Bromination Stabilizes Poly(dG-dC) in the Z-DNA Form under Low-Salt Conditions†

Achim Möller, Alfred Nordheim, Sharon A. Kozlowski, Dinshaw J. Patel, and Alexander Rich*

ABSTRACT: Using circular dichroism studies, Pohl & Jovin (1972) [Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396] demonstrated that poly(dG-dC) undergoes a salt-dependent conformational change characterized by a spectral inversion. The low-salt form corresponds to the right-handed B form of DNA and the high-salt form to the left-handed Z-DNA helix. Modification of poly(dG-dC) by adding bromine atoms to the C8 position of guanine and the C5 position of cytosine residues stabilized this polymer in the Z-DNA form under low-salt conditions. The guanine residues were found to be twice as reactive as the cytosine residues. With a modification of 38% Br^8G and 18% Br^5C, the polymers formed a stable Z-DNA helix under physiological conditions. The bromination produced spectroscopic features very similar to poly(dG-dC) in 4 M NaCl. However, bromination did not freeze the Z structure as was shown by ethidium bromide intercalation studies. Addition of the dye favored an intercalated B-DNA form. The conversion of B- to Z-DNA leads to profound conformational changes which were also seen by a reduced insensitivity to various exo- and endonucleases. Comparative studies showed that the brominated polymers have a high affinity to nitrocellulose filters. In 1 M NaCl, there was virtually no binding of B-DNA, but a substantial binding of Z-DNA was found even at rather low levels of bromination. Unmodified poly(dG-dC) and brominated poly(dG-dC) were investigated by high-resolution phosphorus NMR spectroscopy. The unbrominated segments in the B form gave a single resonance at 4.2 ppm, while the dinucleotide repeat in the Z form gives resonances at 3.0 and 4.5 ppm. With increasing levels of bromination, a new peak appeared at 3.8 ppm. It was suggested that this peak is associated with phosphate perturbation related to bromination of guanine residues.

Z-DNA is the left-handed conformation of a DNA double helix containing alternating purine and pyrimidine residues. The details of the three-dimensional Z-DNA conformation with a dinucleotide repeat were first revealed in a single-crystal atomic resolution X-ray diffraction analysis of a hexanucleotide with the sequence d(CpGpCpGpCpG) (Wang et al., 1979). The left-handed Z-DNA helix has only one groove in contrast to the major and minor grooves found in the right-handed B-DNA conformation. The concave major groove of B-DNA is transformed into the convex outer surface of the Z-DNA helix. In the Z structure, the guanosine residues adopt the C3′ endo pucker of the furanose ring, and all the guanine residues are in the syn conformation. The cytosine residues have the C2′ endo conformation, and the bases are in the anti conformation as in B-DNA. The imidazole ring of the guanine bases is found on the outer part of the molecule with the N7 and C8 positions exposed to the surface. This is in contrast to B-DNA in which the imidazole ring of guanine is in van der Waals contact with the sugar–phosphate backbone.

Z-DNA was first observed by Pohl & Jovin (1972) in the synthetic polynucleotide poly(dG-dC) when the salt concentration was raised to 4 M NaCl. They found a near-inversion of the circular dichroism spectrum under these conditions compared to the low-salt form. The high- and low-salt forms of poly(dG-dC) also have Raman spectra that differ from each other (Pohl et al., 1973). Raman spectra analyses of single crystals which form Z-DNA reveal that they have spectra identical with that of the high-salt form (Thamann et al., 1981). Thus, the high-salt form of poly(dG-dC) is Z-DNA.

The high-resolution proton and phosphorus NMR parameters of poly(dG-dC) in low and high salt are strikingly different and very sensitive to the dinucleotide repeat in the alternating purine–pyrimidine polynucleotide (Patel et al., 1979; Patel, 1979). Specifically, the dGpdC and dCpdG phosphodiesters of poly(dG-dC) in high salt are separated by 1.5 ppm (Patel et al., 1979; Patel, 1979), in agreement with the subsequently revealed zigzag nature of the phosphodiester backbone in Z-DNA oligonucleotide crystals (Wang et al., 1979). Proton nuclear Overhauser effect measurements between base and sugar protons for poly(dG-dC) in high salt (Patel et al., 1982) are in agreement with the observed syn and anti glycosidic torsion angles at the guanosine and cytosine residues in Z-DNA crystals.

We were interested in developing a simple chemical modification method which stabilizes the Z-DNA form of poly(dG-dC) in a physiological salt solution. We have found that bromination of poly(dG-dC) stabilizes Z-DNA in a low-salt solution. This procedure adds bromine atoms predominantly to the C8 position of the guanine imidazole ring and less frequently to the C5 position of cytosine. The bromination procedure has been described briefly (Lafer et al., 1981). The brominated polymer is a powerful immunogen for the development of polyclonal (Lafer et al., 1981) and monoclonal (Möller et al., 1982) antibodies which have been widely used in determining the presence of Z-DNA in a variety of biological systems. Here, we present a full discussion of the bromination method and an analysis of the changes in chemical and physical properties of this molecule which demonstrate that the brominated form of poly(dG-dC) is in the Z-DNA conformation.

Experimental Procedures

Materials

Poly(dG-dC)·poly(dG-dC) and poly(dG)·poly(dC) were purchased from P-L Biochemicals, Inc., and Sigma Chemical

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (A.M., A.N., and A.R.), and Bell Laboratories, Murray Hill, New Jersey 07974 (S.A.K. and D.J.P.). *Received April 15, 1983.* This research was supported in part by the American Cancer Society, the National Science Foundation, and the National Institutes of Health. A.M. and A.N. were supported by the Deutsche Forschungsgemeinschaft.

0006-2960/84/0423-0054$01.50/0

Co., (St. Louis, MO), respectively. Nuclease S1, deoxyribonuclease I, micrococcal nuclease, and calf thymus DNA were obtained from Boehringer Mannheim. Bal 31 was a gift of Prof. J. Wang (Harvard University). Exonuclease III was from New England Biolabs, Inc. Nitrocellulose filters (pore size 0.22 μm) were products of Schleicher & Schuell. Polynucleotides were radioactively labeled by the in vitro nick-translation technique described by Rigby et al. (1977).

Circular dichroism spectra were recorded on a Cary 60 spectrophotometer at ambient temperature. A Zeiss PMQ II spectrometer and a Perkin-Elmer Model 330 spectrometer were used for spectroscopic measurements.

Methods

The 81-MHz phosphorus spectra were recorded with proton broad-band decoupling on a Varian XL-200 spectrometer. The decoupler was left on during data accumulation but was turned off during the delay between pulses in high-salt solution. The phosphorus chemical shifts are referenced relative to internal trimethyl phosphate and are corrected for the temperature and salt dependence of the standard and are relative to the standard in no added salt at 27 °C.

Typical phosphorus spectra were recorded on 50 ODU of polymer in 0.6 mL of buffer in low-salt solution. Following completion of these runs, increasing concentrations of salt were directly added to these samples and spectra recorded after equilibration in high-salt solution for a few hours. The signal to noise ratio of the phosphorus spectra was improved following application of a 4–6-Hz line-broadening contribution.

Sonication of Poly(dG-dC). The polymer was sheared by using a Heat Systems Model W225R Ultrasonics sonicator with a 0.5-in. (1.25-cm) tip attached to the coupler section. Up to 300 A_{260} units of the DNA were dissolved in 6.5 mL of 10 mM tris(hydroxymethyl)aminomethane hydrochloride/ethylenediaminetetraacetic acid (Tris-HCl/1 mM EDTA) (pH 8.0) and sonicated for 1 h (50% duty cycle pulse control and position 6 output control setting) between 0 and 10 °C. The sonication yielded DNA fragments having an average chain length of 150 ± 100 base pairs as measured by acrylamide gel electrophoresis.

