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THE EFFECTS OF RIBOSYLNUCLEOTIDE SUBSTITUTION AND METHYLATION ON THE STRUCTURE OF SYNTHETIC POLYNUCLEOTIDES

City University of New York PH.D. 1985

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THE EFFECTS OF RIBOSYLNUCLEOTIDE SUBSTITUTION AND METHYLATION ON THE STRUCTURE OF SYNTHETIC POLYNUCLEOTIDES

by

HAI-YOUNG WU

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

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ABSTRACT

THE EFFECTS OF RIBOSYLNUCLEOTIDE SUBSTITUTION AND METHYLATION ON THE STRUCTURE OF SYNTHETIC POLYNUCLEOTIDES

by

HAI-YOUNG WU

Advisor : Professor Michael J. Behe

The A conformation of Poly (rG-dC)·Poly (rG-dC) in normal salt conditions is confirmed by its circular dichroic, Raman and NMR spectra. Its characteristic Z-A-Z transition from a very low salt condition (5 mM Tris-HCl) to a high salt condition is also described. In addition, the synthesis of the DNA copolymer Poly (rG-m^5dC)·Poly (rG-m^5 dC) provides an opportunity to study the effect of C-5 position methyl groups on the salt induced conformational transition. The high salt A to Z transition point of Poly (rG-m^5 dC)·Poly (rG-m^5dC) still remains at the 1-2 M NaCl concentration range, unlike the effect of the C-5 position methyl groups on Poly (dG-dC)·Poly (dG-dC), which lowers the B to Z transition point from 2.5 M to 0.7 M (Behe & Felsenfeld 1981). Additionally, the low salt transition of Poly (rG-m^5 dC)· Poly (rG-m^5 dC) requires a bulkier counterion than does the transition for the unmethylated Poly (rG-dC)·Poly (rG-dC). These results indicate that the methylation of cytosine in the C-5 position will not stablize the Z structure of Poly (rG-dC)·Poly (rG-dC), as it does in the Z structure of Poly (dG-dC)·Poly (dG-dC). A

difference between the Z conformation of these two polynucleotides is implied, even though the Z-spectra of these two DNA polymer are very similar.

The C-5 position methyl group on pyrimidine bases has another effect on DNA structure. Both Poly (rG-m^5dC)· Poly (rG-m^5 dC) and Poly (dG-m^5dC)· Poly (dG-m^5 dC) have an alternating structure under normal salt conditions. Two closely spaced but distinct peaks in their ^{31}P NMR spectra indicate two different phosphate atom environments. The same phenomenon is also seen in the ^{31}P NMR spectrum of Poly (dA-dT)· Poly (dA-dT); the C-5 position methyl group is also present on the thymidine residues. It is obvious that the C-5 position methyl groups somehow effect the stacking of nucleotide bases which alters the phosphodiester torsional angle. This characteristic is true of every two nucleotide bases; therefore two distinct phosphate environments are shown. The effect of increasing percentages of methylated pyrimidines on the structure of Poly (dA-dU) · Poly (dA-dU) have been investigated. The results show that methylated pyrimidines play a large role in the stabilization of the "alternating B" conformation of DNA. In addition, the stable A-conformation of Poly(rG-dC) · Poly (rG-dC) in the normal salt condition has been used as a substrate for restriction enzyme Hha I and Hha I methylase. The results show that Hha I methylase works better on the A form than B form, Hha I works somewhat slower on the A-form DNA.

To my parents

and wife

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INTRODUCTION

The steric structure of Deoxyribonucleic Acid (DNA) has been a research topic in DNA biochemistry (reviewed in ref. 1a) ever since the double helix structure was proposed by Watson and Crick (1b). Not only does DNA carry the genetic code of life, but most of the fundamental biochemical and genetic processes in living cells are controlled by interactions between proteins and DNA. However, studies have been limited to the A and B structural families of DNA due to the predominant presence of the right-handed DNA helix *in vivo*. Although Pohl and Jovin found an abnormal structure of Poly (dC-dG) · Poly (dC-dG) in solution (2), the flexibility of DNA structure was not accepted until X-ray crystallography of the $(dC-dG)_3$ hexamer indicated the presence of a left-handed helical structure which was named "Z-DNA" by Wang et al (3) in 1979. It is a completely different DNA-helix with twelve base pairs per helical turn and its left-handed helical structure and phosphate backbone give a zig- zag appearance.

Single-crystal diffraction studies of oligonucleotides have given the largest amount of information about these oligomer structures. This information is free of the kinds of assumptions that typically are made in fiber

diffraction analysis. The oligomers d(CGCG) and d(CGCGCG), crystallized under different conditions (3,4,5), have shown variation in their structures. They have been labelled the Z_I and Z_{II} conformations, respectively. The principle variation in Z_{II} occurs in the orientation of the phosphate residues: They are rotated from their position in the predominant conformation,- Z_I - to lie further outwards and away from the minor groove. The two conformers were identified as gauche(-)-trans and gauche(+)-trans* about the GpC phosphates, respectively. Models for the left-handed Z-DNA polymer in solution existing in a mixture of these two conformations have been described (4). Fibers of certain deoxypolymers yield an X-ray diffraction pattern similar to the patterns of single-crystal X-ray diffraction of oligomers. Alternating purine-pyrimidine sequences are required for the formations of Z-DNA -for example: Poly (dG-dC)· Poly (dG-dC) (2); Poly (dA-dC)·Poly (dG-dT) (6,7,8,9,10); and Poly (dG-m^5dC)· Poly (dG-m^5dC) (11,12).

* are the configurations between the C(3')-C(4')-C(5') bonds. The torsion angle ψ between C(4')-C(5') determines that the conformer is gauche-trans. The symbol (+) indicates a clockwise rotation of the torsion angle ψ' between C(3')-C(4') and (-) indicates a counterclockwise rotation . Since this bond is part of a five-ring system only a limited range of ψ' value is possible (4b).

The evidence for left-handed Z-DNA polymers in solution is based on spectral criteria. The most fundamental spectral evidence is the inverted circular dichroism (CD) spectrum, which was found in the high-salt form of Poly (dG-dC) · Poly (dG-dC) by Pohl and Jovin (2). Besides circular dichroism, ultraviolet absorbance, ^{1}H or ^{31}P nuclear magnetic resonance (NMR)(13), Raman scattering spectroscopy(14), and infrared spectroscopy (15) have been used to measure the transition of DNA structure in solution.

The applications of NMR to the study of nucleic acids have not yet been as extensive as they have been to the study of proteins. The investigations had been limited to single strand polynucleotides (16,17). However, progress has been made in recent years, and now the solution structure, dynamics and binding properties of nucleic acids are all ready to be studied by NMR. Conformational states of double-stranded polynucleotides in solution have been characterized by ^{31}P NMR spectroscopy (18,19,20). A ^{31}P NMR study of the oligomer $d(CG)_8$ revealed two ^{31}P resonance peaks in high-salt solution, whereas only a single peak was found in the low salt solution (13). Since the phosphate groups are found in two different conformations in Z-DNA crystals, this result supports the idea that Z-DNA and the high-salt form of Poly (dG-dC)·

Poly (dG-dC) are the same. The development of High-field NMR makes ^{1}H-NMR the most detailed source of information on the structure of DNA using the Nuclear Overhauser Effect (NOE) (21,22). Furthermore, two dimensional NOE spectroscopy can provide almost all the information that can be provided by X-ray crystallography (23).

Laser-Raman spectroscopy is applicable to both crystals and aqueous solutions and it presents a great amount of information. This information can indicate every conformer in Poly(dG-dC)·Poly (dG-dC) separately - for example: a 682 cm^{-1} band indicates C2'-endo-anti, a band at 665 cm^{-1} is for C3'-endo-anti, 625 cm^{-1} for C3'-endo-syn (24), and 810 cm^{-1} for phosphodiester symmetric stretch (14) - instead of giving an average type of information. These advantages and the small amount of sample required for each scan make this spectroscopy a very convenient and reliable method in DNA structure studies. Infrared spectroscopy is similar to Raman spectroscopy, and does not impose an upper limit on the concentration of the polynucleotide used. It is thus possible to study solutions with very high concentrations, such as gels or hydrated films (15).

The transitions of DNA occuring in solution are likely to be better models in demonstrating the possible conformations for biological contexts. However, left-

handed Z-DNA is relatively less stable than right-handed DNA in physiological solution. Although the methylated Poly (dG-dC)·Poly (dG-dC) was reported to be stablized in the Z-conformation at a relatively low salt concentration (0.7 M Nacl) (11), but it is still not a favorable conformation when equilibrated with right-handed DNA.

The improvement of chemical synthesis methodology for polynucleotides made it possible to study homogenous polynucleotides with different sequences. Those results implied that DNA structure is sequence dependent and is in dynamic equilibrium between any two structures. A particular nucleotide sequence can make the DNA molecule favor a certain structure. On the other hand, certain driving forces provided in its environment can push the equillibrium toward one direction and stabilize a certain structure. The driving forces can be: relative humidity, concentration of counterion, temperature, pH etc.. The functional groups of the nucleoside residues themselves are also important factors in suppling stabilizing forces besides those provided from the surrounding environment. Bromination and methylation of polynucleotides affects the stacking of nucleotide bases and torsional angle of the phosphodiester linkage of the phosphate backbone (25,26).

DNA polymerase I has been used in enzymatic synthesis of polynucleotides <u>in vitro</u> for the last three decades,

since the discovery of DNA polymerase in Escherichia coli in 1956 by Kornberg et al (27). It is apparent, 30 years later, that what is now termed DNA polymerase I is only one of a family of DNA polymerization enzymes and was just the first of these enzymes to be discovered in 1956. The detailed analysis of the structure and mechanism of Escherichia coli DNA polymerase I by Kornberg and his colleagues (28,29) provids a clear picture of enzymatic DNA synthesis and is the experimental framework for much of present-day nucleic acid research. DNA polymerase I is a single polypeptide chain with a molecular weight of 110K. Cleavage with protease yields a large fragment (Klenow fragment) containing the polymerase with 3'-5' exonuclease activity and a small fragment containing only 5'-3' exonuclease activity (30). A template and divalent ion (magnesium) are absolutely required for the polymerization reaction which proceeds in the 5'-3' direction. If the Klenow fragment is used in polynucleotide synthesis in vitro, the 5'-3' exonuclease activity can be prevented; therefore, the synthesized polymers will be stable in a reaction mixture for a long incubation time and a maximum yield of synthetic polynucleotides can be reached.

Enzymatic synthesis of polynucleotides is an efficient way to synthesize large quantities (mg scale) of polynucleotide. However, due to limited template sources,

only homologous polynucleotide with simple repeating sequences can be made. In addition due to the proof reading ability of DNA polymerase I, modified nucleoside analogues are not easily incorporated into the synthetic polynucleotides. Fortunately, for DNA structure studies, highly repeating alternating purine-pyrimidine sequences are those desired for the DNA polymer. Manganese can substitute for magnesium as a required cofactor in all of the reactions of DNA polymerase I (polymerization, 5'-3' exonulease, and 3'-5' exonuclease). When manganese is present at millimolar concentrations, the proof reading ability of DNA polymerase I is dropped dramatically. For example, the frequency of aminopurine misinsertion is increased four fold (31,32). The primary effect of manganese ion is to cause a large decrease in the Km of the mismatched deoxynucleotide for the polymerase-template complex. Based on NMR data with Escherichia coli DNA polymerase I, in the presence of manganese ion the deoxyribose conformations of the binding deoxyribonucleoside triphosphate substrate in the active site of DNA polymerase I may be different from the deoxyribose conformation which is favored in the presence of magnesium ions (33). In this way several different analogues of nucleosides can be inserted into polynucleotides by modifying the conditions of the polymerization reaction of DNA polymerase I.

RNA or RNA-DNA hybrid polynucleotides are in the A conformation (34,35,36,37,38). The ribonucleotides favor the C3'-endo sugar pucker while the alternating C2'-endo-C3'-endo sugar conformation occurs in the Z form Poly (dC-dG)· Poly (dC-dG) crystal (4). C2'-endo is assigned to cytosine residues and C3'-endo is assigned to guanosine residues. According to these facts, a favored Z-conformation can be predicted for a polynucleotide where deoxyribosyl guanosines are substituted with ribosyl guanosines due to the favored of C3'-endo sugar pucker of guanosine residues. Not surprisingly, the mixed ribo-deoxyribo copolymer Poly (rG-dC)· Poly (rG-dC) is in the A conformation in moderate salt conditions. Its A to Z transition point is 1.5 M. Compared to the transition point of the B to Z transition of Poly (dG-dC)· Poly (dG-dC), there is a significant favoring of the transition. Interestingly, a Z conformation was discovered which is very stable in low salt conditions. The synthesis of Poly (rG-dC)· Poly (rG-dC) not only leads to the study of the low salt Z structure but also provides a stable model of A-DNA in moderate salt conditions with a uniform sequence for protein binding and enzyme activity studies.

Turning to another aspect, methylation of DNA is also a very important phenomenon in genetics. The effect of DNA methylation on the expression of genes in eukaryotic cells

has been reviewed several times in the past few years (39, 40,41). Heavy methylation often is associated with gene inactivation, while undermethylation is sometimes required for a gene to be expressed. The methylated sites in chromosomal DNA are most often at the C5 position of cytosine in a -CG- dinucleotide. There has been speculation that the effect of methylating cytosine is to alter DNA conformation since methylation of cytosines in a polymer of the sequence, Poly ($dG-m^5dC$)· Poly ($dG-m^5dC$) was shown to greatly facillitate the B to Z transition. However, no B-Z transition was seen when a plasmid containing the chicken adult beta-globin gene was methylated at its -CCGG- sites by Hpa II methylase (42). The presence of a methylated pyrimidine can cause other conformational variations besides the B-Z transition. It has been observed that the methylated synthetic polymer Poly ($dG-m^5dC$)· Poly ($dG-m^5dC$) is in an "alternating B" conformation in solutions of moderate NaCl concentration but the analogous unmethylated polymer is not(25,43). A similar "alternating A" conformation can also be observed when the mixed ribo-deoxyribo polymer Poly ($rG-m^5dC$)·Poly ($rG-m^5dC$) is dissolved in solutions of moderate NaCl concentration. The alternating B conformation was first proposed by Klug et al (1979) for Poly (dA-dT)· Poly (dA-dT), another polymer containing methylated pyrimidines, and the appearance of a closely spaced doublet (0.2-0.3

ppm separation) in its ^{31}P-NMR spectrum greatly supported the alternating model (19,20,44). Klug et al (1979) speculated that the alternating structure was stabilized by a stacking interaction involving the methyl group of the pyrimidine base(45).

In order to elucidate the dependence of the alternating conformation on methylated pyrimidines, ^{31}P-NMR spectra of several synthetic polynucleotides were observed. The polymers studied include Poly (dA-dU)· Poly(dA-dU), Poly (dA-dT) · Poly (dA-dT), and analogous polynucleotides containing various proportions of dT and dU. These polymers are represented by an expression such as Poly (dA-dU,T)· Poly (dA-dU,T)-60% dT for an alternating purine-pyrimidine polymer in which all of the purine bases are adenosines, 60% of pyrimidine bases are thymine, and 40% of pyrimidine bases are uridine. The separation of peaks in the ^{31}P-NMR spectra of the polymers increases with an increasing percentage of methylated pyrimidines. These results suggest that increasing the percentage of methylated pyrimidines gradually shifts the conformation from a regular to an alternating structure.

The biological role of DNA flexibility has been suggested in several different aspects, especially for Z-DNA. Experiments on the SV-40 transcriptional enhancer provide the most detailed example to date of a specific

biological role that may be carried out by Z-DNA (46). Peck and Wang (47) found that supercoil- induced formation of Z-DNA in an inserted segment results in termination of transcription at the B-Z junction. Other possible roles in recombination (48) and chromatin structure (49,50,51) have been described.

Besides left-handed Z-DNA, other DNA conformations are also possible candidates for regulating biological functions. Vardimon and Rich (52) and Zacharias et al (53) have recently established that the restriction enzyme HhaI and its corresponding methylase do not work on the Z form of an alternating d-(G-C) - sequence in a supercoiled plasmid, but do work when the plasmid is relaxed and the same sequence is in a right-handed conformation. Frederick et al (54) have shown that a portion of the structure of an oligonucleotide when bound to EcoRI in a co-crystal is similar to the A-conformation of DNA. The problem of the effect of substrate conformation on the activity of polynucleotide modifying enzymes is approached by an experiment showing that the mixed ribo-deoxyribo polymer Poly (rG-dC) · Poly(rG-dC), which is in the A conformation at moderate salt concentrations (55), acts as a substrate for HhaI and HhaI methylase.

MATERIALS and METHODS

Materials:

Escherichia coli DNA polymerase I large fragment (Klenow fragment) was purchased from New England Biolabs. or isolated from *E. coli* strain CJ155 by the procedure of Joyce & Grindley (56). Poly (dI-dC)·Poly (dI-dC) and Poly (dA-dT)· Poly (dA-dT) as well as most NTPs and dNTPs were purchased from P-L Biochemicals. 5-methyl-deoxycytidine triphosphate was synthesized by the phosphoramidate procedure as previously described (11). Sephadex G-50, particle size 50-150 u, was purchased from Pharmacia Fine Chemicals. Micrococcal nuclease, DNase I, and Mung Bean Nuclease were purchased from P-L Biochemicals. Most of the restriction enzymes, the methylase and the DNA molecular weight standard (Hae III digested - ØX174 RF DNA) were purchased from New England Biolabs. Bacterial alkaline phosphatase was purchased from Bethesda Research Laboratories, Inc. Acrylamide and N,N'-methylene-bis-acrylamide were purchased from Sigma. (8-^{3}H) Guanosine-5'-triphosphate and Deoxycytidine-5'-(α-^{32}P) triphosphate were purchased from ICN. all other reagents used in this research were either analytical grade or spectro grade. PEI-F cellulose TLC plates were purchased from J.T. Baker.

Preliminary tests for the synthesis of Poly (rG-dC)·Poly (rG-dC) :

The template for the enzymatic synthesis of Poly(rG-dC) ·Poly (rG-dC) is 100 ug of Poly (dI-dC)· Poly (dI-dC) per milliliter. 0.6 mM ribosylguanosine-triphosphate, 0.6 mM deoxyribosylcytidine-triphosphate and 1.25×10^{-2} uM (2uci per milliliter) of (8-^{3}H)-guanosine-5'-triphosphate were added as the substrates of the reaction. The reaction mixture was incubated at 37°C in the presence of 6 mM $MnCl_2$. Various kinds of buffers were tested to prevent the precipitation of Mn^{+2} and to maximize the reaction rate. Once the buffer was selected, an optimal pH was searched for. Each different test was carried out using the same concentration of Klenow fragment (20 units per milliliter). An aliquot of reaction mixture (50 ul) was taken at each time point and stopped with 10 mM (final concentration) Na_2-EDTA The aliquots were then passed through a Sephadex G-50 column in order to separate the large molecular weight DNA polymer from the monomers.

Assay of incorporation rate :

100 ul of sample was taken out from each eluted fraction and put into 5 ml of Beckman Ready-Solv GP scintillation fluid in a scintillation vial. The radioactivities of polymer and monomers present are then counted by scintillation counter. The cpm (count per

minute) of polymer fractions divided by the total cpm (cpm of polymer + cpm of monomers) multiplied by 100% is the incorporation rate of guanosine-5'-triphosphate. The optimal conditions of the synthesis were determined based on the maximization of the incorporation rate.

The synthesis of polynucleotides :

The synthesis of Poly (rG-dC)· Poly (rG-dC) and Poly (rG-m^5dC) · Poly (rG-m^5dC) were carried out as follows: 0.2 ml of reaction media contained 40 mM N,N-bis-(2-hydroxyethyl)-glycine (Bicine) buffer pH 8.95, 1 mM Mercaptoethanol, 6 mM $MnCl_2$, 0.6 mM rGTP, 0.6 mM dCTP (or m^5dCTP), 20 ug of Poly (dI-dC)·Poly (dI-dC), and 4 units of Klenow fragment. After 16 hours of incubation at 37° C the entire mixture was added to one milliliter of reaction media containing the same reactants in the same concentrations except that no Poly (dI-dC)· Poly(dI-dC) was added. After a second 16 hours incubation at 37° C the entire mixture was added to 8 ml of reaction media containing the same reactants in the same concentrations and without the Poly (dI-dC)· Poly (dI-dC) again. After a third 16 hours incubation at 37° C the polymer was precipitated with ethanol, redissolved in 0.4 ml of 10 mM Tris-HCl pH 7.4, extracted twice with phenol and chloroform, and passed through a column of Sephadex G-50 equilibrated with 5 mM Tris-HCl pH 7.4, 0.1 mM EDTA. The

total yield of polymer was about 2 mg.

The synthesis of Poly (dA-dU)·Poly (dA-dU) and its analogous polynucleotides was as follows : In 100 ul of reaction media contained 50 mM Tris-HCl pH7.6, 6mM $MgCl_2$, 1 mM Mercaptoethanol, 10 ug of Poly (dA-dT)·Poly (dA-dT), 3 mM dATP, 3 mM dUTP, and 2 units of Klenow fragment. After 16 hours incubation at 37°C the entire mixture was added to one milliliter of reaction media containing the same reactants in the same concentrations except that no Poly (dA-dT)· Poly (dA-dT) was added. After a second 16 hours incubation at 37°C the entire mixture was added to 10 ml of reaction media , again without adding any Poly (dA-dT) .Poly (dA-dT). The mixture was incubated for another 16 hours at 37°C. The synthesized polymer was precipitated with ethanol, redissolved in 0.4 ml of 10 ml Tris-HCl pH 7.5, extracted twice with phenol and chloroform, and passed through a column of Sephadex G-50 equilibrated with 50 mM Tris-HCl pH 7.5 and 0.1 mM EDTA. The total yield of polymer was about 3 mg. Polymers containing different percentage of dT and dU were synthesized by the same procedures except that dTTP was also added to the reaction media and the total amount of dTTP and dUTP was kept constant at 3 mM.

