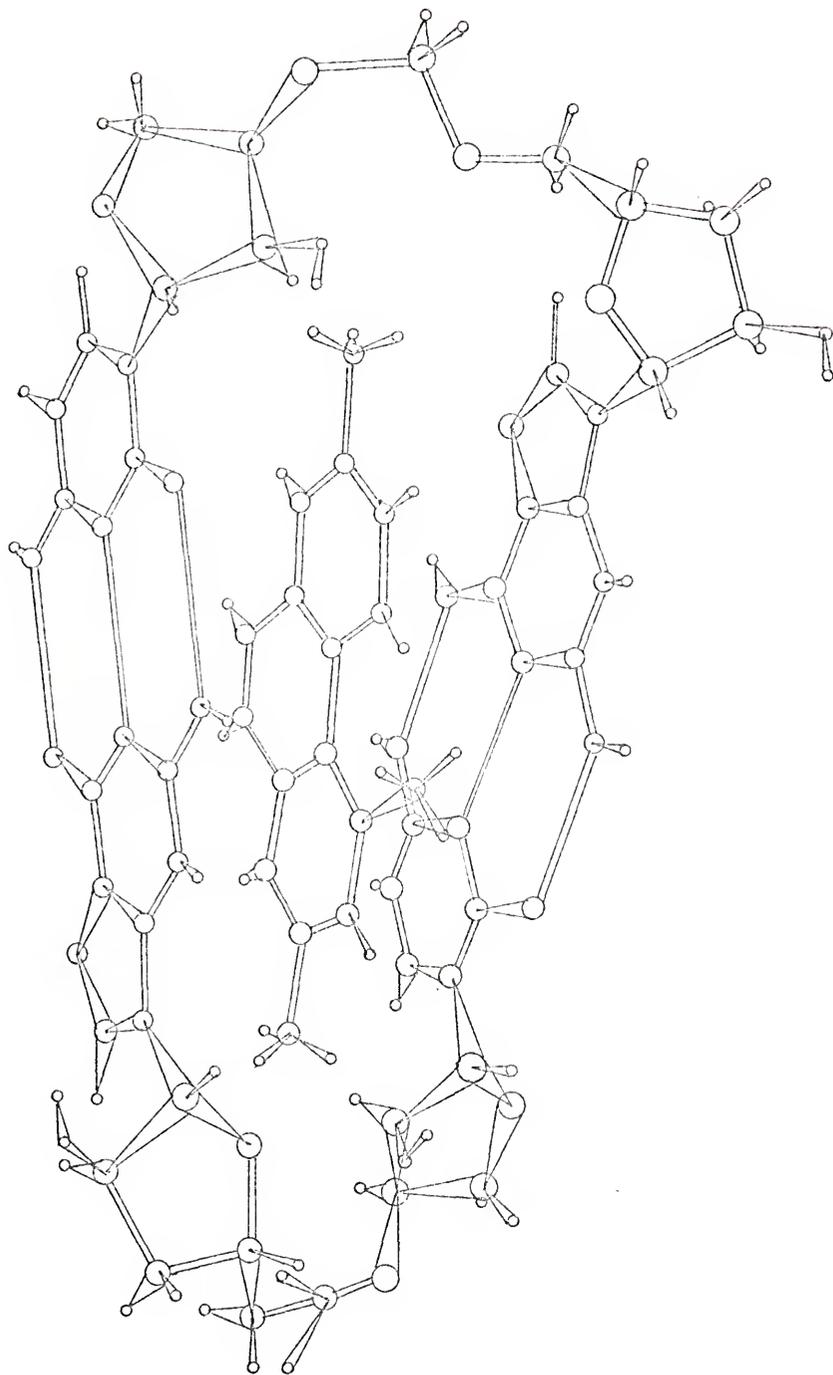


Figure 27. Schematic Illustration of the Possible Intercalation Complexes of 5 to DNA with Long Axis of the Molecule Pointing at Opposite Chains.

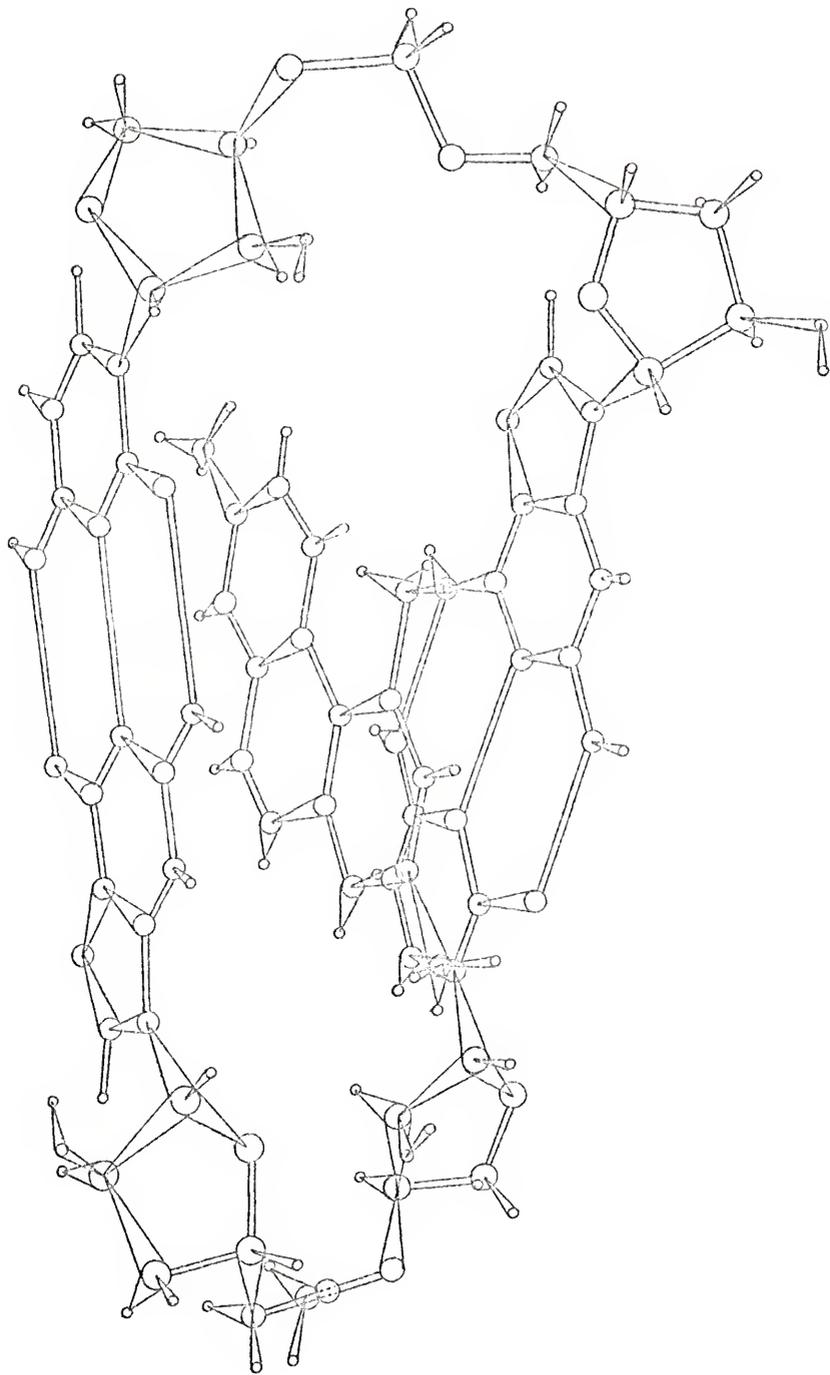


Figure 28. Schematic Illustration of the Possible Intercalation Complexes of 5 to DNA with the Long Axis of the Molecule Pointing at Opposite Grooves.

of the solution.^{85,86} In order to determine the mode of binding of cations 1 to salmon sperm DNA, viscometric titration studies were carried out under the same concentration conditions as those employed for the circular dichroism studies (i.e., 5.60×10^{-4} M DNA P/1 in 0.01 M MES buffer, pH 6.2). It is found that the specific viscosity, η_{sp} , of the solution increases with increasing concentration of 1 and finally reaches a saturation value. The effect of 1 on the specific viscosity is shown in Figure 29. It is noted that the relative specific viscosity increases with increasing concentrations of 1 and finally levels off at a base-pair/cation ratio of 2.32. Similar saturation effects on the specific viscosity of salmon sperm DNA are observed for cations 2-7. The values of base-pair/cation ratio obtained by viscometric titrations are shown in Table 4. It should be noted that these values are almost identical to those obtained by the CD titration technique (i.e., $(\text{base-pair/cation})_{\text{sat}}^{\text{CD}} = (\text{base-pair/cation})_{\text{sat}}^{\text{viscosity}}$). The results are entirely consistent with an intercalation mode of binding for cations 1-9 to DNA. In support of this conclusion, the non-planar compound 10, which contains the 4,7-diphenyl substituents, does not exhibit an induced CD or an increase in viscosity upon binding to DNA.

In order to compare the effective increase in length of the DNA helix by cations 1-7, attempts were made to determine the intrinsic viscosity, $[\eta]$, of the complexes. However, the results are found to be uninformative, since

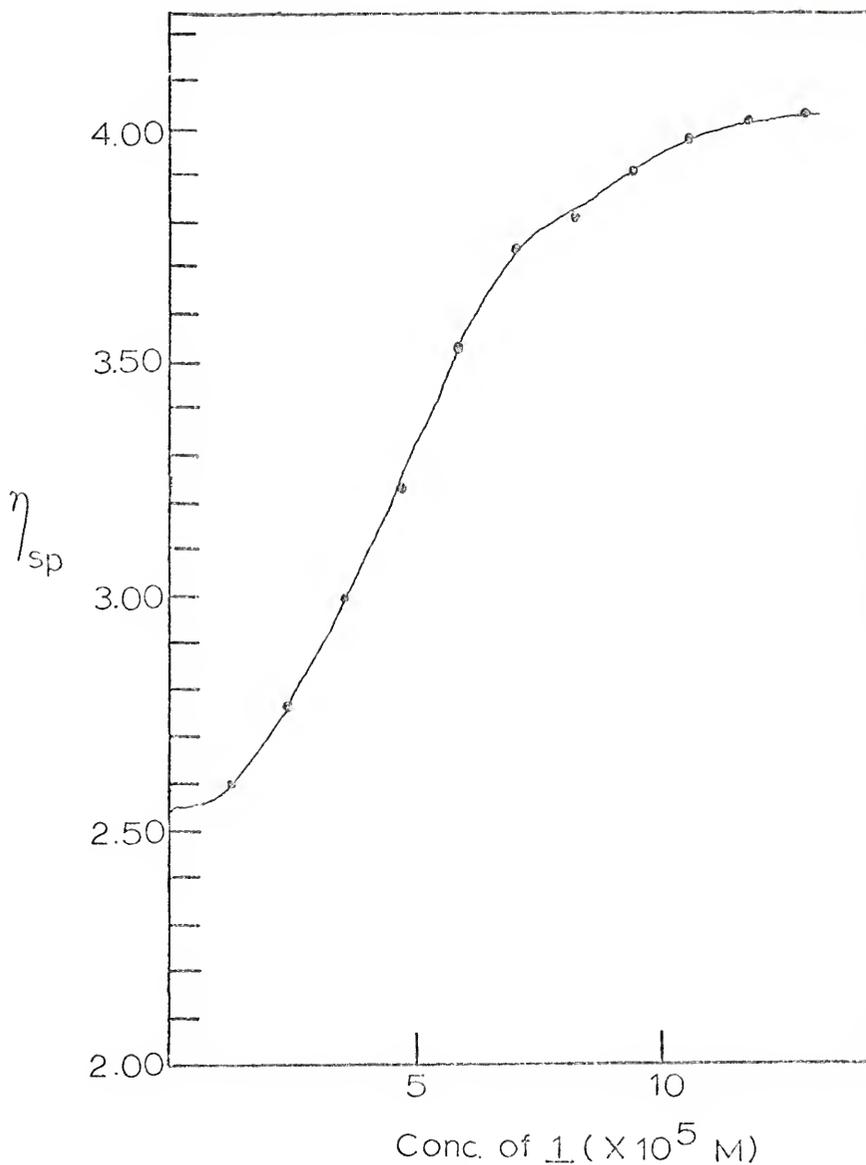
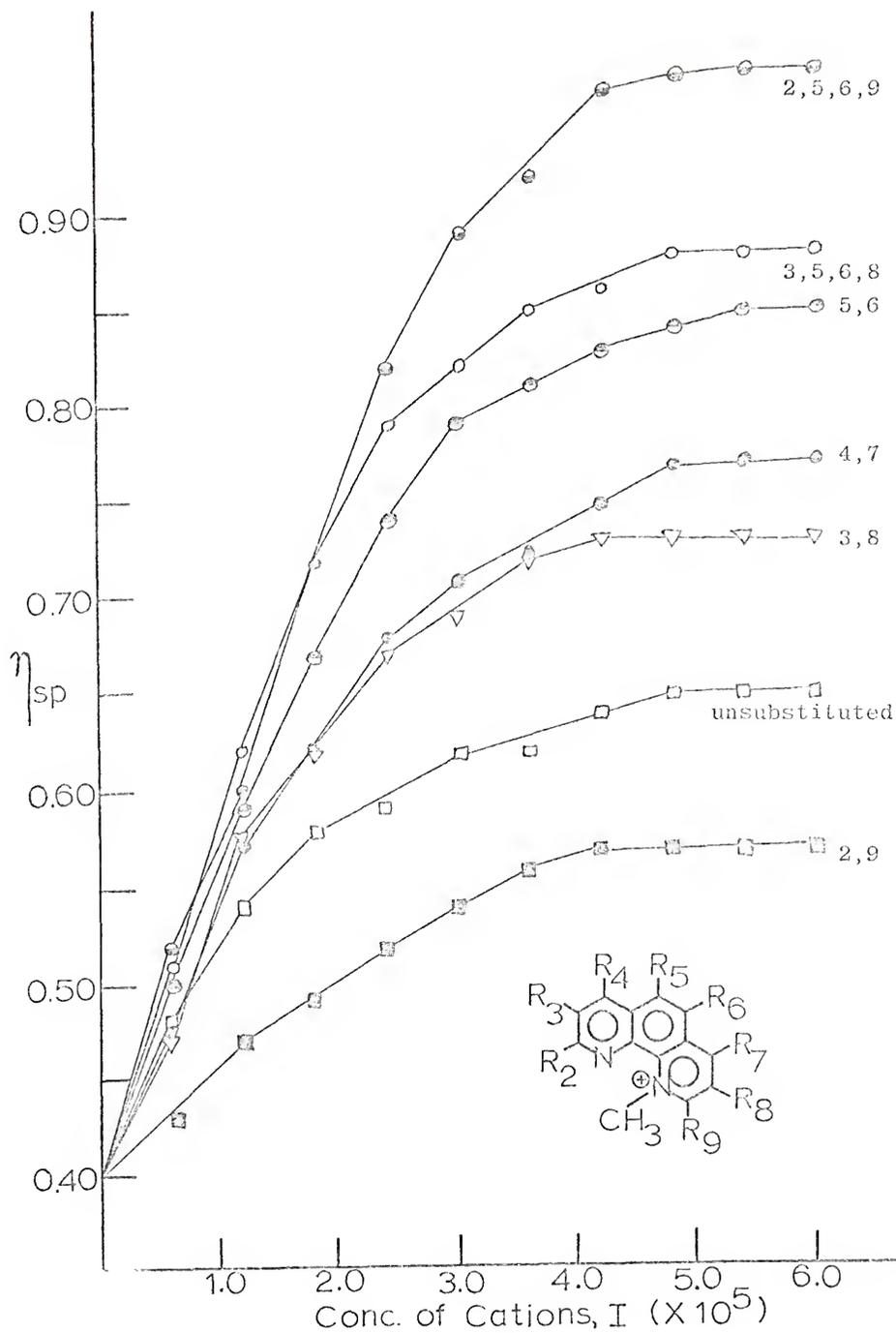


Figure 29. The Effect of Increasing Concentrations of 1 on the Relative Specific Viscosity. The study was conducted in 0.01 M MES buffer, pH 6.2 (0.005 M Na^+) at 37.5°C using 5.60×10^{-4} M P/l of salmon sperm DNA.

the value of the intrinsic viscosity at infinite dilution in the presence of other molecules will and does approach the value of the intrinsic viscosity of free DNA at infinite dilution (i.e., since the binding constant of the small molecule to DNA is finite, the complex will be dissociated at the lower concentrations). Instead, the effect of increasing concentrations of 1-9 on the specific viscosity, η_{sp} , of DNA solution at low concentration of the latter was studied. For instance, at very low DNA concentration, the relative values of η_{sp} upon saturation of the intercalation sites by the cations 1-9 is a close approximation of the relative values of the intrinsic viscosity, $[\eta]$, of the complexes (since by definition $[\eta] = (\eta_{sp}/C)_{C \rightarrow 0}$, where C is the DNA concentration). The effect of increasing concentrations of cations I on the η_{sp} of a solution of 1.0×10^{-4} M salmon sperm DNA P/1 is shown in Figure 30.

