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Covalent Binding of Benzo[*a*]pyrenediol Epoxides to Polynucleotides†

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ABSTRACT: Spectroscopic studies on the *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene- (*anti*-BPDE-) modified synthetic polynucleotide solutions reveal interesting sequence-dependent stereoselective covalent binding of *anti*-BPDE to DNA. Absorption spectral results indicate that the G·C polymers are much more reactive than the A·T polymers toward this metabolite and the homopolymer suffers higher modification than its corresponding alternating polynucleotide. The covalently attached *anti*-BPDE exhibits only a 2–3-nm red shift in the guanine-containing polynucleotide and native DNA solutions as opposed to the 8-nm red shift in poly(G) and none in the A·T polymers. Distinct stereoselectivities are exhibited by poly(dG-dC)·poly(dG-dC) vs. poly(dG)·poly(dC) as suggested by the oppositely signed CD in the pyrene spectral region. Comparison with the *syn*-BPDE modified polynucleotides reveals some interesting differences with its anti diastereomer. Significant contributions from the intercalated *syn*-BPDE are apparent in the modified guanine-containing polynucleotides as indicated by the appearance of 10-nm red-shifted shoulders. In contrast to the strong dependence on polynucleotides for *anti*-BPDE, the rate of hydrolysis of *syn*-BPDE appears to be insensitive to their presence in the solution. *anti*-BPDE modification on the 50 μM hexaamminecobalt-induced Z-form poly(dG-dC)·poly(dG-dC) is much less extensive than its corresponding B form, possibly the consequence of both structural and ionic strength factors. The spectral characteristics of *anti*-BPDE bonded to these two forms are distinctly different, with the Z form resembling more closely those of A·T polymers. Salt titration of the *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) suggests that the "externally" bound moiety becomes intercalated under high-salt condition as judged by the CD spectral red shift and concomitant intensity enhancement.

Polycyclic aromatic hydrocarbons (PAHs) are prevalent in our polluted environment, and some are known to be carcinogenic as well as mutagenic. These relatively inert compounds exert such activities through enzymatic conversion to reactive metabolites which then chemically bind to cellular macromolecules such as DNA (Harvey, 1981). The most widely studied PAH has been benzo[*a*]pyrene (BP), and there is strong evidence to suggest that the most important ultimate carcinogenic metabolites are the bay region epoxides *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE).

There are two diastereomeric forms of BPDE designated as anti and syn in which the benzylic hydroxyl group at C-7 is trans and cis to the epoxide oxygen, respectively (see Figure 1). Although the syn diastereomer is chemically more reactive (Yagi et al., 1977), the anti isomer is much more carcinogenic (Slaga et al., 1977). The weaker potency of the syn isomer has been attributed to the rapid hydrolysis suffered by this isomer as a consequence of intramolecular hydrogen bonding between the C-7 hydroxyl and the 9,10-epoxy group. Of the two enantiomers of *anti*-BPDE, the (+) isomer is a more potent tumor initiator than the (−) variety (Buening et al., 1978; Slaga et al., 1979) and correlates well with the DNA binding ability of these two enantiomers in vitro (Meehan & Straub, 1979).

anti-BPDE has been shown to preferentially form covalent bonds to the exocyclic amino group of guanine (Weinstein et al., ,1976; Jeffrey et al., 1977). Experiments with natural DNA have shown that such covalent binding is quite stereoselective for the duplex DNA but is devoid of such effect for the single-stranded form. Such specificity is not present for

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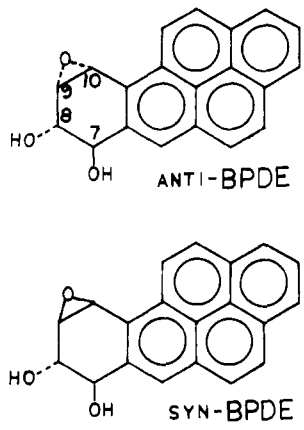

FIGURE 1: Structure drawings for the two diastereoisomers of *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE).

anti-BPDE modification at the adenine sites, and the (+) and (−) enantiomers react equally in either single-stranded or double-stranded DNA (Meehan & Straub, 1979). It has been suggested that such stereoselectivity on covalent binding to the guanine base is the consequence of stereospecific physical binding such as intercalation (Meehan & Straub, 1979).