Length determination of synthesized DNA polymer :

α-^{32}P-deoxycytidine triphosphate was added into the

reaction for the synthesis of Poly (rG-dC)· Poly (rG-dC). After incubation at 37°C for 16 hours the reaction was stopped by adding 10 mM Na_2-EDTA. 1% SDS (Sodium Lauryl Sulfate) was also added to prevent the binding of Klenow fragment to the synthetic polynucleotide. Dephosphorylated Hae III digested-ØX174 RF DNA was end labeled with γ-^{32}P-ATP by first treating with bacterial alkaline phosphatase in 5 mM Tris-HCl pH 8.0 at 37°C for 60 minutes, adding 2% SDS and incubating in 37°C water bath overnight, followed by phenol extraction and ethanol precipitation, drying the precipitated DNA in a vacuum evaporator and dissolving into 200 ul of 5 mM Tris-HCl pH 7.6, 0.1 mM EDTA. This is the so called Bap'd molecular weight standard. 10 ul of Bap'd molecular weight standard was end labeled with γ-^{32}P -ATP on the free 5'-OH end by adding: 7 ul of 1 M Tris-HCl PH 7.4, 10 ul 0.1 M $MgCl_2$, 5 ul 0.1 M Dithiothreotol (DTT), 5 ul γ-^{32}P-ATP (10uci/ul), 1 ul of polynucleotide kinase and enough distilled water to bring the final volumn to 100 ul. Incubation was at 37°C for one hour. The ^{32}P-labeled synthetic polynucletide and the end labaled DNA molecular weight standard were then extracted with phenol and Sevag solution (chloroform:iso-amyl-alcohol;24:1), and precipitated with 2 times the volume of ethanol. The dried DNA samples were then dissloved in TBE buffer (10.8 gm of Tris base; 5.5 gm boric acid, and 0.93 gm of Na_2-EDTA in one liter distilled water) 10% Ficoll and 0.25%

bromophenolblue were added before loading in a 5% acrylamide gel. The acrylamide gel contained 5% acrylamide, 0.25% N,N'-methylene-bisacrylamide, 10 ug per one milliliter of ammonium persulfate and 0.5 ul per milliliter of TEMED (N,N,N',N'-tetramethylene diamine) in TBE buffer. The 0.8 mm thick gel was set up in a BRL vertical gel electrophoresis system and run in 100 volts constant electric current for two and a half hours. After gel electrophoresis the gel was placed in contact with Kodak XAR-5 X-ray film in a Kodak X-omatic cassette including a Kodak lanex regular screen. The whole cassette was then stored at -70°C for 4 hours. The Autoradiographic film was completed after development and fixation in a dark room.

The characteristics of the synthetic polynucleotide :

The sequences of the synthetic polynucleotides were determined by several different methods.

I. Sodium hydroxide hydrolysis : ^{32}P labelled poly (rG-dC) · Poly (rG-dC) and Poly (rG-m^5dC)· Poly (rG-m^5dC) were partial hydrolyzed with 0.3 M NaOH. At the end of the hydrolysis period, an equal amount of 0.3 M acetic acid was added to neutralize the solution. Several different time points were chosen. The hydrolyzed samples plus 40 ug tRNA were then precipitated with ethanol and redissolved

in a solution containing 20 ul DMSO (Dimethyl Sulfoxide), 4 ul 0.1 M phosphate buffer pH 6.6, 6 ul of 8 M ion exchanged glyoxal and 10 ul of distilled water, incubated at 37°C for 30 minutes and then precipitated with ethanol again. The DNA precipitates were then resuspended in 10 ul solution containg 30 mM Tris-Phosphate buffer pH 7.2 , 1.0 mM EDTA, 0.25% bromophenolblue, and 10% Ficoll before loading onto a sequencing gel : 8% acrylamide, 0.4% N,N'-methylene bisacrylamide, 7 M urea in 30 mM Tris-Phosphate buffer pH 7.2 plus 0.1 mM EDTA. The gel was run at 150 volts for 8 hours to prevent raising its temperature. For comparison ^{32}P-labeled Poly (dC-dG)· Poly (dC-dG) was partially digested with Hha I and treated the same as the Poly (rG-dC) · Poly (rG-dC) before loading into the sequencing gel. The gel was run in a BRL vertical sequencing gel electrophoresis system.

II. Nearest neighbor analysis : α-^{32}P-deoxycytidine triphosphate was used as the radioactive label in the synthetic polynucleotide Poly (rG-dC)· Poly (rG-dC). The ^{32}P label must be found in the phosphate between 5'-rGpdC-3'. 75 ug of this ^{32}P labeled polynucleotide was hydrolyzed completely in 0.3 M NaOH for two hours and neutralized with an equal volume of 0.3 M NaOH acetic acid in Tris-HCl buffer pH 7.8. The digested DNA was precipitated with ethanol and redissolved in 540 ul of a solution containing 2 umoles of Tris-HCl pH 8.6, 1 umole of

$CaCl_2$ and 180 units of micrococcal nuclease (57), and incubated in a 37°C water bath for two hours. 25 ul of this double digested DNA sample and 20 ug of unlabeled nucleotide monophosphates (deoxycytidine-3'-monophate and ribosylguanosine-3'-monophate) were applied on a PEI-F cellulose TLC plate and developed using a mobile phase of 1.0 M LiCl. The flourescence spots of the large quantity of unlabeled nucleotide monophosphates was indicated by exposure under short wavelength UV lamp. The spots of digested DNA sample were indicated by autoradiography.

III. Reversed phase High Performance Liquid Chromatography : The percentage of the nucleotide residues of Poly (dA-dU.dT) · Poly (dA-dU.dT) were determined by reversed phase HPLC. Prior to injection, samples were digested to nucleosides by first treating with DNase I in the presence of $MgCl_2$ at 37°C overnight. The DNase I digested samples were then digested with mung bean nuclease in the presence of $ZnCl_2$ at 37°C for 7 hours. 110 units per milliliter of bacterial alkaline phosphatase was then added and another 16 hours incubation at 37°C was carried out (58). The digested samples can be stored at -20°C before injection into the HPLC. 2.5 ug of digested DNA was injected each time into the HPLC (Water Associates) which contains two M-45 solvent delivery systems, one model 660 solvent programmer, a model 440

absorbance detector and a recorder. The column was a Bondapak-C18 and the mobile phase was a linear gradient of 5-15% acetonitrile/1.0% of ammonium acetate pH 5.8. Several standard nucleosides were injected into the same system to indicate the positions of the peaks of deoxyadenosine, deoxythymidine and deoxyuridine. These were the references for identification of the nucleoside peaks of the digested polynucleotides. The ratios of deoxythymidine and deoxyuridine were determined by the weights of the peaks that had been cut from the recording paper, adjusted by a factor according to the extinction coefficient of each nucleoside.

Spectroscopic studies :

Ultraviolet spectra were taken on a Perkin-Elmer 320 UV-Visible spectrophotometer. Spectra were obtained at ambient temperature.

Circular dichroic spectra were taken on a Cary 60 CD-ORD spectrometer set in the CD mode, or taken on a Jasco J 500 spectropolarimeter which is connected to a Jasco data processor. The spectra were stored in the memory of the data processor; the spectra of blank solutions were subtracted out from the spectra of DNA samples.

^{31}P nuclear magnetic resonance (NMR) spectra were obtained with an IBM-Bruker model WP200SY NMR set at 81.01

MHz and were recorded at 25-30° C in the deuterium lock mode with 4000 data points and a spectral window of 4000 Hz. Typically 30,000-40,000 scans were aquired, and a line broadening of 4 Hz was applied prior to fourier transformation. Prior to an NMR experiment, the DNA polymers were sonicated (25) for three hours with a heat system/ultrasonics (Plainview, NY) model w-375 sonicator on pulsed mode, 50% duty cycle, with the out put control set at 6 and the temperature kept at 5-10° C. Polyacrylamide gel electrophoresis showed that the size of the polynucleotides was reduced to 50-250 base pairs by this treatment. The NMR samples contained 2.5-3.0 mg of polynucleotides in 5 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA in 3 ml of 33% D_2O; 1.0 ul of trimethylphosphate was added as an internal standard, and all chemical shifts are measured upfield from it. After the NMR spectrum was recorded in this solution, solid NaCl or CsF was added to the desired concentration and more scans were aquired. The temperature experiments were carried out under the control of a Variable Temperature Unit (IBM) which maintained the probehead at preselected temperatures. Compressed air flowing at a controlled rate over a resistance heater provided high temperatures and evaporated liquid nitrogen flowing over the same resistance heater provided low temperatures. Temperature was monitored and regulated by thermocouple-controlled electronics in the VT 1000

controller.

Raman spectra were run at room temperature with samples in 1 mm i.d. glass capillary tubes. The excitation wavelength was 488 nm, with incident power typically between 150 and 200 mW. Samples were usually 1 mg of DNA polymer in 20 ul of solvent and were run at 5 cm^{-1} spectral resolution. The Raman spectrometer consists of a SPEX 14018 double monochromator with holographically ruled gratings, a cooled RCA C31034A phototube, and pacific precision instruments photon counting detection. Spectra were collected and stored on floppy disks on a system consisting of an Apple computer and interactive structures A/D and D/A converters.

HhaI methylase assay :

75 ul reaction mixtures were incubated at 22° C contained 100 mM Tris-HCl (pH 7.8), 10 mM Na_2EDTA, 20 mM dithiothreitol, 0.3 ug DNA polymer, 108 $_p$moles ^{3}H-SAM (S-[methyl-^{3}H]-adenosyl-L-methionine) and 50 units of HhaI methylase. 5 ul samples were taken at intervals (every 10 minutes) and the incorporation of radioactive methyl groups was monitored by acid insolubility. 5 ul of each sample was put on a Whatman GF/F glass fiber disc (2.4 cm) and stopped immediately by adding 2-3 drops of cold 5% Trichloroacetic acid. The disc was allowed to flow on the surface of a ice-cold 5% TCA solution and then go down

into the solution after having absorbed enough of it after ten minutes. it was washed twice with fresh 5% TCA solutions, ten minutes each wash, then transfered to ice-cold ethyl alcohol - ether 1:1 mixture, and finally transfered into ice - cold ether for ten minutes(59). After drying in the air the discs were put in scintillation vials and ten milliliter of Econofluor-2 scintillation fluid added. The radioactivity (Count Per Minute) of samples was read with a Beckman scintillation counter (model:LS7500). The cpm of tritium label on the acid insoluble DNA polymer divided by the total cpm of tritium in solution will be the methylation rate of the C5-methyl group on the nucleotide base of cytidine.

RESULTS

According to the DNA polymerase I assay (29), the synthesis of Poly (dC-dG)·Poly (dC-dG) in the presence of phosphate buffer pH 7.4 and magnesium ion can be achieved by using Poly (dI-dC)·Poly (dI-dC) as template. However, once manganese ions were added to the reaction instead of magnesium ions, precipitation occurred, stopping the reaction. Goodman et al (32) have pointed out that Mn^{+2} can be the divalent ion for DNA polymerase I. Several buffers were tested for precipitation formation in the polymerization reaction. Hepes (N-2-Hydroxyethyl piperazine-N'-2-Ethane Sulfonic acid) buffer, TES [N-tris(Hydroxymethyl) methyl-2-aminoethane-sulfonic acid] buffer and Bicine [N-N',bis(2-Hydroxyethyl) Glycine] buffer were suitable and better than phosphate buffer for the synthesis of Poly (dG-dC)· Poly (dG-dC) in the presence of magnesium ion. However, Hepes and TES buffers caused precipitations in the presence of Manganese ion, where Bicine buffer did not, its manganese ion solution kept clear for a long incubation period at 37°C. Unfortunately the incorporation rate of the nucleotide triphosphates in the synthesis of Poly (rG-dC)·Poly (rG-dC) in Bicine buffer pH 7.4 was very low (only 1.4% incorporation rate resulted). The enzymatic reaction was very pH dependent. A

series of polymerization reactions assayed at different pH's (Fig.1) showed that the optimal pH of this reaction is pH 8.95. The incorporation rate of ^{3}H-rGTP of the reaction at pH 8.95 after 16 hours incubation at 37^{o} C reached nearly 26%. A maximum 32% incorporation rate was reached after 24 hours incubation at 37^{o}C in the same reaction conditions; after 24 hours incubation time the concentration of the synthetic DNA polymer was stable and kept constant up to sixty hours in the 37^{o}C water bath. (Fig.2) The advantage of using Klenow fragment is obvious - no 5'-3' exonuclease activity present in solution allowed the synthetic DNA polymers to be maintained for a long incubation period without any degradation and a maximum yield to be achieved. Considering a risk of microorganisms growth in the reaction mixture, the reasonable reaction time was decided to be 20-24 hours. The concentration of manganese ion in the mM range seemed to have no significant effect on the incorporation rate of this reaction (Fig.3) It was obvious, however, that 0.1 mM manganese ion was unable to support the normal synthetic reaction rate.

The length of the synthetic DNA polymers were in the range of 700 to 1000 base pairs which was determined by gel electrophoresis with the molecular weight marker HaeIII digested ØX174-RF DNA. The ribosyl-deoxyribosyl

alternating character of the synthetic DNA polynucleotides was shown by the ladder-like band patterns on a sequencing gel of 0.3 M NaOH partially hydrolyzed synthetic polymer (Fig. 4) The mechanism of NaOH hydrolysis is illustrated in Fig.5. Nearest neighbor analysis also showed that the ribosylguanosine is right next to deoxyribosylcytidine on both strands of the synthetic polynucleotides (Fig. 6). The total yield was about 2 mg of polymer in a 10 ml reaction mixture(Table 1).

Brahms et al (60) have synthesized Poly (rG-dC). Poly (rG-dC) by a different method and have shown it to be in the A conformation at intermediate salt levels. We confirm this result (see below).

Methylated Poly (rG-dC) · Poly (rG-dC) was also synthesized using the same reaction conditions as in the synthesis of Poly (rG-dC)·Poly (rG-dC), except that C5-methyl deoxycytidine triphosphate was used instead of deoxycytidine triphosphate as one of the substrates in the reaction. The total yield of Poly (rG-m^5dC).Poly (rG-m^5dC) was also about 2 mg in a 10 ml reaction.

Poly (dA-dU)· Poly (dA-dU) and analogous polynucleotides containing various proportions of dT and dU were synthesized. The proportions of pyrimidine bases were determined by HPLC as 68% thymine, 49% thymine and 40% thymine, with contain 32% uridine, 51% uridine and 60%

uridine respectively. The polymers were represented by an expression such as Poly (dA-dU,T)·Poly (dA-dU,T) - 68% dT, for an alternating purine-pyrimidine polymer in which all of the purine bases are adenosines, 68% of pyrimidine bases are thymine, and 32% of pyrimidine bases are uridine.

Spectroscopic studies of the synthetic polymer Poly (rG-dC)·Poly (rG-dC):

Circular Dichroism : The dependence of the CD spectrum of Poly (rG-dC)·Poly (rG-dC) on NaCl concentration is shown in Figs. 7 and Fig. 8. At low salt (5 mM Tris), the polymer gave a spectrum characteristic of Z-form DNA, with a large negative band at 292 nm followed by a positive peak at 265 nm (Fig.7, spectrum A). At the intermediate salt concentration of 0.2 M NaCl, the CD spectrum (Fig.7, spectrum B) looks like that of A-form DNA : the negative peak is much reduced from that of the low-salt spectrum, and the positive peak has increased, when the concentration of NaCl was increased to 2 M, the CD spectrum reverted back to that of Z-form DNA (Fig.7, spectrum C). Fig.8 is a plot of the molar ellipticity of the polymer at 292 nm vs. salt concentration. Fig.8A shows that the band decreases noncooperatively over the range 0-0.1 M NaCl. Fig.8B shows that it stays constant at NaCl concentrations from 0.1 to 1.3 M and then increases cooperatively from

1.3 to 1.8 M.

Raman spectroscopy : Raman spectra of Poly (rG-dC)· Poly (rG-dC) and of Poly (dG-dC)·Poly (dG-dC) are shown in Fig. 9. In 0.1 M NaCl Poly (rG-dC)·Poly (rG-dC) exhibits a strong peak at 811 cm^{-1} (Fig. 9, spectrum B), characteristic of the A form of polynucleotides (61). Poly (dG-dC) · Poly (dG-dC) shows a peak at 831 cm^{-1} (Fig. 9, spectrum D), which is a marker for the B conformation (61). When the NaCl concentration was increased to 2.5 M, the 683 cm^{-1} peak of Poly (dG-dC)·Poly (dG-dC) disappeared and a peak at 624 cm^{-1} appeared (Fig. 9, spectra C and D). This has been assigned to a guanine ring vibration (14). With the hybrid polymer Poly (rG-dC)·Poly (rG-dC) in 2.5 M NaCl, a similar phenomenon occurred: the peak at 669 cm^{-1} disappeared and was replaced by a new peak at 637 cm^{-1} (Fig. 9, spectra A and B).

To obtain a raman spectrum of Poly (rG-dC)· Poly (rG-dC) under true low-salt conditions when the polymer is present at very high concentration, special precautions must be taken. The polymer sample was first passed through a sephadex G-50 column equilibrated with distilled water to remove excess counterions. The sample was then lyophilized and dissolved in 20 ul of 5 mM Tris-HCl, pH 7.5/0.1 mM EDTA. The low-salt raman spectrum of poly (rG-dC). Poly (rG-dC) is shown in Fig. 10. As in the high-salt spectrum,

the low-salt spectrum shows that the peak at 811 cm^{-1} and 669 cm^{-1} have disappeared, while a peak at 630 cm^{-1} appears, consistent with a low-salt A-Z transition.

^{31}P NMR : Spectra of the polymer at different NaCl concentrations are shown in Fig. 11. In low-salt solutions (5 mM Tris), the spectrum shows three peaks: the first is at -3.1 ppm; the second, at -4.1 ppm; and the third, less well resolved, at -4.6 ppm (Fig. 11, spectrum A). When NaCl was added to a concentration of 0.2 M, the spectrum collapsed to a single peak at -4.1 ppm (Fig. 11, spectrum B). But when the salt concentration was increased to 2 M, the spectrum showed two peaks, the first peak being at -2.9 ppm and the second peak being at -4.1 ppm (Fig. 11, spectrum C).

Effect of other ions on the DNA polymer Poly (rG-dC)· Poly (rG-dC):

The effect of ions other than Na^+ on the conformation of the polymer are listed in Table 2. Only monovalent cations seemed capable of causing the high-salt transition without aggregation. Divalent ions also seemed able to promote the high-salt transition, but the polymer then immediately aggregates, so it is difficult to confirm this observation. Most surprising is the inability of hexamine cobalt (III) $\{[Co(NH_3)_6]^{3+}\}$ to promote the high-salt

transition in view of its ability to induce the B-Z transition in Poly (dG-dC)·Poly (dG-dC) at micromolar concentration (11).

Oligovalent ions (+2 to +4) are very efficient promotors of the low-salt transition. As seen in Table 2, micromolar concentrations of Mg^{2+}, $[Co(NH_3)_6]^{3+}$, or $spermine^{4+}$ were enough to change the CD spectrum from a Z-like to an A-like spectrum.

Spectroscopic studies of the synthetic polymer Poly (rG-m^5dC)·Poly (rG-m^5dC):

Circular Dichroism : The CD spectra of Poly (rG-m^5dC)·Poly(rG-m^5dC) under several salt conditions are shown in Fig. 12. In 5 mM triethanolamine buffer (Fig. 12A) the polymer has a CD spectrum that is very similar to the CD spectra of the Z forms of Poly (dG-dC)·Poly (dG-dC) (2) and Poly (dG-m^5dC)·Poly (dG-m^5dC) (11), with a negative peak at 292 nm and a positive peak at 263 nm. As the salt concentration is increased to 50 mM (Figs. 12B and 13) the CD spectrum changes cooperatively. The negative peak decreases and shifts to 286 nm, the positive peak increases and shifts to 267 nm, and a new negative peak develops at 248 nm. As the salt concentration is increased further to several molar (Figs. 12C and 14) the positive peak decreases monotonically and remains centered at 267

nm, the negative peak at 245 nm also decreases monotonically and does not shift, and the negative peak at 286 nm remains unchanged.

Raman spectra : The Raman spectrum of Poly ($rG\text{-}m^5dC$)· Poly ($rG\text{-}m^5dC$) in 0.2 M NaCl is shown in Fig. 15. At this salt concentration a strong peak is seen at 811 cm^{-1} indicative of the A conformation (61). Additionally, there is a peak at 671 cm^{-1}, which arises from guanosine residues that are in the C3'-endo-anti conformation (24). This sugar conformation might be expected since the guanosines of the polymer are ribonucleotides.

^{31}P-NMR : The ^{31}P-NMR spectra of Poly ($rG\text{-}m^5$ dC).Poly ($rG\text{-}m^5$ dC) in 5 mM Tris at several NaCl concentrations are shown in Fig. 16A-F. At 0 M NaCl (Fig. 16A) the spectrum shows a single peak at -4.2 ppm. As the NaCl concentration is increased to 30 mM (Fig. 16B) the single peak resolved into two closely spaced peaks at -3.8 and -4.3 ppm. The spectrum remains the same until 1 M NaCl where downfield peak begins to appear at -2.7 ppm (Fig. 16E). At 2 M NaCl (Fig. 16F) the transition is complete, with two peaks of equal intensity at -2.7 and -4.1 ppm.

The above spectra were taken in the presence of 5 mM Tris buffer, 0.1 mM EDTA. As shown in Fig. 16A, in the absence of NaCl in this buffer a single peak is seen.

However, if the buffer is changed to 25 mM triethanolamine, 0.1 mM EDTA, the spectrum shown in Fig. 17 is obtained. Two widely separated peaks are seen at -2.9 and -4.6 ppm.