A number of interesting observations can be made. (1) The limiting values of the η_{sp} of DNA solution upon saturation of the intercalating sites are dependent on the number of and position of methyl group substituents on the aromatic ring of I. The order of increasing η_{sp} at saturation is found to be: 2,9- < unsubstituted < 3,8- < 4,7- < 5,6- < 3,5,6,8 < 2,5,6,9. (2) Since the study was carried out at near infinite dilution of the DNA, the relative values of η_{sp} at saturation are close approximations of the relative values of the intrinsic viscosity of DNA-I complexes. Moreover, the intrinsic viscosity of a rod-like molecule is directly proportional to $L^{1/3}$, where L is the length of the

Figure 30. The Effect of Increasing Concentrations of Cations I on the Specific Viscosity of Near Infinitely Dilute Solution of Salmon Sperm DNA. The salmon sperm DNA concentration is 1.0×10^{-4} M P/l.



rod.⁸⁷ Therefore, the order of increasing η_{sp} for the DNA-I complexes also reflects the order of increasing effective length of the helix. Hence, the helix length of salmon sperm DNA-6 complex is found to be greater than that of the DNA-1 complex, when all the intercalating sites are filled (Figure 30). Such an effect may arise by two separate mechanisms. (i) There are more intercalation sites on DNA available for the binding of 6 as compared to 1. The results of the equilibrium dialysis studies are consistent with this interpretation. For example, Scatchard-type treatment of the binding data shows one strong binding site per 4.35 and 2.96 base-pairs of salmon sperm DNA for cations 1 and 6, respectively (Table 5). However, such an explanation does not account for the higher value of the η_{sp} at saturation obtained upon intercalation of 1 as compared to 2, since the number of strong binding sites per base-pairs of salmon sperm DNA are found to be 4.35 and 2.86, respectively. Similar discrepancies between the values of η_{sp} at saturation and the maximum number of binding sites for cations I are also noted (Figure 11 and Table 3). (ii) Differences in steric interaction between cations I and the base-pairs of the intercalating site may also lead to differences in helical length. For example, the larger Van der Waal radii of the four methyl group substituents on the aromatic ring of 6, as compared to the H-substituents of 1, can also account for the observed higher η_{sp} of the DNA-6 complex, as compared to the DNA-1 complex. This argument, however,

TABLE 5

Summary of the Scatchard-Type Treatment of the Binding Studies of Cations I to Various Nucleic Acids^a

Cation	Salmon Sperm DNA		Poly d(A-T)-Poly d(A-T)		Micrococcus luteus DNA	
	$K_a \times 10^{-4}$	\bar{n}_{\max}^b	$K_a \times 10^{-4}$	$1/2\bar{n}_{\max}^b$	$K_a \times 10^{-4}$	$1/2\bar{n}_{\max}^b$
1	4.03	0.123	2.83	0.122	4.10	0.174
2	4.94	0.173	-----	-----	-----	-----
4	14.30	0.208	-----	-----	-----	-----
6	18.50	0.165	16.0	0.180	2.78	0.235
7	8.17	0.192	-----	-----	-----	-----

Cation	Calf Thymus DNA		Poly dG-poly dC		Deproteinized Micrococcus luteus DNA	
	$K_a \times 10^{-4}$	\bar{n}_{\max}^b	$K_a \times 10^{-4}$	$1/2\bar{n}_{\max}^b$	$K_a \times 10^{-4}$	$1/2\bar{n}_{\max}^b$
1	2.61	.114	0	0	3.72	.148
6	16.90	.154	1.93	.180	19.4	.222

^aEquilibrium dialysis studies were carried out in 0.01 M MES buffer, 0.020 M in Na⁺, pH 6.2, using 5.53x10⁻⁴ M, 5.26x10⁻⁴ M, 2.95x10⁻⁴ M, 4.95 x 10⁻⁴ M, 4.51x10⁻⁴ M, and 3.88x10⁻⁴ M P/I of salmon sperm DNA, poly d(A-T)-poly d(A-T), Micrococcus luteus DNA, calf thymus DNA, poly dG-poly dC and deproteinized Micrococcus luteus DNA, respectively. Reporter concentrations were varied from 2x10⁻⁵ to 6x10⁻⁵ M. Duplicate measurements on each of the above systems were carried out and the average values are reported in the table. Deviations of not more than ±4 and ±7% of the \bar{n}_{\max} and K_a values are observed, respectively. $1/2\bar{n}_{\max}$ values represent the minimum number of base-pairs per binding site.

does not explain the lower η_{sp} observed for the DNA-2 complex (2 contains a 2,9-dimethyl substituent), as compared to the DNA-1 complex, nor does it account for the observed order of η_{sp} for the various DNA-I complexes at saturation.

Clearly, the observed order of increasing helical length of the various DNA-I complexes cannot be explained in terms of either mechanisms (i) or (ii) or a combination of the two. A model which fits the observed data is shown in Figure 31. It is based on the supposition that the "thickness" of the aromatic ring of I is not uniform and, moreover, is dependent on the position of substitution of the methyl groups. For example, the Van der Waals contact distance of a CH_3 group is larger than the in-plane contact distance of an aromatic ring (i.e., 2.1 and 1.7 angstroms for the former and latter, respectively).⁸⁸ Thus, the in-plane structure of N-methyl-1,10-phenanthroline cation, 1, should be considered as a "wedge" of varying thickness. Therefore, intercalation of 1 between base-pairs of DNA will not only lead to lengthening of the helix, but also to slight bending of the rod at the point of intercalation (Figure 31). The first effect will lead to an increase in the η_{sp} , while the second will lead to a decrease. Bending of the helix will be expected to occur to a larger extent if greater variation in thickness of the "wedge" from one end of the molecule to the other exists. Thus, the results shown in Figure 30 can be readily understood, since the order of increasing η_{sp} at saturation by cations I (i.e., 2,9 < unsubstituted < 3,8 4,7 < 5,6 < 3,5,6,8 < 2,5,6,9) is also the expected order

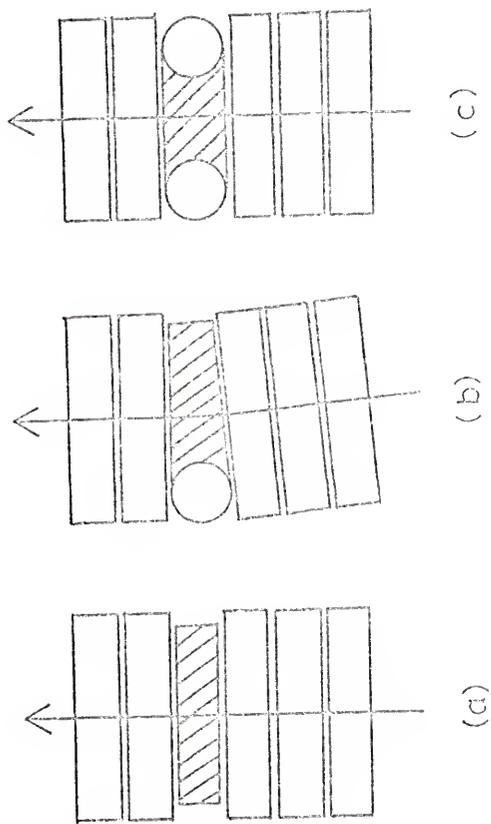


Figure 31. Schematic Illustrations of the Possible Complexes of I to DNA Showing Lengthening (a) and (c) as well as Bending of the Helix (b) at the Intercalation Site.

of decreasing variation in thickness of the aromatic ring along the short axis of the molecule. For example, substitution of methyl groups at the 5 and 6 positions of the ring will lower the difference in thickness across the short axis of the aromatic cation, I, and, therefore, bending of the helix will be minimal. However, bending of the helix will be expected to increase as substitution of the ring of I by dimethyl groups progressively gets nearer to the nitrogen atoms.⁸⁹ This model, in conjunction with mechanisms (i) and (ii), adequately explains the viscometric data.

Equilibrium Dialysis Studies

The results of T_m (helix-coil transition), proton magnetic resonance (pmr), induced circular dichroism, and viscometric studies strongly suggest a common mode of binding of cations I to DNA (i.e., intercalation between base-pairs). In addition, the induced CD and viscometric data indicate that differences in the binding "geometry" of the intercalated cation I exist. In order to further understand the interaction specificities of N-methyl-1,10-phenanthroline cation, binding studies to nucleic acids of various base compositions were carried out using equilibrium dialysis techniques. In these studies, the nucleic acid concentration was kept constant, and the concentrations of the cations were varied from 2×10^{-5} M to 6×10^{-5} M. The concentrations of free cations I were determined directly by uv absorption (Table 3). The data obtained

were analyzed by the Scatchard technique⁹⁰ according to the following equation,

$$\bar{n} = \bar{n}_{\max} - 1/K_a \frac{\bar{n}}{R_f}$$

where \bar{n} is the number of moles of I bound per mole of DNA phosphate, \bar{n}_{\max} represents maximal binding, K_a is the association constant for the DNA-I complex, and R_f is the concentration of unbound cation. A plot of \bar{n}/R_f versus \bar{n} gives the values of \bar{n}_{\max} (x-axis intercept) and $\bar{n}_{\max}K_a$ (y-axis intercept). The results of this study are shown in Figures 32 and 33 (for cations 1 and 6, respectively) and the data are summarized in Table 5. Several interesting observations may be made. (1) Differences in binding affinities, K_a , and maximum number of strong binding sites are noted for cations I with salmon sperm DNA. For example, the following order of increasing affinity is observed:

$$\underline{1} < \underline{2} < \underline{7} < \underline{4} < \underline{6}$$

With one exception, a similar order of increasing number of strong binding sites is noted (i.e., 1 < 6 < 2 < 7 < 4). Although the binding affinity results are consistent with the T_m data (which also show the same order of increasing stabilization of the helical structure), they are, nonetheless, surprising. For example, greater steric hindrance and, hence, lower affinity is expected for the DNA-6

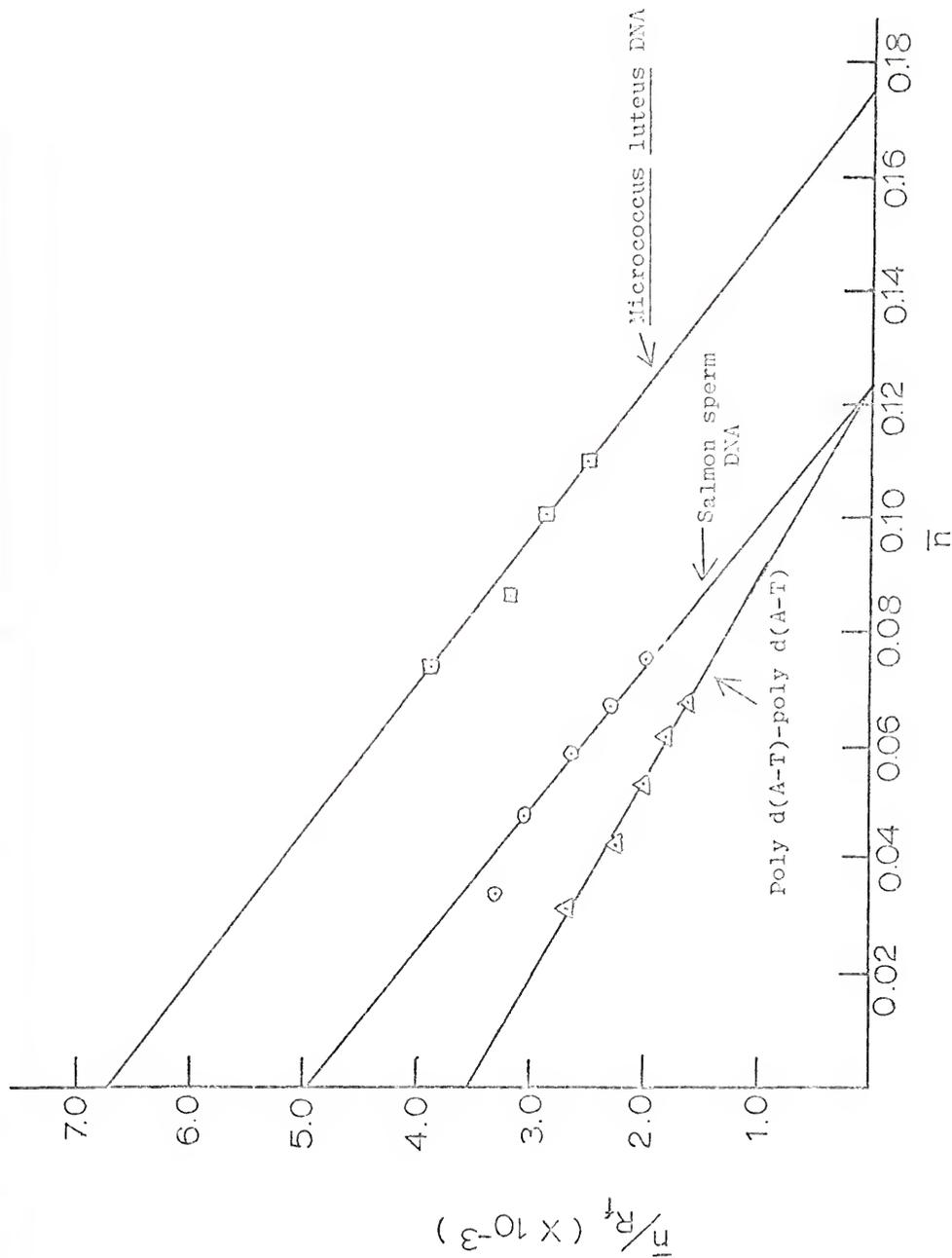


Figure 32. The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of Cation I with Poly d(A-T)-poly d(A-T), Salmon Sperm DNA, and Micrococcus luteus DNA.