Bromination of the Polymers. The polymers were dissolved in 20 mM sodium citrate (pH 7.2), 1 mM EDTA, and 4 M NaCl. The solution was kept at room temperature to facilitate the B- to Z-DNA transition. The high-salt concentration lowered the pH to 6.4. Aqueous bromine reagent was prepared by adding bromine (Fisher Scientific Co.) to distilled water and mixing thoroughly until the water became saturated with bromine at room temperature. The bromine-saturated water was added to the polymers (3 mM in nucleotide) in different bromine/nucleotide ratios. The reaction was allowed to proceed at room temperature for 10 min with occasional mixing by inversion. Excess bromine was subsequently removed by bubbling air through the reaction mixture (kept in an ice–water bath) for 10 min. The solution was then extensively dialyzed against 10 mM phosphate (pH 7), 150 mM NaCl, and 1 mM EDTA. One milligram of Br–poly(dG-dC) has an A_{260} of 14.4.

Determination of the Extent of Bromine Modification. To analyze the extent of modification, the polymers were precipitated with ethanol. The dried pellets were resuspended in 10 μL of perchloric acid (72%) and treated at 100 °C for 1 h to remove the bases. The solution was neutralized, and insoluble products were removed by centrifugation. The bases were separated by passage through a Waters Associates μBondapak C18 column used with a Beckman liquid chromatograph (Model 100A pump, Model 210 sample injector, and Model 153 detector operating at a wavelength of 254 nm). The bases were eluted with a methanol gradient (0–50%) in 20 mM sodium hydrogen phosphate buffer at pH 4.5 (Figure 1). The flow rate for the column was 2 mL/min. The mole fraction of each base was calculated by determining the peak area with the formula (peak height × peak width at half-height)/2.

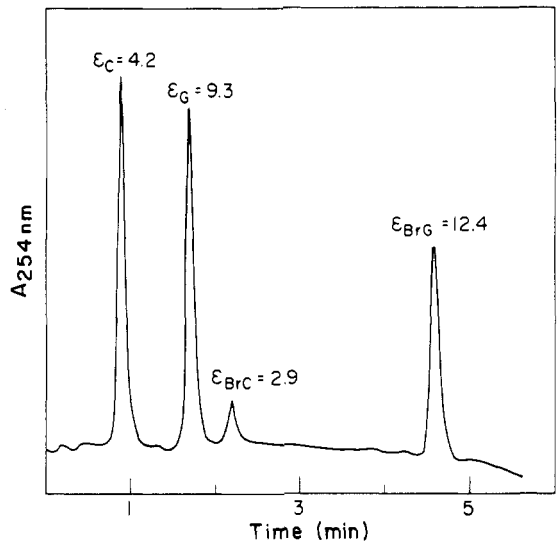

FIGURE 1: High-pressure liquid chromatographic analysis of hydrolysis products from brominated poly(dG-dC) ($\theta = 1$). The extinction coefficients for the individual bases at 254 nm and pH 4.6 are indicated.

Filter Binding Studies. Aliquots (0.5 mL) of unmodified or modified polymers at concentrations of 0.4 mM nucleotide in 10 mM phosphate buffer (pH 7.0) and various amounts of NaCl were incubated at 37 °C for 40 min and then filtered through nitrocellulose filters. The filters were washed 3 times with a solution containing the same salt concentration and dried, and the radioactivity was determined.

The nuclease assays were carried out under the following conditions: S1 nuclease, 30 mM sodium acetate (pH 4.6), 50 mM NaCl, 1 mM $ZnSO_4$, and 5% glycerol; DNase I, 30 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$; micrococcal nuclease, 50 mM Tris-HCl (pH 8.5) and 2 mM $CaCl_2$; exonuclease III, 66 mM Tris-HCl (pH 8.0), 0.66 mM $MgCl_2$, and 1 mM β-mercaptoethanol; Bal 31, 20 mM Tris-HCl (pH 8.1), 12.5 mM $MgSO_4$, 12.5 mM $CaCl_2$, 200 mM NaCl, 1 mM EDTA, and 1 mg/mL bovine serum albumin. The *nuclease digestions* were carried out in a volume of 0.1 mL with polymer concentrations of 0.5 μM in nucleotide. After a 1-h incubation at 37 °C (30 °C for Bal 31), the reaction mixture was chilled, and 0.1 mL of 5% perchloric acid was added. After 5 min at 0 °C, the mixture was centrifuged, and the radioactivity of the supernatant was determined.

Results

Spectroscopic Studies. The influence of bromination on the spectroscopic properties of poly(dG-dC) was monitored by measuring both the circular dichroism and the UV spectra (Figure 2) under low-salt conditions (0.15 M NaCl, 1 mM EDTA, and 10 mM phosphate, pH 7.0). Figure 2A shows the circular dichroism spectra of unmodified poly(dG-dC) and two different levels of modification. The solid curve represents B-DNA, while the interrupted lines are those typical of partially ($\theta = 0.6$) and fully converted ($\theta = 1.0$) Z-DNA. The dashed curve which shows the near-inversion of the circular dichroism spectrum is produced by a polymer in which 38% of the guanine residues and 18% of the cytosine residues are brominated. Further bromination does not change the circular

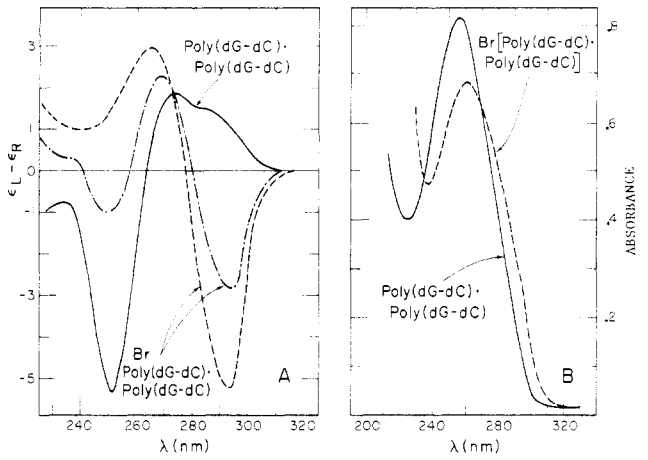

FIGURE 2: Circular dichroism spectra (A) of poly(dG-dC) in the B form (—) and brominated polymers with $\theta = 0.6$ (-·-) and $\theta = 1$ (---) in 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, and 1 mM EDTA. Absorption spectra (B) of poly(dG-dC) (B form) (—) and brominated poly(dG-dC) (Z form, $\theta = 1$) (---) under the same ionic conditions as listed under (A).

dichroism spectrum. The degree of transition (θ), measured at 290 nm, is defined by $\theta = (\alpha_B - \alpha)/(\alpha_B - \alpha_Z)$ where α is the specific ellipticity of the solution and α_Z and α_B are the constants for the Z and B forms, respectively (Pohl & Jovin, 1972). The intermediate curve with a 60% inversion is found for a polymer in which 22% of the guanine residues and 8% of the cytosine residues are brominated. Figure 2B shows the ultraviolet absorption spectra of the unmodified and the brominated polymer which is fully converted ($\theta = 1$). It can be seen that bromination reduces the intensity of the absorption band at 260 nm and leads to an increase of the absorption band at 295 nm. It should be noted that the change in the absorbance spectrum of the brominated polymer compared to the nonbrominated polymer is much greater than the change that is seen in the nonbrominated polymer in going from 0.15 to 4 M NaCl. When poly(dG-dC) forms the Z-DNA structure in 4 M NaCl, there is a small decrease in the absorbance maximum at 257 nm and a considerable increase in the absorbance at 295 nm. The change in these values is exaggerated when Z-DNA is stabilized by bromination due to the fact that the extinction coefficients of the brominated nucleotides are lower at 257 nm than the unmodified ones.