Effect of oligovalent ions on the conformation of the polymer:

Neither Mg^{2+} nor hexamine $cobalt^{3+}$ could induce the high salt transition in Poly (rG-m^5dC)·Poly (rG-m^5dC) at any concentration before aggregation of the polymer takes place, as monitored by ^{31}P-NMR (data not shown).

The characteristics of the synthetic polymer Poly (dA-dU,T)·Poly (dA-dU,T) - with different percentage of dT:

Poly (dA-dT)· Poly (dA-dT) has been seen to give two closely spaced peaks in its ^{31}P-NMR spectrum (19,20) at moderate salt concentrations, and this has been interpreted as strong evidence for an alternating B conformation (45). The separation of the peaks can be increased by the addition of high concentrations of CsF (19,44). In our hands this behavior of Poly (dA-dT)·Poly (dA-dT) is reproduced, as seen in Fig. 18A-C. The separation of the peaks is 0.19 ppm in 0.1 M NaCl and increases to 0.51 ppm in 3 M CsF. In the case of the totally unmethylated polymer Poly (dA-dU)·Poly (dA-dU), a single peak is seen at moderate salt concentrations (Fig.

19A-B) and resolves into two peaks only at very high CsF levels (Fig. 19C-D). For polymers containing both dU and dT, the separation of the two peaks increases with increasing %dT (Fig. 20-22).

The separation of the peaks for the various polymers is plotted vs. CsF concentration in Fig. 23. It can be seen that for all polymers except Poly (dA-dU) · Poly (dA-dU), the separation of the two phosphorous resonances increases with increasing %dT of the polymer. In Fig. 24, with the CsF concentration held constant at 3 M, the chemical shift of the downfield and upfield peaks of each polymer is plotted vs. %dT of the polymer. As the percentage of methylated pyrimidines increases from 0% to 100% the downfield peak gradually shifts by 0.1 ppm to lower negative values and the upfield peak gradually shifts by 0.2 ppm to higher negative values.

The characteristic of the analogous brominated polymer Poly (dA-Br^5dU)·Poly (dA-Br^5dU):

The ability of the analogous brominated polymer, Poly (dA-Br^5dU)· Poly (dA-Br^5dU), to exist in an alternating conformation was investigated by observing its ^{31}P-NMR spectrum. As seen in Fig. 25, the brominated polymer gives a single peak at low salt concentration that resolves into two peaks at 3 M CsF separated by 0.29 ppm. In contrast, at this salt level Poly (dA-dT)·Poly (dA-dT) shows two

resonances separated by 0.51 ppm.

The methylation activities of HhaI methylase on the synthetic polynucleotides in different conformations:

The ability of a restriction methylase to use the mixed ribo-deoxyribo polymer as a substrate. As shown in Fig. 26, HhaI methylase not only can methylate poly (rG-dC)·Poly (rG-dC), but does so at a faster rate (2-3 times faster) and to a greater extent (about 3 times more methyl groups incorporated) than it does for the B form Poly (dG-dC)·Poly (dG-dC).

DISCUSSION

Conformations of Poly (rG-dC)·Poly (rG-dC):

CD spectroscopy of Poly (rG-dC)· Poly (rG-dC) at intermediate salt concentrations (0.1-1.0 M NaCl) shows a distinctly A-like spectrum, with a large positive peak at 260 nm (Fig. 7,spectrum B). It is definitely distinct from a B-like spectrum (positive peak at 280 nm followed by a negative peak at 250 nm); however, the small negative peak at 290 nm has a certain Z-like flavor to it. To be sure that the polymer was in the A form, we took a Raman spectrum of it in 0.1 M NaCl (Fig. 9, spectrum B). This clearly showed a peak at 811 cm^{-1}, characteristic of an A conformation (61). These results were previously reported by Brahms et al. (60), and on that basis they assigned the polymer to the A conformation. We confirm their results. However, we also observed that at the two extremes of monovalent cation concentration, 5 mM Tris and 2 M NaCl, CD showed a Z-like spectrum for Poly (rG-dC)·Poly (rG-dC). To confirm these assignments, we again took Raman spectra and compared them to the Raman spectra of Poly (dG-dC)·Poly (dG-dC) under identical conditions. Two regions of the Raman spectrum are diagnostic. In the first region, 800-840 cm^{-1}, A form shows a peak around 810 cm^{-1} (61), B form shows a broader, weaker peak at about 830-840 cm^{-1} (61), and the Z form has no features (14). Upon going

from 0.1 to 2.5 M NaCl, the peak of poly (rG-dC).Poly(rG-dC) at 811 cm^{-1} collapses (Fig. 9, spectra C and D), which is the behavior that is expected for an A-Z transition. In the second region, 620-685 cm^{-1}, Poly (dG-dC) · Poly (dG-dC) shows a band at 683 cm^{-1} in the B form that shifts to 624 cm^{-1} in the Z form (Fig. 9, spectra C and D). This band has been assigned to a guanine ring vibration (14). Poly (rG-dC)·Poly (rG-dC) in 0.1 M NaCl shows a band in the same area, 669 cm^{-1}, that shifts to 637 cm^{-1} in 2.5 M NaCl (Fig. 9, spectra A and B). These Raman spectra strongly support the contention that we are observing a high-salt A-Z transition in Poly (rG-dC)·Poly (rG-dC).

The Raman spectrum of Poly (rG-dC)· Poly (rG-dC) in 5 mM Tris, pH 7.5/0.1 mM EDTA (Fig. 10) is very similar to its high-salt spectrum and, thus, confirms that a low-salt Z conformation of the polymer exists. To further elucidate this surprising result, we turned to ^{31}P-NMR. Fig. 11, spectrum A shows that, in 5 mM Tris buffer, the polymer has phosphorous resonances at -3.1 and -4.1 ppm. These are very similar to the position of phosphorous resonances of the Z forms of Poly (dG-dC)·Poly (dG-dC) (13), Poly (dG-m^5 dC).Poly (dG-m^5dC) (25), and the high-salt Z form of Poly (rG-dC)· Poly (rG-dC) in NaCl (see below). However, Fig. 11, spectrum A also shows a third phosphorous resonance at -4.6 ppm.

Although the CD, Raman, and ^{31}P-NMR results, taken together, argue strongly that the low-salt conformation is Z form, clearly it is different from the high-salt Z conformations of other polymers. A possible explanation for the ^{31}P NMR results may be found in the initial x-ray crystallographic studies of Z-DNA oligonucleotides (3,4,5). Rich and co-workers (4) observed that in a single crystal of $d(CpG)_3$, even in the same oligonucleotide chain, different phosphate conformations could exist between GpC residues. The two conformers they identified were gauche(-)-trans and gauche(+)-trans about the GpC phosphates, which they dubbed the Z_I and Z_{II} conformations, respectively. They further suggested that left-handed Z DNA polymers in solution may exist in a mixture of conformations ranging from pure Z_I through mixtures of Z_I and Z_{II} to pure Z_{II} . It seems likely that Poly (rG-dC)·Poly (rG-dC) in low-salt solutions exists as a mixture of Z conformations. The peak at -4.6 ppm in the phosphorous NMR spectrum (Fig. 11, spectrum A) would then correspond to an altered GpC phosphate conformation, possibly the Z_{II} structure. CD results are consistent with this interpretation. Since the relative positions of the bases are identical in the Z_I and Z_{II} conformations, identical CD spectra are expected for the two forms, and this is what is seen (Fig. 7, spectra A and C).

^{31}P NMR at different salt concentrations offers further confirmation of conformational assignments. In 0.2 M NaCl (Fig. 11, spectrum B) the low-salt phosphorous resonances collapse into a single resonance at -4.1 ppm, similar to that for Poly (dG-dC)·Poly (dG-dC) in the same buffer (20), and a single peak is expected for a regular A helix. In 2.0 M NaCl (Fig. 11, spectrum C) the phosphorous resonance splits into two peaks, at -2.9 and -4.1 ppm, identical to the resonances of the Z forms of Poly (dG-dC)·Poly (dG-dC) and Poly (dG-m^{5}dC)·Poly (dG-m^{5}dC) in NaCl solutions (25,43), and exactly the behavior expected for a high-salt Z conformation.

Nature of the transitions of Poly (rG-dC)·Poly (rG-dC):

A priori one might expect major differences between an A-Z and a B-Z transition. Both the A and the Z forms of DNA are less hydrated than the B form (62), so that one would expect solvent structure to play less of a role in an A-Z vs. a B-Z transition. On the other hand, because the difference in the linear charge density is greater between the A and Z forms than the B and Z forms, one might expect electrostatic forces to play a greater role in an A-Z transition than they play in the B-Z transition. We have shown that Poly (rG-dC).Poly (rG-dC) undergoes an A-Z transition at 1.3 - 1.8 M NaCl. Compared to the B-Z transition in Poly (dG-dC). Poly (dG-dC), the above

transition occurs at lower-salt concentration and is some what less cooperative. Possible explanations for these observations are: (i) the transition is less cooperative because there is less of a hydration difference between the A and Z forms, and (ii) the transition occurs at less-stringent salt conditions [1.3-1.8 M NaCl vs. 2.3-2.5 M for Poly (dG-dC)·Poly (dG-dC)] because the 2'-OH group of rG favors entry of the polymer into the left-handed conformation. It may do this, as we first thought, because ribonucleotides favor the C3'-endo sugar pucker, and the Z conformation consists of alternating C2'-endo-C3'-endo sugar conformations (4).

As we have shown, Poly (rG-dC)· Poly (rG-dC) also undergoes a noncooperative conformational transition at low Na concentration in which increased counterion concentration actually disfavors the Z form. Additionally, it is seen in Table 2 that oligovalent cations (+2 to +4) immediately effectuate the Z-A transition once their concentration is on the same order as the concentration of polymer phosphate residues. Since this seems to be a counterion condensation phenomenon (63), where the oligovalent counterions condense onto the DNA and reduce its residual charge spacings that govern counterion condensation are farther apart in the low-salt Z form than in the A form. This observation fits very well with the suggestion that the low-salt Z form contains Z_{II}

conformers. The distance of closest approach of phosphates (across the minor groove) in a Z_{II} helix is greater than in a Z_I helix:8.6 vs. 7.7 Å, respectively (4). Therefore, one would expect a low-salt Z helix to be predominantly Z_{II}. Additionally, the distance of closest approach of phosphates in an A helix (across the major groove) is between 7.8 and 8.5 Å (64). So if the electrostatic forces arising from these interactions governed counterion condensation and the conformational transitions, one would expect to see a Z_{II}-to-A-to-Z_I transition as a function of increasing salt concentration.

The real situation is probably somewhat more complicated. Other forces besides electrostatics undoubtedly play a part. There are hydration differences between the Z_I and Z_{II} structures (4), and the exact distance of closest approach of phosphate groups in solution for the three forms of Poly (rG-dC)·Poly (rG-dC) is not exactly known. And since there are three ^{31}P NMR peaks, the low-salt conformation is probably a mixture of Z_I and Z_{II} forms, not pure Z_{II}. Additionally, it should be emphasized that the mixture must occur within a single double helix, not on separate double helices. Whether there is a regular pattern of the Z_I and Z_{II} conformations in a helix or a random pattern, however, cannot be deduced at this time.

An "alternating A" conformation assignment for Poly (rG-m^5dC)·Poly (rG-m^5dC):

An "alternating B" conformation was proposed for the synthetic polynucleotide Poly (dA-dT)·Poly (dA-dT) by Klug et al (45) to explain a number of unusual properties of the polymer that had been described in the literature. The main differences between normal B and alternating B in their model were in the sugar-phosphate backbone, specifically in the phosphodiester torsion angles. A strong piece of evidence supporting their model was the appearance of two closely spaced resonances in the ^{31}P-NMR spectrum of Poly (dA-dT)·Poly (dA-dT), indicating non-equivalent phosphate enviroments (19,20,44). The two peaks are separated by about 0.5-1.0 ppm, with the peaks going non-cooperatively further apart at extremely high CsF concentration (44).

Two other alternating purine-pyrimidine co-polymers, Poly (dA-dU)·Poly (dA-dU) (65) and Poly (dG-dC)·Poly (dG-dC) (25), give a single ^{31}P-NMR peak at low and moderate salt concentrations. Thus they are probably not in an alternating B structure. However, a second methylated polymer, Poly (dG-m^5dC).Poly (dG-m^5dC), does exhibit two closely spaced, but distinct, peaks at all salt concentrations when in the B form. This has been ascribed

to an alternating B structure (25,43).

We provide evidence that alternating structures can also occur in the A conformation. The Raman spectrum of Poly (rG-m^{5}dC)·Poly (rG-m^{5}dC) in 0.2 M NaCl (Fig. 15) clearly shows a strong peak at 811 cm^{-1}, which is a marker for the A conformation (61). The CD spectrum of the polymer (Fig. 12B), is not typical of A form DNA. However, polyribonucleotides in the A conformation have been seen to give a negative peak at 280-290 nm as well as a positive peak at 260-270 nm (66). Thus the CD spectrum does not conflict with the Raman data and we conclude that the polymer is in the A conformation at moderate salt concentration.

At 0 M NaCl in Tris buffer the ^{31}P-NMR spectrum of Poly (rG-m^{5}dC)·Poly (rG-m^{5}dC) shows a single peak at -4.2 ppm (Fig. 16A). As the NaCl concentration is increased to 30 mM (Fig. 16B) the peak splits into two peaks at -3.8 and -4.3 ppm. No changes in the CD spectrum are seen over this range. We interpret this as a salt dependent conformational change from a regular to an alternating A conformation in which the helix now has a dinucleotide repeat. The peak at -4.2 ppm (Fig. 16A) would then arise from the phosphates in the regular A helix. This is consistent with the ^{31}P-NMR spectrum of the unmethylated polymer which gives a single peak at -4.1 ppm (55) in the

0.01-1 M NaCl concentration range, due to an A helix. As the salt concentration is raised to 300 mM, both peaks shift downfield by about 0.1 ppm, but the separation between the peaks stays constant at about 0.53 ppm. Although the range of salt concentrations is limited here, this trend is similar to that reported by Chen & Cohen (19) for Poly (dA-dT)·Poly (dA-dT) in the presence of increasing concentrations of Na^+.

It now seems clear from this work and from past reports that a methylated pyrimidine is a strong promoter of alternating structure in either the A or B conformations. For the A conformation it has been shown here that, although Poly (rG-dC)·Poly (rG-dC) gives only a single ^{31}P-NMR peak at moderate salt concentration (55), when the cytosine is methylated two peaks are seen (Fig. 16C), indicative of an alternating structure. For the B conformation two methylated polymers, Poly (dG-m^5dC)·Poly (dG-m^5dC) (25,43) and Poly (dA-dT)·Poly (dA-dT) (19,20,44) give two peaks, but the analogous unmethylated polymers, Poly (dG-dC).Poly (dG-dC) (25) and Poly (dA-dU).Poly (dA-dU) (65), do not.

<u>Poly (rG-m^5dC)·Poly (rG-m^5dC) undergoes low and high salt transitions to a Z conformation:</u>

Poly (rG-dC)·Poly (rG-dC) is in a left hand helical Z conformation in 5 mM Tris buffer (55). The methylated

polymer, Poly (rG-m^5dC)·Poly (rG-m^5dC), however, gives a single phosphorous resonance in its ^{31}P-NMR spectrum in 5 mM Tris buffer (Fig. 16A), which is not the behavior of a Z form and which, we have argued above, reflects a regular A conformation. But when the methylated polymer is in a solution containing only triethanolamine cation as the counterion (Fig. 17) two widely separated peaks are seen at -2.9 and -4.6 ppm, which is the behavior expected for a Z form. The CD spectrum of the polymer in this solution (Fig. 12A) is very similar to the CD spectra of the Z forms of Poly (dG-dC)·Poly (dG-dC) (2) and Poly (dG-m^5dC)·Poly (dG-m^5dC) (11). The CD spectrum changes cooperatively as the concentration of NaCl (Fig. 13) or Tris (data not shown) is increased past 30 mM. We are led to believe from the CD and ^{31}P-NMR data that the methylated polymer converts to a left hand helix in the presence of triethanolamine. The effectiveness of triethanolamine may be explained by observing that the triethanolamine cation is a larger, bulkier ion than Tris and, therefore, may be less effective in screening the phosphate - phosphate charge repulsions in polynucleotides. So triethanolamine buffer can be thought of as a "lower salt" condition than Tris buffer.

As the salt concentration is increased to 1 M a high salt A-Z transition begins to take place (Fig. 16E). At 2

M NaCl the polymer gives two phosphorous resonances separated by 1.4 ppm (Fig. 16F). The resonances at - 2.7 and -4.1 ppm are of equal intensity. This behavior is very similar to that of Poly (dG-dC)·Poly (dG-dC) (13), Poly (dG-m^5 dC)·Poly (dG-m^5dC) (25,43), and Poly (rG-dC)· Poly (rG-dC) (55) in high salt solutions, all of which are known to be in the Z conformation at the given conditions. Since the negative CD peak of the polymer at 286 nm does not change at high salt and the peak at 267 nm decreases monotonically with increasing salt concentration (Fig. 21), CD can not be used here to monitor the conformational change. Therefore we must rely on ^{31}P-NMR to conclude that the high salt transition occurs between 1 and 2 M NaCl.

Neither the high salt nor the low salt transition to a Z form is more facile in the methylated polymer than it is in poly (rG-dC)·poly (rG-dC). The high salt transition occurs between 1 and 2 M NaCl in the methylated polynucleotide (Fig. 16 E & F) compared to a transition range of 1.3-1.8 M NaCl for the analogous unmethylated polymer (55). And the low salt transition of poly (rG-m^5 dC).Poly (rG-m^5dC) requires a bulkier counterion than does the transition for the unmethylated polymer. Thus the methyl group at the 5 position of cytidine does not have the large effect on an A-Z transition as it has on the B-Z transition (11). This must be because it either stablizes the A conformation more than the B conformation, or does

not stabilizes the Z conformation of Poly (rG-m^5dC). Poly (rG-m^5dC) as much as the Z form of Poly (dG-m^5dC). Poly (dG-m^5dC). The latter possibility would imply some differences between the Z conformations of the fully deoxy and the mixed ribo- deoxyribo polymer.

Flexibility of the Poly (rG-m^5dC)· Poly (rG-m^5dC):

The sequence dependence of DNA conformation has been amply demonstrated in the past few years. In the salt dependent behavior of Poly (rG-m^5dC)· Poly (rG-m^5 dC), however, the great conformational flexibility of polynucleotides is manifested. Within a modest change in solvent conditions the polymer goes from a low salt Z form to a regular A form to an alternating A form, and at higher salt concentration reverts again to a Z form. Other conformations of the polymer may exist that have not been observed under the solvent conditions and with the techniques employed here. Although there is no sequence of Poly (rG-m^5 dC) · Poly (rG-m^5dC) that occurs *in vivo*, nonetheless the conformations available to this synthetic polymer may reflect conformational states available to DNA sequences *in vivo* under the correct conditions of counterion concentration and type, helical strain, or specific protein binding.

Methylation of pyrimidine bases contributes to the

formation of alternating right-handed DNA conformations:

Methylated sites in eukaryotic DNA are thought to have a role in the regulation of gene function (39,40). There are several ways that methylation can be thought to regulate genes. The first is by a regulatory protein recognizing the methylated sequence directly. Presumably the DNA is in a more or less normal B conformation and the extra contacts provided by the methyl groups are sufficient for the protein to distinguish it from the unmethylated sequence. A second possibility is that methylation of DNA causes a change in the conformation of the DNA itself. The altered conformation can then either be bound by a regulatory protein or is itself a better or worse template for a polymerase.

The second alternative has gotten much attention in the past few years since it was shown that methylation can sometimes affect the conformation of DNA quite dramatically. Behe and Felsenfeld (1981) demonstrated that methylation of the synthetic polynucleotide Poly (dG-dC). Poly (dG-dC) at the 5 position of cytosne, which is often the site of eukaryotic methylation, can lower the concentration of counterions required to flip the polymer into the Z conformation, so that the methylated polymer could exist in the Z form at physiological ionic strength(11). However, the methylation of DNA can

apparently have other effects on structure. Klug et al (1979) proposed a model for the structure of Poly (dA-dT)· Poly (dA-dT) which they dubbed the "alternating B" conformation. In this model the two sequences ApT and TpA differed in base stacking and phosphodiester conformation. It was suggested (45) that the altered structure occurred to maximize the stacking of thymine's methyl group on the base below it. Physical evidence for the model was provided by ^{31}P-NMR studies (19,20,44) of Poly (dA-dT)· Poly (dA-dT) which showed two closely spaced, but distinct, phosphorous resonances, reflecting the two phosphorous environments of the polymer. Eckstein & Jovin (1983) showed that the downfield peak was due to ApT while the upfield peak was due to TpA(67). Patel et al (1981) showed that the separation of the peaks could be increased by large concentrations of CsF (44).

The alternating purine-pyrimidine polymer Poly (dG-dC)· Poly (dG-dC) did not give two phosphorous resonances except at very high salt concentration (25). However, the analogous methylated polymer, Poly (dG-m^{5}dC)·Poly (dG-m^5 dC), did give two closely spaced peaks even at low salt concentration (25,43), which was interpreted as reflecting an alternating B conformation for the polymer. And the two mixed ribo-deoxyribo co-polymers Poly (rG-dC)·Poly (rG-dC) (55) and Poly (rG-m^{5}dC).Poly (rG-m^{5}dC) (68) are in the A conformation at moderate salt concentrations. Although the

Poly (rG-dC). Poly (rG-dC) gives a single ^{31}P-NMR peak, the analogous methylated polymer gives two closely spaced peaks. It has been interpreted as evidence for an "alternating A" conformation. Thus it appeared to us, as Klug et al (1979) speculated, that a methylated pyrimidine was a promotor of an alternating conformation (45). We then decided to investigate the effect of methyl groups on structure by making a series of polymers containing varing ratios of methylated to unmethylated pyrimidines.