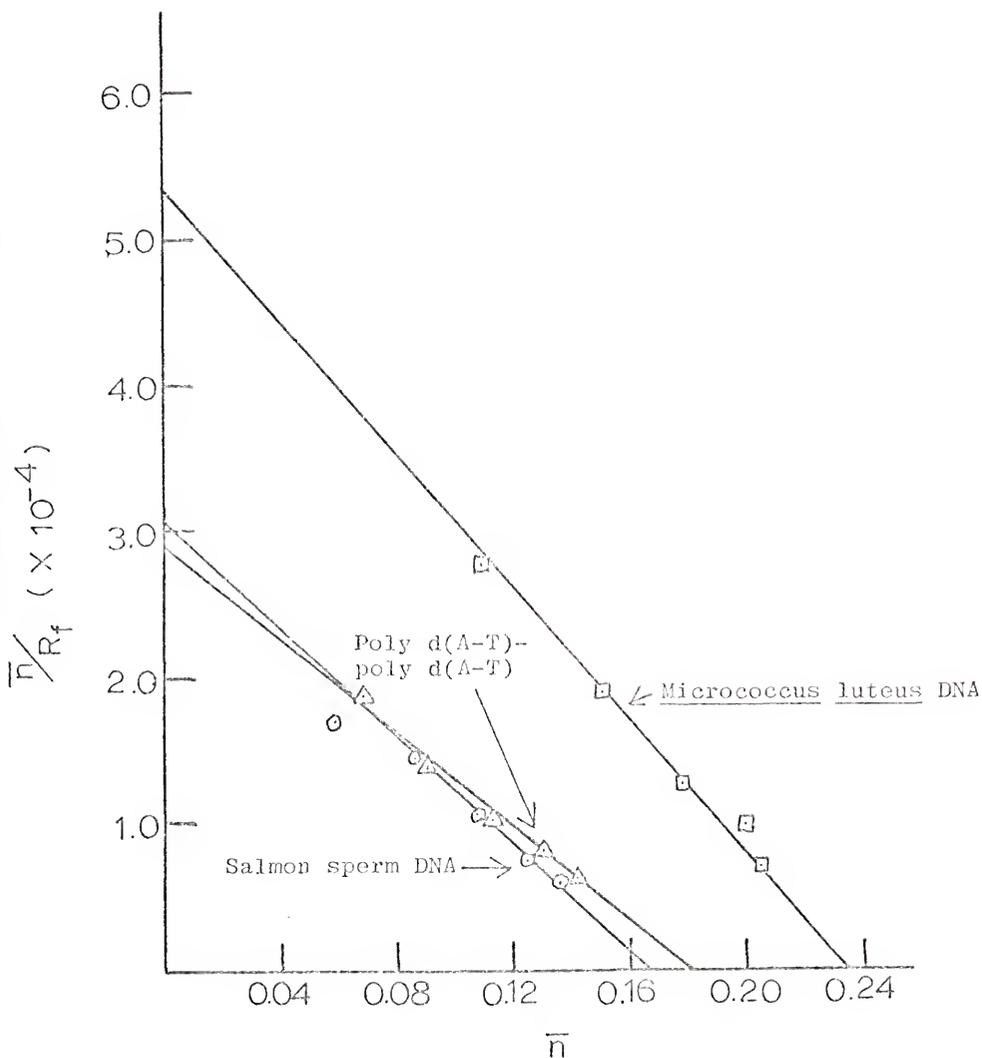


Figure 33. The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of Cation \bar{G} with Poly d(A-T)-poly d(A-T), Salmon Sperm DNA, and Micrococcus luteus DNA.

complex, as compared to the DNA-1 complex, due to the presence of the 3,5,6,8-tetramethyl groups in the former. It is clear, however, that this is not the case. Moreover, the maximum number of strong binding sites for the methylated cations, 1, (i.e., 2, 4, 5, and 7) is found to be higher than the unsubstituted cation 1. Therefore, it is concluded that adjacent base-pairs of DNA may readily separate by distances greater than 6.8 angstroms in order to accommodate a bulky intercalating cation, (e.g., 6). In the latter case, a separation distance of at least 7.6 angstroms is required.

(2) The effect of nucleic acid base-composition on the apparent binding constant and the maximum number of binding sites was studied for cations 1 and 6 (Table 5). The results show the following order of increasing affinity of 1 to nucleic acids, poly d(A-T)-poly d(A-T) (100% A-T) < Micrococcus luteus DNA (28% A-T) \approx salmon sperm DNA (58% A-T), and the following order of increasing affinity of 6 to nucleic acids, poly d(A-T)-poly d(A-T) < salmon sperm DNA < Micrococcus luteus DNA. In addition, it is noted that G-C rich DNA (i.e., Micrococcus luteus DNA) shows a higher maximum number of strong binding sites for 1 and 6 than the other nucleic acids. Steric hindrance to intercalation between A-T sites is one possible explanation for the above observation. For example, the separation distance required for intercalation of 6 between base-pairs composed of A-T sites may be as high as 8.4 angstroms, if the CH₃ group of thymine is in an eclipsed conformation with respect to a

CH_3 substituent of the intercalating cation 6. Such effects are illustrated in Figure 34.

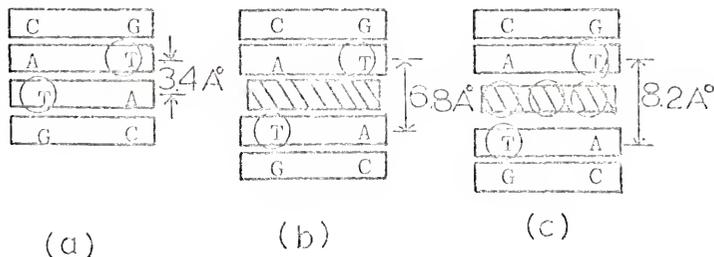


Figure 34. Schematic Illustrations of the Complexes of I to DNA Showing the Possible Separation Distances Between Base-Pairs Required to Accommodate Unsubstituted (b) and Methyl Substituted N-Methyl-1,10-Phenanthroline Cation (c).

In summary, systematic studies of the interaction specificities of methyl substituted N-methyl-1,10-phenanthroline cations, I, with nucleic acid of various base compositions have been carried out. In all cases, a common mode of binding is observed (i.e., intercalation between base-pairs of DNA). Selective interactions of I with DNA are noted as a function of the position and number of methyl substituents on the N-methyl-1,10-phenanthroline ring. For example, the more highly

substituted systems exhibit (1) higher affinity, (2) greater stabilization of the helix, and (3) higher viscosity upon binding to DNA. Moreover, selective binding to G-C sites (and/or combined G-C/A-T sites) by the more highly substituted aromatic cations is observed. These, as well as other effects, are discussed and the results can be accounted for in terms of reasonable structural models.

Results and Discussion--II

Indirect evidence has been obtained which suggests that the 2,4-dinitroaniline ring of the reporter molecule, 11 (Figure 35), intercalates between base-pairs of DNA, and the

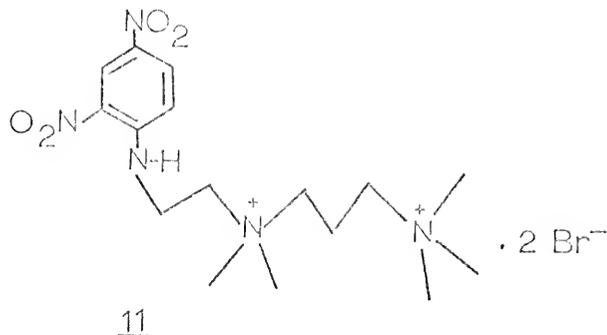


Figure 35. The 2,4-Dinitroaniline Reporter Molecule.

dispositively charged side chain lies in the minor groove⁹¹ (see introduction section). Recently, x-ray studies by Rosenberg et al.,⁹² showed that Na^+ lies in the minor groove of a Watson-Crick H-bonded dinucleoside phosphate. If the

above conclusions are correct, then increasing Na^+ concentration should lower the maximum number of DNA binding sites for reporter 11. Moreover, if basic proteins bind to the minor groove, then this effect should be reflected in the binding of reporter 11 to DNA and DNA-protein complexes.

The effect of increasing sodium ion concentration on the binding of 11 to salmon sperm DNA is shown in Figure 36, and the data summarized in Table 6. Several interesting

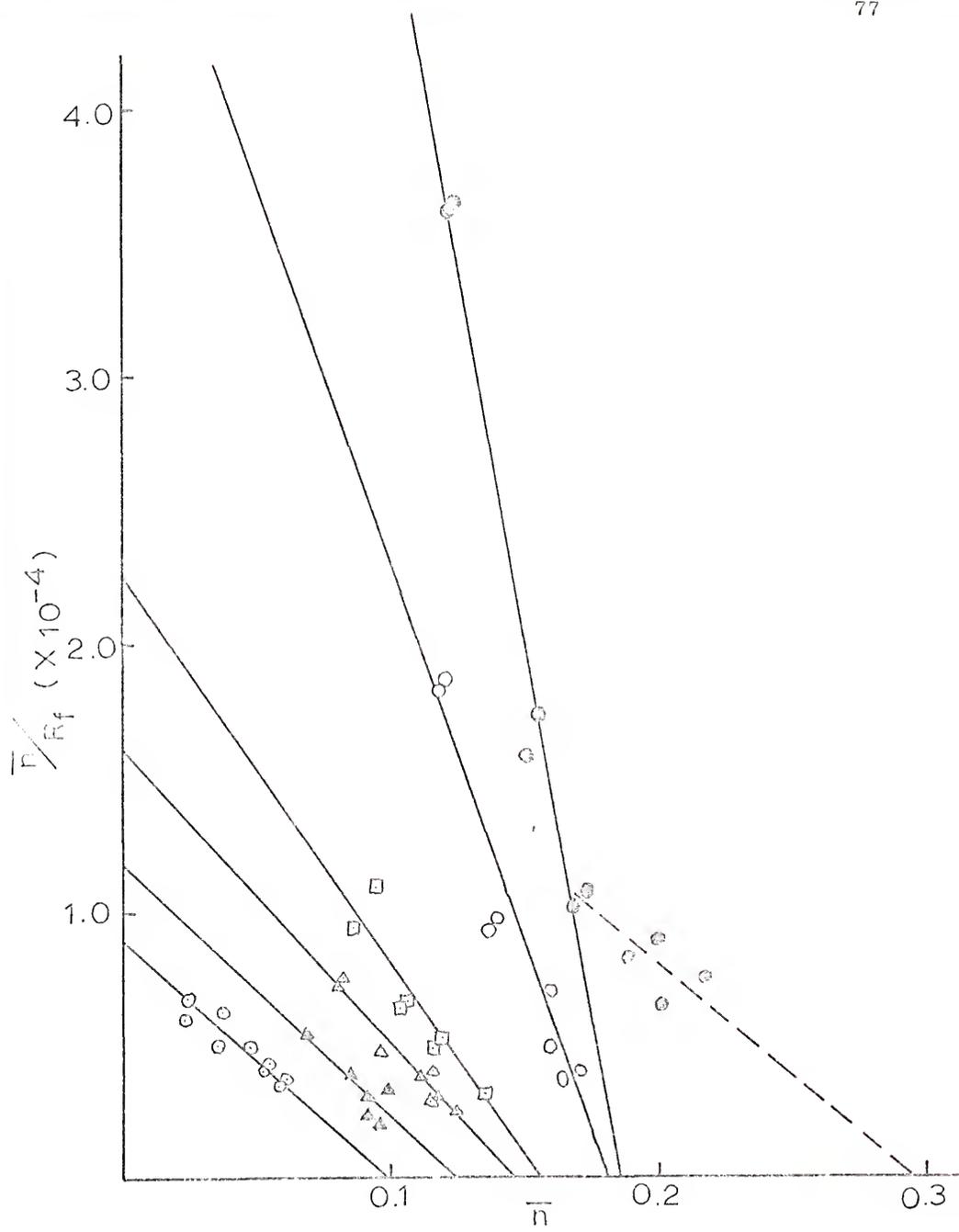
TABLE 6
The Effect of Sodium Ion on the
Binding of Reporter 11 to Salmon Sperm DNA.^a

Na^+	\bar{n}_{max}	K_a	$1/2\bar{n}_{\text{max}}^b$
0.005 M	0.190	612,000	2.63
0.01 M	0.182	280,000	2.77
0.02 M	0.155	144,000	3.22
0.03 M	0.147	101,000	3.40
0.04 M	0.125	96,000	4.00
0.05 M	0.100	89,500	5.00

^aEquilibrium dialysis studies were carried out in 0.01 M 2-(N-morpholino)ethane sulfonic acid buffer (MES) pH 6.2, using 2.43×10^{-4} M P/1 salmon sperm DNA. Reporter concentrations were varied from 2×10^{-5} to 6×10^{-5} M. ^bThe $1/2\bar{n}_{\text{max}}$ values represent the minimum number of base-pairs per binding site.

observations can be made. (1) At low salt concentration (0.005 M Na^+) the reporter molecule 11 exhibits two types of binding to salmon sperm DNA, namely, strong binding ($K_a = 612,000$), and weak binding ($K_a = 80,000$). The strong

Figure 36. The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of 11 with Salmon Sperm DNA at Varying Na^+ Concentrations, 0.005 M ($\circ-\circ-\circ$), 0.01 M ($\circ-\circ-\circ$), 0.02 M ($\square-\square-\square$), 0.03 M ($\Delta-\Delta-\Delta$), 0.04 M ($\Delta-\Delta-\Delta$), and 0.05 M ($\circ-\circ-\circ$).



binding is due to intercalation of the aromatic ring of 11 between base-pairs of DNA, and the low binding is presumably due to an external electrostatic mode of interaction.⁹³ In line with this interpretation is the fact that at higher salt concentrations (≥ 0.01 M Na^+) the secondary weak binding is not observed. Similar ionic strength effects have been reported for the interaction of DNA with ethidium bromide by Atkepisi and Kindelis.⁹⁴ (2) The binding affinity, K_a , and the maximum number of strong binding sites on salmon sperm DNA is dependent on the Na^+ concentration (Table 6). For example, K_a varies from 6.12×10^5 (in 0.005 M Na^+) to 8.95×10^4 (in 0.05 M Na^+) and the maximum number of strong binding sites, \bar{n}_{max} , varies from 0.19 to 0.10 (i.e., 1 reporter per 2.6 base-pairs to 1 reporter per 5.0 base-pairs). The above results indicate that Na^+ competes with reporter 11 for DNA binding sites (in the minor groove).

The interaction of 11 with DNA-basic protein complexes were examined. The results of the binding studies of reporter 11 to DNA and DNA-poly-L-lysine complexes are shown in Figure 37, and are summarized in Table 7. Several interesting observations may be made. (1) Poly-L-lysine affects the binding affinity, K_a , of 11 to salmon sperm DNA. For example, K_a at 0.005 M Na^+ is observed to be 6.12×10^5 . In the presence of 16 and 24 $\mu\text{g/ml}$ of poly-L-lysine, K_a is lowered to 2.12×10^5 and 2.05×10^5 , respectively. Similarly, the value of \bar{n}_{max} decreases from 0.19 (in the absence of

Figure 37. The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of 11 with Salmon Sperm DNA ($\square-\square-\square$), Salmon Sperm DNA + $16 \mu\text{g/ml}$ of Poly-L-lysine ($\circ-\circ-\circ$), and Salmon Sperm DNA + $24 \mu\text{g/ml}$ of Poly-L-lysine ($\Delta-\Delta-\Delta$).