There is, indeed, evidence to indicate that the predominant mode of physical binding between *anti*-BPDE and the duplex DNA is that of intercalation (Geacintov et al., 1981). This led to the suggestion that the covalent linkage is preceded by intercalative physical binding (Meehan et al., 1982). There is also strong spectroscopic evidence to suggest that the covalently linked *anti*-BPDE resides externally to DNA and presumably in the minor groove (Geacintov et al., 1980; Lefkowitz et al., 1982). It is thus puzzling that if intercalative physical binding precedes chemical lesion, the covalent adducts should exhibit external binding characteristics. It is to be noted, however, that others have presented results to argue that the pyrene moieties in the covalent adducts are intercalated between the bases in a wedgelike fashion (Hogan et al., 1981).

The nature of the binding site and the effect of such covalent binding on the structure of DNA are not yet clear. A melting study on DNA solutions with various extent of *anti*-BPDE modification has led to the conclusion that covalent attachment of *anti*-BPDE at guanine results in local denaturation (Pulkrabek et al., 1977). Others have presented evidence to argue otherwise (Hogan et al., 1981). Kakefuda & Yamamoto (1978) have provided evidence from their circular DNA studies to suggest that binding to adenine causes local denaturation of DNA, whereas the more than 10-fold greater binding to guanine does not create such denaturation.

To help answer some of these questions, we have earlier carried out physical binding studies with synthetic polynucleotides using pyrene (Chen, 1983) and 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene (*anti*-BPT) (Chen, 1984) as model compounds for *anti*-BPDE. Specifically, we were interested in finding out the possible base sequence preference on such binding since if intercalation is the dominant binding mode it should depend on π-electron interactions between the pyrene chromophore and the sandwiching bases. These studies reveal that in neutral pH the intercalative binding modes of pyrene and *anti*-BPT are important in poly(dA-dT)·poly(dA-dT), much less so in poly(dG-dC)·poly(dG-dC) and poly(dA-dC)·poly(dG-dT), and negligible in poly(dG)·poly(dC) and poly(dA)·poly(dT) solutions. On the basis of these observations as well as the fact that intercalative binding of *anti*-BPDE to the duplex DNA accelerates its hydrolysis to tetraols (Geacintov et al., 1980), we have earlier speculated that in neutral solutions intercalation may have little, if any, to do with the chemical lesion of *anti*-BPDE to the guanine base of DNA. This may, on the contrary, provide an efficient pathway for detoxification at the intercalating (dA-dT and/or dT-dA) sites through accelerated hydrolysis. Chemical lesions presumably occur mainly at the external sites, and consequently the covalent adducts exhibit external spectral characteristics.

To further clarify the interplay between the physical binding and chemical lesion, covalent binding studies of *anti*-BPDE to synthetic polynucleotides are hereby carried out. Since the physical binding is sequence specific, it is thus important to see if the chemical lesion also exhibits such a property and to what extent each polynucleotide is being modified stereoselectively. These results along with those of comparative studies with *syn*-BPDE are presented in this paper.

Materials and Methods

Poly(G) was purchased from Sigma Chemical Co., and an extinction coefficient of 9000 cm^{-1} M^{-1} at 260 nm was used for its concentration determination. The sources and the extinction coefficients for the calf thymus DNA and other synthetic polynucleotides used have previously been described (Chen, 1983). Sodium phosphate buffer (10 mM) of pH 7.0 containing 0.01 M NaCl and 1 mM EDTA was used throughout unless stated otherwise.

anti- and *syn*-BPDE were purchased from NCI Chemical Carcinogen Reference Standard Repository, a function of the Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, MD 20205. The fine crystals were dissolved in tetrahydrofuran (THF) and their concentrations determined by using extinction coeffcients (in 95% ethanol) at 344 nm of 48 600 and 47 300 M^{-1} cm^{-1} for *anti*- and *syn*-BPDE, respectively. Covalently modified polynucleotide solutions were prepared by adding 13.7 μL of 4.6 mM BPDE stock solution to an amber vial containing 2.5 mL of polynucleotide solution to result in a final BPDE concentration of 25 μM. The mixture was then incubated for at least 2 h and subsequently extracted with ether (buffer saturated) for about 25 times to ensure the removal of noncovalently bound tetraols. The extent of covalent modification was calculated by using the extinction coefficient of 30 000 M^{-1} cm^{-1} for the BPDE spectral maximum in the 343–346-nm region. The polynucleotide concentrations in ether-extracted solutions were calculated with the appropriate extinction coefficients and the BPDE-corrected absorbances in the DNA region. The Z-form DNA was obtained either by 50 μM hexaamminecobalt (HAC) or 3.6 M NaCl in a poly(dG-dC)·poly(dG-dC) solution.