We anticipated two possible extreme situations before we began. The first was that the occurrence of the alternating structure was a very localized phenomenon, with methylated base pairs locked into the alternating form, but that the alternating structure did not extend further into the rest of the helix. The second case was that the regular to alternating transition was highly cooperative. In the first situation one would expect to see a third ^{31}P-NMR peak, corresponding to the regular B conformation, increasing in intensity and the two original peaks decreasing as the percentage of methyl groups was decreased. In the second case, the highly cooperative situation, one would expect two peaks of equal intensity for all polymers down to a critical percentage of methylated residues, and a single peak after that. (This is what is seen in the B-Z transition as a function of

salt concentration.) As seen in Fig. 18-22, neither of these postulated cases occurs. Instead, as seen in Fig. 23 & 24, at any one CsF concentration, as the percentage of methylated bases decreases the separation between the phosphorous resonances decreases. This suggests that the conformation of the polymer is gradually progressing from a regular helix to an alternating helix as the percentage of methylated pyrimidines increases from 0% to 100%. This further implies that a continuum of conformations, stabilized by an increasing percentage of methylated pyrimidines, is stable between a regular and an alternating helix. This interpretation is supported by studies of the effect of CsF (19,44) on peak separation in an alternating structure. As CsF is continuously increased the separation of phosphorous resonances of Poly (dA-dT)· Poly (dA-dT) and the polymers reported here continuously increases, strongly suggesting the gradual shift to a more pronounced alternating structure, as Chen & Cohen (1983) argued in the case of Poly (dA-dT)·Poly (dA-dT) (19). This study was conducted on polymers of the general form Poly (dA-dU,T)· Poly (dA-dU,T) because, among other reasons, they do not have a propensity to flip to a Z form at high salt concentrations like Poly (dG-dC) ·Poly (dG-dC). Discussion of the results obtained here, then, should be limited to sequences of dA-dT residues. Nonetheless, It is tempting to speculate that methylation of -CG- sequences

in vivo is having a similar effect as on the synthetic polymers, shifting a region of DNA toward a more alternating structure, and that regulatory proteins are recognizing such subtle conformational changes.

We have observed that several other factors besides methylation that a priori might have been thought to favor the alternating structure are insufficient to do so. The first is the sugar conformations of the polynucleotide. In the alternating B model of Klug et al (1979) for Poly (dA-dT)·Poly (dA-dT), the deoxyribose of dA is in the C3'-endo conformation and the sugar of dT is C2'-endo(45). As mentioned above, Poly (rG-dC)·Poly (rG-dC) does not show an alternating structure (55) even though the purine residues are expected to be in the C3'-endo conformation since ribonucleotides favor that form. The analogous methylated polymer, however, is in an alternating conformation at moderate salt concentration (68).

A second factor which we expected to favor the alternating B form was the presence of a bromine at the C5 position of dU. Klug et al (1979) made the conjecture that Poly (dA-Br^5dU)·Poly (dA-Br^5dU), since it binds the lac repressor more tightly than Poly (dA-dT)·Poly (dA-dT) and much more tightly than heterogeneous sequence DNA (69), would favor the alternating B form more than Poly (dA-dT)·Poly(dA-dT) (45). However, Fig. 25 shows that the

brominated polymer gives a single ^{31}P-NMR peak at low salt, and a much smaller separation of peaks in CsF solution than Poly (dA-dT)·Poly (dA-dT). This suggest that Poly (dA-Br^5 dU)·Poly (dA-Br^{5}dU) binds the lac repressor more tightly than heterogeneous sequence DNA for some reason other than an alternating structure.

Action of enzymes on an A form polynucleotide:

DNA can exist in a variety of conformations (70). The B form apparently is the predominant conformation for most sequences near physiological conditions, but perturbing factors can cause the left hand helical Z form or the right hand helical A form to occur. The Z form can be the predominant conformation under mild solvent conditions if the DNA is highly methylated (11) or under supercoiling tension (71,72,73). However, only certain sequences (alternating purine-pyrimidine, G-C rich) are candidates to take up the Z form. The A form of DNA has not yet been seen in solution under mild conditions, but almost any sequence of DNA can assume the A form if appropriate solvent conditions are employed (74).

There have been a number of reports which indicate that Z-DNA occurs in vivo and has a physiological function. Anti-Z antibodies bind to discrete regions of polytene chromosomes from several insect species (75,76), and specific Z-DNA binding proteins have been discovered (77).

The occurrence of A-DNA in vivo has much less evidence to support it. However A-DNA, since it is right hand helical like B-DNA, would also be much more difficult to find as a transient minority of a larger B-DNA population. Recently Frederick et al (54) have seen that the DNA flanking the enzyme recognition site in an EcoR1-oligonucleotide co-crystal is in an A-like conformation. A possibility raised by this is that the enzyme induces the DNA it binds to in vivo into the A form. Because this would involve a physiological occurrence and role for A-DNA, we decided to investigate the ability of several deoxyribonucleases and a restriction methylase to act on the A conformation of a polynucleotide.

As a model for the A conformation of DNA we used the mixed ribo-deoxyribo co-polymer Poly (rG-dC)·Poly (rG-dC), where ribo and deoxyribonucleotide residues alternate on both strands. This polymer is useful because it is in the A conformation at moderate salt concentrations (55,60), its two strands are identical, and its base sequence is a potential substrate for several restriction enzymes. The drawback, of course, is that all of the guanosines have a 2'-OH group, which may inhibit an enzyme that normally works on DNA independent of the conformation of the polynucleotide. Nonetheless, if a positive result is obtained we can conclude that the A conformation of the

polymer is a substrate for the particular enzyme.

Because of the recent negative results with Z-DNA (52,53), we were anxious to see if the restriction enzyme HhaI could cut a polymer in the A conformation. The restriction enzyme HhaI did cut Poly (rG-dC)·Poly (rG-dC) at a somewhat reduced rate compared to B-form Poly (dG-dC) ·Poly (dG-dC) as the substrate(Sehyoon, Wu, & Behe, unpublished results). Molloy and Symons (78) have previously shown that a DNA-RNA hybrid of cucumber mosaic virus RNA and its cDNA, which presumably is in the A form, is cut by HhaI, but only at enzyme levels 20-50 times higher than that required for duplex DNA. This may be because the occurrence of a strand containing all ribonucleotides inhibits the enzyme more than alternating ribo-and deoxyribo- nucleotides. Another restriction enzyme that can recognize the polymer sequence, FnuDII, was unable to cut the polymer (Sehyoon, Wu, & Behe, unpublished results). It is not known if this is due to its inability to act on an A conformation or if the guanosine 2'-OH inhibits the enzyme. The two other endodeoxynucleases that we studied also gave varying results. While staphylococcal nuclease is able to cut Poly (rG-dC)·Poly (rG-dC) at a rate comparable to the analogous fully deoxy polymer, pancreatic DNaseI can't digest the polymer at all (Sehyoon, Wu, & Behe, unpublished results). This latter result apparently conflicts with an earlier

report (79) that said DNaseI cleaved crab d(A-T) polymer, which is reported to contain occasional ribonucleotides, after rG residues. That report may be reconciled to this paper if the occasional ribonucleotide of crab d(A-T) polymer is insufficient to force it into the A conformation. In that instance it might mean that DNaseI could cut near a ribonucleotide that is in the B conformation, but could not cut a polynucleotide in the A conformation. We were also test an enzyme other than a nuclease on the mixed polymer. HhaI methylase was the enzyme of choice since not only is its respective nuclease able to act on the A conformation, but the methylase probes a different region of the helix, the major groove. HhaI methylase was not only able to transfer a methyl group from S-adenosyl methionine to the A form polymer, but it could also do it at a faster rate and to a greater extent than with Poly (dG-dC)·Poly (dG-dC) as a substrate (Fig. 26).

Conformation of polynucleotides bound to enzymes:

What do these results imply about the conformational state of other substrates of the enzymes that do work on Poly (rG-dC)· Poly (rG-dC)? The first possibility is that the binding sites of the enzymes are flexible enough to recognize both the B and the A conformations. This may be the case for the comparatively non-specific staphylococcal

nuclease, but it is much less likely in the case of the highly sequence specific HhaI and HhaI methylase. Sufficient flexibility to recognize the greatly different B and A conformations would almost surely mean decreased sequence specificity. Another possibility is that the enzymes work on B form DNA and force Poly (rG-dC)· Poly (rG-dC) into a B form before acting on it. This also seems unlikely. Forcing the polymer into a B form would cost binding energy that ultimately would be reflected in the reaction rate. But the three enzyme (HhaI, HhaI methylase, staphylococcal nuclease) work at rates similar to those using B form Poly (dG-dC)· Poly (dG-dC) as a substrate. By the same reasoning one could argue against the A form being the substrate and B form polynucleotides forced into the A conformation before reaction can occur. It seems to us that there are two possible explanations. The first is that A-DNA is the substrate of these enzymes, but that the 2'-OH on the guanosines of our model polymer slows the action of the enzymes down to that for the unfavorable B conformation. The second possibility is that the enzyme bound substrate conformation is somewhere between the solution B and A forms so that, fortuitously, the binding of B or A form polymers is equally productive. Obviously, much more work needs to be done. But the results reported here raise higher the possibility that A-DNA may be a biologically active form of DNA.

Figure 1

The incorporated rate of ^{3}H-rGTP of Poly (rG-dC) · Poly (rG-dC) synthesis reaction , after 16 hrs incubation at $37^{o}C$, Vs. pH of the reaction mixture.

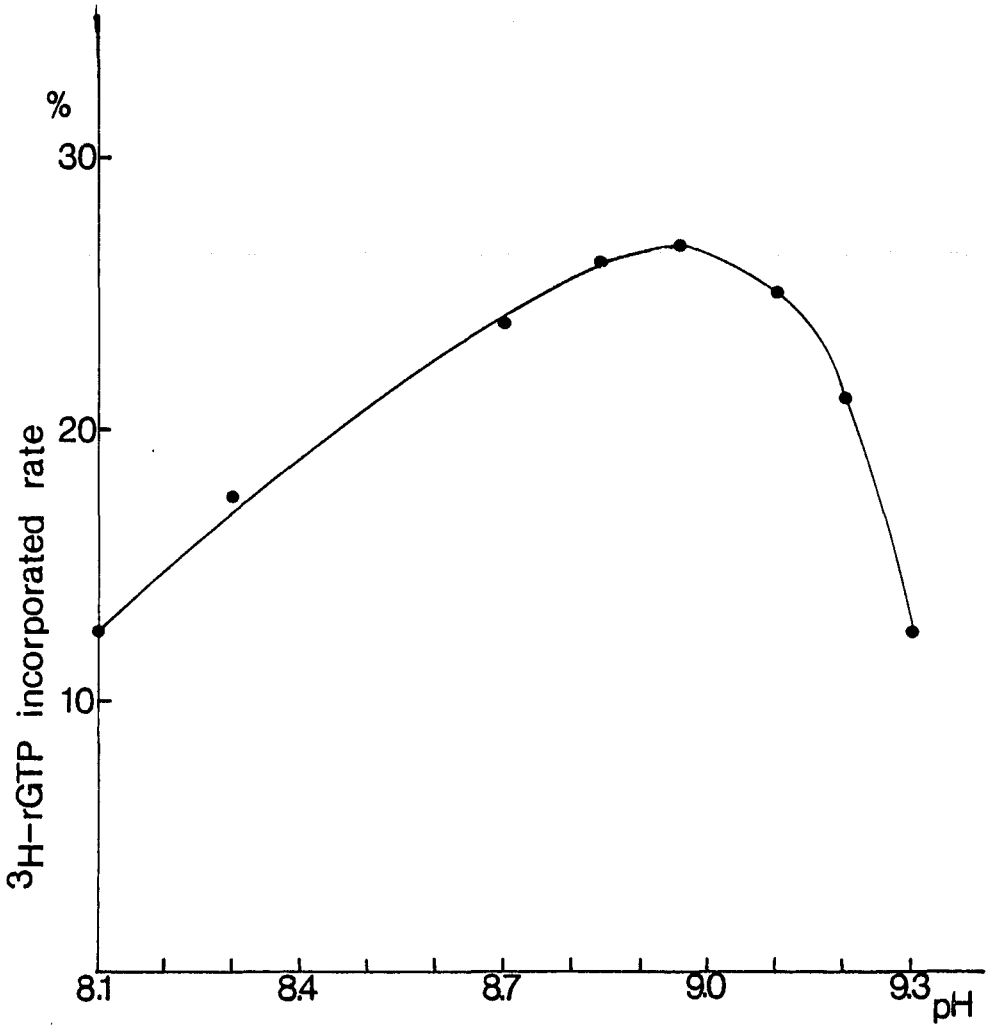

%
30
20
10
^{3}H-rGTP incorporated rate
8.1
8.4
8.7
9.0
9.3
pH

Figure 2

The incorporated rate of ^{3}H-rGTP of Poly (rG-dC) • Poly (rG-dC) synthesis at pH 8.95 Vs. reaction time.

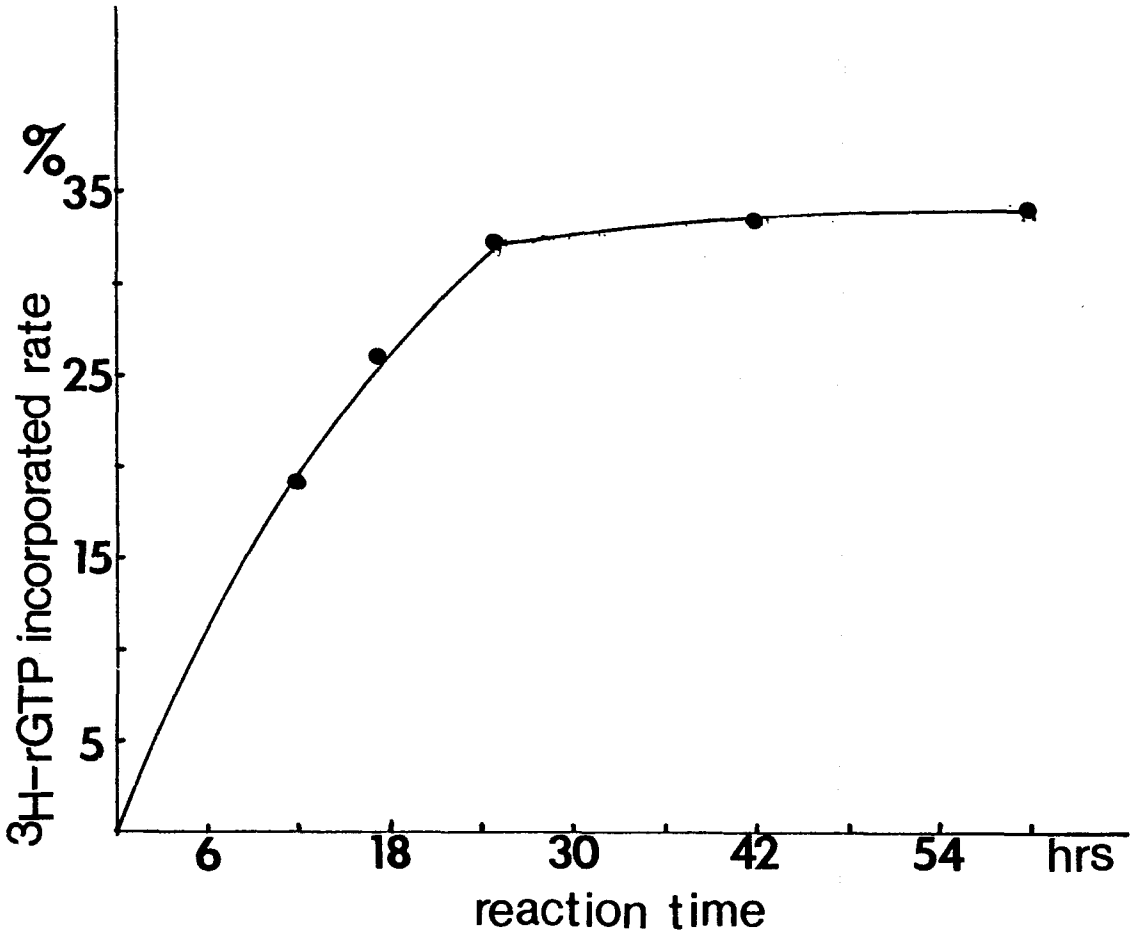

%
35
25
15
5
^{3}H-rGTP incorporated rate
6
18
30
42
54
hrs
reaction time

Figure 3

The incorporated rate of ^{3}H-rGTP of Poly (rG-dC) ·Poly (rG-dC) synthesis in the presence of different concentration of manganese ion : (—▲———▲—) 6 mM; (—□———□—) 3 mM; and (—●———●—) 0.1 mM, Vs. reaction time.

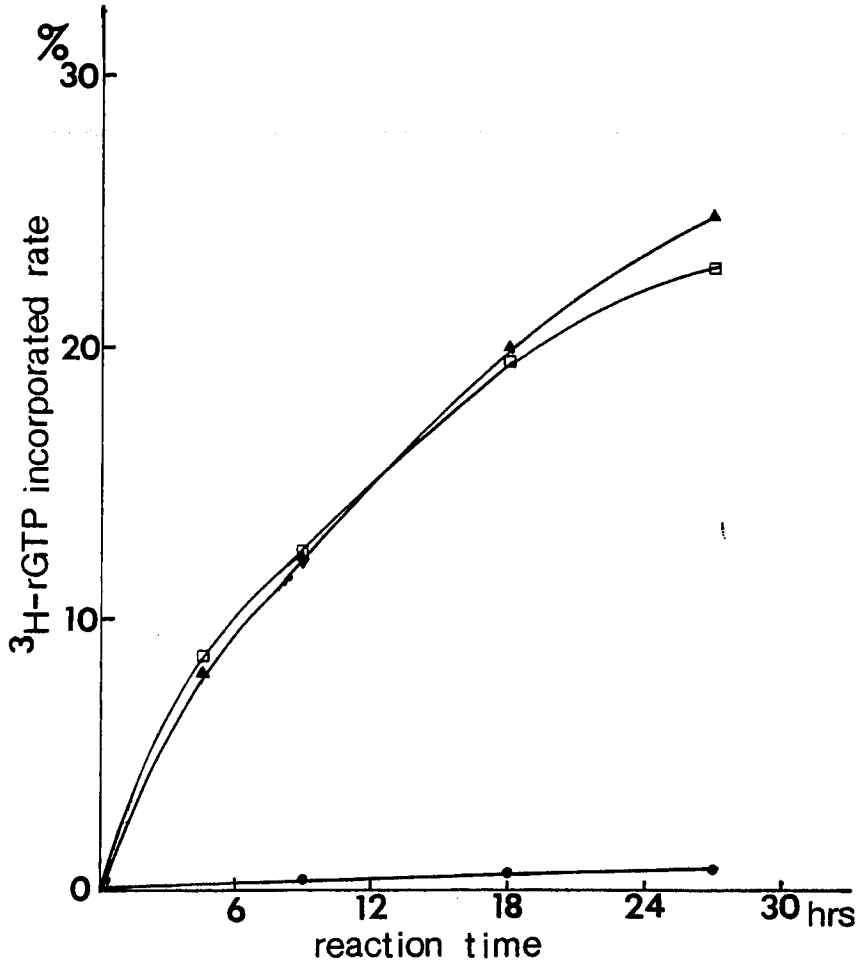

%
30
20
10
0
^{3}H-rGTP incorporated rate
6
12
18
24
30 hrs
reaction time

Figure 4

Autoradiography of partially base hydrolyzed Poly (rG-dC) · Poly (rG-dC) and partially HhaI restriction digested Poly (dG-dC)· Poly (dG-dC) on a sequencing gel. (a) HhaI (1 unit/10 ul) digested Poly (rG-dC)· Poly (rG-dC); 2 hrs incubation at 37°C (b-f) Poly (dG-dC) Poly (dG-dC) digested with HhaI (1 unit/10 ul) at 37° C for 1 hr, 1.5 hr, 1.75 hr, 2 hr, and 2.5 hr respectively. (g-j) 0.3 M NaOH hydrolyzed Poly (rG-dC) Poly (rG-dC) incubated at 37°C for 20 minutes, 10 minutes, 5 minutes respectively (K) Poly (dG-dC)·Poly (dG-dC) incubated in 0.3 M NaOH solution at 37°C for 20 minutes.