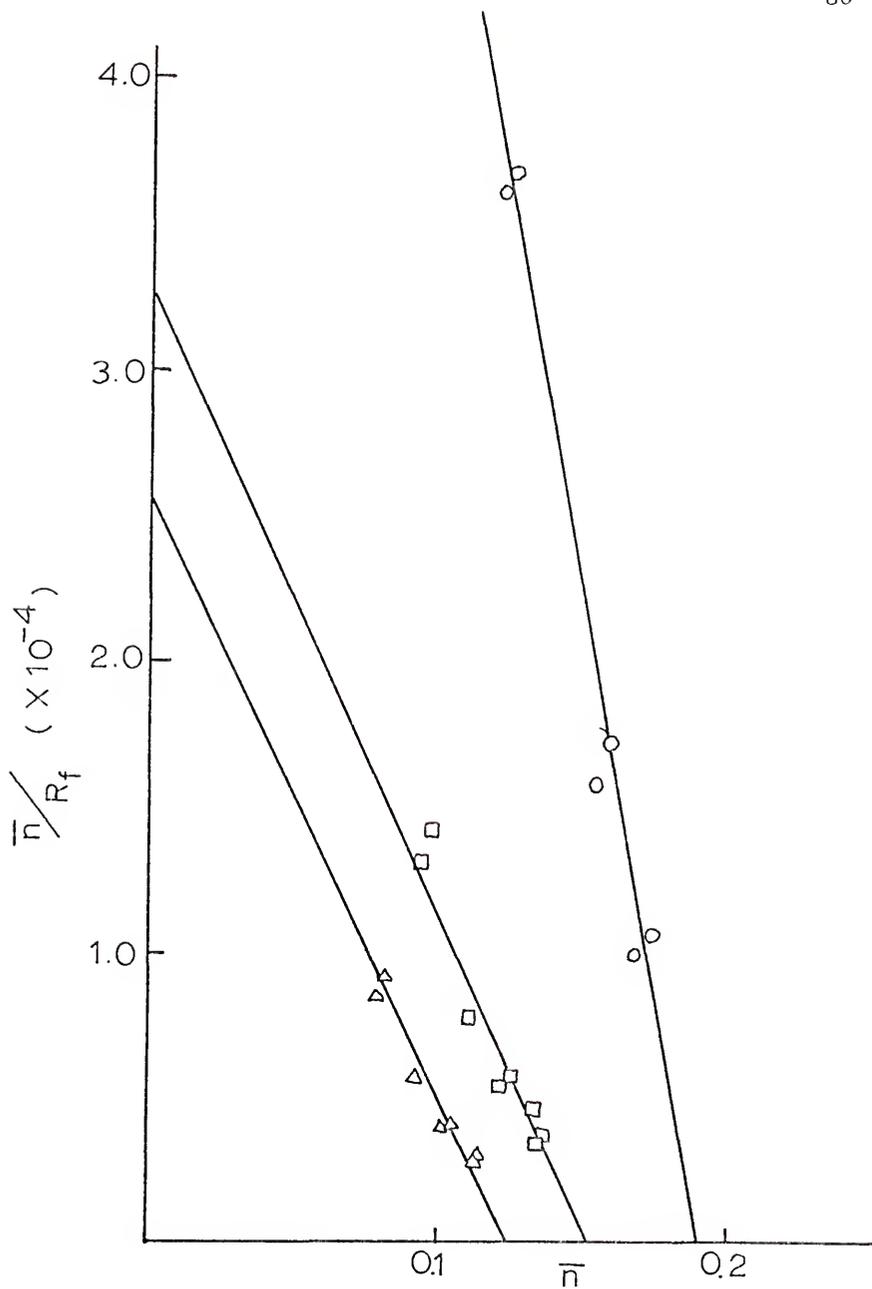


TABLE 7

The Effect of Basic Proteins on the Binding of Reporter 11 to Salmon Sperm and Calf Thymus DNA. ^a

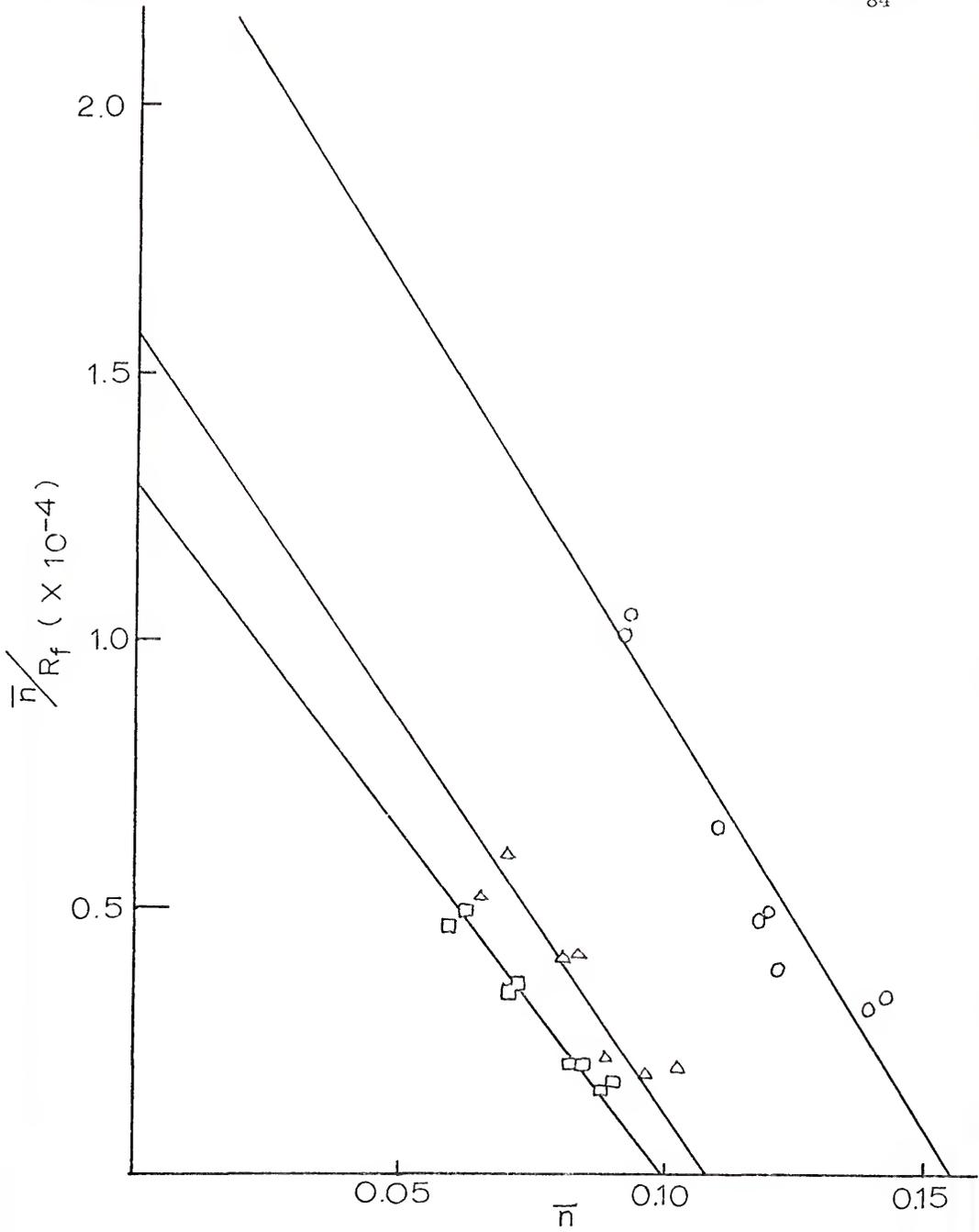
System	Na ⁺ Conc	Salmon Sperm DNA		Calf Thymus DNA	
		$\frac{1}{i_{\max}}$	$\frac{K_a}{1/2i_{\max}}$	$\frac{1}{i_{\max}}$	$\frac{K_a}{1/2i_{\max}}$
-	0.005	0.190	612,000	-	-
Polylysine(16 μ g/ml)	0.005	0.148	212,000	-	-
Polylysine(24 μ g/ml)	0.005	0.124	205,000	-	-
-	0.02	0.155	144,000	0.155	146,000
Histone II (60 μ g/ml)	0.02	0.100	140,000	0.098	132,000
Histone III(60 μ g/ml)	0.02	0.095	148,000	0.098	133,000
Histone IV (60 μ g/ml)	0.02	0.092	147,000	0.107	145,000

^aEquilibrium dialysis studies were conducted in the same conditions as in Table 6.

poly-L-lysine) to 0.124 in the presence of 24 $\mu\text{g/ml}$ of poly-L-lysine (i.e., from a maximal binding of 1 reporter molecule per 2.6 base-pairs in free DNA to 1 reporter in 4.1 base-pairs in the DNA-poly-L-lysine complex). (2) The effect of calf thymus histones (II, III, and IV) on the binding of II to calf thymus DNA (Figure 38 and Table 7) and to salmon sperm DNA (Table 7) shows similar results to those obtained with poly-L-lysine. For example, in 0.02 M Na^+ , maximal binding of 11 to calf thymus DNA, \bar{n}_{max} , decreases from a value of 0.155 to approximately 0.10 in the absence and presence of 60 $\mu\text{g/ml}$ of calf thymus Histones II, III, and IV. These \bar{n}_{max} values correspond to 1 reporter per 3.2 base-pairs of free DNA and 1 reporter per 5.0 base-pairs of DNA-histone complexes. The decrease in the value of \bar{n}_{max} in the presence of histones corresponds to a 35% loss in the maximum concentration of strong binding sites.

This work shows clearly that the basic proteins (poly-L-lysine and calf thymus Histones II, III, and IV) compete with reporter 11 for DNA binding sites. However, it is still unclear whether the observed lower number of maximum DNA-reporter binding sites in the presence of the basic protein arise from competition for the same site (minor groove) and/or via indirect interaction of the basic proteins that may cause structural changes in the DNA helix which might hinder the binding (i.e., intercalation) of 11 from the minor groove.

Figure 38. The Scatchard Plots of the Binding Studies Data Obtained by Equilibrium Dialysis Technique for Interactions of 11 with Calf Thymus DNA (O-O-O), Calf Thymus DNA + 60 $\mu\text{g/ml}$ Histone II (□-□-□), and Calf Thymus DNA + 60 $\mu\text{g/ml}$ Histone IV (Δ-Δ-Δ).



When native chromatin and reconstituted chromatin were subjected to the equilibrium dialysis technique, it was found that graphs of \bar{n}/R_f versus \bar{n} resulted in a localization of points through which a straight line could not possibly be drawn with any certainty. Because of this, the binding constant for each data point was calculated according to the following equation:

$$K = \frac{R_b}{(P_t - R_b)R_f}$$

where R_b represents the amount of bound reporter, R_f is the free reporter concentration, and P_t is the total phosphate concentration of DNA. Table 8 shows the constants and averages for native and reconstituted chromatin and free DNA. It is noted that the average binding constants are well within the range of each other in experimental error. No definitive conclusions can, therefore, be reached from these data.

An attempt was also made to study S chromatin, M chromatin, and free DNA from Hela S-3 cells with reporter 11 by equilibrium dialysis techniques. It was found (as in the native and reconstituted chromatin experiments) that a binding constant had to be calculated for each data point. A time study was done because it was found that no two experiments gave reproducible results. In fact, it was observed that in two separate time studies on S and M

TABLE 8

Binding Data of Reporter 11 to Native and Reconstituted Chromatin and Free DNA^a

<u>Native Chromatin</u>	<u>Reconstituted Chromatin</u>	<u>Free DNA</u>
<u>K</u>	<u>K</u>	<u>K</u>
2820	4720	3140
2240	2280	3040
2460	2460	2880
2370	2300	2780
2060	3650	4120
1320	2380	3550
1720	4200	2840
2890	2810	2950
2850	4240	5400
		5400
$K_{av} = 2410 \pm 520$	$K_{av} = 3230 \pm 870$	$K_{av} = 3610 \pm 820$

^aEquilibrium dialysis studies were carried out at 0°, in 0.01 M MES buffer, pH 6.2 and 0.02 M in Na⁺.

chromatin, over periods of 10 days, no reproducible data were obtained. It was found that reporter 11 had a consistently higher binding constant with S rather than M chromatin. This is in agreement with the concept that the DNA in S chromatin is more open and/or exposed than the DNA in M chromatin.

This effect is of some interest in that so many researchers use these chromatins. Yet, these chromatins will not give reproducible results with a proven technique. There are at least two explanations which will account for this phenomenon.

(1) Chromatin is not really "soluble" in aqueous solutions. If shaken thoroughly, a solution of chromatin will seem to be soluble, although possibly a little turbid or cloudy. In a twelve-hour time span, it is found that there is a very noticeable aggregation of molecules at the bottom of the solution. Such heterogeneity may account for the erratic results. (2) In the work-up procedures for isolating chromatin, it is very likely that proteolytic enzymes are left mixed with the chromatin. Slow degradation of the proteins in chromatin may also account for the erratic data.

In summary, it is found that poly-L-lysine and calf thymus Histones II, III, and IV affect the binding of reporter 11 to salmon sperm and calf thymus DNA. Whether there is a direct competition for the same site (minor groove) and/or via indirect interaction of the basic proteins to the major groove is undetermined.

Results and Discussion--III

In order to further understand the biological roles in which histones might be involved (e.g., the packaging of DNA and the repressing of certain genetic information), a series of DNA-basic protein complexes were made and studied by viscometric techniques. The effects of increasing concentration of poly-L-lysine and calf thymus Histones II, III, and IV on the viscosity of solutions of salmon sperm and calf thymus DNA were studied. The results are shown in Figures 39 and 40. Two interesting observations can be made. (1) The basic proteins cause considerable lowering of the viscosity of the DNA solutions. For example, 25 $\mu\text{g/ml}$ of poly-L-lysine decreases the relative specific viscosity, $\eta_{\text{sp}}^{\text{complex}}/\eta_{\text{sp}}^{\text{DNA}}$, of salmon sperm and calf thymus DNA to a value of 0.10. Calf thymus Histones III and IV at 60 $\mu\text{g/ml}$ also decrease the relative specific viscosity of calf thymus DNA to a value of 0.10. The magnitude of the decrease in relative specific viscosity (approaching the specific viscosity of buffer) is supporting evidence for the role in which histones might play in compacting and packaging DNA. Sanford⁹⁵ observed that L-lysyl-L-phenylalanine amide (6.0×10^{-4} M) was effective in decreasing the relative specific viscosity of salmon sperm DNA to 0.55 (see Figure 16). A non-classical intercalation model was proposed whereby the aromatic residue is partially inserted between base-pairs, causing a slight bending of the DNA molecule at the point of complexation.

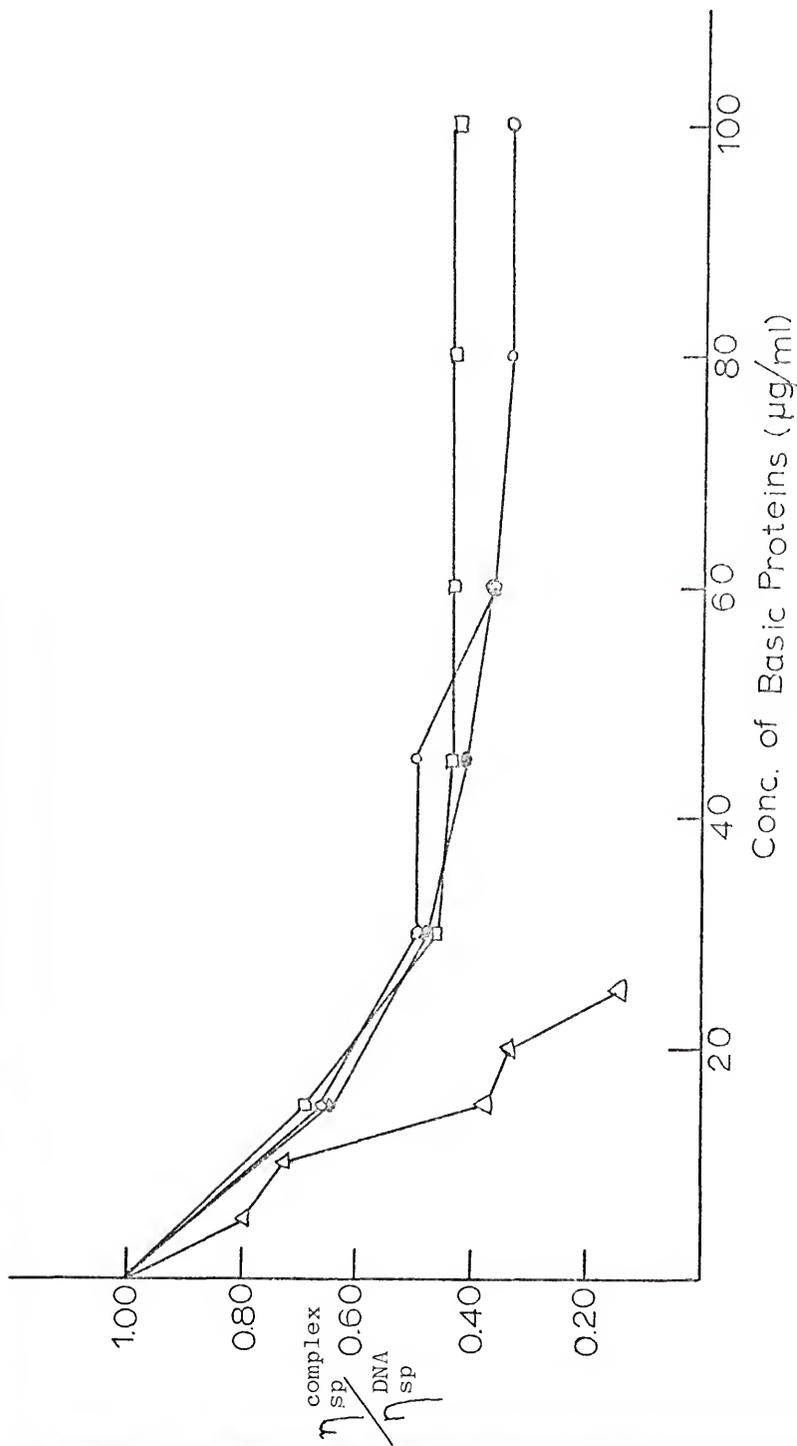


Figure 39. The Effect of Increasing Concentrations of Basic Proteins on the Relative Specific Viscosity of a 2.44×10^{-4} M P/1 Salmon Sperm DNA Solution. The study was performed in 0.01 M MES, pH 6.2 and 0.02 M Na^+ . Poly-L-lysine (Δ - Δ - Δ), Histone II (\circ - \circ - \circ), Histone III (\square - \square - \square), Histone IV (\circ - \circ - \circ).