A study was made to determine the effects of bromination on the $A_{295/260}$ absorbance ratios and on the degrees of transition (θ) as measured by circular dichroism. Two different polymers were used having chain lengths of approximately 1500 ± 500 and 150 ± 100 nucleotides, respectively. It can be seen (Figure 3) that different amounts of bromine are required in the reaction mixture in order to produce a change in the absorbance ratios in either the long or the short polymers. Lower levels of bromine per nucleotide in the reaction mixture are required to stabilize the long polymer in the Z form, whereas higher levels are required for the shorter polymer. The bromination reactions are carried out in 4 M NaCl, under which conditions both the short and the long polymers have the same circular dichroism and absorption spectra as shown in Figure 2. Thus, the difference in the amount of bromination required for Z stabilization is not likely to be attributed to significant differences in the amount of Z-DNA found in the two different preparations. The difference in the two curves suggests that there may be some significant cooperativity in the Z-DNA stabilization by bromination with an important destabilization effect at the ends of the double helix which may revert more readily to B-DNA

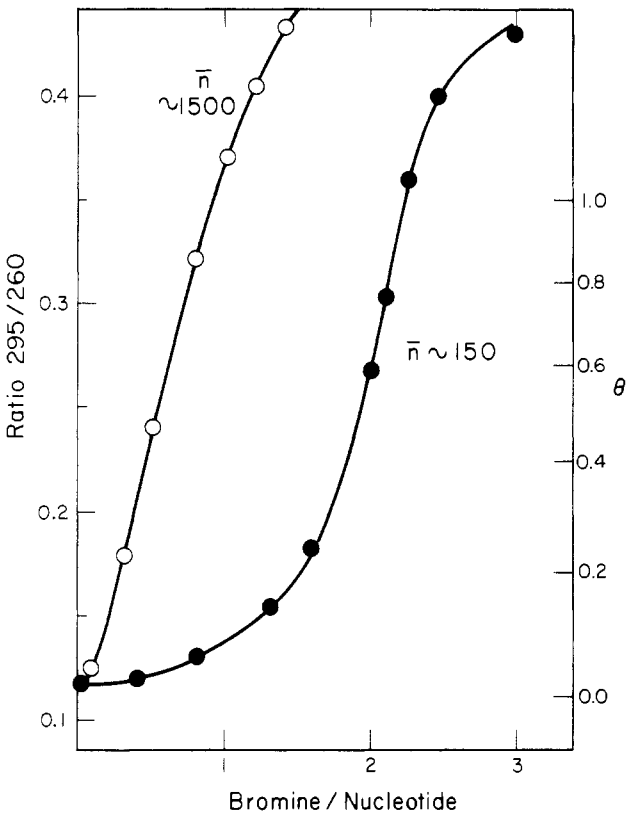

FIGURE 3: Bromine/nucleotide ratios in the reaction mixture plotted against the $A_{295/260}$ ratios and the corresponding degree of transition (θ) values measured in low-salt buffer conditions. Two different polymers were used with chain lengths of n ~1500 bp (○) and n ~150 bp (●). Note that the degree of transition (θ) scale is not linear, and the absorbance continues to change with added bromine even after the transition is complete ($\theta = 1$).

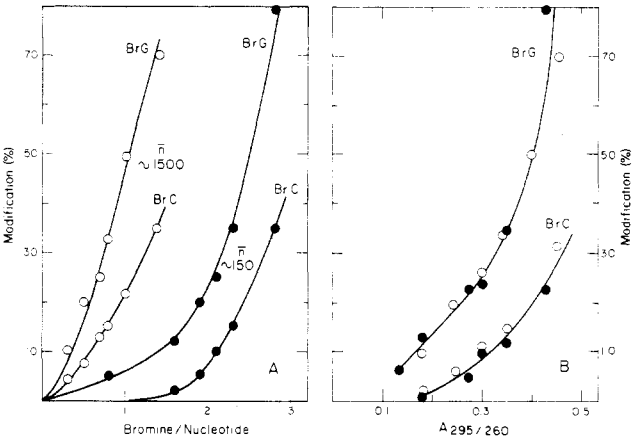

FIGURE 4: Extent of modifications for two polymers of different chain lengths [n ~1500 bp (○) and n ~150 bp (●)] seen in relation to the bromine to nucleotide ratios in the reaction mixtures (A). In (B), the amount of modification can be determined by measuring the absorbance ratios $A_{295/260}$. (○) Modified bases in the longer polymer (n ~1500 bp) and (●) in the short polymer (n ~150 bp).

once the NaCl is dialyzed out of the solution.

The relationship between the ratio of bromine per nucleotide in the reaction mixture and the percent of modification is shown for the two different lengths of polymers in Figure 4A. It can be seen that the extent of modification is considerably greater in the guanine residues than in the cytosine residues. In general, the extent of guanine modification is slightly more than twice that observed for cytosine.

The percentage of modification of guanine and cytosine residues is shown in Figure 4B as a function of the $A_{295/260}$

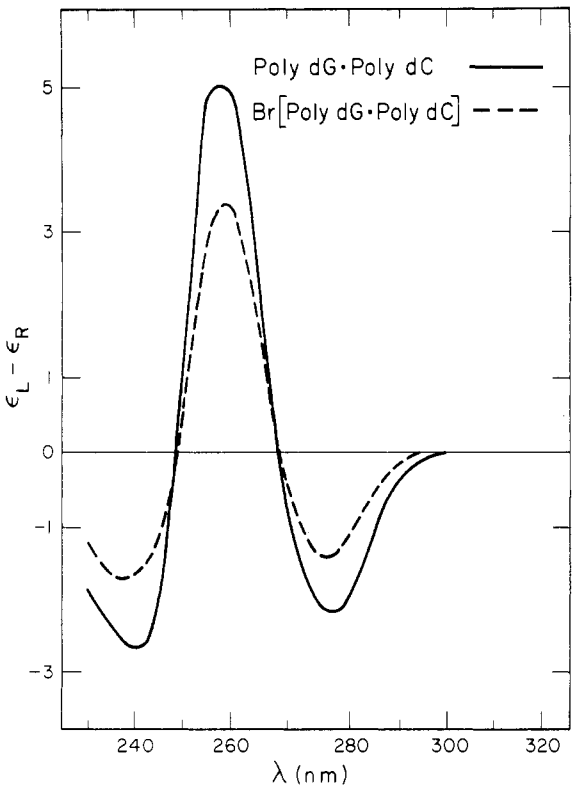

FIGURE 5: Circular dichroism spectra of unmodified poly(dG)·poly(dC) (—) and modified by bromination (---) in 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, and 1 mM EDTA.

absorbance ratios. At an absorbance ratio of $A_{295/260}$ = 0.35, the polymers are fully stabilized, and the degree of transition is equal to 1.0. The data presented in Figure 4A,B make it possible to estimate the level of modification which would be produced for a given ratio of bromine per nucleotide in the reaction mixture by simply measuring the $A_{295/260}$ absorbance ratio for the modified polymer.

Control experiments were carried out with poly(dG)·poly(dC), which is not likely to form Z-DNA because it does not have alternating purine–pyrimidine residues. Figure 5 presents the circular dichroism spectra of poly(dG)·poly(dC) when unmodified (solid line) or modified by bromination to the extent of 57% Br^8G and 28% Br^5C (dashed line). It can be seen that there is no change in the shape of the circular dichroism spectrum despite the somewhat larger extent of modification in this polymer.