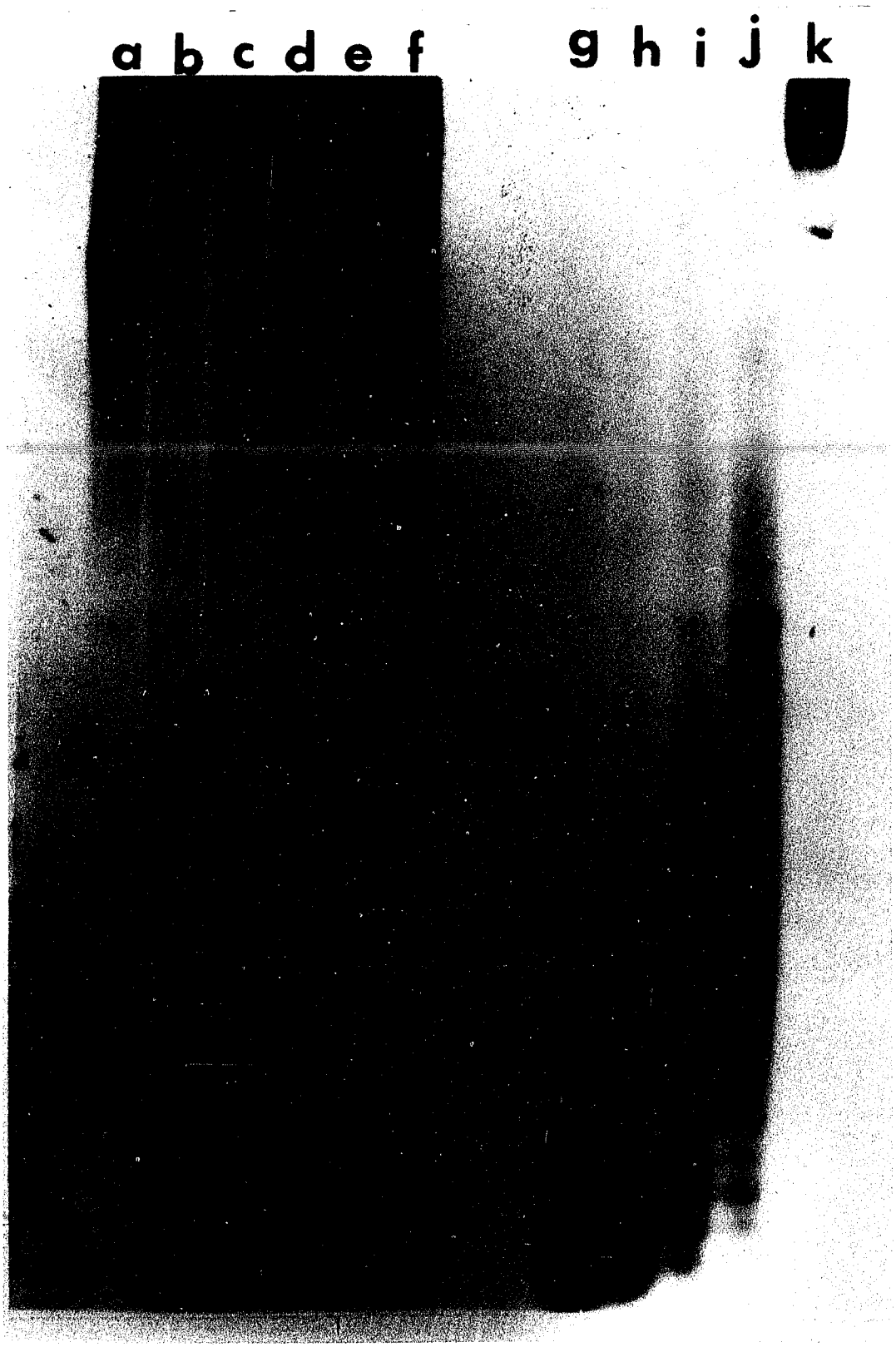

a b c d e f
g h i j k

Figure 5

The illustration of base hydrolysis of Poly (rG-dC)·Poly (rG-dC).

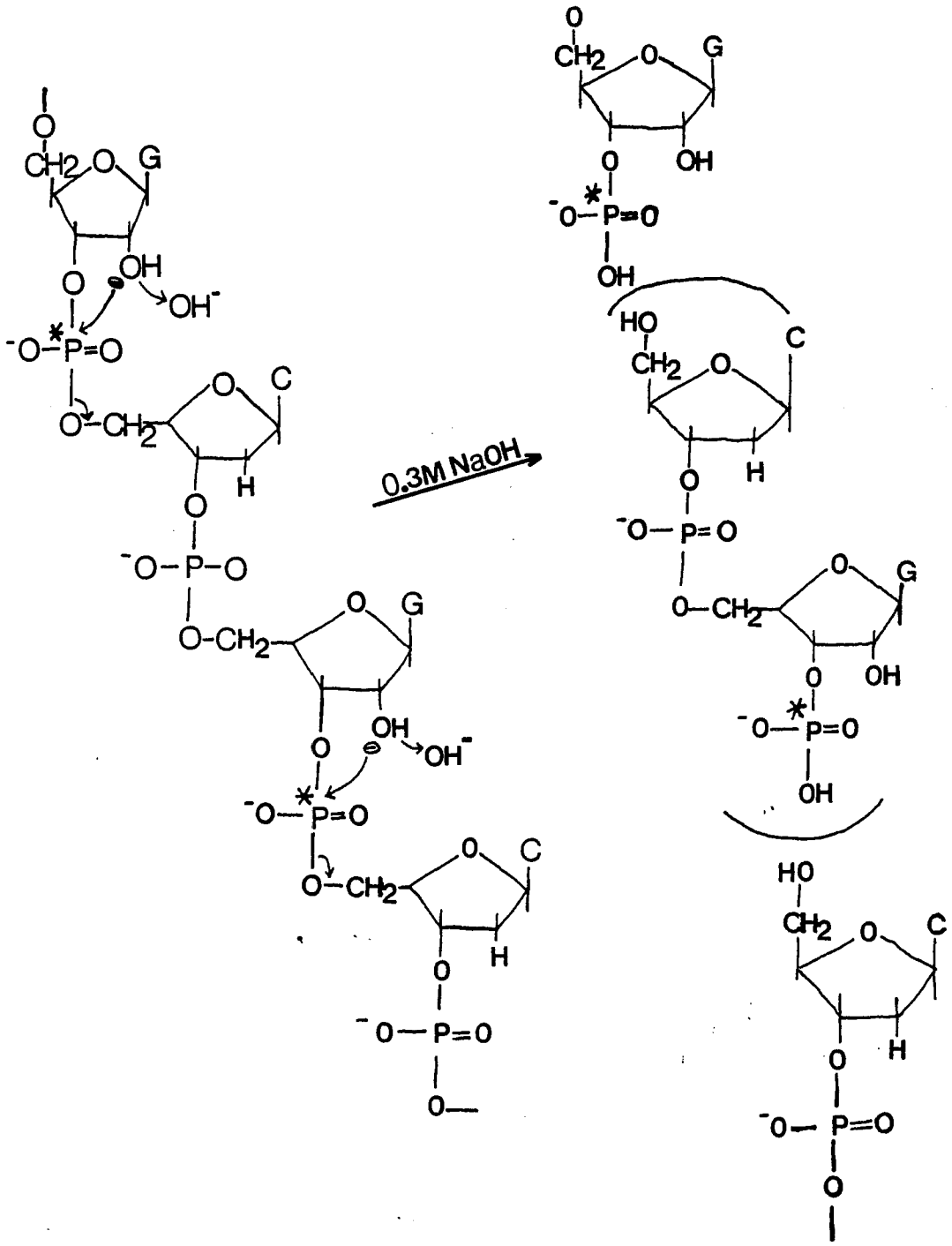

0.3M NaOH

Figure 6

The Thin Layer Chromatography of 0.3 M NaOH and Micrococcal nuclease double digested Poly (rG-dC)· Poly (rG-dC). (Lane 1) the cold 2'-deoxycytidine-3'-mono-phosphate (Lane 2) the ^{32}P labelled digested Poly (rG-dC)·Poly(rG-dC) (Lane 3) The cold guanosine-3'-monophosphate.

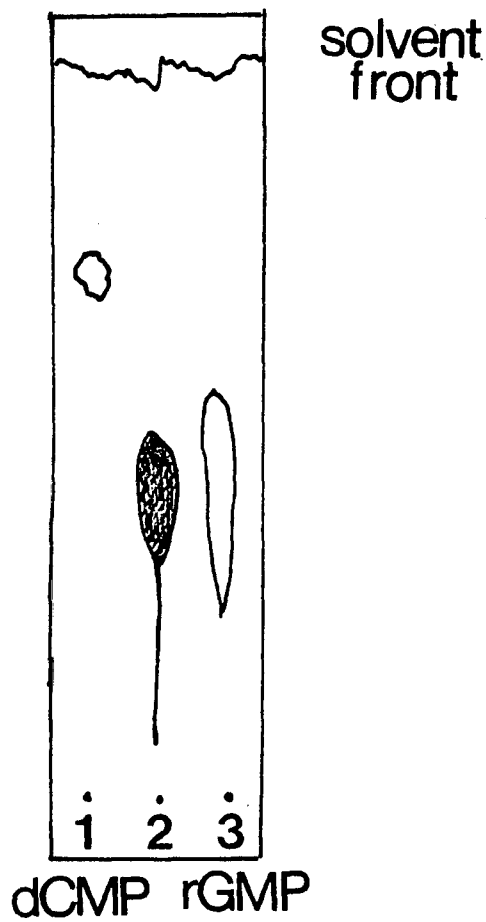

solvent
front
1
2
3
dCMP
rGMP

Figure 7

Circular dichroism spectrum of Poly (rG-dC)· Poly (rG-dC) in 5 mM Tris, pH 7.5/0.1 mM EDTA containing 0 M NaCl (spectrum A), 0.2 M NaCl (spectrum B), or 2.0 M NaCl (spectrum C).

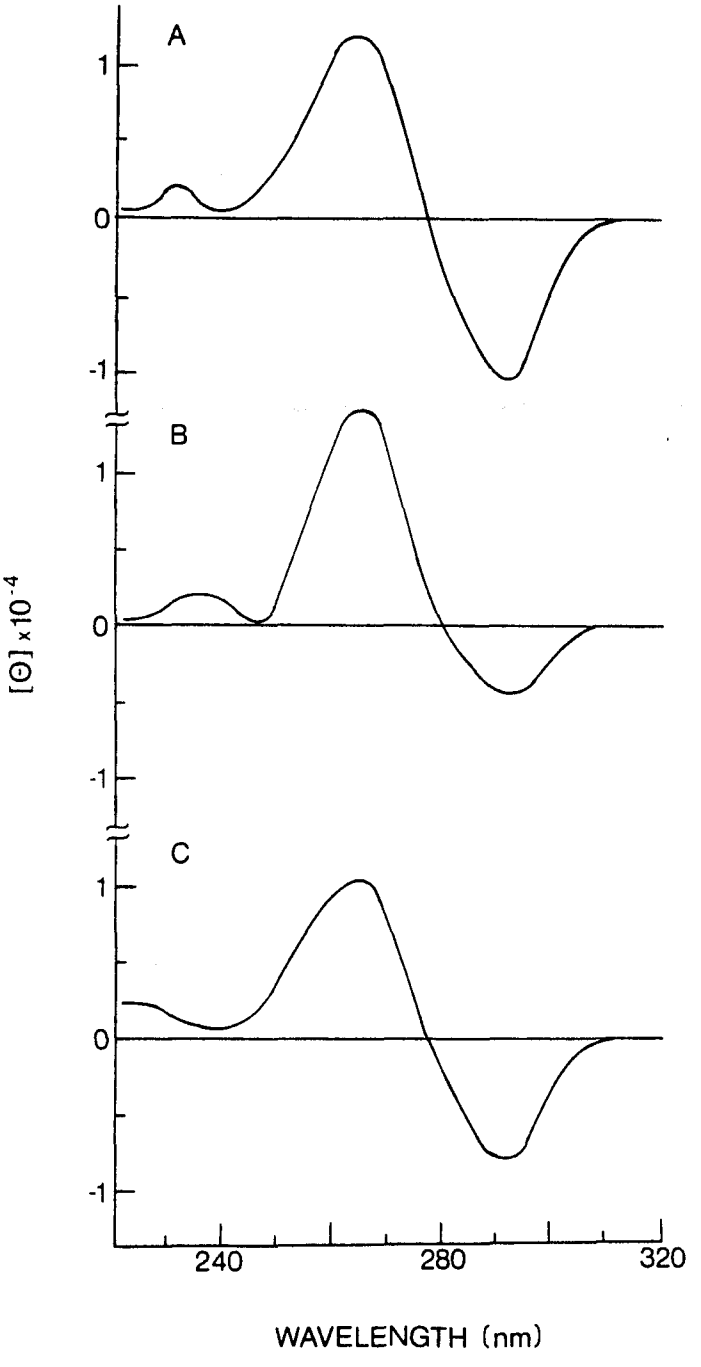

A
B
C
1
0
-1
[Θ] x10^-4
240
280
320
WAVELENGTH (nm)

Figure 8

Molar ellipticity of Poly (rG-dC)·Poly (rG-dC) at 292 nm as a function of NaCl concentration.

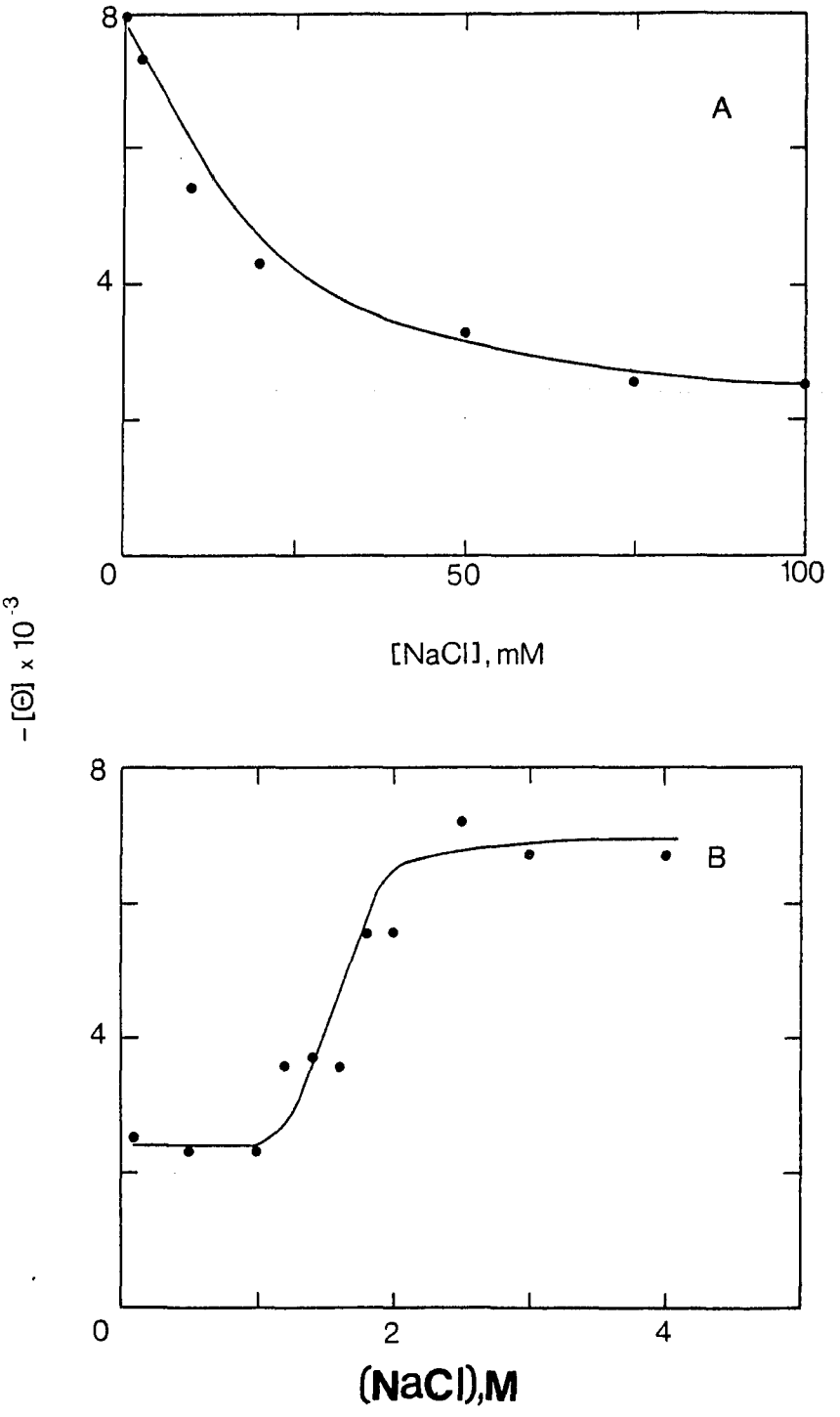
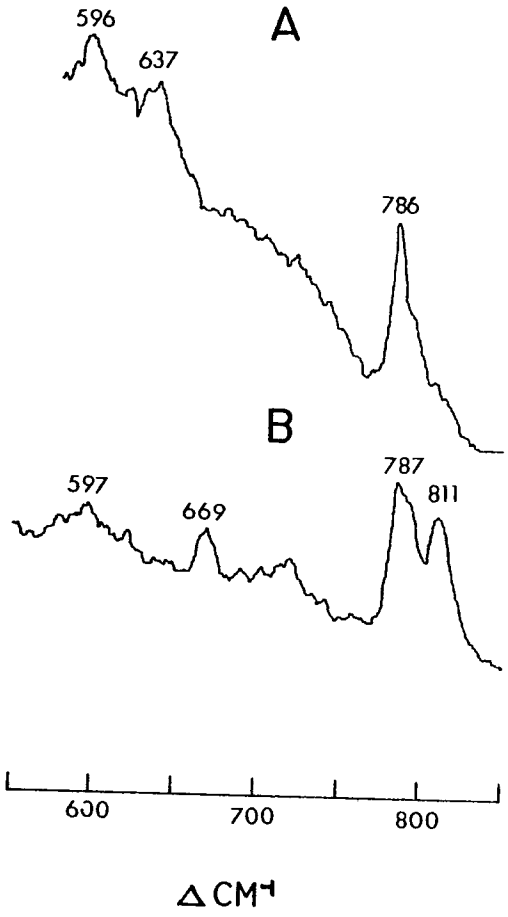

8
4
0
50
100
A
[NaCl], mM
$-[\Theta] \times 10^{-3}$
8
4
0
2
4
B
(NaCl),M

Figure 9

Raman spectra of Poly (rG-dC)· Poly (rG-dC) (spectra A and B) and Poly (dG-dC)·Poly (dG-dC) (spectra C and D) in 5 mM Tris, pH 7.5/0.1 mM EDTA containing 2.5 M NaCl (spectra A and C) and 0.1 M NaCl (spectra B and D).


596
637
A
786
B
597
669
787
811
600
700
800
Δ CM⁻¹

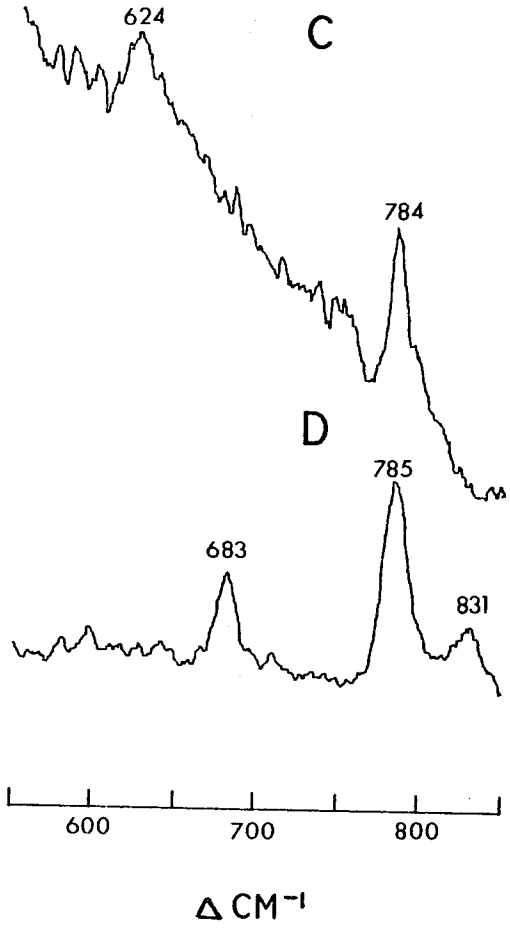

624
C
784
D
683
785
831
600
700
800
Δ CM⁻¹

Figure 10

Raman spectrum of Poly (rG-dC). Poly (rG-dC) (A) in 5 mM Tris, pH 7.5/0.1 mM EDTA (B) plus 0.1 M NaCl.

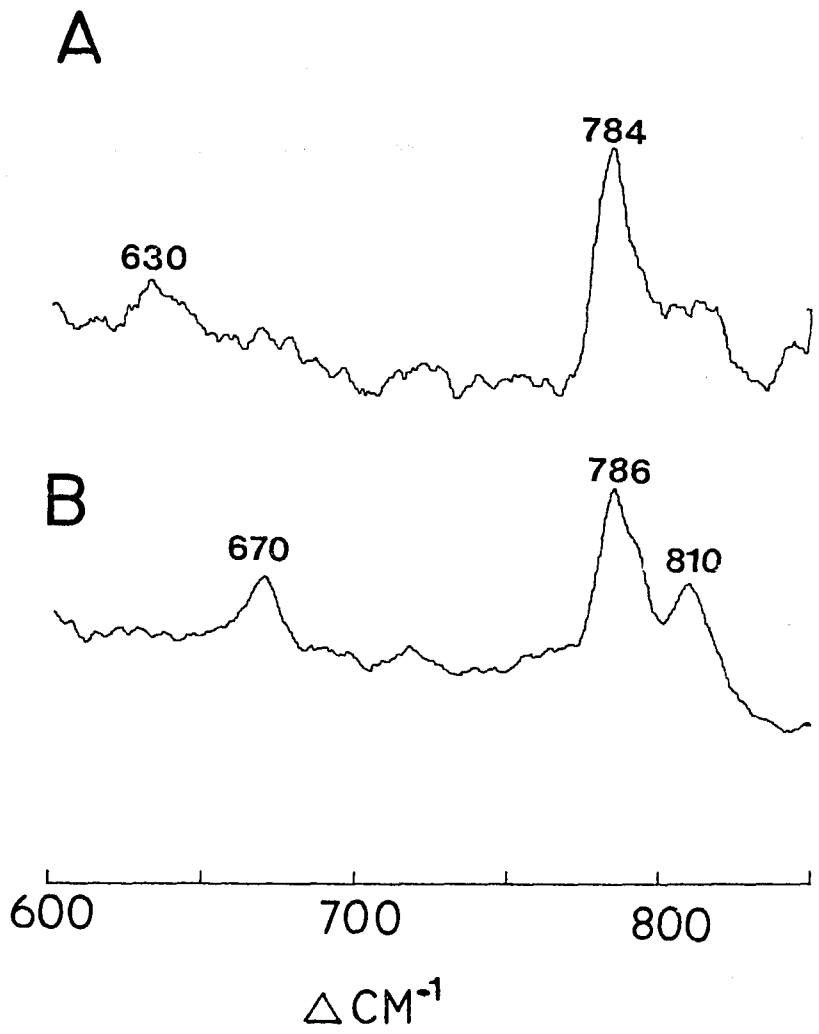

A
784
630
B
786
670
810
600
700
800
△CM-1

Figure 11

^{31}P NMR spectra of Poly (rG-dC)· Poly (rg-dC) in 5 mM Tris, pH 7.5/0.1 mM EDTA containing 0 M NaCl (spectrum A), 0.2 M NaCl (spectrum B), or 2.0 M NaCl (spectrum C). All solutions also contained 1 ul of trimethyl phosphate as a reference standard, which gives rise to the peaks at 0 ppm.