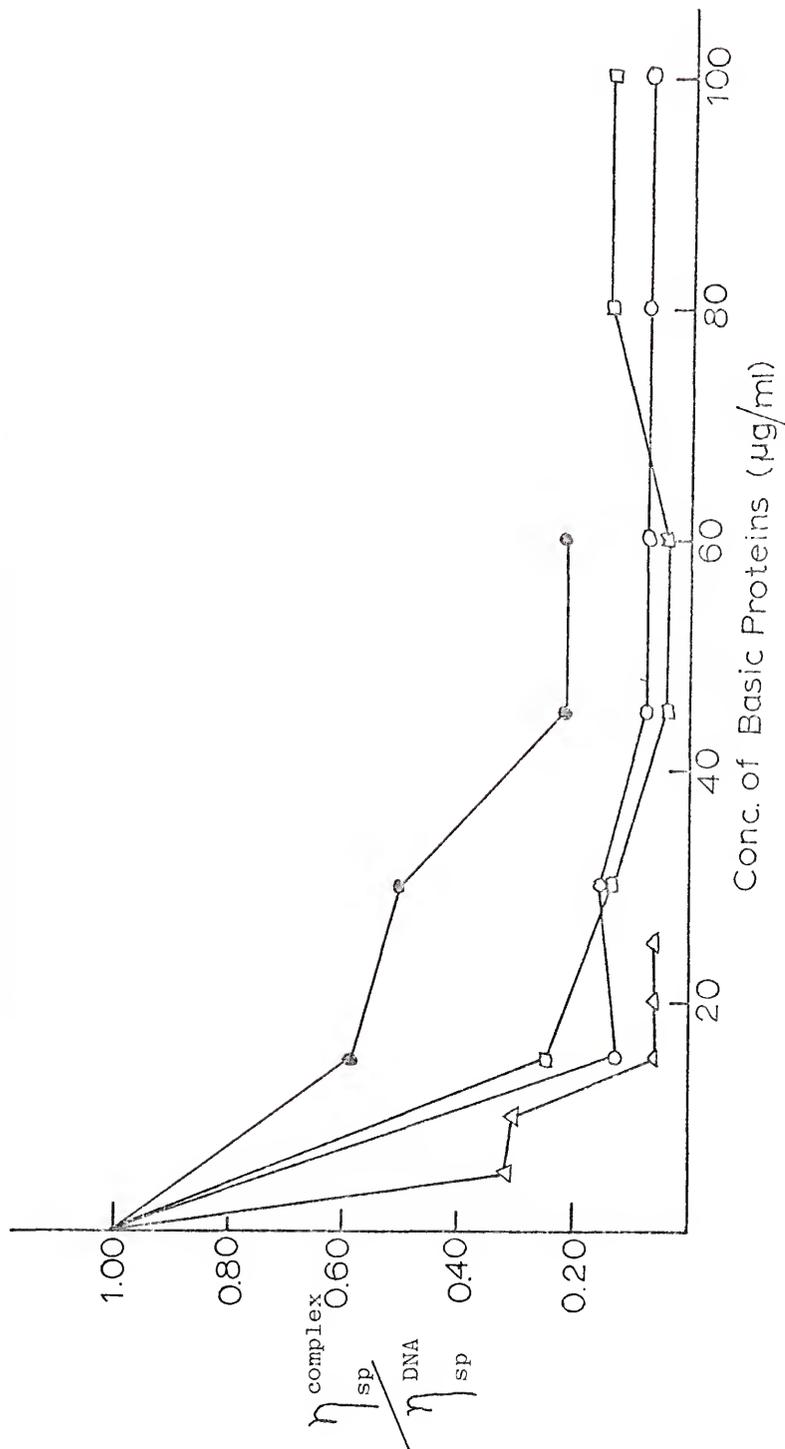


Figure 40. The Effect of Increasing Concentrations of Basic Proteins on the Relative Specific Viscosity of a 2.84×10^{-4} M P/1 Calf Thymus DNA Solution. The study was performed in 0.01 M MES, pH 6.2 and 0.02 M Na^+ . Poly-L-lysine (Δ - Δ - Δ), Histone II (\bullet - \bullet - \bullet), Histone III (\square - \square - \square), Histone IV (\circ - \circ - \circ).

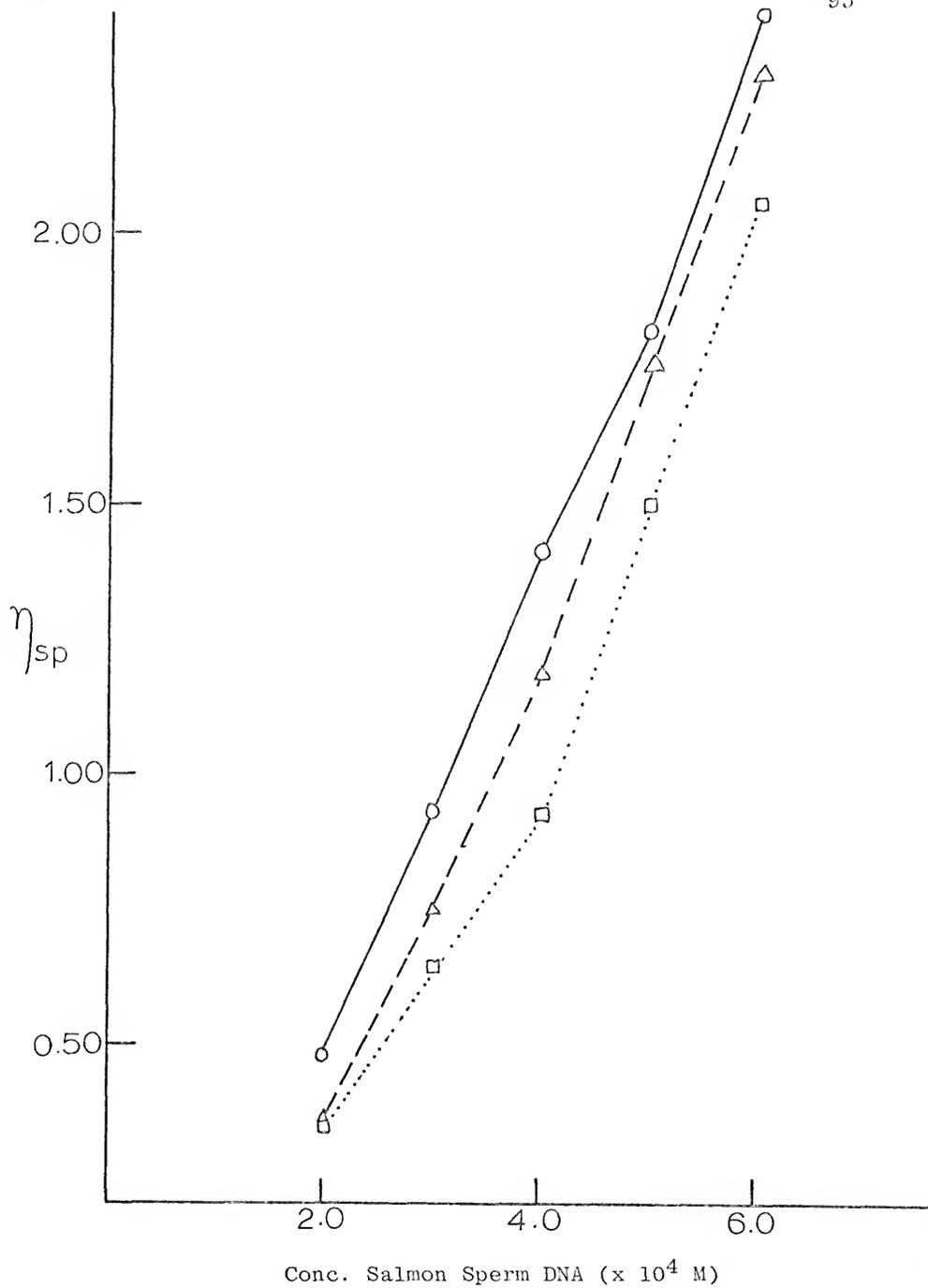
The overall effect is a reduction in the length of DNA which results in a decrease in the specific viscosity of the solution. The present studies show that poly-L-lysine and the calf thymus Histones II, III, and IV are more effective than a dipeptide in decreasing the relative specific viscosity of DNA. The magnitude of the decrease can be explained in terms of electrostatic constriction of the polymer and/or via a bending mechanism. (2) The calf thymus Histones II, III, and IV do not affect the relative specific viscosity of calf thymus and salmon sperm DNA to the same extent. For example, 60 $\mu\text{g}/\text{ml}$ of Histone III decreases the relative specific viscosity of salmon sperm and calf thymus DNA to 0.40 and 0.10, respectively. The differing effects can be explained in at least three ways. (i) Histone III is able to recognize different sequences in the two DNAs. Such specific recognition might lead to differences in the folding or bending of the double helix and, hence, to different relative viscosities. Some researchers^{96,97,98} have suggested that an aromatic amino acid (e.g., phenylalanine or tyrosine) of a protein may intercalate between base-pairs in DNA and may serve as an "anchor" to prevent slippage of the protein along the DNA helix. Work by Gabbay et al.,⁹⁹ who studied the interactions of 70 different di-, tri-, and tetrapeptides and di-, tri-, and tetrapeptide amides to DNA of various base compositions, supported this "anchoring" mechanism. In addition, the results of pmr, viscosity, CD, Tm, and equilibrium

dialysis suggested that not only site-specific intercalation of the aromatic residue of the peptides, but also a dependence on the primary structure, was involved. On this basis, a "selective bookmark" hypothesis was proposed whereby the "bookmarks" (i.e., the aromatic residues of the proteins) could recognize the "pages of the book" (i.e., the intercalating sites). A "selective bookmark" mechanism would adequately explain the histones' capability of recognizing DNA sequences and, therefore, affecting relative specific viscosities. Such selective recognition of the DNA helix by histones supports the "repressing" role in which histones might be involved. (ii) The differing effects may be explained by different molecular weights of the DNAs. For example, if calf thymus DNA is of a much higher molecular weight than salmon sperm DNA, then a folding of the higher molecular weight DNA will lead to a greater decrease in viscosity than a similar folding of the lower molecular weight DNA. However, there are at least two objections to this explanation. (a) Poly-L-lysine affects both calf thymus and salmon sperm DNA to the same extent. (b) The specific viscosities, η_{sp} , of calf thymus and salmon sperm DNA at the same concentration are the same. The viscosity of a DNA solution is dependent on the molecular size and conformation of DNA. Since the conformation of salmon sperm and calf thymus DNA is assumed to be the rod-like Watson-Crick-Wilkins double helix, then the molecular size of the salmon sperm and calf thymus DNA must be similar. (iii) The differences in relative specific

viscosities may also be attributed to different binding associations of the histones to DNA (i.e., Histone III may be bound to a different extent to salmon sperm and calf thymus DNA). The extent to which poly-L-lysine and Histones II, III, and IV are bound to calf thymus and salmon sperm DNA is presently being studied.

In order to investigate the exchange of protein between DNA helices, various DNA and DNA-protein solutions were studied by viscosity (as described in the Introduction--Problem III). The effects of increasing salmon sperm and calf thymus DNA with a constant Histone III concentration ($30 \mu\text{g/ml}$) on the specific viscosity (at 10° , in 0.01 M MES , $\text{pH } 6.2$ and 0.02 M Na^+) are shown in Figures 41 and 42, respectively. The solid lines represent the η_{sp} of the DNA alone and the dotted lines represent the η_{sp} of the DNA-Histone III complex. The points comprising the dashed lines represent the same concentrations of the points comprising the dotted line, but made differently. For example, uncomplexed DNA was added to a DNA-basic protein complex just prior to the viscosity reading. Therefore, if there was immediate exchange of Histone III between DNA helices, the dotted and dashed lines would overlap. It is evident that at 10° , in 0.01 M MES , $\text{pH } 6.2$ and 0.02 M Na^+ , Histone III does not exchange completely between either salmon sperm or calf thymus DNA. Under the same conditions except at 37.5° (Figures 43 and 44), it is evident that complete exchange does not occur. Unfortunately, the extent to which exchange might take place cannot be determined by this method.

Figure 41. The Effect of Increasing Concentration of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 (—) and 30 $\mu\text{g}/\text{ml}$ (---- and.....) Histone III. The exchange study was performed in 0.01 M MES, pH 6.2, 0.02 M Na^+ and at 10° .



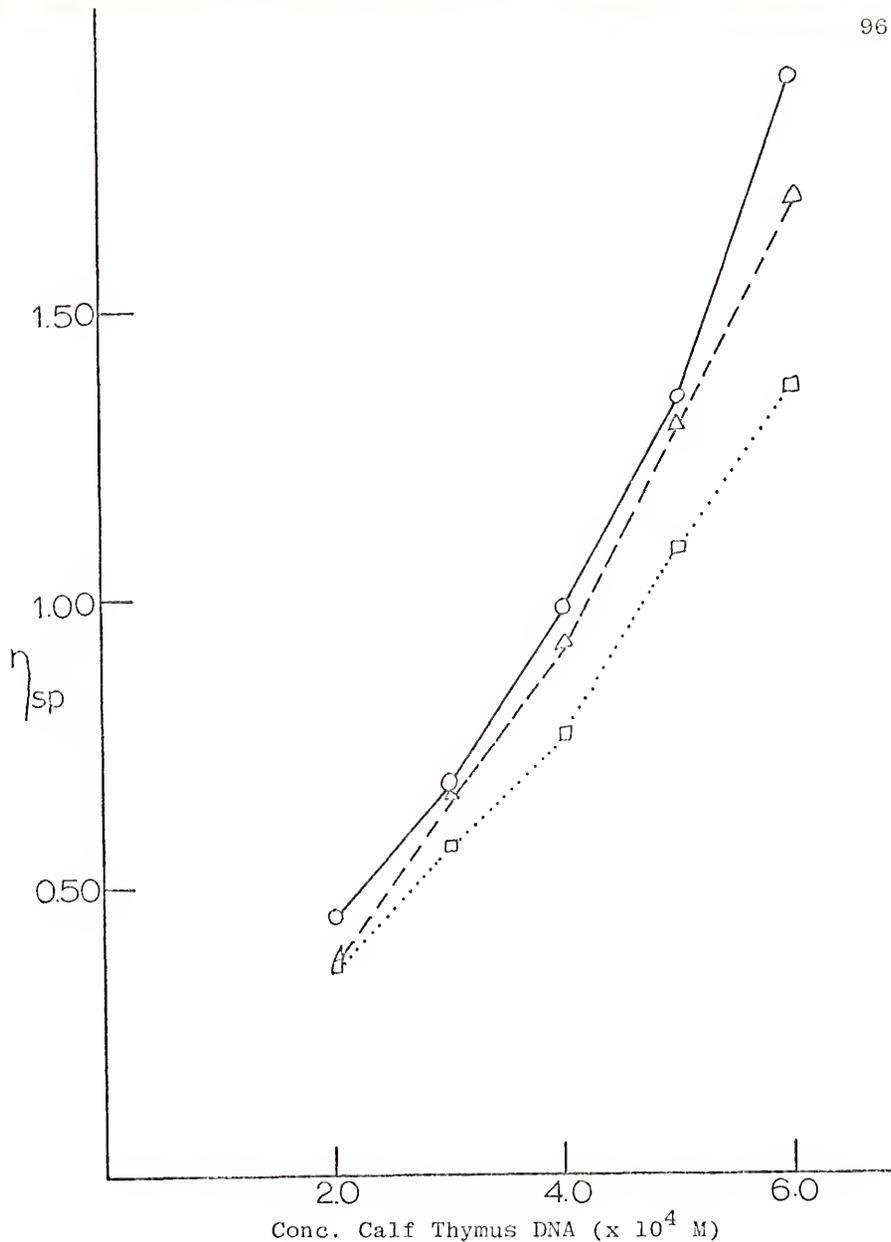


Figure 42. The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 (—) and 30 $\mu\text{g/ml}$ (----and.....) Histone III. The exchange study was performed in 0.01 M MES, pH 6.2, 0.02 M Na^+ and at 10° .