Absorption spectra were measured at 24 °C with a Cary 210 spectrophotometric system using cuvettes of 1-cm path length. Kinetic measurements were made by monitoring the absorbance increase at 342 nm after adding 6.8 μL of 4.6 mM BPDE stock to 1.25 mL of a desired solution. CD spectra were measured with a JASCO J-500A recording spectropolarimeter at room temperatures.

Results

Absorption Spectra of Covalently Bound BPDE

anti-BPDE-Modified Polynucleotides. Absorption spectra of *anti*-BPDE chemically bound to various polynucleotide solutions are shown in Figure 2. Interesting features to be noted are the following: (1) poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) exhibit the highest binding abilities, with the homopolymer being slightly more effective, and the

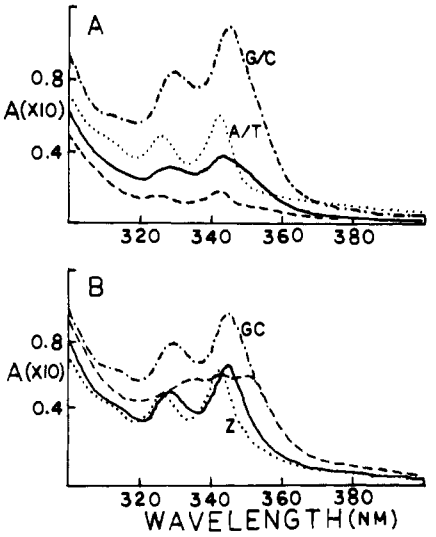

FIGURE 2: (A) Absorption spectra of covalently attached *anti*-BPDE in 0.1 mM poly(dG)·poly(dC) (---), 0.1 mM poly(dA)·poly(dT) (···), 0.1 mM poly(dA-dC)·poly(dG-dT) (—), and 0.1 mM poly(dA-dT)·poly(dA-dT) (---) and in (B) 0.1 mM poly(dG-dC)·poly(dG-dC) (---), 0.1 mM Z DNA (···) containing 50 μM hexaamminecobalt(III), 0.2 mM natural DNA (—), and 0.05 mM poly(G) (---). Respective unmodified DNA spectrum has been subtracted out. All BPDE modifications in this work have been accomplished by the presence of 25 μM BPDE in the solutions.

absorbance maxima of both are about 2–3-nm red shifted from the corresponding tetraols; (2) significant binding occurs in the poly(dA)·poly(dT) solution and is more effective than in the solution of its sequence isomer poly(dA-dT)·poly(dA-dT) with no shift in absorption maxima observed for either; (3) the spectrum of *anti*-BPDE in poly(dA-dC)·poly(dG-dT) solution is broadened, and the amplitudes are slightly lower than in the poly(dA)·poly(dT) solution; (4) the spectrum of *anti*-BPDE adduct of natural DNA exhibits a 2–3-nm red shift, similar to those of dG·dC polymers; (5) *anti*-BPDE attached to poly(G) exhibits almost a featureless spectrum, with major contribution from the 350–352-nm (8–10-nm red shifted) band being quite evident; (6) *anti*-BPDE can chemically bind to Z-form poly(ddG-dC)·poly(dG-dC) (induced by 50 μM hexaamminecobalt), and the extent of binding and spectral features (no 2–3-nm red shift) are almost identical with those of poly(dA)·poly(dT) but not to the B form of poly(dG-dC)·poly(dG-dC).

Higher binding abilities of poly(G) and the two G·C polymers are consistent with the well-known fact that *anti*-BPDE is quite guanine specific in natural DNA. The 2–3-nm red shifts of pyrenyl absorption maxima in these polynucleotides are in line with earlier calf thymus DNA observations (Geacintov et al., 1982; Undeman et al., 1983). Significant *anti*-BPDE modification on the two A·T polymers are unexpected as previous studies indicate adenine adducts to be only minor products formed between *anti*-BPDE and native DNA (Meehan & Straub, 1979). The reduced binding to poly(dA-dC)·poly(dG-dT) as compared to the G·C polymers is understandable in terms of descreased guanine content and the lower binding ability of the alternating dA polymer. The spectral similarity of chemically reacted *anti*-BPDE in natural DNA and G·C polymers is consistent with its guanine specificity.