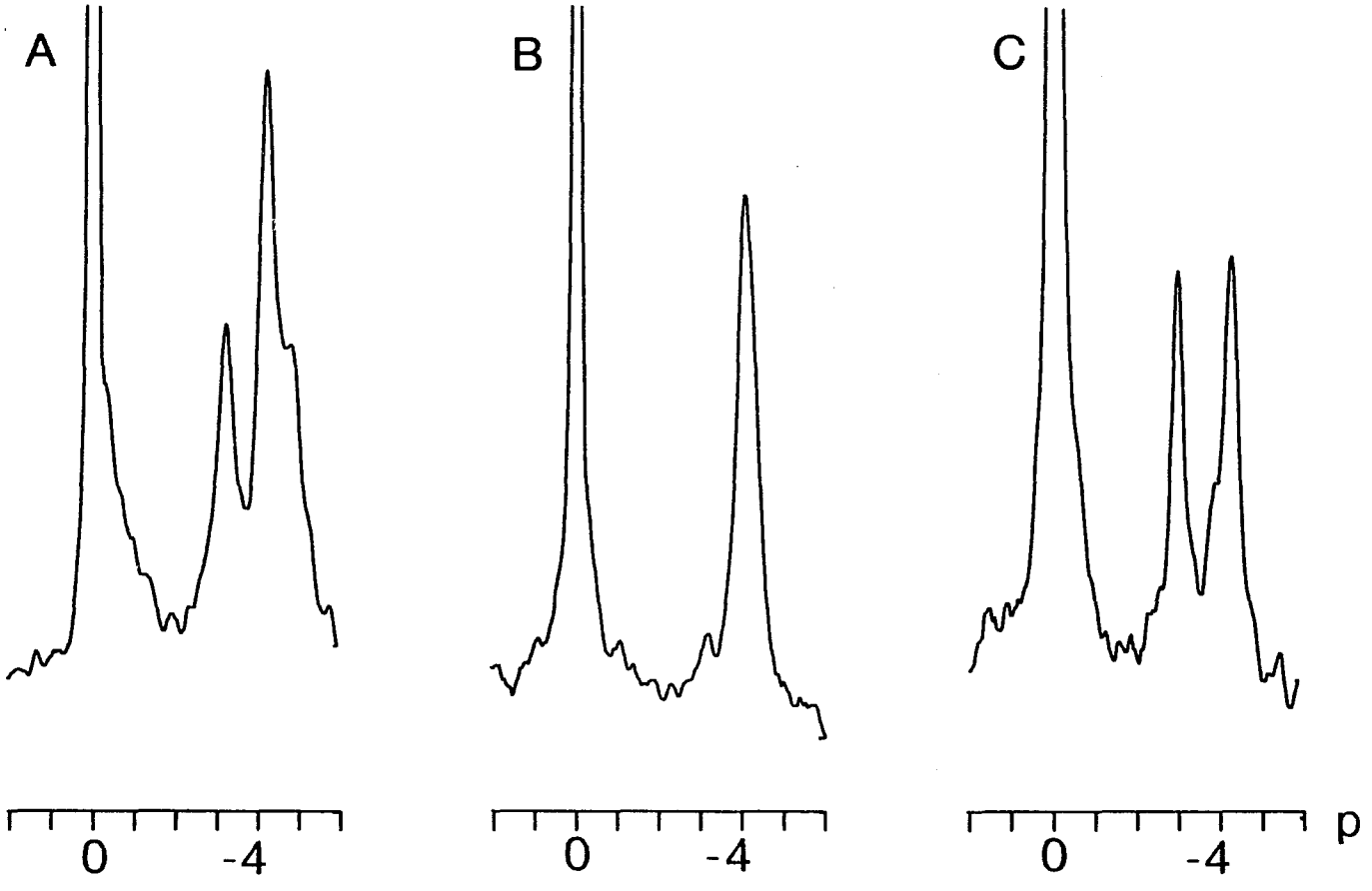

A
B
C
0
-4
0
-4
0
-4
p

Figure 12

Circular dichroism spectra of Poly (rG-m^5dC)· Poly (rG-m^5dC) in 5 mM triethanolamine-HCl pH 7.5, 0.1 mM EDTA, plus : (A) 0 M NaCl; (B) 50 mM NaCl; (c) 3 M NaCl.

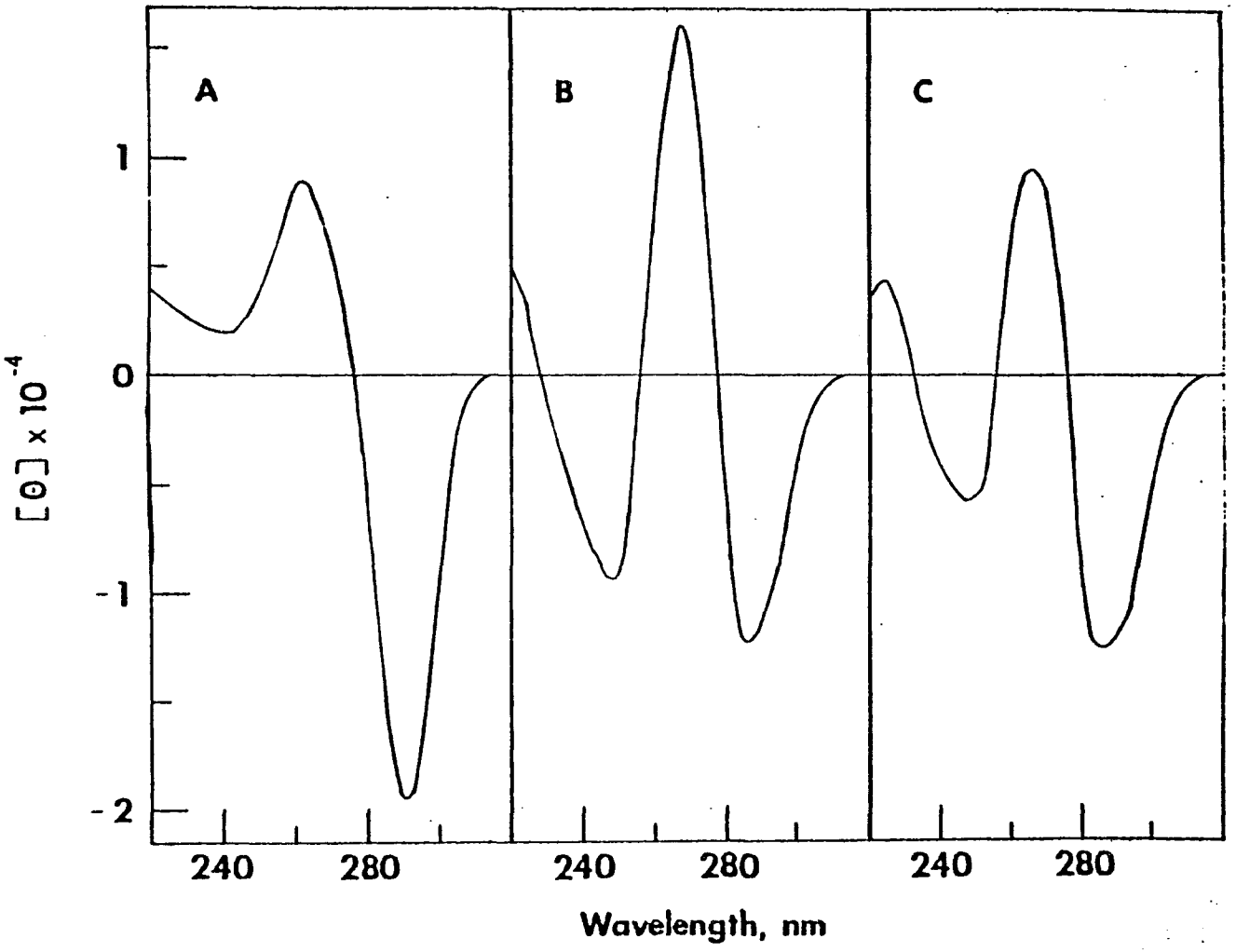

A
B
C
1
0
-1
-2
[Θ] x 10^-4
240
280
240
280
240
280
Wavelength, nm

Figure 13

Molar ellipticity of Poly (rG-m^5dC) · Poly (rG-m^5dC) at 292 nm as a function of NaCl concentration.

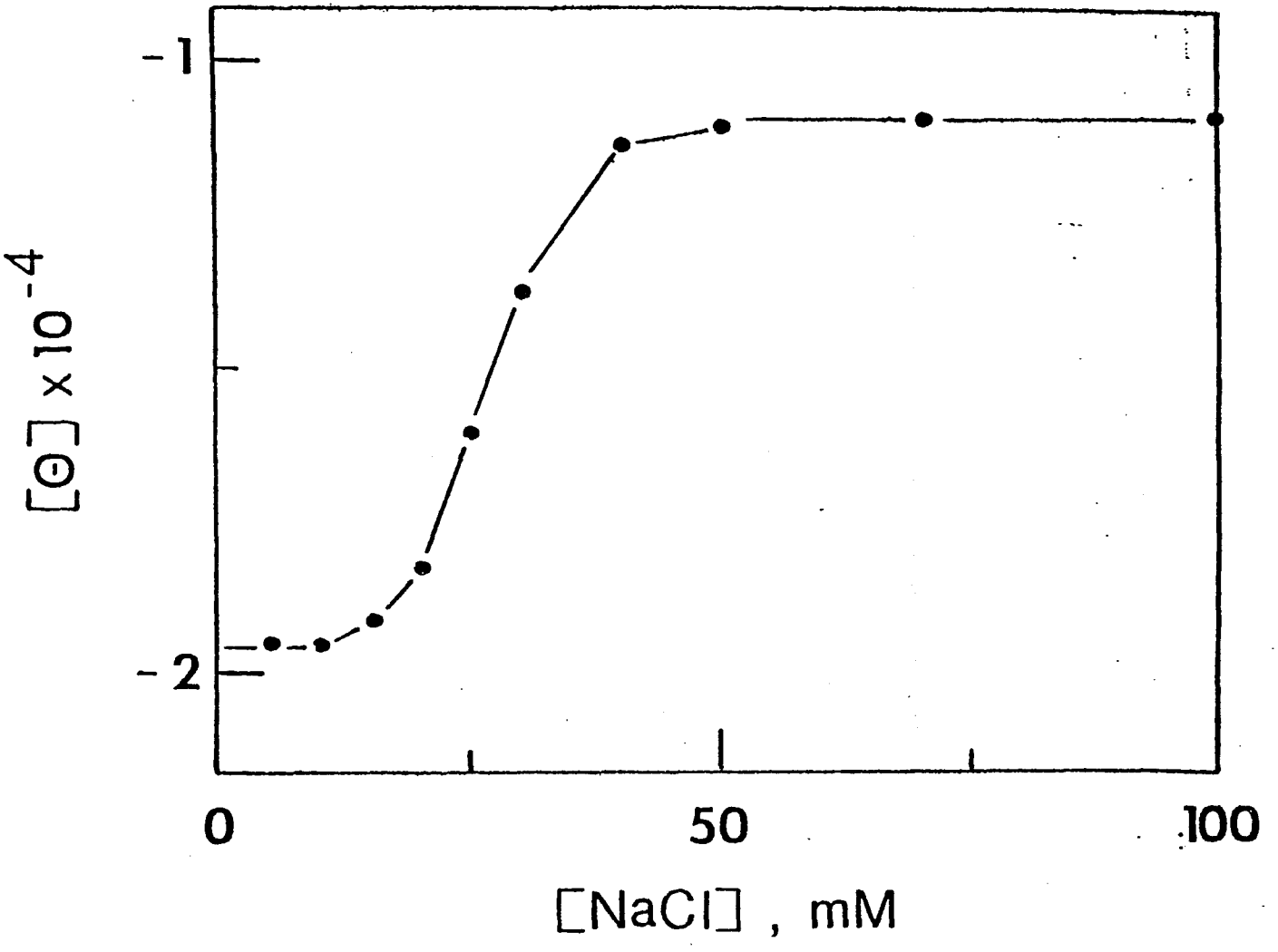

-1
-2
[Θ] x 10^-4
0
50
100
[NaCl] , mM

Figure 14

Molar ellipticity of Poly (rG-m^5dC)· Poly (rG-m^5dC) at 267 nm as a function of NaCl concentration.

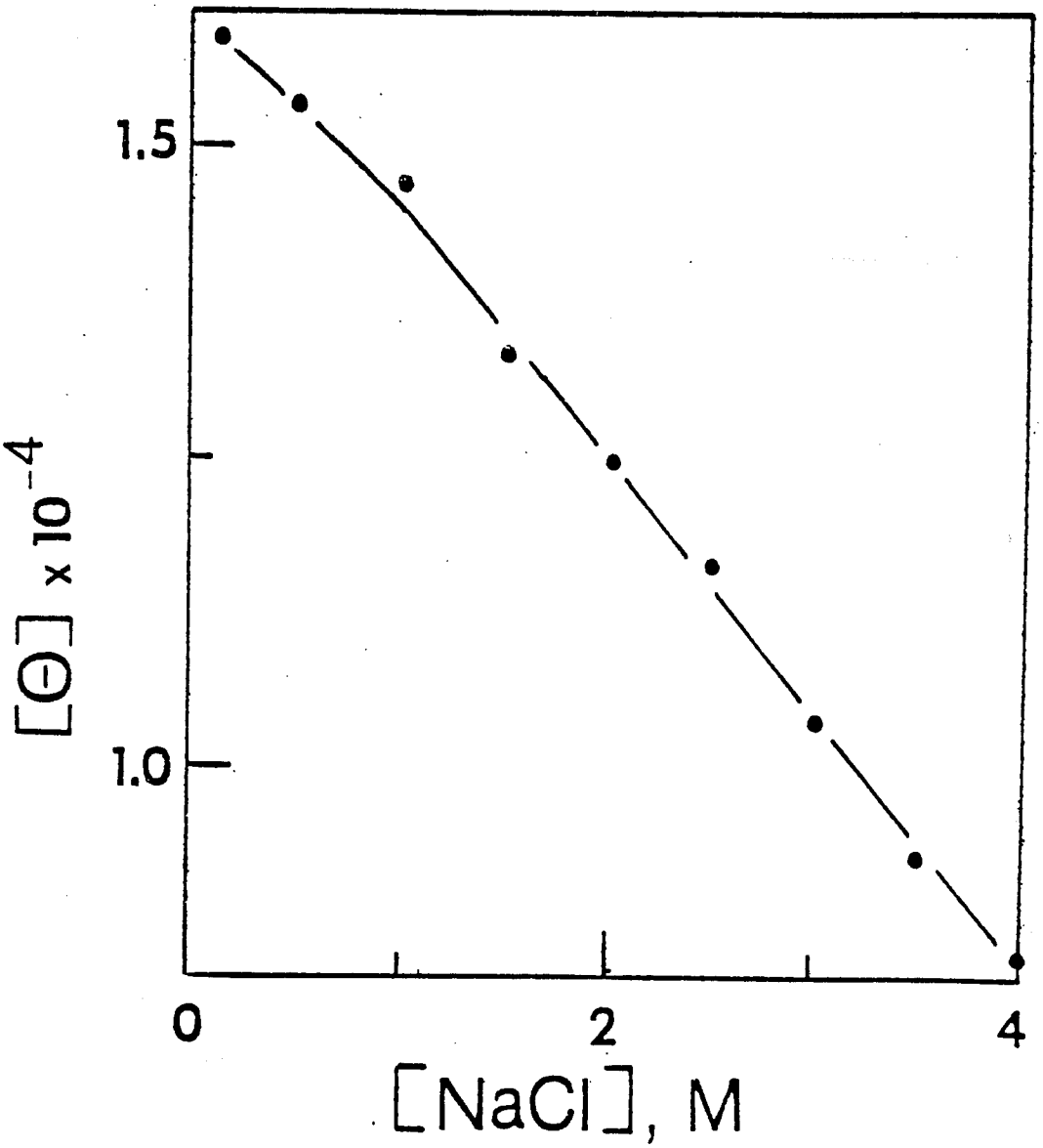

1.5
1.0
[⊖] x 10⁻⁴
0
2
4
[NaCl], M

Figure 15

Raman spectrum of Poly ($rG\text{-}m^5dC$)· Poly ($rG\text{-}m^5dC$) in 0.2 M NaCl, 5 mM Tris pH 7.5, 0.1 mM EDTA.

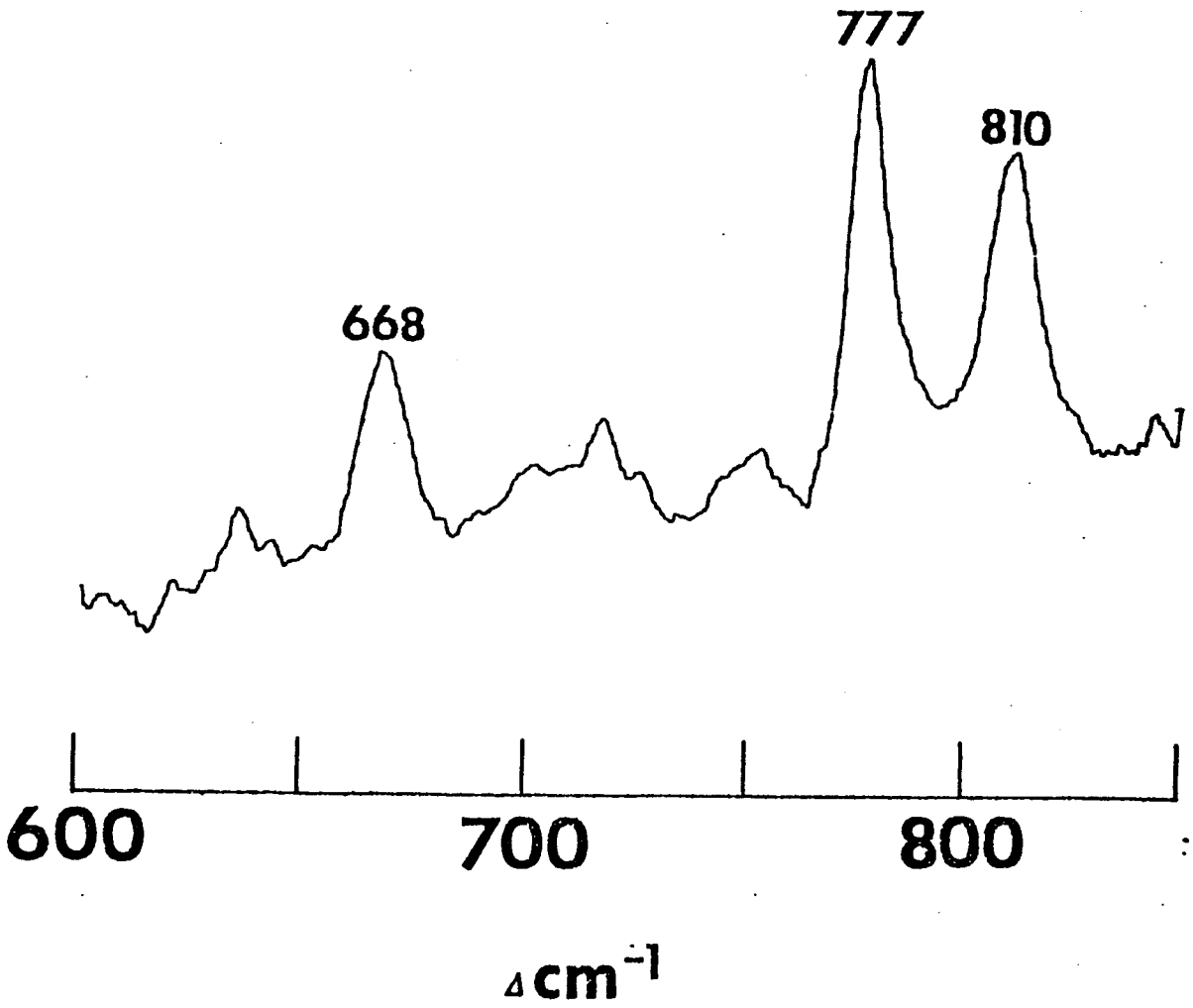

777
810
668
600
700
800
Δ cm⁻¹

Figure 16

^{31}P-NMR spectra of Poly (rG-m^{5}dC)·Poly (rG-m^{5}dC) in 5 mM Tris pH 7.5, 0.1 mM EDTA, plus : (A) 0 M NaCl; (B) 0.03 M NaCl; (C) 0.1 M NaCl; (D) 0.3 M NaCl; (E) 1 M NaCl; (F) 2 M NaCl.