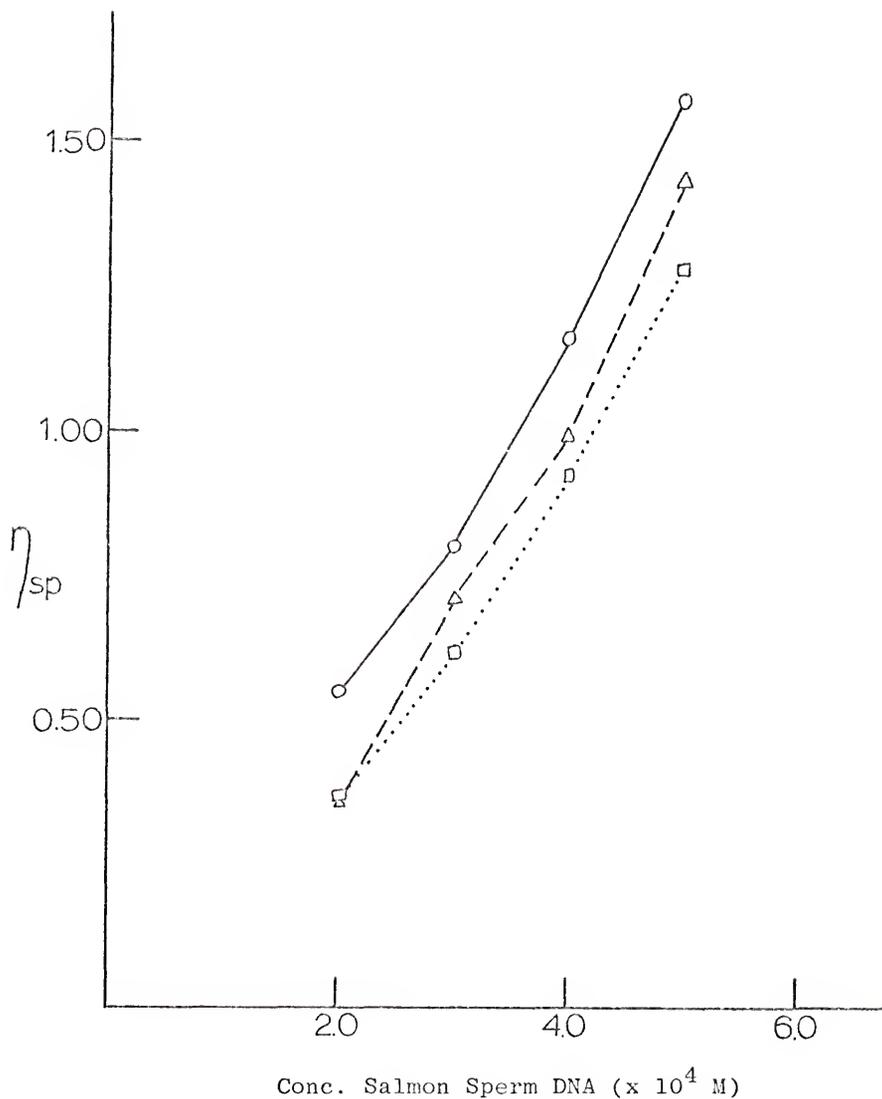


Figure 43. The Effect of Increasing Concentration of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 (—) and 30 $\mu\text{g/ml}$ (---and....) Histone III. The exchange study was performed in 0.01 M MES, pH 6.2, 0.02 M Na^+ and at 37.5°.

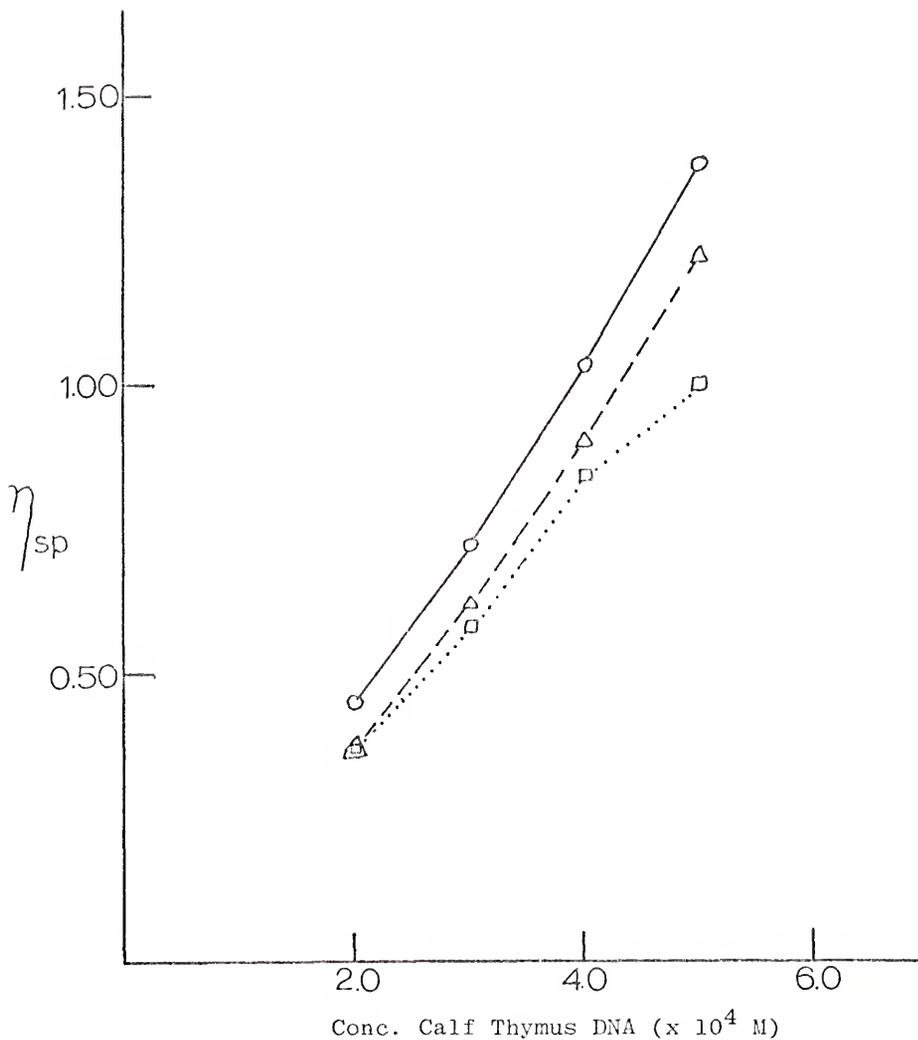


Figure 44. The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 (—) and 30 $\mu\text{g/ml}$ (---and....) Histone III. The exchange study was performed in 0.01 M MES, pH 6.2, 0.02 M Na^+ and at 37.5°.

It was necessary to establish whether a polycation (poly-L-lysine) would exchange under the conditions of the experiment. The effects of increasing calf thymus and salmon sperm DNA with a constant poly-L-lysine concentration of $10 \mu\text{g/ml}$ on the specific viscosity (at 10^0 , in 0.01 M MES, pH 6.2 and 0.02 M Na^+) are shown in Figures 45 and 46. It is evident that for both DNAs, the dashed and dotted lines overlap and, therefore, complete exchange of poly-L-lysine between DNA helices has occurred. This lends support to the assumption that histones, with aromatic and/or hydrophobic residues which can intercalate between base-pairs of DNA, are decidedly more "sticky" and are less apt to exchange between DNA helices than a polycation which binds principally via electrostatic interaction.

In order to establish the effect of Na^+ concentration on the DNA-Histone III complex, an exchange study was performed at 0.10 M Na^+ . The effects of increasing DNA concentration with a constant Histone III concentration of $30 \mu\text{g/ml}$ on the specific viscosity (at 10^0 , in 0.01 M MES, pH 6.2 and 0.10 M Na^+) are shown in Figures 47 and 48. It is evident that Histone III freely exchanges between calf thymus DNA helices, but not between salmon sperm DNA helices. It is reasonable to assume that the increased Na^+ concentration weakens the binding of Histone III to calf thymus DNA to a greater extent than to salmon sperm DNA.

In summary, viscosity studies, involving various poly-L-lysine and histone-DNA complexes, showed selective interactions

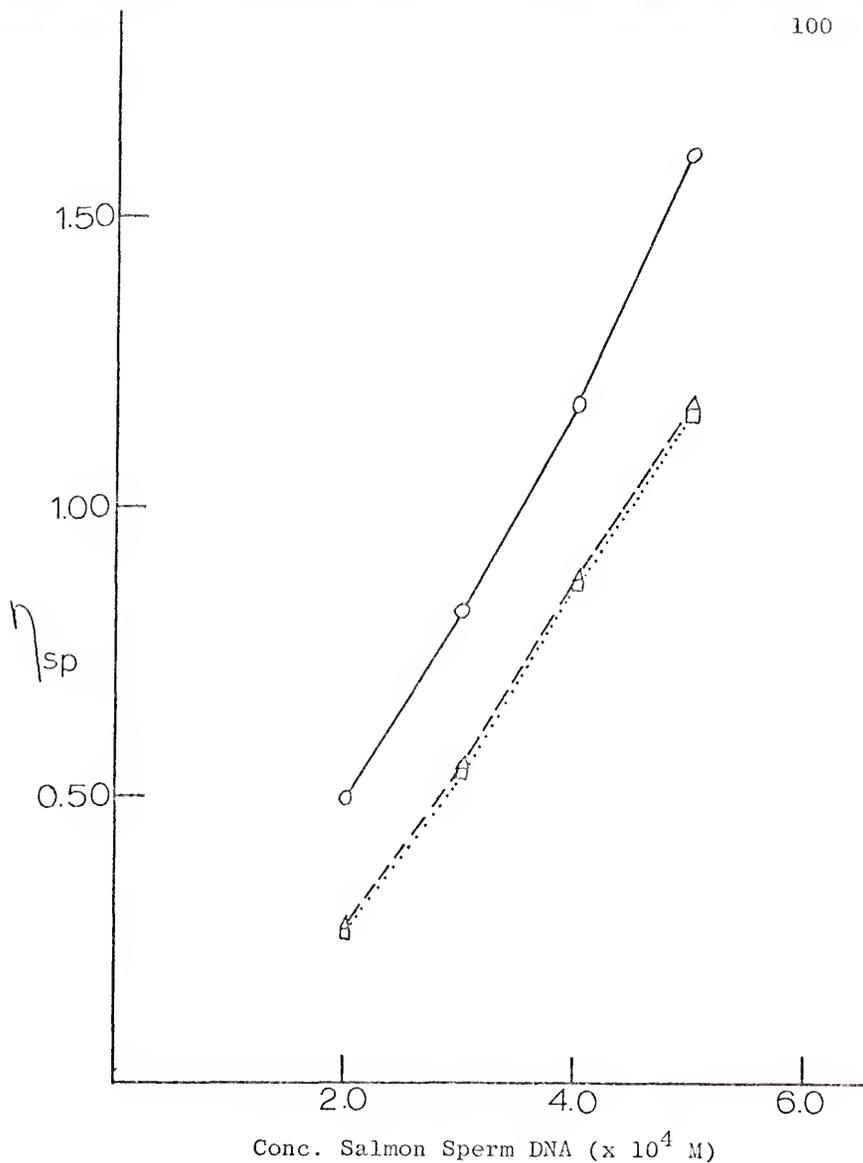


Figure 45. The Effect of Increasing Concentration of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 (—) and 10 $\mu\text{g/ml}$ (- - - and) Poly-L-lysine. The exchange study was performed in 0.01 M MES, pH 6.2, 0.02 M Na^+ and at 10° .

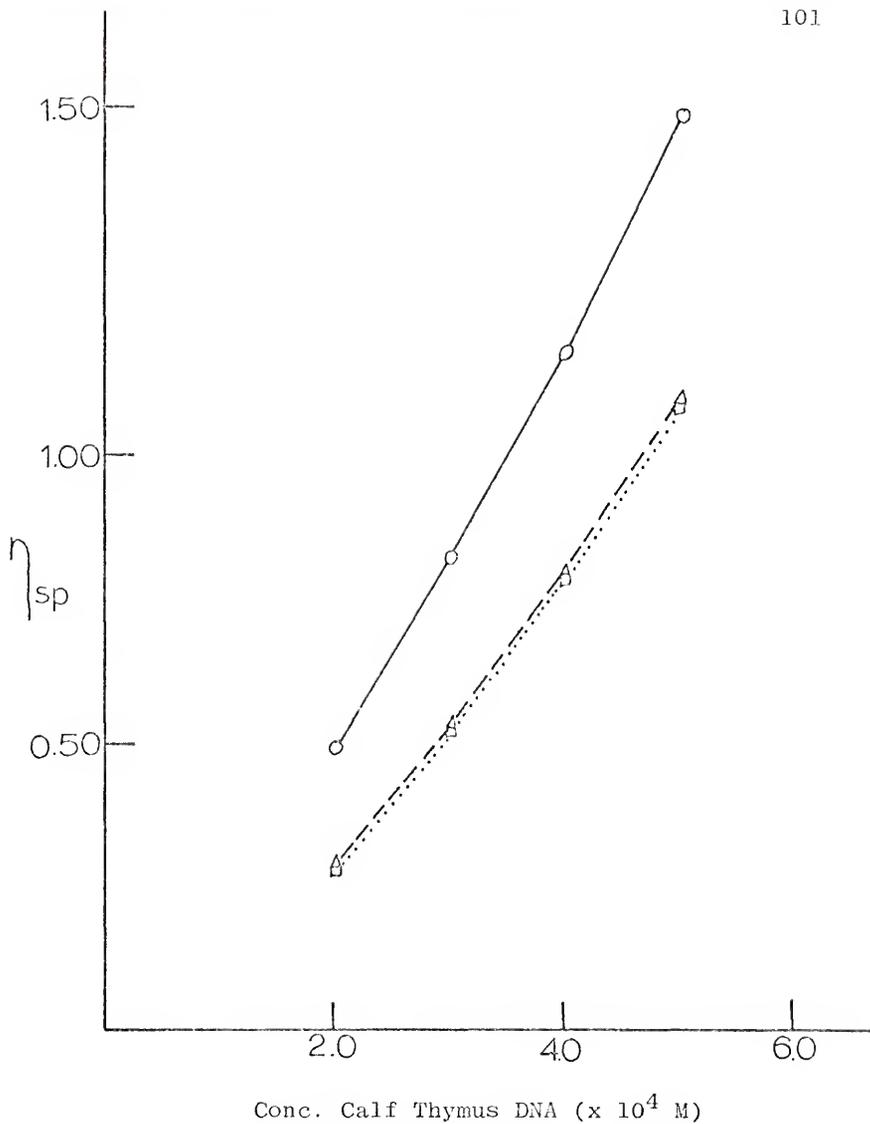


Figure 46. The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 (—) and 10 $\mu\text{g/ml}$ (- - - and ····) Poly-L-lysine. The exchange study was performed in 0.01 M MES, pH 6.2, 0.02 M Na^+ and at 10^0 .