The prominent display of the 8–10-nm red-shifted shoulder in poly(G) solution is consistent with the notion that stacking interactions between the pyrene moiety and guanine base occur in the *anti*-BPDE-modified poly(G). The absence of such large red shifts in the *anti*-BPDE-modified natural and synthetic

Table I: Comparison on the Extent of *anti*-BPDE Covalent Modification to Various Polynucleotides

polynucleotide	concn[a] (mM)	max[b] (nm)	% modification[c]
poly(dA)·poly(dT)	0.1	343	1.7
poly(dA-dT)·poly(dA-dT)	0.1	343	1.1
poly(dA-dC)·poly(dG-dT)	0.1	344	>1.2
poly(dG)·poly(dC)	0.1	346	3.1
poly(dG-dC)·poly(dG-dC)	0.1	346	2.6
Z-form poly(dG-dC)·poly(dG-dC)	0.1	343	2.0
Z DNA (3.6 M NaCl)	0.1	343	0.6
calf thymus DNA	0.2	345	0.9
poly(G)	0.05	343	>3.1

[a] Concentrations are expressed per nucleotide. Solutions are buffered at pH 7.0 with 10 mM sodium phosphate containing 1 mM EDTA and 0.01 M NaCl. Z-form poly(dG-dC)·poly(dG-dC) is induced by the addition of 50 μM hexaamminecobalt or 3.6 M NaCl. [b] Wavelength of the spectral maximum used for the bonded BPDE concentration determination. [c] Expressed as percent of total nucleotide modified. The values estimated for poly(dA-dC)·poly(dG-dT) and poly(G) are the lower limits as consequences of spectral broadening. Such underestimation is more serious for poly(G) as significant contribution from the 351-nm shoulder is readily apparent from Figure 2B.

duplex DNAs suggests that the pyrene moiety does not intercalate into DNA in the classical sense (Geacintov et al., 1982). The 2–3-nm red shift of the absorbance maxima and the presence of broad spectral features around 350 nm for the guanine-containing polynucleotides do, however, indicate some stacking interactions with the guanine base. In contrast, no red shift is observed for the modified Z DNA, suggesting that the pyrene moiety is in a microenvironment which is less perturbed by the bases as compared to the adducts of B-form poly(dG-dC)·poly(dG-dC). Although the comparison between the deoxypolynucleotides and poly(G) may not be valid due to the peculiar structure of poly(G) in which a four-stranded helix with N7 and O6 of one base being hydrogen bonded to hydrogens of N1 and N2 of another has been proposed (Zimmerman et al., 1975), it does serve to illustrate that large spectral red shifts can occur through stacking interactions.

A comparison on the extent of modification among various polynucleotides is presented in Table I. Binding to Z DNA in 3.6 M NaCl is also included to indicate the much reduced reactivity in the high-salt condition (Michaud et al., 1983; Geacintov et al., 1984a). These numerical data should only be used for qualitative comparison since spectral shifts and broadening vary greatly among solutions which can result in rather inaccurate concentration estimations.

syn-BPDE-Modified Polynucleotides. The absorption spectra of *syn*-BPDE attached to natural and synthetic DNAs are shown in Figure 3. The covalent binding abilities of these polynucleotides toward *syn*-BPDE are approximately half of the corresponding abilities toward *anti*-BPDE. Quantitative comparisons between these two metabolites and among polynucleotides are complicated by the distinctly different spectral features of some; thus, no numerical comparison in tabular form has been attempted.

The most outstanding difference between the spectra of *syn*-BPDE and those of *anti*-BPDE is the prominent appearance of the 352-nm shoulders in the guanine-containing polynucleotides for the former, suggesting important contribution from the intercalated adducts. Such shoulders, however, are absent in the two A·T polymers. The similarity between the spectra of *syn*-BPDE covalently attached to poly(dA)·poly(dT) and those of Z-form poly(dG-dC)·poly(dG-dC) is again evident. The differing features in the pyrene spectral region between the modified B-form and Z-form poly(dG-

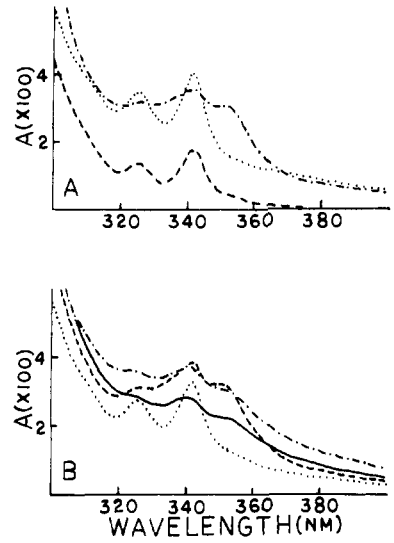

FIGURE 3: Absorption spectra in the pyrene spectral region of some *syn*-BPDE-modified polynucleotide solutions. (A) 0.1 mM poly(dG)·poly(dC) (···); 0.1 mM poly(dA)·poly(dT) (···); 0.1 mM poly(dA-dT)·poly(dA-dT) (---). (B) 0.1 mM poly(dG-dC)·poly(dG-dC) (···); 0.1 mM Z DNA (···) containing 50 μM hexaamminecobalt(III); 0.05 mM poly(G) (---); 0.2 mM DNA (—).