Effects of NaCl Concentration on the Degree of Transition. When the NaCl concentration of a solution of poly(dG-dC) is raised, the molecule starts to convert to Z-DNA as shown by an inversion of the circular dichroism spectrum and a change in the $A_{295/260}$ absorbance ratio (Pohl & Jovin, 1972). Figure 6 shows the changes in the absorbance ratios as a function of the NaCl concentration for polymers which have been brominated to various extents. For the unmodified polymer, a sharp cooperative transition can be seen near a NaCl concentration of 2.4 M. As noted above, a θ value of 1.0 corresponds to a level of bromination (38% Br^8G, 18% Br^5C) which produces the maximal change in the circular dichroism spectrum. At this level of modification, the entire polymer is in the form of Z-DNA as shown by the fact that exposure to increasing amounts of NaCl does not result in any further changes in the $A_{295/260}$ absorbance ratios. Intermediate levels of modification for θ values of 0.2, 0.4, and 0.6 show that they can be fully converted to Z-DNA as measured by increases in the absorbance ratios at elevated levels of NaCl.

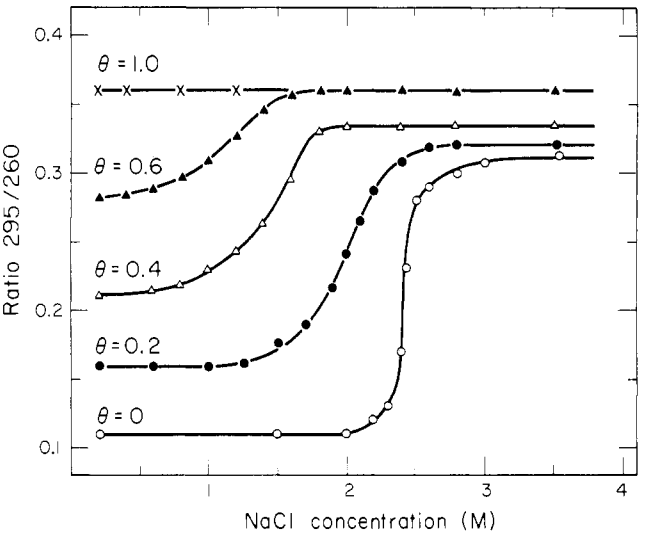

FIGURE 6: Poly(dG-dC) brominated to produce various levels of transition (θ). The level of bromination is for θ = 0.2 (10% Br^8G and 4% Br^5C), θ = 0.4 (18% Br^8G and 5% Br^5C), θ = 0.6 (22% Br^8G and 8% Br^5C), and θ = 1.0 (38% Br^8G and 18% Br^5C). The change in the absorbance ratios 295/260 is plotted as a function of varying levels of NaCl concentration in the solution.

It should be noted that the amount of NaCl necessary for the conversion to Z-DNA decreases steadily as the value of θ increases. The midpoint for the transition curve using the polymer with a θ = 0.2 is at 2 M, while it is near 1.2 M for a polymer with θ = 0.4. Thus, less NaCl is required to stabilize Z-DNA due to the fact that the partial bromination has also contributed to the Z-DNA stabilization. As the level of bromination increases, there is a steady increase in the value of the absorbance ratio which plateaus as the NaCl concentration is increasing. As mentioned above, this is due to the fact that the brominated nucleotides modify the absorbance spectrum of the polymer compared to the unmodified nucleotides.

Evidence has been presented by Kuhnlein et al. (1980) which showed that a salt-induced transition of supercoiled PM2 DNA changes it from a form which passes through nitrocellulose filters to a form which is retained by the filters. The transition occurs between 2.5 and 3.5 M NaCl, and this was suggested to indicate that Z-DNA may be retained by nitrocellulose while B-DNA passes through. The transition can also be observed with linear DNA but requires a much higher salt concentration. We have tested the filter binding abilities of poly(dG-dC) when the polymer was unmodified or brominated to various levels. The results are presented in Figure 7. Radioactive polymers were passed through nitrocellulose filters, and the percentage of binding to the filter is plotted as a function of the NaCl concentration. The unmodified polymer (θ = 0) shows a binding curve which is similar to the salt-dependent change in the absorbance ratios as shown in Figure 6. The midpoints of the curves are at 2.4 M NaCl in both cases. It can be seen that polymers are bound to the filter at progressively lower concentrations of NaCl as the amount of bromination increases. In this case, a small change in the level of bromination (θ = 0.2) results in a very rapid change in the filter binding characteristics so that there is now a midpoint close to slightly more than 1 M NaCl. This effect is much more dramatic than the change in the amount of NaCl required to produce a change in the absorbance ratios shown in Figure 6. Further increases in the level of bromination produced progressively smaller changes in filter binding. However, it should be noted that the modified polymer with

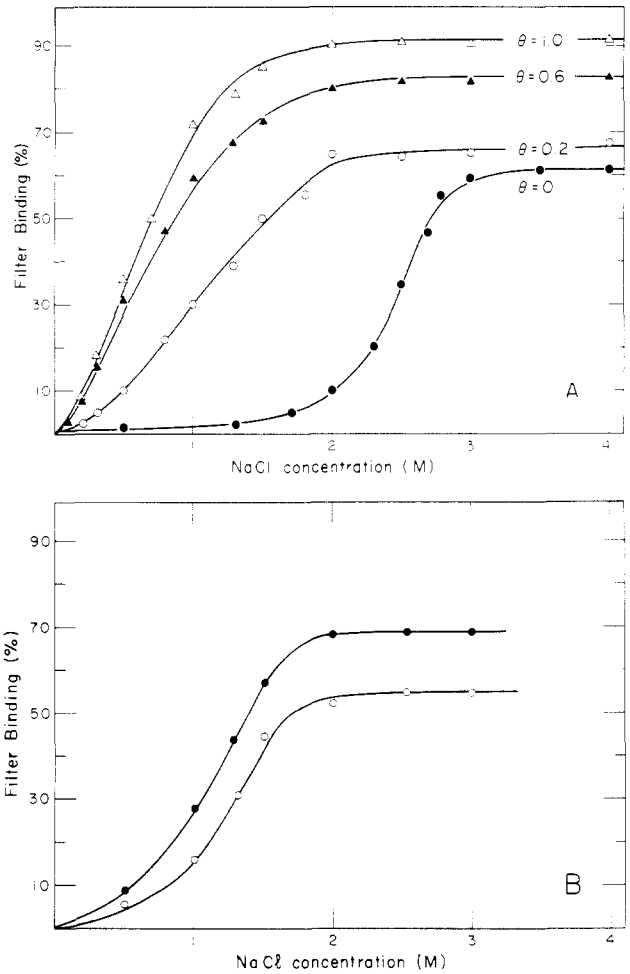

FIGURE 7: (A) Retention of unmodified poly(dG-dC) (●) and brominated polymers [modified to different extents: θ = 0.2 (○); θ = 0.6 (▲), and θ = 1.0 (△)] by nitrocellulose filters in the presence of varying concentrations of NaCl. For the level of bromination, see the legend to Figure 6. (B) The binding to filters for poly(dG)·poly(dC) (○) and its brominated form (●) as a function of the NaCl concentration is shown.

a θ value equal to 1.0 still requires a considerable amount of NaCl in order to be retained on the filter. This is quite unlike the effect of NaCl on the absorbance ratio of the fully modified polymer (Figure 6).

In a control experiment, unmodified and brominated poly(dG)·poly(dC) was bound to filters as a function of the NaCl concentration. As can be seen in Figure 7B, the midpoints for both polymers are very similar. Thus, the modification of the polymers by bromination is not responsible for the different filter binding characteristics. Why bromination increases the plateau level is not understood.