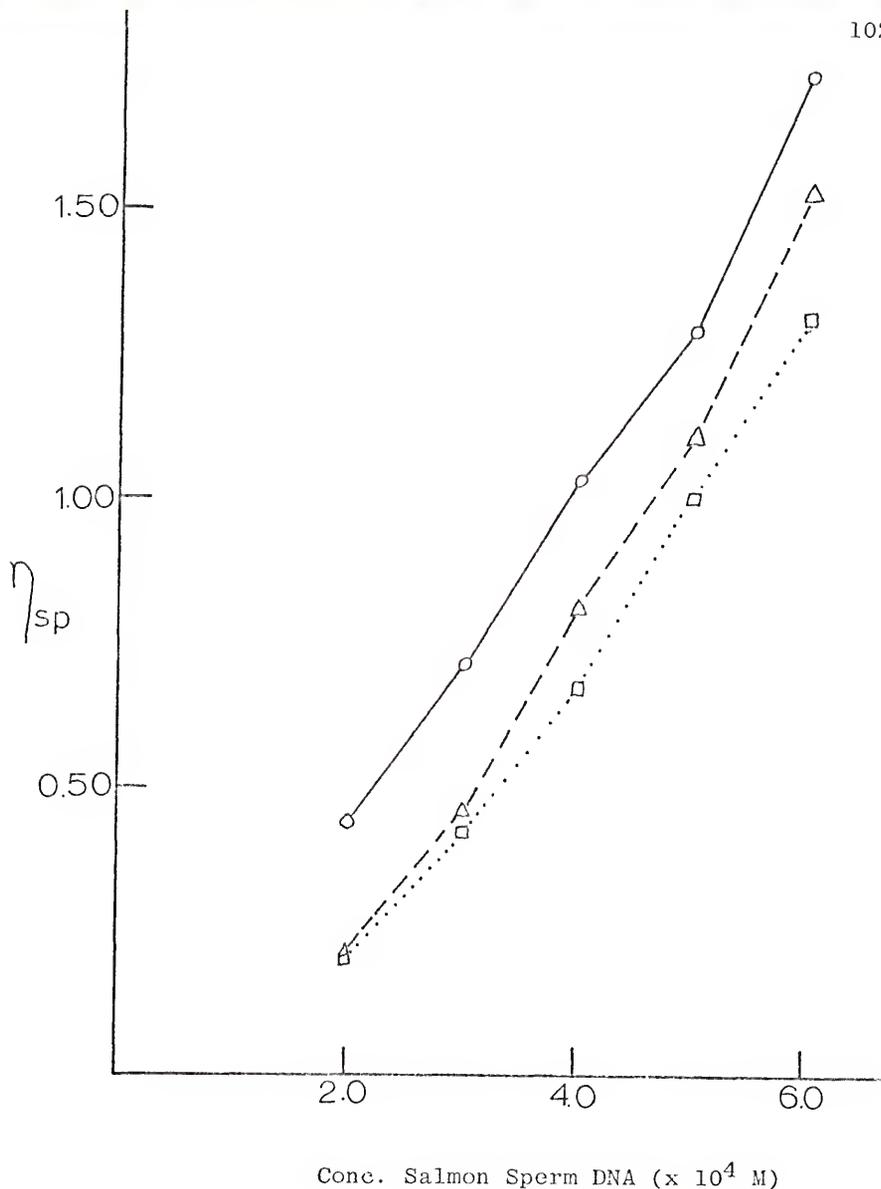


Figure 47. The Effect of Increasing Concentrations of Salmon Sperm DNA on the Specific Viscosity of Solutions with 0 (—) and 30 $\mu\text{g/ml}$ (---and····) Histone III. The exchange study was performed in 0.01 M MES, pH 6.2, 0.10 M Na^+ and at 10° .

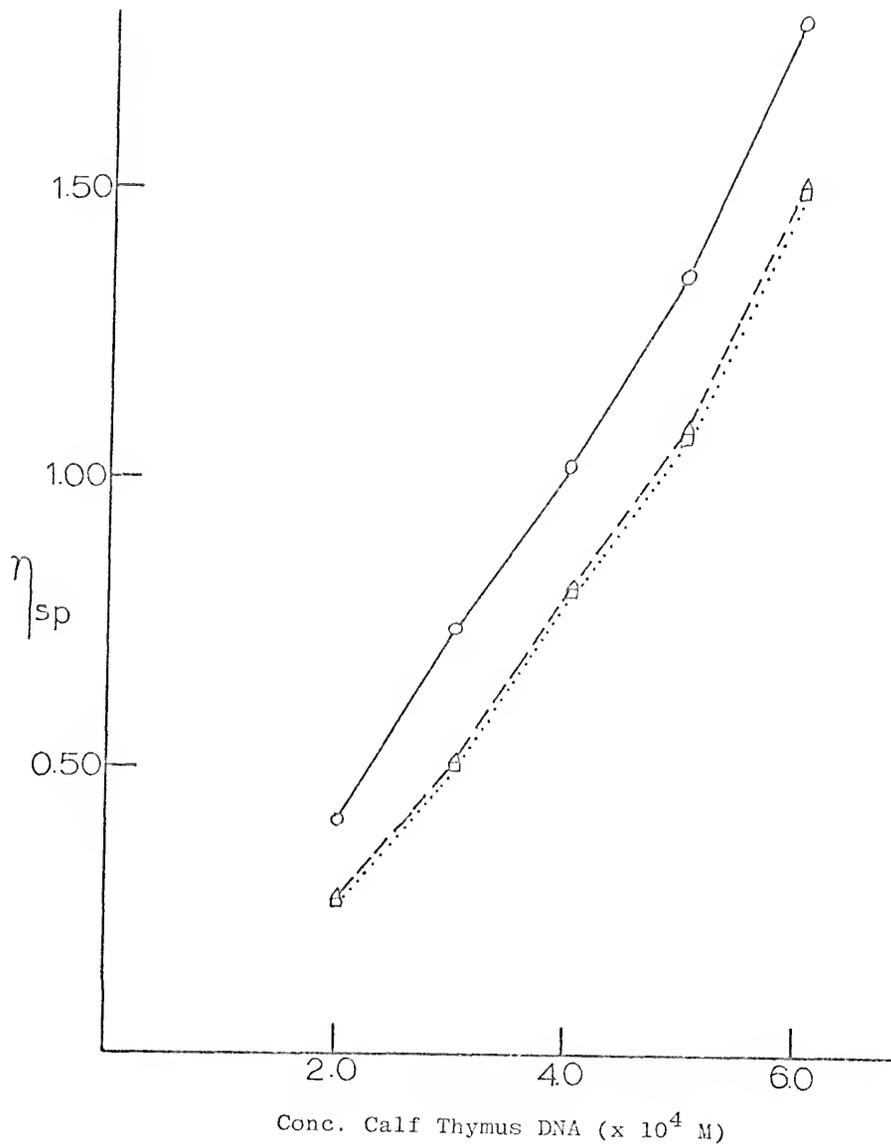


Figure 48. The Effect of Increasing Concentration of Calf Thymus DNA on the Specific Viscosity of Solutions with 0 (—) and 30 $\mu\text{g/ml}$ (- - - and ····) Histone III. The exchange study was performed in 0.01 M MES, pH 6.2, 0.10 M Na^+ and at 10°.

of the basic proteins to DNA as evidenced by the different decreases in relative specific viscosities. Exchange of Histone III and poly-L-lysine between DNA helices was monitored by a viscometric technique. It was found that at 10° , in 0.01 M MES, pH 6.2 in 0.02 M Na^{+} , poly-L-lysine freely exchanged, but Histone III did not. Further evidence for selective interactions of proteins for DNA was found when exchange studies at 10° , in 0.01 M MES, pH 6.2 in 0.10 M Na^{+} , showed Histone III freely exchanged between calf thymus DNA but not between salmon sperm DNA helices.

EXPERIMENTAL

Viscosity studies were performed on a low-shear Zimm Viscometer (Beckman Instrument Company). A number of viscometric experiments were performed by Mr. William S. Barksdale, III. Circular dichroism spectra were taken on a Jasco J-20 spectropolarimeter and the molar ellipticities were measured relative to a standard ellipticity of a D-camphor sulfonic acid solution. Melting temperature studies (helix to coil) were performed on a Gilford 240 spectrometer equipped with a temperature programmer (Neslab Instruments), a Honeywell recorder, and a Leeds and Northrup potentiometer. Proton magnetic resonance (pmr) spectra were recorded on either a Varian XL-100 (spectra were run by Dr. C. S. Baxter) or a Varian A60-A, both equipped with a variable temperature probe. Tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentane sulfonate (DSS) was used as standards in non-aqueous and aqueous solutions, respectively. A Beckman Zeromatic II was used in the determination of pH. Infrared spectra were taken on either a Perkin-Elmer 137 or 337 or a Beckman IR-10 infrared spectrophotometer. Ultraviolet and visible absorption work to determine extinction coefficients and concentrations were done on either a Cary 15 or a Gilford 240. Absorption studies involving the equilibrium dialysis technique were done on the Gilford 240. Melting

points of compounds were taken on either a Mel-Temp or a Thomas Hoover apparatus and were uncorrected. Compounds sent for carbon and hydrogen analysis (Atlantic Microlabs, Incorporated) were first dried on a Virtis freeze dryer.

Salmon sperm and calf thymus DNA were bought from the Worthington Biochemical Corporation. Micrococcus luteus and poly dG-poly dC were bought from Miles Laboratories, Incorporated. Poly d(A-T)-poly d(A-T) and Histones II, III, and IV were purchased from the Sigma Chemical Company. Poly-L-lysine (MW 38,000) was obtained from Pilot Chemicals, Incorporated.

The DNAs were stored at 0°C. For a given study, a stock solution of the DNA was prepared (2.5 mg/ml) in a 0.01 M 2(N-morpholino) ethane sulfonic acid (MES) buffer, pH 6.2, 0.005 M Na⁺ and stored at 0°C. This stock solution was then diluted to the desired concentration.

The native, reconstituted, S and M chromatins were obtained from Dr. Gary Stein.

The N-methyl-1,10-phenanthroline cations, I (as the chloride salts), were synthesized from the respective parent phenanthroline system by alkylation with methyl iodide, followed by conversion of the iodide to the chloride.¹⁰⁰ The parent 1,10-phenanthrolines were synthesized according to previously published procedures.^{101,102,103}

ReactionsSynthesis of N-Methyl-1,10-Phenanthroline Chloride, 1

In a sealed tube, 1.0 gm (5.5 mmole) of 1,10-phenanthroline (Aldrich Chemical Company, Incorporated) was placed with methyl iodide, 2.35 gm (16.5 mmole), and heated to 90° for 12 hours. The excess methyl iodide was evaporated under a stream of nitrogen. The solid was taken up in a minimum of hot ethanol and a few drops of ethyl ether were added until the solution became turbid. The solution was allowed to cool and 1.5 gm (4.7 mmole) of yellow solid were collected by filtration: mp 200-201° (lit. value, 200-203°);¹⁰⁴ yield 85%. Anal. calculated for C₁₃H₁₁N₂I: C, 48.46; H, 3.45. Found: C, 48.52; H, 3.58.

To a stirring solution of 1.0 gm (3.1 mmole) of N-methyl-1,10-phenanthroline iodide in ethanol was added an excess of freshly precipitated silver chloride. (The silver chloride was precipitated from a mixture of silver nitrate and sodium chloride solutions). The solution was stirred for 30 minutes and then filtered. To the ethanol solution was added a few drops of ethyl ether and the solution cooled. After one hour, 700 mg (3.0 mmole) of white solid, 1, were collected by filtration: mp 171-172°; yield 98%. The ultraviolet spectrums in H₂O showed maxima at 218 nm ($\epsilon = 3.85 \times 10^4$) and 271 nm ($\epsilon = 3.55 \times 10^4$). The XL-100 pmr spectrum of the N-methyl cation, 1, showed an eight-proton multiplet centered at

820 Hz (aromatic protons) and a three-proton singlet at 531 Hz (N-methyl protons). Anal. calculated for $C_{13}H_{11}N_2Cl \cdot O \cdot 1H_2O$: C, 67.14; H, 4.86. Found: C, 66.90; H, 4.95.

Synthesis of N-Methyl-2,9-Dimethyl-1,10-Phenanthroline Chloride, 2

As described for compound 1, 1.0 gm (4.8 mmole) of neocuproine (2,9-dimethyl-1,10-phenanthroline), purchased from the G. Frederick Smith Chemical Company, was treated with methyl iodide, 1.95 gm (14.4 mmole). Upon work-up, 1.4 gm (4.0 mmole) of the iodide salt was collected by filtration: mp 195-196^o; yield 83%. Anal. calculated for $C_{15}H_{15}N_2I$: C, 51.44; H, 4.33. Found: C, 51.39; H, 4.28.

To 1.0 gm (2.9 mmole) of N-methyl-2,9-dimethyl-1,10-phenanthroline iodide stirring in ethanol was added freshly precipitated silver chloride as described for compound 1. Work-up yielded 700 mg (2.7 mmole) of white solid, 2: mp 181-182^o; yield 93%. The ultraviolet spectrum in H_2O showed maxima at 220 nm ($\epsilon = 3.63 \times 10^4$) and 282 nm ($\epsilon = 3.16 \times 10^4$). The XL-100 pmr spectrum of 2 showed a six-proton multiplet centered at 800 Hz (aromatic protons), a three-proton singlet at 499 Hz (N-methyl protons), and two three-proton singlets at 285 and 313 Hz (ring methyl protons). Anal. calculated for $C_{15}H_{15}N_2Cl \cdot O \cdot 3H_2O$: C, 68.19; H, 5.97. Found: C, 67.98; H, 5.74.

Synthesis of N-Methyl-5,6-Dimethyl-1,10-Phenanthroline Chloride, 3

Methyl iodide 1.95 gm (14.4 mmole) was added to 1.0 gm (4.8 mmole) of 5,6-dimethyl-1,10-phenanthroline as described for compound 1. All of the iodide was converted to 800 mg (3.1 mmole) of the chloride salt, 3: mp 162-163^o; yield 65%. The ultraviolet spectrum in H₂O showed maxima at 209 nm ($\epsilon = 3.16 \times 10^4$), 223 nm ($\epsilon = 2.93 \times 10^4$), and 283 nm ($\epsilon = 3.09 \times 10^4$). The XL-100 pmr spectrum of 3 showed a six-proton multiplet centered at 850 Hz (aromatic protons), a three-proton singlet at 510 Hz (N-methyl protons), and two three-proton singlets at 261 and 269 Hz (ring methyl protons). Anal. calculated for C₁₅H₁₅N₂Cl·1.1H₂O: C, 64.67; H, 6.18. Found: C, 64.58; H, 6.20.

Synthesis of N-Methyl-2,5,6,9-Tetramethyl-1,10-Phenanthroline Chloride, 4

As described for compound 1, 1.0 gm (4.2 mmole) of 2,5,6,9-tetramethyl-1,10-phenanthroline was alkylated with methyl iodide, 1.78 gm (12.6 mmole), and converted to 950 mg (3.3 mmole) of the chloride salt, 4: mp 175-176^o; yield 79%. The ultraviolet spectrum in H₂O showed maxima at 222 nm ($\epsilon = 3.15 \times 10^4$), 229 nm ($\epsilon = 3.28 \times 10^4$), and 292 ($\epsilon = 3.47 \times 10^4$). The XL-100 pmr spectrum of 4 showed a four-proton multiplet centered at 815 Hz (aromatic protons), a three-proton singlet at 481 Hz (N-methyl protons), and four three-proton singlets at 307, 278, 255, and 252 Hz (ring methyl protons). Anal. calculated for C₁₇H₁₉N₂·O·3H₂O: C, 69.87; H, 6.76. Found: C, 69.89; H, 6.61.

Synthesis of N-Methyl-3,8-Dimethyl-1,10-Phenanthrolium Chloride, 5

As described for compound 1, 1.0 gm (4.8 mmole) of 3,8-dimethyl-1,10-phenanthroline was alkylated with methyl iodide, 1.95 gm (14.4 mmole), and converted to 825 mg (3.2 mmole) of the chloride salt, 5: mp 171-172^o; yield 67%. The ultraviolet spectrum in H₂O showed maxima at 212 nm ($\epsilon = 3.31 \times 10^4$), 226 nm ($\epsilon = 3.38 \times 10^4$), and 274 nm ($\epsilon = 3.30 \times 10^4$). The XL-100 pmr spectrum of 5 showed a six-proton multiplet centered at 820 Hz (aromatic protons), a three-proton singlet at 510 Hz (N-methyl protons), and two three-proton singlets at 262 and 273 Hz (ring methyl protons). Anal. calculated for C₁₅H₁₅N₂Cl·O·9H₂O: C, 65.53; H, 6.12. Found: C, 65.56; H, 6.11.

Synthesis of N-Methyl-3,5,6,8-Tetramethyl-1,10-Phenanthrolium Chloride, 6

As described for compound 1, 1.0 gm (4.2 mmole) of 3,5,6,8-tetramethyl-1,10-phenanthroline was alkylated with methyl iodide, 1.78 gm (12.6 mmole), and converted to 750 mg (2.6 mmole) of the chloride salt, 6: mp 175-176^o; yield 62%. The ultraviolet spectrum in H₂O showed maxima at 218 nm ($\epsilon = 3.06 \times 10^4$), 223 nm ($\epsilon = 3.54 \times 10^4$), and 288 nm ($\epsilon = 3.83 \times 10^4$). The XL-100 pmr spectrum of 6 showed a four-proton multiplet centered at 840 Hz (aromatic protons), a three-proton singlet at 488 Hz (N-methyl protons), and four three-proton singlets at 262, 270, 276, and 289 Hz (ring methyl protons). Anal. calculated for C₁₇H₁₉N₂Cl·O·7H₂O: C, 68.16; H, 6.82. Found: C, 68.26; H, 6.81.