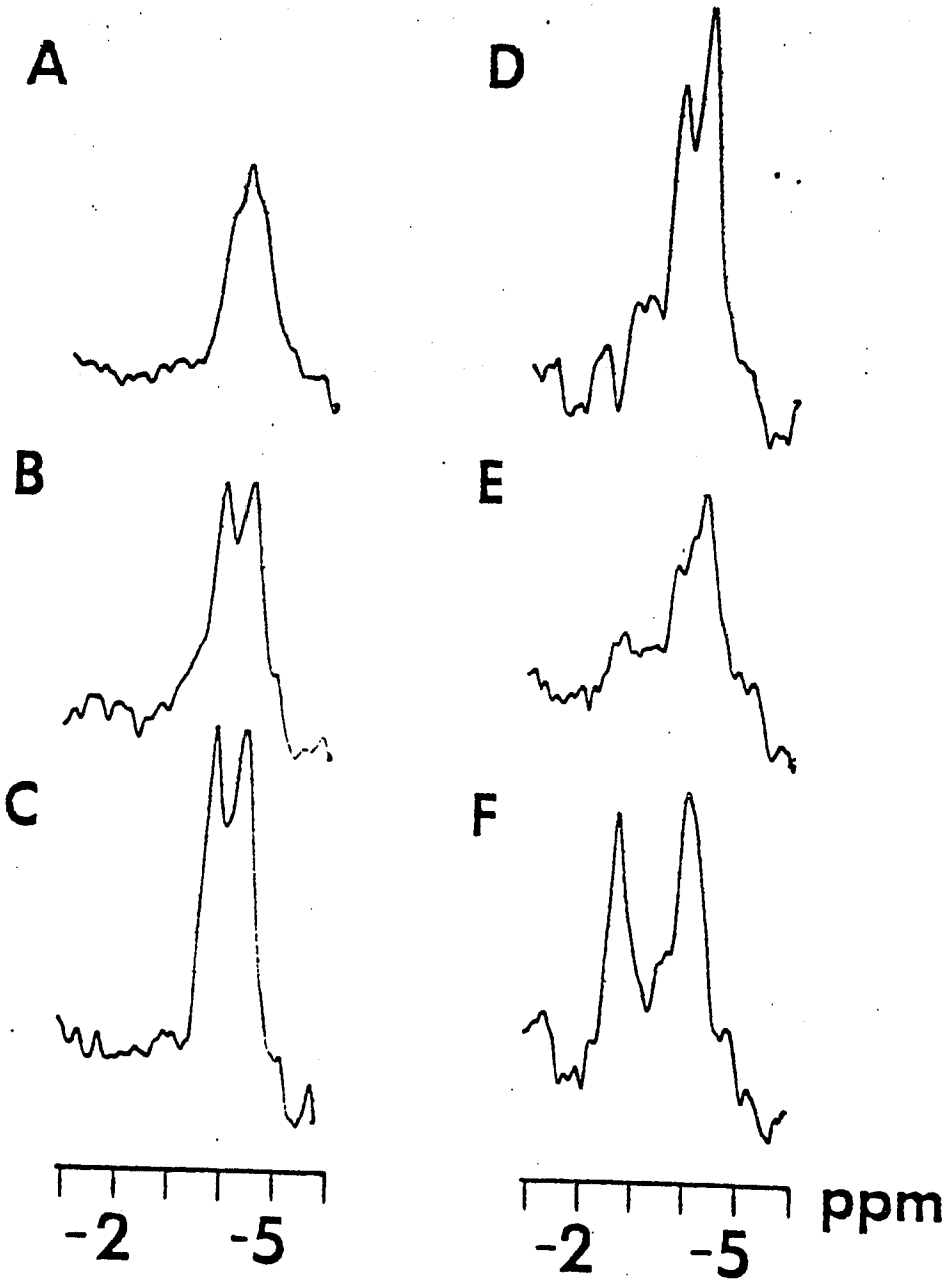

A
B
C
D
E
F
-2
-5
-2
-5
ppm

Figure 17

^{31}P-NMR spectrum of Poly (rG-m^{5}dC)· Poly (rG-m^{5}dC) in 25 mM triethanolamine pH 7.5, 0.1 mM EDTA.

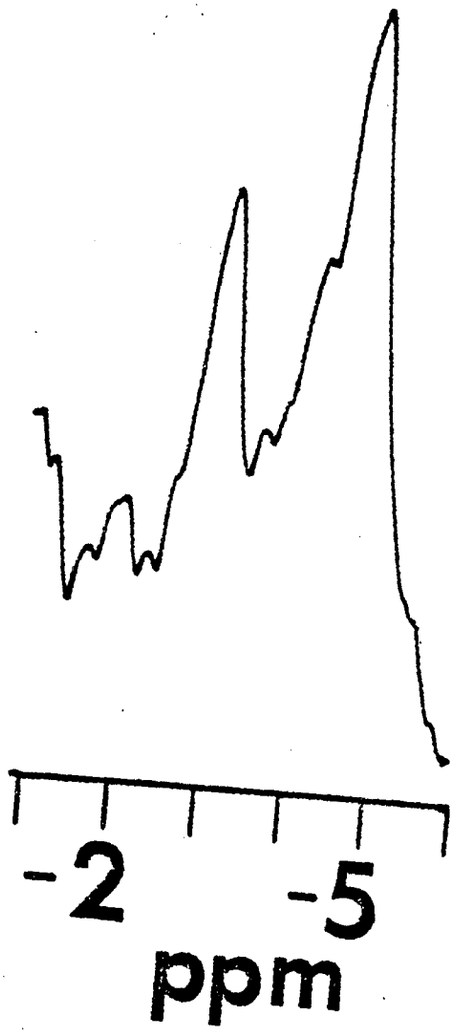

-2
-5
ppm

Figure 18

^{31}P-NMR spectra of Poly (dA-dT)·Poly (dA-dT) in low salt buffer plus : (A) 0 M CsF; (B) 1 M CsF; (C) 3 M CsF.

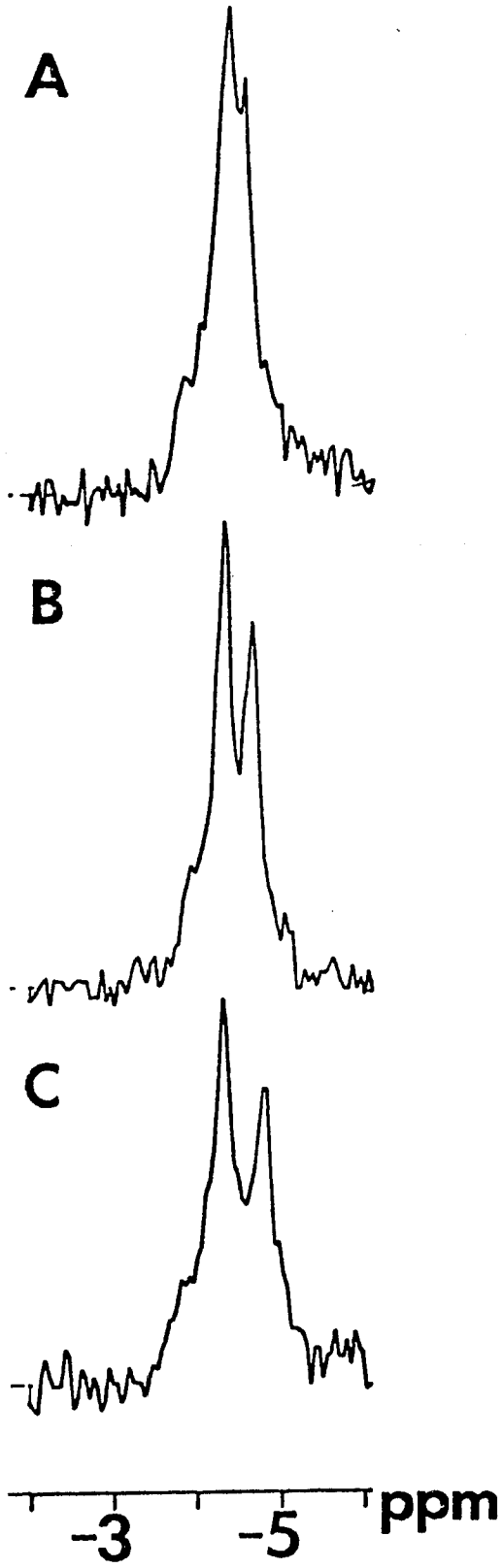

A
B
C
-3
-5
ppm

Figure 19

^{31}P-NMR spectra of Poly (dA-dU)$\cdot$ Poly (dA-dU) in low salt buffer plus : (A) 0 M CsF; (B) 1 M CsF; (C) 2 M CsF; (D) 3 M CsF.

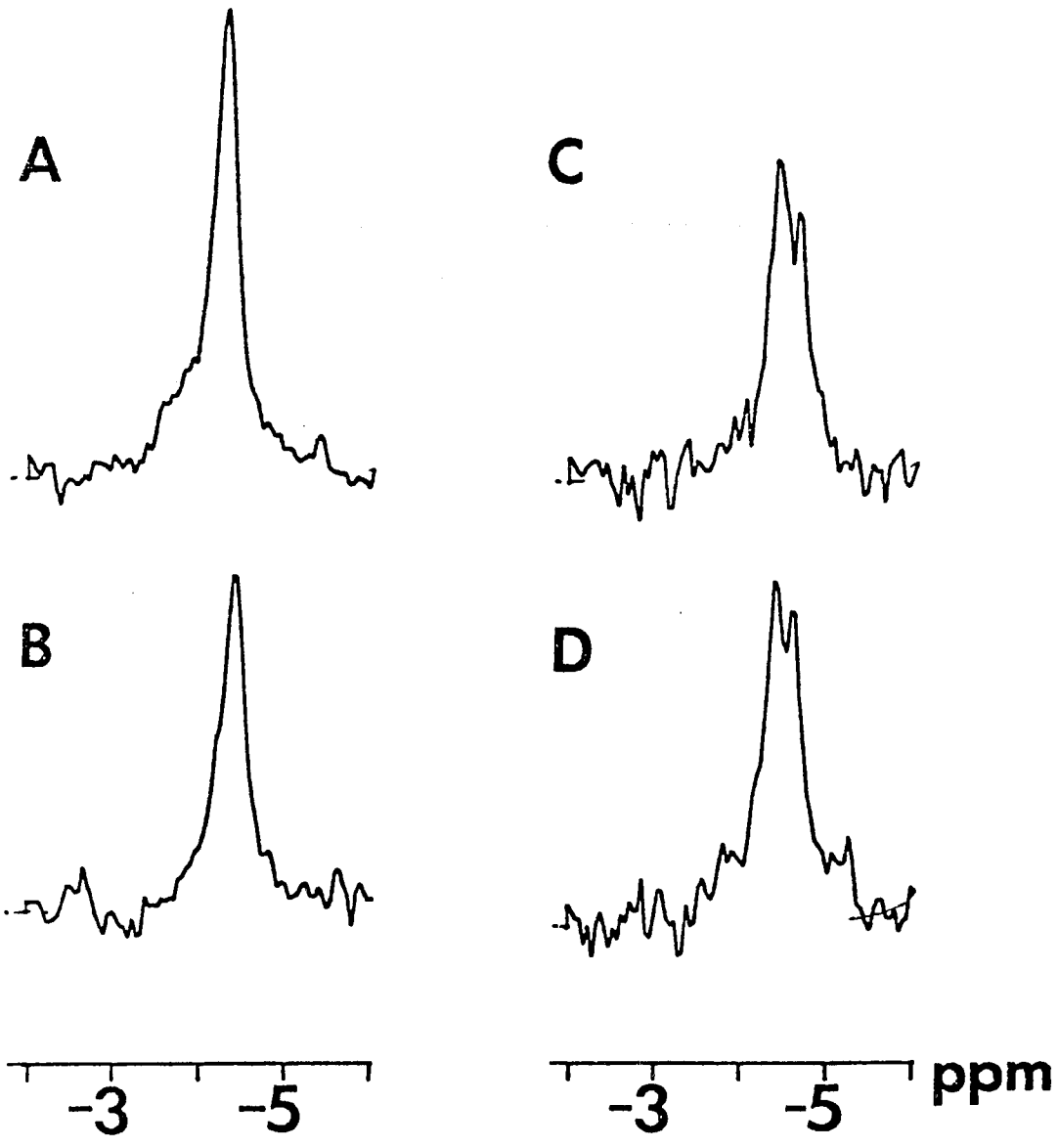

A
C
B
D
-3
-5
-3
-5
ppm

Figure 20

^{31}P-NMR spectra of Poly (dA-dU,T)·Poly (dA-dU,T) - 40% dT in low salt buffer plus : (A) 0 M CsF; (B) 1 M CsF; (C) 2 M CsF; (D) 3 M CsF.

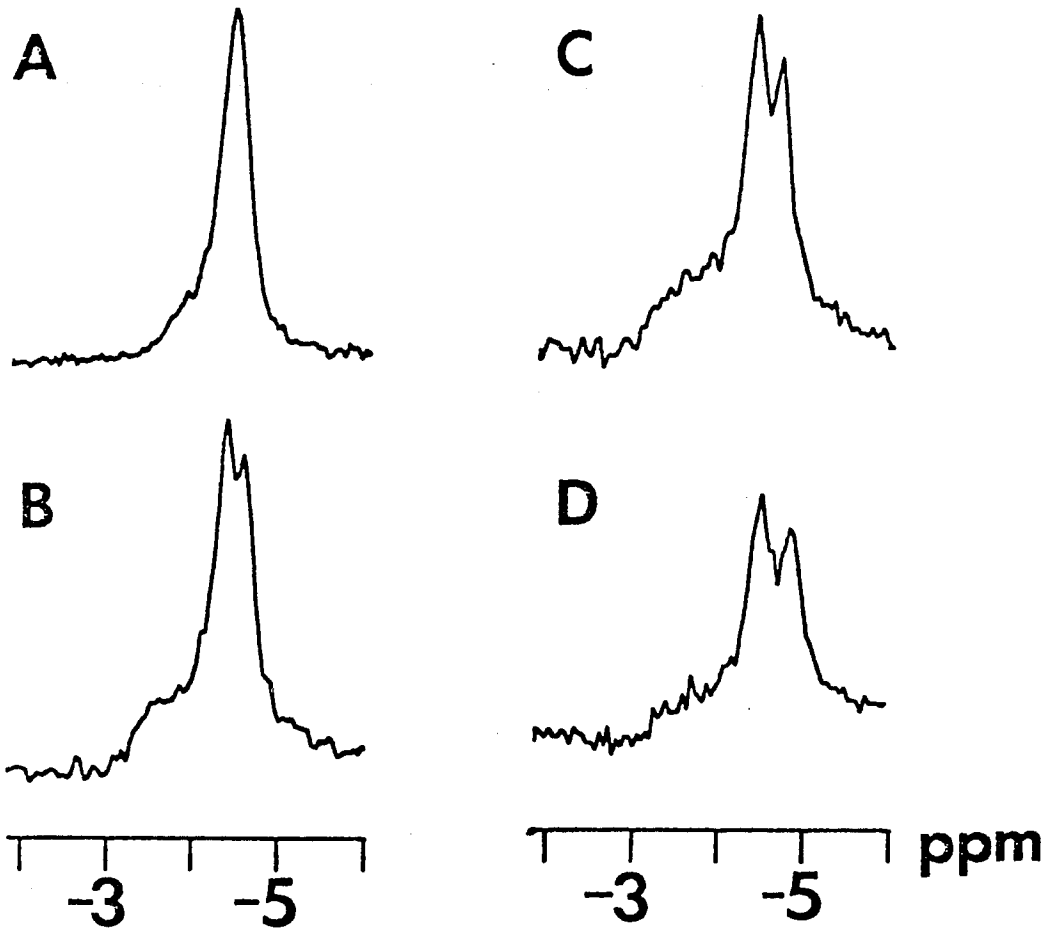

A
C
B
D
-3
-5
-3
-5
ppm

Figure 21

^{31}P-NMR spectra of Poly (dA-dU,T)·Poly (dA-dU,T) - 49% dT in low salt buffer plus : (A) 0 M CsF; (B) 1 M CsF; (C) 2 M CsF; (D) 3 M CsF.

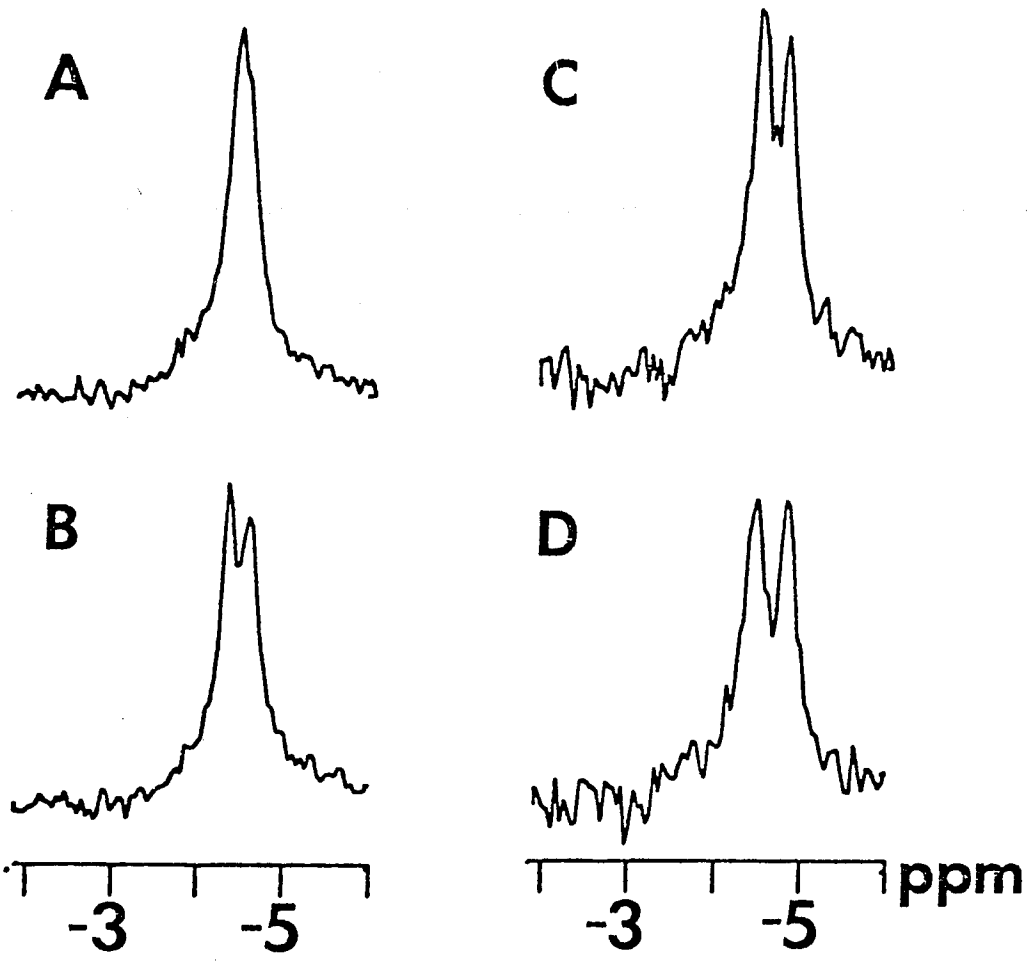

A
C
B
D
-3
-5
-3
-5
ppm

Figure 22

^{31}P-NMR spectra of Poly (dA-dU,T)·Poly (dA-dU,T) - 68% dT in low salt buffer plus : (A) 0 M CsF; (B) 1 M CsF; (C) 2 M CsF; (D) 3 M CsF.

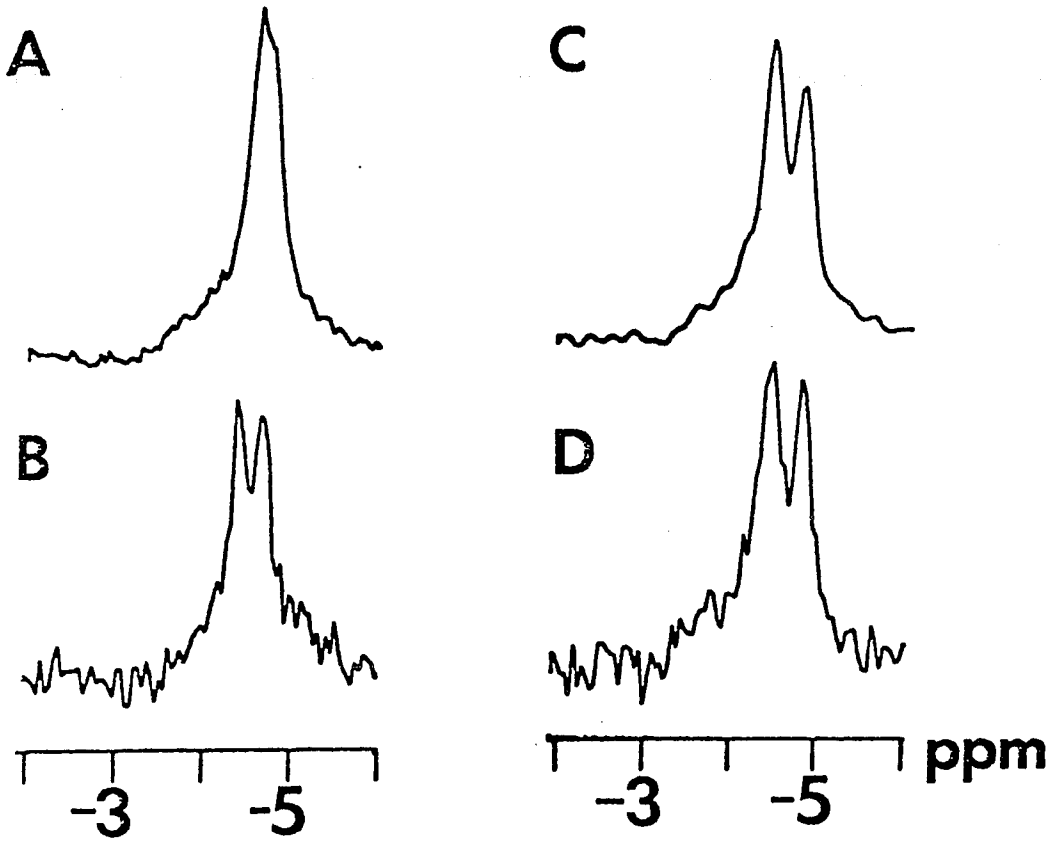

A
B
C
D
-3
-5
-3
-5
ppm

Figure 23

Separation of peaks as a function of [CsF] in the ^{31}P-NMR spectra of :——●——, Poly (dA-dT)·Poly (dA-dT);——○——, Poly (dA-dU,T)·Poly (dA-dU,T) - 68% dT;——■——, Poly (dA-dU,T)·Poly (dA-du,T) - 49% dT;——□——, Poly (dA-dU,T)·Poly (dA-dU,T) - 40% dT;——▼——, Poly (dA-dU)·Poly (dA-dU).