Effect of the Intercalator Ethidium Bromide. Pohl et al. (1972) have shown that the effect of simple ions on the conformation of poly(dG-dC) can be reversed by the addition of ethidium bromide; i.e., it converted Z-DNA back to an intercalated B-DNA form. Experiments were accordingly carried out (Figure 8) to test the effect of varying concentrations of ethidium bromide on the circular dichroism spectra of unmodified poly(dG-dC) under high-salt conditions and brominated poly(dG-dC) under low-salt conditions. Addition of the drug to 23 μM results in a partial transformation of the brominated polymer's spectrum so that it has lost some of its Z-DNA character but it has not yet fully converted to the intercalated B-DNA spectrum (Figure 8A). At a concentration of 50 μM, ethidium bromide yields a spectrum which is similar to the curve shown in Figure 8B; it is the circular

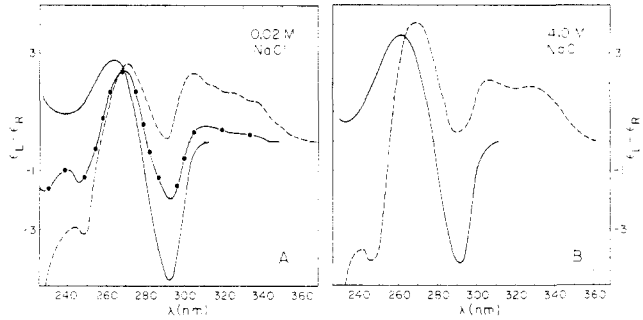

FIGURE 8: Circular dichroism spectra of brominated poly(dG-dC) (θ = 1) in 20 mM NaCl in the presence of different ethidium bromide concentrations: (—) 0 μM dye; (●) 23 μM dye; (---) 50 μM dye. (B) Spectra of poly(dG-dC) are shown in the absence (—) and presence of 50 μM ethidium bromide (---) in 4 M NaCl.

dichroism spectrum of an intercalated B-DNA structure. When isoamyl alcohol is added, the ethidium bromide can be extracted, and this leads again to a circular dichroism spectrum equal to that of the solid line in Figure 8A (data not shown). The brominated polymer thus shifts back to the Z-DNA conformation upon removal of the intercalator. These experiments show that the brominated polymers have the same property as that of the high-salt-stabilized, unbrominated polymer with respect to structural Z–B interconversion induced by the ethidium bromide intercalator.

Nuclease Sensitivity. The conversion of B-DNA to Z-DNA is a profound conformational change, and one might anticipate that there would be considerable differences in the sensitivity to nucleases relative to the B-DNA structure. The effectiveness of various exo- or endonucleases and single-stranded nucleases was examined on both the unmodified and brominated forms of poly(dG-dC). The results of these experiments are shown in Table I where the percentage of acid-soluble nucleotides was determined after nuclease digestion. Nuclease S1 is reactive on single-stranded DNA. This is demonstrated by the fact that it makes 96% of the nucleotides of denatured calf thymus DNA acid soluble. However, poly(dG-dC) and the brominated polymers appear to be equally insensitive to nuclease S1.

Micrococcal nuclease completely digests poly(dG-dC) when it is in the B form. For the brominated polymers with degrees of transition (θ) of 0.6 and 1.0, it can be seen that progressively fewer nucleotides are made acid soluble by the enzyme as the degree of transition increases. At a degree of transition of 0.6, 64% of the nucleotides are acid soluble, while at θ = 1.0, only 42% are acid soluble. These results cannot be due solely to the possible insensitivity of micrococcal nuclease to brominated nucleotides because control experiments were carried out with both unmodified and brominated poly(dG)·poly(dC) (59% Br^8G and 28% Br^5C). Both of these polymers were digested equally well by the enzyme. The decreased sensitivity of the brominated poly(dG-dC) by micrococcal nuclease must be due to the conformational change in the B-DNA to Z-DNA transition. It should be pointed out that increasing the concentrations of micrococcal nuclease leads to higher levels of acid-soluble nucleotides. This suggests that although the Z-DNA form is a less effective substrate to micrococcal nuclease than B-DNA, nonetheless it is able to cut the molecule, and the reaction is affected by increased levels of the enzyme.

These results are in marked contrast to the endonuclease DNase I cleavage. DNase I is able to solubilize virtually all of the nucleotides in poly(dG-dC). However, conversion to Z-DNA results in almost complete DNase I inactivation. When the material is fully converted to Z-DNA (θ = 1), only

Table I: Sensitivity of Poly(dG-dC) and Brominated Poly(dG-dC) to Various Nucleases

enzyme	polymer	degree of transition, θ	% acid-soluble nucleotides
nuclease S1	denatured calf thymus DNA		96
	poly(dG-dC)	0	3
	brominated poly(dG-dC)	0.2	2
		0.8	3
		1.0	1
micrococcal nuclease	poly(dG-dC)	0	99
	brominated poly(dG-dC)	0.6	64
	brominated poly(dG-dC)	1.0	42
	poly(dG)·poly(dC)		80
	brominated poly(dG)·poly(dC)		90
DNase I	poly(dG-dC)	0	98
	brominated poly(dG-dC)	0.6	49
	brominated poly(dG-dC)	1.0	10
	poly(dG)·poly(dC)		82
	brominated poly(dG)·poly(dC)		77
exonuclease III	poly(dG-dC)	0	92
	brominated poly(dG-dC)	0.6	31
	brominated poly(dG-dC)	1.0	1
	poly(dG)·poly(dC)		89
	brominated poly(dG)·poly(dC)		61
Bal 31 nuclease	poly(dG-dC)	0	76
	brominated poly(dG-dC)	0.6	17
	brominated poly(dG-dC)	1.0	3
	poly(dG)·poly(dC)		57
	brominated poly(dG)·poly(dC)		41

10% of the nucleotides are acid soluble. However, partial conversion to Z-DNA results in increasing nuclease sensitivity. In contrast to micrococcal nuclease, increasing the amounts of DNase I does not have any further effect on the percentage of acid-soluble nucleotides produced by DNase I on the brominated polymer which is fully converted to Z-DNA. This difference in the substrate specificity for Z-DNA between the micrococcal nuclease and DNase I has also been observed by Behe et al. (1981) in their studies of the 5-methyl derivative of poly(dG-dC) which forms Z-DNA. Z-DNA was shown to be insensitive to DNase I, but it was partially sensitive to micrococcal nuclease.

Two different exonucleases, exonuclease III and Bal 31, were studied for their ability to cut B-DNA and brominated Z-DNA. The unmodified polymer is a good substrate for both enzymes, while no cleavage could be found for the brominated Z form. Again, the partly converted molecule has partial sensitivity. This insensitivity is not due to bromination, as shown by the fact that exonuclease and Bal 31 are capable of digesting most of the brominated poly(dG)·poly(dC) into acid-soluble nucleotides (Table I).

^{31}P Nuclear Magnetic Resonance Experiments. One of the strongest physical indications of Z-DNA formation in solution is the unique ^{31}P NMR resonance spectrum of $(dG\text{-}dC)_n$ in 4 M NaCl solution. Two phosphorus resonances separated by 1.5 ppm were observed for the alternating oligomer (Patel et al., 1979) and polymer (Patel, 1979b) in high-salt solution. The fact that the two peaks were equal in area suggested that there were two different conformations associated with the phosphate groups in Z-DNA. Accordingly, ^{31}P NMR experiments were undertaken to determine the effect of bromination on sonicated poly(dG-dC), in both low- and high-salt solutions.