Synthesis of N-Methyl-4,7-Dimethyl-1,10-Phenanthroline Chloride, 7

As described for compound 1, 1.0 gm (4.8 mmole) of 4,7-dimethyl-1,10-phenanthroline was alkylated with methyl iodide, 1.98 gm (14.4 mmole) and converted to 880 mg (3.4 mmole) of the chloride salt, 7: mp 169-170°; yield 71%. The ultraviolet spectrum in H₂O showed maxima at 210 nm ($\epsilon = 3.07 \times 10^4$), a shoulder at 228 nm and at 273 nm ($\epsilon = 3.58 \times 10^4$). The XL-100 pmr spectrum of 7 showed a six-proton multiplet centered at 820 Hz (aromatic protons), a three-proton singlet at 508 Hz (N-methyl protons), and two three-proton singlets at 277 and 300 Hz (ring methyl protons). Anal. calculated for C₁₅H₁₅N₂Cl·O·4H₂O: C, 67.73; H, 6.00. Found: C, 67.71; H, 6.11.

Synthesis of N-Methyl-5-Nitro-1,10-Phenanthroline Chloride, 8

In a three-necked round-bottom flask equipped with reflux condenser, thermometer, and dropping funnel, 4.0 gm (22.2 mmole) of 1,10-phenanthroline and 40 ml of concentrated sulfuric acid were heated to 100°. To this mixture, a solution of 8 ml fuming nitric acid and 8 ml concentrated sulfuric acid was added via the dropping funnel. The reaction mixture was allowed to stir for two hours, poured over ice, and neutralized to a pH of 6.0. The yellowish solid was collected by filtration and 2.2 gm (9.8 mmole) of 5-nitro-1,10-phenanthroline were recrystallized from ethanol: mp 199-201° (lit. value, 200-201°);¹⁰⁵ yield 45%. The ir

spectrum showed the characteristic nitro group absorptions at 1510 and 1340 cm^{-1} . The A60-A pmr spectrum showed a multiplet at 460 Hz (aromatic protons) which was different from the aromatic multiplet of 1,10-phenanthroline. Anal. calculated for $\text{C}_{12}\text{H}_7\text{N}_3\text{O}_2 \cdot 0.2\text{H}_2\text{O}$: C, 62.98; H, 3.26. Found: C, 63.07; H, 3.35.

As described for compound 1, 1.0 gm (4.4 mmole) of 5-nitro-1,10-phenanthroline was alkylated with methyl iodide, 1.85 gm (13.2 mmole), and converted to 810 mg (2.9 mmole) of the chloride salt, 8: mp 146-147 $^{\circ}$; yield 66%. The ultraviolet spectrum in H_2O showed maxima at 242 nm ($\epsilon = 1.85 \times 10^4$) and 272 nm ($\epsilon = 2.77 \times 10^4$). The XL-100 pmr spectrum of 8 showed a seven-proton multiplet at 860 Hz (aromatic protons) and a three-proton singlet at 540 Hz (N-methyl protons). Anal. calculated for $\text{C}_{13}\text{H}_{10}\text{N}_3\text{O}_2 \cdot 0.6\text{H}_2\text{O}$: C, 54.49; H, 3.95. Found: C, 54.45; H, 3.97.

Synthesis of N-Methyl-5-Nitro-3,8-Dimethyl-1,10-Phenanthroline Chloride, 9

In a three-necked round-bottom flask equipped with a reflux condenser, thermometer and dropping funnel, was placed 2.0 gm (9.6 mmole) of 3,8-dimethyl-1,10-phenanthroline and 10 ml of 30% SO_3 in concentrated sulfuric acid and the temperature brought to 150 $^{\circ}$. To this mixture was added, dropwise, 5.5 ml of concentrated nitric acid, so that the temperature did not exceed 170 $^{\circ}$. The reaction mixture was stirred at 165 $^{\circ}$ for one hour and then poured over ice and neutralized

with a solution of saturated NaOH. The yellow solid was collected and 1.0 gm (3.9 mmole) of 5-nitro-3,8-dimethyl-1,10-phenanthroline was recrystallized from ethanol: mp 213-215^o; yield 41%. The A60-A pmr spectrum showed a five-proton multiplet centered at 510 Hz (aromatic protons) and a six-proton singlet at 157 Hz (ring methyl protons). Anal. calculated for C₁₄H₁₁N₃O₂: C, 59.60; H, 5.08. Found: C, 59.74; H, 5.50.

As described for compound 1, 1.0 gm (3.9 mmole) of 5-nitro-3,8-dimethyl-1,10-phenanthroline was alkylated with methyl iodide, 1.65 gm (11.7 mmole), and converted to 740 mg (2.4 mmole) of the chloride salt, 9: mp 216-217^o; yield 62%. The ultraviolet spectrum in H₂O showed maxima at 210 nm ($\epsilon = 2.65 \times 10^4$), 220 nm ($\epsilon = 2.53 \times 10^4$), and at 282 nm ($\epsilon = 2.28 \times 10^4$). The XL-100 pmr spectrum of 9 showed a five-proton multiplet centered at 900 Hz (aromatic protons), a three-proton singlet at 527 Hz (N-methyl protons), and two three-proton singlets at 269 and 280 Hz (ring methyl protons). Anal. calculated for C₁₅H₁₄N₃O₂·0.3H₂O: C, 58.27; H, 4.77. Found: C, 58.28; H, 5.07.

Synthesis of N-Methyl-4,7-Diphenyl-1,10-Phenanthrolinium Chloride, 10

As described for compound 1, 1.0 gm (3.0 mmole) of 4,7-diphenyl-1,10-phenanthroline was alkylated with methyl iodide, 1.27 gm (9.0 mmole), and converted to 650 mg (1.7 mmole) of the chloride salt, 10: mp 97-98^o; yield

57%. The ultraviolet spectrum in H_2O had maxima at 217 nm ($\epsilon = 3.71 \times 10^4$) and 231 nm ($\epsilon = 4.39 \times 10^4$). The XL-100 pmr spectrum of 10 showed a sixteen-proton multiplet at 800 Hz (aromatic protons) and a three-proton singlet at 538 Hz (N-methyl protons). Anal. calculated for $C_{25}H_{19}N_2Cl$: C, 70.16; H, 5.66. Found: C, 70.66; H, 5.61.

Viscosity Studies

Viscometric work was done on a low-shear Beckman Viscometer (Beckman Instrument Company) with a thermal jacket kept at 37.5° by a Haake FJ constant-temperature water circulator. A rotor, with a steel pellet inside, was placed in the stator and the solution to be run was added so as to float the rotor to a predetermined height. When the magnetic bars were rotating freely, the rotor turned at a constant rate. The rotor was timed for twenty revolutions with a Lab-Chron 1400 Timer.

The time for twenty revolutions of the rotor in buffer is defined as t_0 . The time for twenty revolutions of the rotor in a DNA solution is defined as t . The relative viscosity, η_{rel} , is equal to t/t_0 , and the specific viscosity, η_{sp} , is defined as $\eta_{sp} = \eta_{rel} - 1$. A number of viscometric experiments were done by Mr. William S. Barksdale, III.

Viscometric titration of salmon sperm DNA (at 5.60 and 1.00×10^{-4} M P/l) by successive additions of μl increments of 10^{-2} M N-methyl-1,10-phenanthroline cations were performed in 0.01 M MES, pH 6.2 and at 37.5° .

For the histone titration studies, a 2.84×10^{-4} M and 2.44×10^{-4} M P/1 of calf thymus and salmon sperm DNA were used in 0.01 M MES, pH 6.2 and 0.02 M Na^+ . The DNA-poly-L-lysine (5,10,15,20 and 25 $\mu\text{g}/\text{ml}$ in poly-L-lysine) and DNA-histone (15,30,45,60,80 and 100 $\mu\text{g}/\text{ml}$ in histone) were prepared in 0.5 M Na^+ in order to avoid precipitation of the complex and, subsequently, the solutions were dialyzed to 0.01 M MES, pH 6.2 and 0.02 M Na^+ .

The salmon sperm and calf thymus DNA concentrations used in the viscometric exchange studies were 2.0, 3.0, 4.0, 5.0 and 6.0×10^{-4} M P/1. The DNA-Histone III complexes were prepared using a constant Histone III concentration of 30 $\mu\text{g}/\text{ml}$ in 0.5 M Na^+ and varying DNA concentrations. The solutions were subsequently dialyzed to 0.01 M MES, pH 6.2 and either 0.02 M or 0.10 M Na^+ . In addition, a stock solution of 4.0×10^{-4} M P/1 DNA and 60 $\mu\text{g}/\text{ml}$ Histone III was prepared in 0.5 M Na^+ and dialyzed to 0.01 M MES, pH 6.2 in either 0.02 M or 0.01 M Na^+ . The stock solution of the DNA-Histone III complex was diluted 1:1 with appropriate buffer and either 0.0, 1.0, 2.0, 3.0 or 4.0 ml of 10^{-3} M P/1 DNA (with additional buffer) added to reach final concentrations of 2.0, 3.0, 4.0, 5.0 and 6.0×10^{-4} M P/1 DNA, respectively, and 30 $\mu\text{g}/\text{ml}$ of Histone III. The 10^{-3} M DNA was added to the diluted complex stock solution immediately before the viscometric measurements.

The DNA-poly-L-lysine complexes were made exactly like the DNA-Histone III complexes except that a concentration of

10 $\mu\text{g}/\text{ml}$ of poly-L-lysine was used. The DNA-poly-L-lysine complex stock solution was, therefore, 4.0×10^{-4} M P/l DNA and 20 $\mu\text{g}/\text{ml}$ poly-L-lysine.

Circular Dichroism Studies

Circular dichroism spectra were run on a Jasco J-20 spectropolarimeter. The circular dichroism spectra salmon sperm DNA solutions of 5.60×10^{-4} M P/l (0.01 M MES buffer, pH 6.2) were taken on the spectropolarimeter between 400 and 300 nm, and μl additions of reporter molecules were added until further addition of reporter gave no further change in signal. The results are shown as a graph of observed ellipticity, $(\theta)_{\text{obs}}$ versus amount of reporter added.

Proton Magnetic Resonance Studies

PMR studies were carried out using low molecular weight salmon sperm DNA in order to avoid high viscosity. The DNA was sonicated in a Bio-Sonic IIA apparatus (Bronwill Scientific Company). Three grams of salmon sperm DNA were dissolved in 100 ml of 0.01 M sodium phosphate buffer (0.01 M Na^+), pH 7.0, and 100 ml of deionized water. Nitrogen was bubbled through the solution to remove oxygen, and the beaker placed in an ice bath. The solution was sonicated for three-minute intervals for two hours. The solution was filtered (0.8 micron millipore filter membrane) by suction filtration and then freeze dried to yield 2.6 gm of sonicated DNA.

A stock solution of sonicated DNA was prepared by dissolving 75 mg in 1 ml of D₂O (containing 1 mg of DSS). The stock solution (0.32 M P/1) was diluted with 0.2 ml of reporter in D₂O-DSS solution to give a solution of 0.16 M P/1 DNA and 0.2 M reporter. XL-100 pmr spectra were taken by Dr. C. S. Baxter.

Melting Temperature Studies

Melting temperature studies of the helix-coil transition was monitored by a Gilford 240 spectrometer equipped with a Neslab temperature programmer and a Honeywell recorder. The sample compartment was thermostated by a Haake FJ constant-temperature water circulator. The temperature of the cell compartment was measured directly with a thermocouple connected to a Leeds-Northrup Company Millivolt Potentiometer.

The salmon sperm DNA (8.40×10^{-5} M P/1) and poly d(A-T)-poly d(A-T) (1.14×10^{-4} M P/1) were prepared in 0.01 M MES buffer at pH 6.2. Base-pair to reporter ratios (base pair/reporter) studied with salmon sperm DNA were: 4.2, 2.1, 1.4, and 1.1. The ratios (base-pair/reporter) studied with poly d(A-T)-poly d(A-T) were: 2.0 and 1.0.

Equilibrium Dialysis Studies

About 300 20 cm strips of Visking dialysis tubing (26/100 ft NOJAX casings) were soaked in deionized water for one hour. The membranes were then boiled in a solution of 4.5 gm ethylene diaminetetraacetic acid (EDTA), 12.6 gm of NaHCO₃, 1500 ml deionized water, and 1500 ml of

ethanol, three times, each time for two hours, and subsequently washed and boiled in deionized water three times, each time for two hours. The membranes were stored in deionized water at 0° with a few drops of formaldehyde to inhibit bacterial growth.

The dialysis experiments were performed in a pair of plexiglass blocks with ten cylindrical holes cut into each half. A clean cellulose membrane was placed between the blocks. The blocks were secured by ten screws and four C clamps. On one side of the membrane a DNA solution (in the order of 2 to 3 x 10⁻⁴ M P/1) was introduced. To the other side of the membrane a reporter solution (2 to 12 x 10⁻⁵ M) was added. The tops of the ten cells were covered by a strip of parafilm. After 18 hours, the side of the cells containing only reporter molecule was pipetted into a curvette and the absorbance at the appropriate wavelength maximum of the reporter recorded. Blank determinations showed that no DNA passed through the membrane. Equilibration was allowed to proceed for 18 hours, although it was shown (by a reporter versus buffer cell) that equilibration of reporter was complete in 8 hours.

The DNA (salmon sperm DNA, calf thymus DNA, poly d(A-T)-poly d(A-T), poly dG-poly dC, deproteinated Micrococcus luteus DNA, and native Micrococcus luteus DNA) were used at concentrations of 6.08 x 10⁻⁴ M, 4.95 x 10⁻⁴ M, 5.26 x 10⁻⁴ M, 4.51 x 10⁻⁴ M, 3.88 x 10⁻⁴ M, and 2.95 x 10⁻⁴ M P/1,

respectively. In all cases, the equilibrium dialysis studies were carried out in 0.01 M MES buffer, pH 6.2, and 0.02 M in Na^+ .

The DNA, native, reconstituted, S and M chromatin solutions were dialyzed into 0.01 M MES, pH 6.2 and 0.02 M Na^+ . The solutions were diluted to 2.38×10^{-4} M, 2.20×10^{-4} M, 2.35×10^{-4} M, 2.34×10^{-4} M, and 2.22×10^{-4} M P/1, respectively, and the diluted solutions used for equilibrium dialysis. The concentrations of reporter 11 used for the equilibrium dialysis studies were 11.9×10^{-5} M, 9.96×10^{-5} M, 7.72×10^{-5} M, 5.80×10^{-5} M, and 3.86×10^{-5} M. The S and M chromatin solutions were subjected to time studies. The chromatin solutions were subjected to equilibrium dialysis on the day the chromatin was received from Dr. Gary Stein and also after seven and ten days storage at 0°C .

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BIOGRAPHICAL SKETCH

Rolfe Eaton Scofield was born on August 6, 1946, at White Plains, New York. In June of 1964, he graduated from White Plains High School, White Plains, New York. In June of 1969, he was graduated from the University of New Hampshire, Durham, New Hampshire, with a Bachelor of Arts, with a major in Chemistry. During his senior year at New Hampshire, he served as President of the American Chemical Society Student Affiliates. In September of 1969, he enrolled in the Graduate School of the University of Florida, Gainesville, Florida. He worked as a teaching assistant from September, 1969, until September, 1970, and as a research assistant from September, 1970, until August, 1973, when he completed his work for a Doctor of Philosophy in Chemistry.

Rolfe Eaton Scofield married Carol Ann Sheldon on September 7, 1968. On September 18, 1972, they were blessed with a daughter, Paige Bethany Scofield.

He is a member of the American Chemical Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Edmond J. Gabbay, Chairman
Associate Professor of Chemistry

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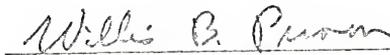
George Butler
Professor of Chemistry

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Paul Tarrant
Professor of Chemistry

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August, 1973

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