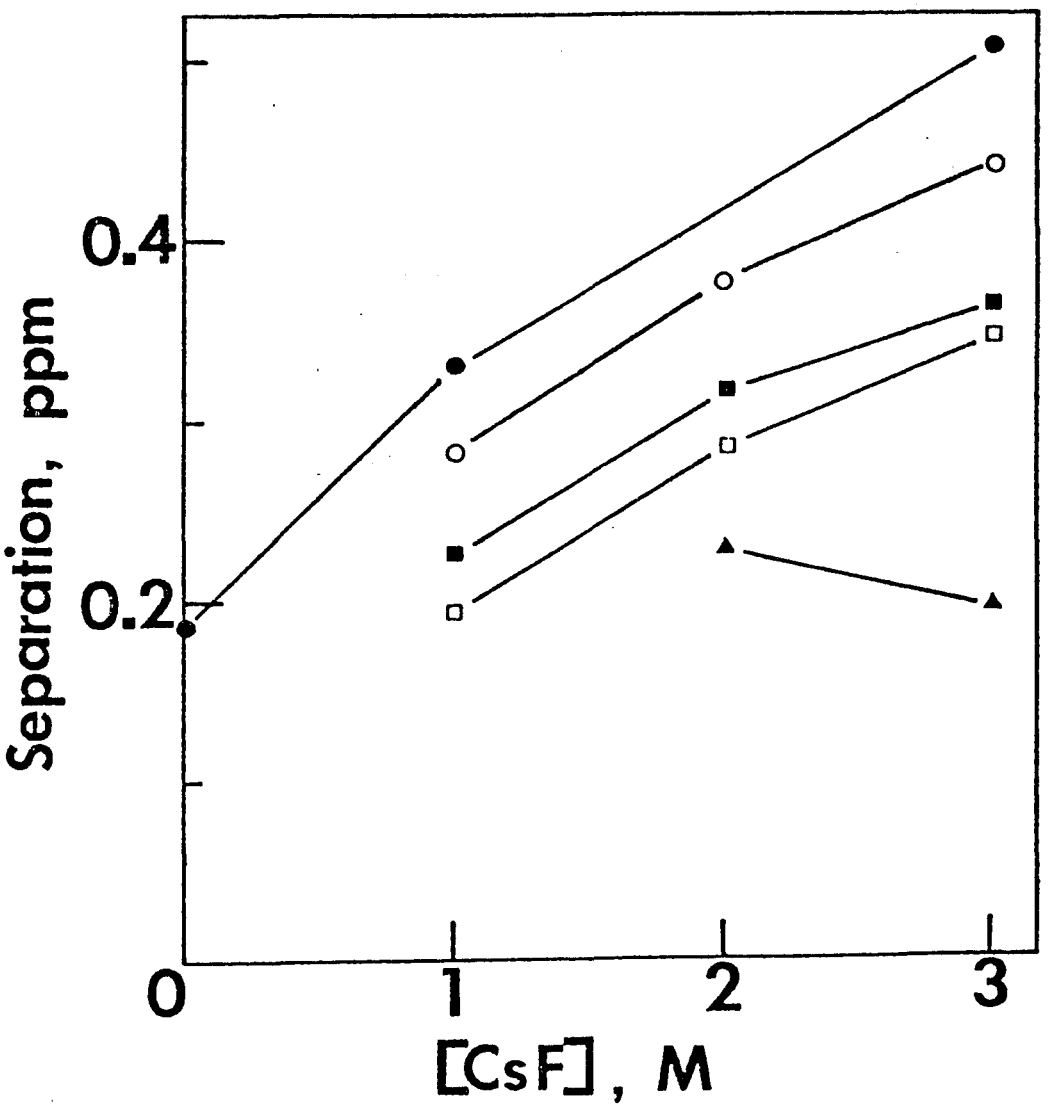

Separation, ppm
0.4
0.2
0
1
2
3
[CsF], M

Figure 24

Chemical shift of the downfield and upfield ^{31}P-NMR peaks of the polymers in 3 M CsF vs. %dT

Chemical Shift, ppm
-4.8
-4.6
-4.4
-4.2
0
50
100
%T

Figure 25

^{31}P-NMR spectra of Poly (dA-Br^{5}dU)· Poly (dA-Br^{5}dU) in low salt buffer plus : (A) 0 M CsF; (B) 3 M CsF.

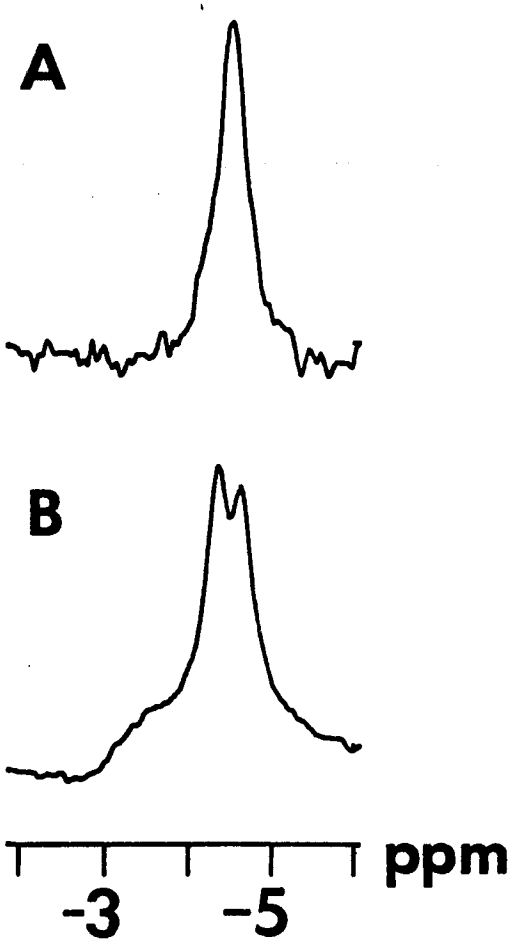

A
B
ppm
-3
-5

Figure 26

Picomoles of ^{3}H-methyl groups transferred to Poly (dG-dC)·Poly (dG-dC) (—●———●—) and Poly (rG-dC)·Poly (rG-dC) (—o———o—) by HhaI methylase versus time.

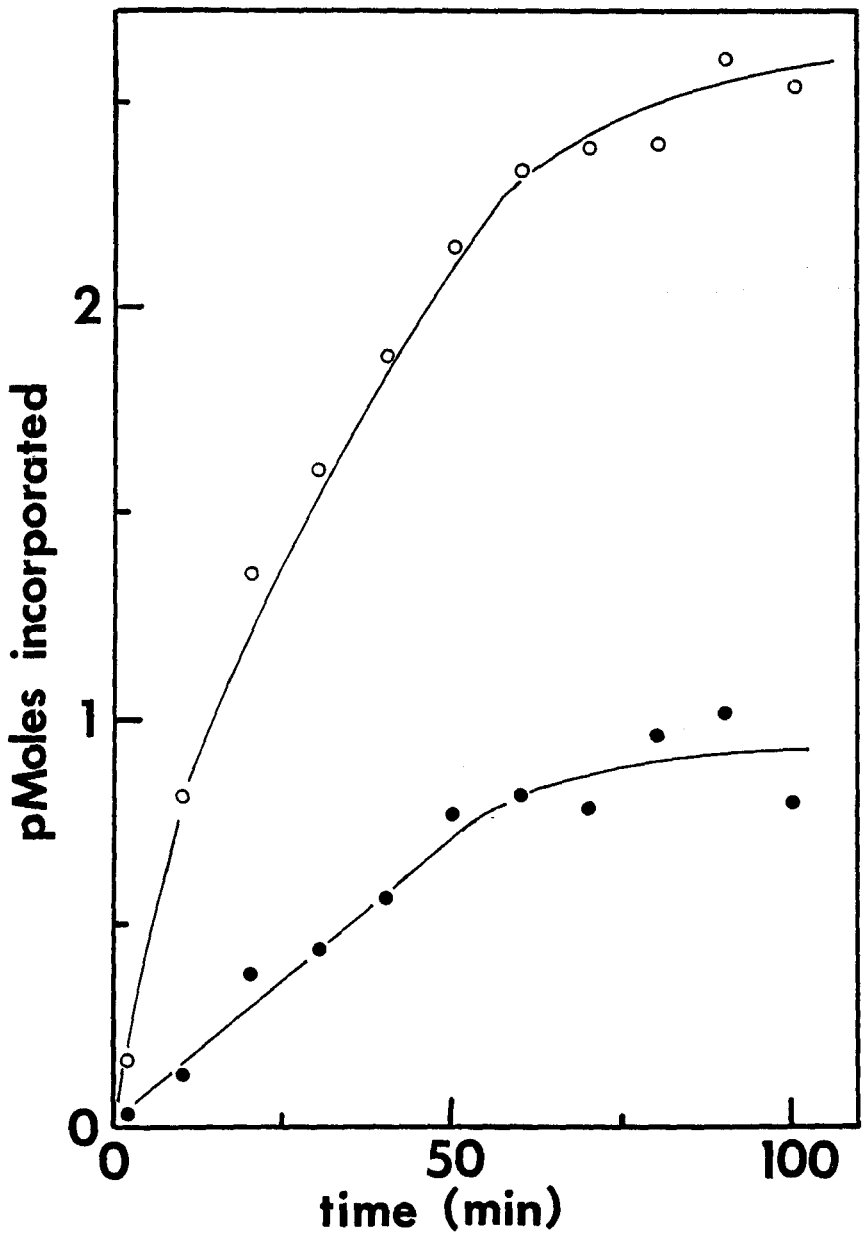

pMoles incorporated
0
1
2
0
50
100
time (min)

Table 1. Components of the reaction mixture of Poly (rG-dC)•Poly (rG-dC) synthesis

Bicine-HCl pH 8.95	40 mM
Mercaptoethanol	1 mM
$MnCl_2$	6 mM
Poly (dI-dC)•Poly (dI-dC)	2 A_{260} /ml
ribosyl-guanosine-5'-triphosphate	0.6 mM
deoxyribosyl-cytidine-5'-triphosphate	0.6 mM
Klenow fragment	20 units/ml

* only added in first 100 ul reaction, the synthesized polynucleotide can be the template for the expanded reactions.

Table 2.Concentration of cations at the transition midpoints

Ion	Low-salt transition	high-salt transition
Na^{+}	25.0	1500
Li^{+}	10.0	2000
Mg^{2+}	0.100	*
$[Co(NH_3)_6]^{3+}$	0.017	*
$spermine^{4+}$	0.003	*

Ion concentrations are given as mM. All ions were added as the chlorides. All solutions contain 5 mM Tris, pH 7.5/0.1 mM EDTA /50 uM Poly (rG-dC)·Poly (rG-dC) (as DNA phosphate.

*Produces A form CD spectrum until increasing cation concentration causes aggregation.

BIBLIOGRAPHY

1a. Dickerson, R. E., Drew, H. R., Conner, B. N., Kopka, M. L., Pjura, P. E. (1983) Cold Spring Harbor Symp. Quant. Biol. 47: 13-24

1b. Watson, J. D.,and Crick, F.H.C. (1953) Nature (London) 171 : 737

2. Pohl, F. M., and Jovin, T. M. (1972) J. Molec. Biol. 67 : 375-396

3. Wang A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford J. L., Van Boom J. H., Van der Marel, G. and Rich, A. (1979) Nature(London) 282 : 680-686

4a. Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Van der Marel, G., Van Boom, J. H. and Rich, A. (1981) Science 211 : 171-176

4b. Sundaralingam, M. Biopolymer 7: 821-839

5. Drew H. R., Takano, T., Tanaka, S., Itakura, K. D. and Dickerson, R. E. (1980) Nature (London), 567-573

6. Arnott, S., Chandrasekaran, R., Birdsall, D. L., Leslie, A. G. W. and Ratcliff, R. L. (1980) Nature (London) 283 : 743-745

7. Hamada, H., and Kakunaga, T. (1982) Nature (London) 298 : 396-398

8. Rich, A. (1982) Cold Spring Harbor Symp. Quant. Biol. 1, 1-12

9. Nordheim, A. and Rich, A. (1983) Proc. Natl. Acad. Sci. U.S.A. 80 : 1821-1825

10. Haniford, D. B. and Pulleyblanck, D. E. (1983) Nature (London) 302 : 632-634

11. Behe, M. and Felsenfeld, G. (1981) Proc. Natl. Acad. Sci. U.S.A. 78 : 1619-1623

12. Behe, M., Zimmerman S., and Felsenfeld, G. (1981) Nature (London) 293 : 233

13. Patel, D. J., Canuel, L. L., and Pohl, F. M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76 : 2508-2511

14. Thamann, T. J., Lord, R. C., Wang, A. H.-J., and

Rich, A. (1981) Nucleic Acids Res. 9 : 5443-5457

15. Taillandier, E., Taboury J. A., Adam, S., and Liquier J. (1984) Biochemistry 23 : 5703-5706

16. Jardetzky, O. (1964) Adv. Chem. Phys. 7 : 499

17. McDonald, C. C., Phillips, W. D., and Penman, S. (1964) Science 144 : 1234

18. Cohen, J. S., Wooten, J. B., and Chatterjee, C. L. (1981) Biochemistry 20 : 3049-3055

19. Chen, C.-W. and Cohen, J. S. (1983) Biopolymers 22 : 879-893

20. Shindo, H., Simpson, R. T., and Cohen, J. S. (1979) J. Biol. Chem. 254 : 8125-8128

21. Redfield, A. G., Roy, S., Sanches, V., Tropp, J., and Figueroa, N. (1981) in 2nd Biomolecular Stereodynamics Conference (Sarma, R., Ed.) pp. 195-208, Academic Press, New York.

22. Patel, D. J., Kozlowski, S. A., Marky, L. A., Broka, C., Rice, J. A., Hakura, K., and Breslauer, K. J. (1982) Biochemistry 21 : 428-436

23. Scheek R. M., Boelens, R., Russo, N., Van Boom, J. H. and Kaptein, R. (1984) Biochemistry 23 : 1376-1383

24. Nishimura Y., Tsuboi, M., Nakano, T., Higuchi, S., Sato, T., Shida, T., Uesugi, S., Ohtsuka, E., and M. Ikehara (1983) Nucleic Acids Res. 11 : 1579-1588

25. Patel, D. J., Kozlowski, S. A., Nordheim, A. and Rich, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79 : 1413-1417

26. Möller, A., Nordheim, A., Kozlowski, S. A., Patel, D. J., and Rich, A. (1984) Biochemistry 23 : 54-62

27. Kornberg, A., Lehman, I. R., Bassman, M. J., and Simms, E. S. (1956) Biochim. Biophys. Acta 21 : 197

28. Kornberg, A. (1969) Science 163 : 1410

29. Kornberg, A. (1974) DNA synthesis - Freeman, San Francisco. pp. 67-121

30. Jacobsen, H., Klenow, H., and Overgaard-Hansen K.

(1974) Eur. J. Biochem. 45 : 623-627

31. Seal, G., Shearman, C. W., and Loeb, L. A. (1979) J. Biol. Chem. 254 : 5229-5237

32. Goodman, M. F., Keener, S., Guidotti, S., and Branstomb, E. W. (1983) J. Biol. Chem. 258 : 3469-3475

33. Sloan, D. L., Loeb, L. A., Mildvan, A. S., and Feldmann, R. J. (1975) J. Biol. Chem. 250 : 8913-8920

34. Selsing, E., and Wells R. D. (1979) J. Biol. Chem. 254 : 5417-5422

35. Selsing, E., and Wells R. D. (1979) J. Biol. Chem. 254 : 5410-5416

36. Wu, H. M., Dattagupta N., and Crothers D. M. (1981) Proc. Natl. Acad. Sci. U.S.A. 78 : 6808-6811

37. Wang, A. H.-J., Fujii, S., Van Boom, J. H., Van der Marel, G. A., Van Boeckel, S. A. A. & Rich, A. (1982) Nature 299 : 601-604

38. Zimmerman S. B., and Pheiffer B. H. (1981) Proc. Natl. Acad. Sci. 78 : 78-82

39. Bird, P. A. (1984) Nature(London) 307 : 503

40. Felsenfeld G., and McGhee (1982) Nature 296 : 602

41. Lindahl, T. (1981) Nature (London) 290 : 363-364

42. Nickol, J., and Felsenfeld, G. (1983) Cell 35 : 467-477

43. Chen, C.-W., Cohen, J. S., and Behe, M. J. (1983) Biochemistry 22 : 2136-2142

44. Patel, D. J., Kozlowski, S. A., Suggs, J. W., and Cox, S. D. (1981) Proc. Natl. Acad. Sci. U.S.A. 78 : 4063-4067

45. Klug, A., Jack, A., Viswamitra, M. A., Kennard, O., Shakked, Z., and Steitz, T. A. (1979) J. Mol. Biol. 131 : 669-680

46. Rich, A., Nordheim, A., and Wang, A. H.-J. (1984) Ann. Rew. Biochem. 53 : 791-846

47. Peck, L. J. and Wang, J. C. (1985) Cell 40 : 129-137

48. Nordheim, A. and Rich, A. (1983) Proc. Natl. Acad. Sci. U.S.A. 80 : 1821-1825

49. Miller, F. D., Rattner, J. B., and Van de Sande, J. H. (1983) Cold Spring Harbor Symp. Quant. Biol. 47 : 571-576

50. Varshavsky, A. T., Sundin, O., and Bohn, M. (1981) Nucleic Acids Res. 5 : 5931-5938

51. Scott, W.A. and Wigmore, D. J. (1978) Cell 15 : 1511-1518

52. Vardimon, L. and Rich, A. (1984) Proc. Natl. Acad. Sci. U.S.A. 81 : 3268-3272

53. Zacharias, W., Larson, J. E., Kilpatrick, M. W. and Wells, R. D. (1984) Nucleic Acids Res. 12 : 7677-7684

54. Frederick, C. A., Grable, J., Melia, M., Samudzi, C., Jen-jacobsen, L., Wang, B.-C., Greene, P., Boyer, H. W., and Rosenberg, J. M. (1984) Nature (London) 309 : 327-331

55. Wu, H.-Y. and Behe, M. J. (1984) Proc. Natl. Acad. Sci. U.S.A. 81 : 7284-7287

56. Joyce, C. M. and Grindley, N. D. F. (1983) Proc. Natl. Acad. Sci. U.S.A. 80 : 1830-1834

57. Josse, J. and Swartz M. (1963) Methods in Enzymology 6 : 743

58. Kuo, K. C., McCune, R. A., and Gehrke, C. W. (1980) Nucleic Acids Res. 8 : 4763-4776

59. Nishimura, S., Jacob, T. M., and Khorana, H. G. (1964) Proc. Natl. Acad. Sci. U.S.A. 52 : 1494-1499

60. Brahms, S., Vergne, J., Brahms, J. G., DiCapua, E., Bucher, P., and Koller, T. (1982) J. Mol. Biol. 162 : 473-493

61. Thomas, G. A. and Peticolas, W. L. (1983) J. Am. Chem. Soc. 105 : 986-992

62. Drew, H. R. and Dickerson, R. E. (1981) J. Mol. Biol. 151 : 535-556

63. Manning, G. S. (1978) Q. Rev. Biophys. 11 : 179-246

64. Conner, B. N., Takano, T., Tanaka, S., Itakura, K., and Dickerson, R. E. (1982) Nature (London) 295 : 294-299

65. Wu, H.-Y. and Behe, M. J. (1985) Biochemistry (in press)

66. Uesugi, S., Ohkubo, M., Ohtsuka, E., Ikehara, M. Kobayashi, Y., Kyogoku, Y., Westerink, H., Van der Marel, G. A., Van Boom, J. H., and Haasnoot, C. A. (1984) J. Biol. Chem. 259 : 1390-1393

67. Eckstein, F. and Jovin, T. M. (1983) Biochemistry 22 : 4546-4550

68. Wu, H.-Y. and Behe, M. J. (1985) Nucleic Acids Res. 13 : 3931-3940

69. Lin, S.-Y. and Riggs, A. D. (1971) Biochem. Biophys. Res. Comm. 45 : 1542-1547

70. Zimmerman, S. B. (1982) in Ann. Rev. Biochemistry, Small, E. E., Boyer, P. D., Meister, A. and Richardson, C. C. Eds. 51 : 395-428, Annual Reviews Inc., Palo Alto.

71. Singleton, C. K., Klysik, J., Stirdivant, S. M. and Wells, R. D. (1982) Nature (London) 299 : 312-316

72. Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, B. D., and Rich, A. (1982) Cell 31 : 309- 318

73. Stirdivant, S. M., Klysik, J., and Wells, R. D. (1982) J. Biol. Chem. 257 : 10159-10165

74. Ivanov, V. I., Minchenkova, L. E., Minyat, E. E., Frank-Kamenetskii, M. D. and Schyolkina, A. K. (1974) J. Mol. Biol. 87 : 817-833

75. Jovin, T. M., McIntosh, L. P., Arndt-Jovin, D. J., Zarling, D. A., Robert-Nicoud, M., Van de Sande, J. H., Jorgenson, K. F., and Eckstein, F. (1983) J. Biomol. Struc. Dyn. 1 : 21-57

76. Nordheim, A., Pardue, M. L., Lafer, E. M., Moller, A., Stollar, B. D. and Rich, A. (1981) Nature (London) 294 : 417-421

77. Nordheim, A., Tesser, P., Azorin, F., Kwon, Y. H.,

Möller, A., and Rich, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79 : 7729-7733

78. Molloy, P. L. and Symons, R. H. (1980) Nucleic Acids Res. 8 : 2939-2945

79. Pruch, J. M. and Laskowski, M. (1980) J. Biol. Chem. 255 : 9409-9412