The ^{31}P NMR spectra (in 0.1 M NaCl) of poly(dG-dC) with various levels of bromination are shown in Figure 9A–E. At zero bromination, poly(dG-dC) presumably in the B-DNA form has one major peak which resonates near 4.2 ppm (Figure 9A). We observe a shoulder at 3.8 ppm downfield from the main 4.2 ppm resonance when the level of bromination has been raised such that 10% of the guanine residues and 5% of the cytosine residues are brominated (Figure 9B). At 25% Br^8G and 12% Br^5C modification, it can be seen that two new peaks characteristic of Z-DNA arise (indicated in Figure 9E by the asterisks at 3.0 and 4.5 ppm). At the same time,

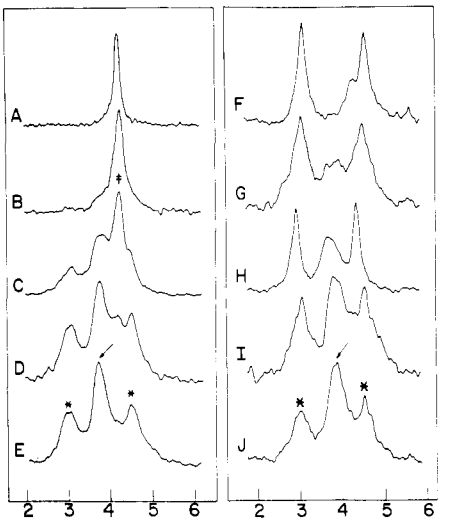

FIGURE 9: Proton noise decoupled 81-MHz ^{31}P NMR spectra of poly(dG-dC) and its brominated analogue in 5 mM phosphate, 0.5 mM EDTA, and D_2O in low and high salt. The left panel contains spectra recorded in 0.1 M NaCl added salt with no bromination of the polymer in spectrum A while (B) contains 10% Br^8G and 5% Br^5C bromination, (C) contains 25% Br^8G and 12% Br^5C bromination, (D) contains 38% Br^8G and 18% Br^5C bromination, and (E) contains 55% Br^8G and 25% Br^5C bromination. The proton noise decoupling was on during the accumulation and the delay between pulses (probe temperature 46 °C). The right panel (spectra F–J) contains spectra recorded following addition of 4.0 M NaCl to the low-salt samples, the spectra of which are presented in the left panel (spectra A–E). The proton noise decoupling was on during the accumulation and off during the delay between pulses (probe temperature 52 °C). The ^{31}P chemical shifts are corrected for NaCl and temperature dependence of internal trimethyl phosphate and are plotted relative to the standard in a no added salt solution at 27 °C. The peaks from the unbrominated segments of the polymer in the B-DNA conformation are designated with a double dagger, and in the Z-DNA conformation, they are designated by asterisks. The peaks from the brominated segments of the polymer are designated by an arrow.

another peak at 3.8 ppm designated by an arrow begins to accumulate at the position of the shoulder which was present in the 10% dG bromination curve (Figure 9B). When the bromination is increased to 38% Br^8G and 18% Br^5C, the Z-DNA resonances at 3.0 and 4.5 ppm and the peak at 3.8 ppm increase in intensity while the B-DNA resonance at 4.2 ppm has decreased considerably (Figure 9D). At an even higher level of bromination, 55% Br^8G and 25% Br^5C, the B-DNA resonance at 4.2 ppm has disappeared completely. The Z-DNA resonances at 3.0 and 4.5 ppm (designated by asterisks) are prominently separated and exhibit a combined area comparable to the 3.8 ppm resonance designated by an arrow (Figure 9E).

The two peaks marked with the asterisks are believed to derive from the unmodified parts of poly(dG-dC) which are forming Z-DNA. These segments steadily increase until they reach a relative maximum of 38% guanine bromination. As noted above, at this level of bromination, the majority of the polymer has converted to Z-DNA as determined by optical spectroscopic criteria. It is interesting that the 3.8 ppm resonance, designated by an arrow, has a relative area which closely approximates the percentage bromination of the dG residues in poly(dG-dC). This suggests that the 3.8 ppm resonance is associated with the phosphodiester backbone related to bromination of guanine residues.

This interpretation of the low-salt ^{31}P spectra (Figure 9A–E) is reinforced by inspection of the corresponding ^{31}P spectra in 4 M NaCl solution (Figures 9E–J). The control spectrum of unbrominated poly(dG-dC) in 4 M NaCl reveals two peaks at 3.0 and 4.5 ppm characteristic of Z-DNA (Figure 9F). It is only with bromination of polymers that one can observe an accumulation of a resonance in the intermediate spectral region (3.8 ppm) as shown in the spectrum containing 10% Br^8G and 5% Br^5C (Figure 9G). As the level of bromination increases, the intensity of the 3.8 ppm resonance ascribed to the perturbations due to the brominated guanine bases increases (Figure 9H), and the increase is continued to two higher levels of bromination (Figure 9I,J). The spectra in 4 M NaCl (Figure 9F–J) differ significantly from their counterparts in 0.1 M NaCl (Figure 9A–E) in that the peak at 4.2 ppm attributed to B-DNA in the unbrominated segments in the low-salt spectra is never found in the 4 M NaCl spectrum. This is in agreement with the fact that all the unbrominated segments are forming Z-DNA in high-salt solution.

Another set of experiments was carried out to determine the effect of increasing salt concentrations on poly(dG-dC) which was brominated to low levels (25% Br^8G, 12% Br^5C). In Figure 10A (0.1 M NaCl), the 3.0 and 4.5 ppm peaks due to the Z-DNA phosphates of the unbrominated segments are designated by asterisks. A significant intensity is found at the 4.2 ppm peak due to the B-DNA phosphates of the unbrominated segments and in another peak at 3.8 ppm associated with the brominated dG segments (Figure 10A). Raising the salt concentration to 1.1 M results in a significant decrease of the 4.2 ppm peak. However, there is no increase in the size of the peak at 3.8 ppm which we have attributed to brominated dG segments (Figure 10B). Increasing the salt concentration to 2.1 M NaCl results in further enhancement of the Z-DNA peaks at 3.0 and 4.5 ppm at the expense of the 4.2 ppm B-DNA peak without any perturbation of the 3.8 ppm peak attributed to brominated dG segments (Figure 10C). The ^{31}P NMR data in Figure 10 are comparable to the optical data in Figure 6 showing the effect of increasing NaCl concentration on the $A_{295/260}$ absorbance ratio at a value $\theta = 0.6$. It can be seen that the midpoint of the conversion from B- to

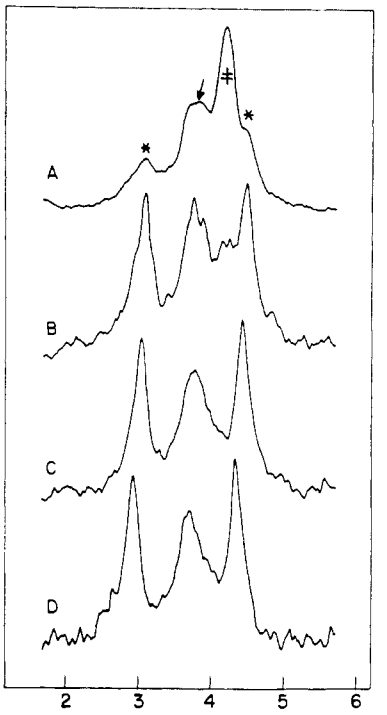

FIGURE 10: Proton noise decoupled 81-MHz ^{31}P NMR spectra of poly(dG-dC) with 25% of dG and 12% of dC residues brominated. The solution contained D_2O with 5 mM phosphate and 0.5 mM EDTA at 48 ± 2°. The concentration of added salt was (A) 0.1 M NaCl, (B) 1.1 M NaCl, (C) 2.1 M NaCl, and (D) 3.1 M NaCl. The ^{31}P chemical shifts are corrected for the NaCl and temperature dependence of internal trimethyl phosphate and are plotted relative to the standard in a no added salt solution at 27 °C. The peaks from the unbrominated segments of the polymer in the B-DNA conformation are designated by a double dagger and by asterisks in the Z-DNA conformation. The peaks from the brominated segments of the polymer are designated by an arrow.

Z-DNA is approximately 1 M NaCl and that the conversion is virtually complete by 2 M NaCl (Figure 6). This is consistent with the ^{31}P NMR spectra which show that the spectrum is virtually completely converted to Z-DNA in 2.1 M NaCl (Figure 10C). Raising the salt concentration further does not result in any further enhancement of the two Z-DNA peaks nor is there a change associated with the peak which we attribute to the perturbation due to the brominated dG segments (Figure 10D).

Discussion

The material presented in this paper provides information on how to stabilize the Z-DNA conformation in poly(dG-dC) through the use of a simple chemical modification which brominates predominately the 8-position of guanine residues and, to a lesser extent, the 5-position of cytosine residues. Figures 2 and 3 present data which show that the Z-DNA conformation is stabilized when approximately one-third of the guanine (C8) residues and one-fifth of the cytosine (C5) residues are brominated. At this level of bromination, poly(dG-dC) is stabilized in the Z conformation in a low-salt (150 mM NaCl) solution. However, even at 5 mM NaCl, the brominated polymer shows all the spectroscopic characteristics of the Z form. It is important to note that bromination produces a stabilizing effect on the Z-DNA conformation of poly(dG-dC) which is similar to the effect of 4 M NaCl. It is clear that this effect is due to a conformational Z-DNA stabilization and not due to bromination per se since brominated poly(dG)·poly(dC) (Figure 5) does not produce an inversion in the circular dichroism spectrum. The reason for

the effectiveness of bromination is interpreted to be stereochemical. The proton on the C8 position of guanine in B-DNA is in van der Waals contact with the sugar–phosphate backbone. In contrast to this, the proton at the 8-position of guanine in Z-DNA is on the outside of the helix where there are no constraints on the size of the groups which can be attached there. Substituting a bromine atom with a van der Waals radius of 2.0 Å for the hydrogen atom (van der Waals radius 1.0 Å) results in a strong stabilization of the syn conformation which is found in the guanosine residues of Z-DNA. The effect of bromination has been illustrated by a large number of studies (Uesugi et al., 1982; Bugg & Thewalt, 1969; Tavale & Sobell, 1970). The syn conformation of deoxyguanosine was first seen in a crystal structure involving a hydrogen-bonded complex containing deoxyguanosine and 5-bromodeoxycytidine (Haschemeyer & Sobell, 1965). It has been pointed out that unmodified purines can exist in either syn or anti conformations as there is no steric block (Haschemeyer & Rich, 1967). In solution, they exist in an equilibrium. The effect of bromination is simply to alter the equilibrium between syn and anti conformations for deoxyguanosine in favor of the syn conformation through the use of a bulky substituent for which there is inadequate room in the anti conformation. There have been studies by Michelson et al. (1970) on poly(8-bromoguanilic acid). This polymer shows a variety of physicochemical changes which have been interpreted as indicating that the purine residues in poly(8-bromoguanilic acid) do not exist in the anti conformation but rather in the syn conformation. It is interesting that this brominated polymer does not form a double helix with either poly(C) or poly(dC). This suggests that stabilizing the guanine residues in the syn conformation is inadequate to stabilize a left-handed B-DNA helix.

It should be stressed that there is an equilibrium between the B- and Z-DNA conformations. The effect of bromination is simply to alter the equilibrium constant. This is seen for the individual monomers directly as in NMR studies of 8-bromodeoxyguanosine (Pless et al., 1978) and from other ethidium bromide intercalation experiments (Figure 8). Bromination does not freeze the polymer in one structure. There is an active equilibrium, and the insertion of an intercalating agent into the brominated Z-DNA polymer modifies the equilibrium sufficiently so that the intercalated B-DNA is more favorable than the intercalated Z-DNA form.

It is likely that bromination of cytosine on the 5-position also stabilizes the Z conformation. Behe & Felsenfeld (1981) have shown that poly(dG-m^{5}dC) is stabilized in the Z conformation. It has been noted that methylation in this position fills a small depression in the surface of Z-DNA, as discerned from the three-dimensional crystal structure of (m^{5}dC-dG)$_3$ (Fujii et al., 1982). Studies on poly(Br^{5}dC-dG) have shown that this molecule requires only 5 mM Tris buffer to be stabilized in the Z conformation (Malfoy et al., 1982).

The available data suggest that bromination of guanosine residues on the 8-position as well as the C5 position of cytidines leads to stabilization of the Z-DNA conformation. We are unable, however, to estimate which of these modifications is more important for stabilization. The actual extent of modification in this procedure is roughly 2 to 1; that is, twice as many guanine residues are brominated as cytidine residues. This probably reflects a difference in reactivity associated with the ready access of bromine to the guanine imidazole ring.

In these experiments, we have been using the degree of transition (θ) as a rough measure of the amount of Z-DNA in solution. It is simply a measurement of the change in the circular dichroism at the wavelength of 290 nm as a function of different amounts of bromination. The end points are well-defined; that is, when θ is 0, the polymer is simply B-DNA; when it is 1.0, the circular dichroism spectrum has inverted maximally, and presumably the molecule is entirely in the form of Z-DNA. Since there is not at present an adequate quantitative theory regarding the circular dichroism spectrum, it is difficult to interpret θ in a quantitative sense as indicative of the amount of Z-DNA for values between 0 and 1. Significant end effects are expected to make θ a nonlinear function of the amount of Z-DNA if we were to compare a shorter stretch of Z-DNA with a very long stretch. In a polymer with short stretches of Z-DNA, there would be a number of B–Z transitions which have an unknown effect on the circular dichroism. With very long stretches of Z-DNA, the end effects would be less important. For this reason, one cannot make an accurate quantitative assessment of θ. It is interesting, however, that there is a rough proportionality between the extent of bromination and the degree of transition. Intermediate values for θ of 0.2, 0.4, and 0.6 corresponds to 8%, 18%, and 22% of bromoguanosine, respectively. This suggests that even though there is not a theoretical basis for assuming that θ is a direct measure for the amount of Z-DNA, nonetheless the analytical data suggest that they are related.

The filter binding experiments in Figure 7 suggest that Z-DNA binds to nitrocellulose filters. In 1 M NaCl there is virtually no binding of B-DNA, but a substantial binding of Z-DNA is found even at rather low levels of bromination. It will be interesting to see whether this method can be used to separate DNA species from each other based on their Z-DNA content, as for example in plasmids with various levels of negative supercoiling (Nordheim et al., 1982).

PM2 DNA modified by 2-(*N*-acetoxyacetylamino)fluorene also has the property of binding to nitrocellulose filters. This modification results in filter binding at lower salt concentrations (Kuhnlein et al., 1980). 2-(*N*-Acetoxyacetylamino)-fluorene is known to be bind also on the 8-position of guanine and similarly stabilizes the Z-DNA conformation of poly(dG-dC) (Sage & Leng, 1980; Santella et al., 1981).

The results of the nuclease digestion studies indicate that a conformational change from a B to a Z structure results in considerable changes in nuclease sensitivity for a variety of enzymes. The B-DNA seems to be a better substrate for both the tested endo- and exonucleases than is the Z-DNA formed by bromination of poly(dG-dC). The data suggest that micrococcal nuclease cleaves the brominated poly(dG-dC) only at high enzyme concentrations. A similar effect has been observed for the Z-DNA form of poly(dG-m^{5}dC) in which cleavage occurs only with 20-fold higher levels of micrococcal nuclease (Behe et al., 1981). It is unclear at present whether the conformation which is cleaved is the Z-DNA conformation or the small amount of B-DNA conformation which is in equilibrium in it. Because of the fact that high concentrations of the enzyme are required, it is tempting to believe that perhaps it is the B-DNA conformation which is cleaved and that the higher levels of micrococcal nuclease are used to stabilize that conformation long enough so that it can be cleaved.

Singleton et al. (1982) found that nuclease S1 recognizes and cleaves some structural feature at the junction between neighboring right- and left-handed DNA regions. Under our assay conditions, we could not detect acid-soluble nucleotides with S1 nuclease digestion. None of the enzymes tested in Table I appears to have Z-DNA as its normal substrate. It would be interesting to know whether there are any nucleases

in vivo for which Z-DNA is the normal substrate rather than right-handed B-DNA.

^{31}P Nuclear Magnetic Resonance Experiments. We have been able to monitor separately by high-resolution phosphorus NMR spectroscopy the unmodified and modified forms of poly(dG-dC), respectively; the latter brominated to different extents. The unbrominated segments in the B form give a single resonance at 4.2 ppm while the Z form with a dinucleotide repeat gives resonances at 3.0 and 4.5 ppm. Recent studies by Jovin and collaborators (Jovin et al., 1983) on selectively labeled thiophosphate analogues of poly(dG-dC) demonstrated that the 3.0 and 4.5 ppm resonances may be assigned to dGpdC and dCpdG, respectively, in the zigzag backbone of Z-DNA. The phosphorus NMR data in Figures 9 and 10 clearly demonstrate that partial bromination of poly(dG-dC) results in a switch from B- to Z-DNA of adjacent unbrominated segments. Thus, at high levels of bromination, 55% Br^8G and 25% Br^5C, the B- to Z-DNA transition of unmodified segments is complete in 0.1 M NaCl (Figure 9E). Adding 4 M NaCl has no additional effect on the phosphorus spectra (Figure 9J). Alternatively, at low levels of bromination, 25% Br^8G and 12% Br^5C, the B- to Z-DNA transition of unmodified segments occurs at lower NaCl concentrations (Figure 10) than that observed by Pohl & Jovin (1972) for poly(dG-dC) in solution.

We have observed a new phosphorus peak at 3.8 ppm with increasing levels of bromination of poly(dG-dC). The relative intensity of this peak closely approximates the percent bromination of dG residues in poly(dG-dC). This suggests that the resonance at 3.8 ppm is associated with phosphate perturbations related to bromination of guanine residues. Inspection of a Z-DNA model shows that there are two phosphate groups roughly equidistant from the C8 position of guanine which are brominated. A further indication that both phosphate groups at the modification site are perturbed is the fact that the two peaks at 3.0 and 4.5 ppm remain roughly equal in area to each other at all levels of bromination (Figure 9). If only one of the phosphate peaks were perturbed by bromination, the phosphate of either dGpdC or dCpdG, then one would develop an inherent asymmetry in the molecule which would be expressed by a relative preponderance of one of the two peaks at 3.0 and 4.5 ppm originating in the Z-DNA conformation of the unmodified segments.

We might ask what is the relationship between the 3.8 ppm resonance for the dGpdC and dCpdG phosphodiesters in the brominated segment of the polymer with the characteristic Z-DNA resonances at 3.0 ppm for dGpdC and 4.5 ppm for dCpdG in the unbrominated segment of the polymer. Is it likely that the entire molecule is in the Z-DNA form with two phosphates perturbed slightly where the imidazole ring of guanine is brominated, or is it possible that these phosphate groups at the modification site are not in the Z-DNA conformation but are in yet another conformation? Phosphorus NMR does not provide an answer to this question since we do not know the effects of halogenation on the chemical shifts of proximal phosphodiester groups. The nuclease experiments, however, suggest that both the brominated and unbrominated segments of poly(dG-dC) adopt a Z-DNA conformation. If there were significant segments at the bromination site in a conformation other than Z-DNA, one might expect differentiation of nuclease sensitivity based on the level of bromination. In general, this is not seen in the data presented.

Acknowledgments

We acknowledge the help of Robert Beckman in the initial experiments.

Registry No. Poly(dG-dC), 36786-90-0; poly(dG)·poly(dC), 25512-84-9; ethidium bromide, 1239-45-8.

References

Behe, M., & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1619–1623.

Behe, M., Zimmerman, S., & Felsenfeld, G. (1981) *Nature (London) 293*, 233–235.

Bugg, C. E., & Thewalt, U. (1969) *Biochem. Biophys. Res. Commun. 37*, 623–628.

Fujii, S., Wang, A. H.-J., van der Marel, G., van Boom, J. H., & Rich, A. (1982) *Nucleic Acids Res. 10*, 7879–7892.

Haschemeyer, A. E. V., & Sobell, H. M. (1965) *Acta Crystallogr. 19*, 125–130.

Haschemeyer, A. E. V., & Rich, A. (1967) *J. Mol. Biol. 27*, 369–384.

Jovin, T. M., van de Sande, J. H., Zarling, D. A., Arndt-Jovin, D. J., Eckstein, F., Füldner, H. H., Greider, C., Grieger, I., Hameri, E., Kalisch, B., McIntosh, L. P., & Robert-Nicoud, M. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 143–154.

Kuhnlein, U., Tsant, S. S., & Edwards, J. (1980) *Nature (London) 287*, 363–364.

Lafer, E. M., Möller, A., Nordheim, A., Stollar, B. D., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3546–3550.

Malfoy, B., Rousseau, N., & Leng, M. (1982) *Biochemistry 21*, 5463–5467.

Michelson, A. M., Mowry, C., & Kapuler, A. M. (1970) *Biochim. Biophys. Acta 217*, 7–17.

Möller, A., Gabriels, J. E., Lafer, E. M., Nordheim, A., Rich, A., & Stollar, B. D. (1982) *J. Biol. Chem. 257*, 12081–12085.

Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, B. D., & Rich, A. (1982) *Cell (Cambridge, Mass.) 31*, 309–318.

Patel, D. J. (1979) *Stereodynamics of Molecular Systems* (Sarma, R. H., Ed.) pp 397–435, Pergamon Press, Elmsford, NY.

Patel, D. J., Canuel, L. L., & Pohl, F. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2508–2511.

Patel, D. J., Kozlowski, S. A., Nordheim, A., & Rich, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1413–1417.

Pless, R., Dudycz, L., Stolarski, R., & Shugar, D. (1978) *Z. Naturforsch. C: Biosci. 33C*, 902–907.

Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375–396.

Pohl, F. M., Jovin, T. M., Baehr, W., & Holbrook, J. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3805–3809.

Pohl, F. M., Ranade, A., & Stockburger, M. (1973) *Biochim. Biophys. Acta 335*, 85–92.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.

Sage, E., & Leng, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6076–6080.

Santella, R. M., Grunberger, D., Weinstein, J. B., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1451–1455.

Singleton, C. K., Klysik, J., Stirdivant, S. M., & Wells, R. D. (1982) *Nature (London) 299*, 312–316.

Tavale, S. S., & Sobell, H. M. (1970) *J. Mol. Biol. 48*, 109–123.

Thamann, T. J., Lord, R. C., Wang, A. H.-J., & Rich, A. (1981) *Nucleic Acids Res. 9*, 5443–5457.

Uesugi, S., Shida, T., & Ikehara, M. (1982) *Biochemistry 21*, 3400–3408.

Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.