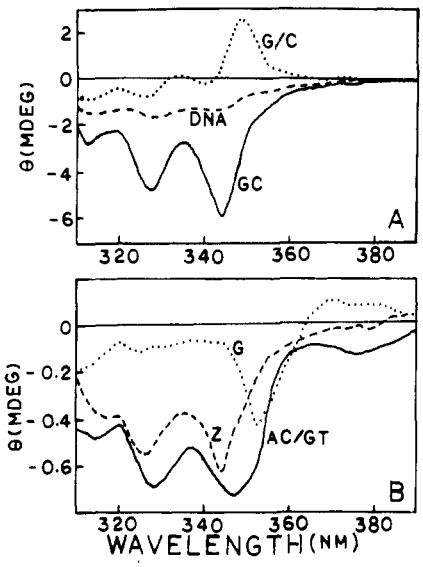

FIGURE 4: CD spectra in the pyrene spectral region of some *anti*-BPDE-modifed polynucleotide solutions. (A) 0.1 mM poly(dG)·poly(dC) (···); 0.1 mM poly(dG-dC)·poly(dG-dC) (—); 0.2 mM DNA (---). (B) 0.1 mM poly(dA-dC)·poly(dG-dT) (—); 0.1 mM Z DNA (---); 0.05 mM poly(G) (···). Notice the 10 times smaller ellipticity scale in (B). A 2-cm cell was used.

dC)·poly(dG-dC) are more apparent for the *syn*-BPDE as manifested by the respective presence and absence of the 352-nm band. No significant difference on the extent of covalent modification between poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) has been observed for *syn*-BPDE.

CD Spectra of BPDE Covalently Bound to Polynucleotides

Interesting Sequence-Dependent Covalent Binding of anti-BPDE. Although the extent and the absorption spectral characteristics of *anti*-BPDE modification on the two G·C polymers are quite similar, their corresponding CD specta are strikingly different as can be seen in Figure 4A. CD spectrum of *anti*-BPDE bound to poly(dG-dC)·poly(dG-dC) exhibits strong negative maxima at 344 and 328 nm whereas that of poly(dG)·poly(dC) shows positive long wavelength maximum

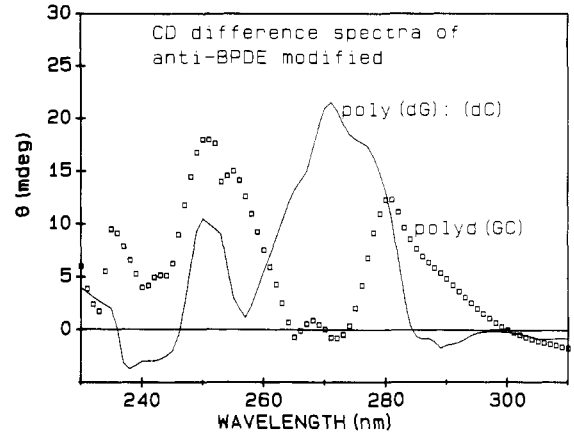

FIGURE 5: Difference CD spectra between the *anti*-BPDE-modifed and unmodified DNA solutions in the shorter wavelength region. 0.1 mM poy(dG-dC)·poly(dG-dC) (□) and 0.1 mM poly(dG)·poly(dC) (—) solutions. A 2-cm cell was used.

with a definite red shift. This red shift most likely arises from partial spectral cancellation from the negative vibronic progression of slightly shorter wavelengths [similar to those of poly(dG-dC)·poly(dG-dC)] as evidenced by the appearance of a weak 328-nm negative maximum. CD spectrum for the *anti*-BPDE bound to natural DNA is similar to that of poly(dG-dC)·poly(dG-dC) but with much reduced intensity and can partly be attributed to the spectral cancellation of these two contributions.

The CD spectra of *anti*-BPDE bound to other polynucleotides are at least an order of magnitude smaller than those of G·C polymers, and the ones displaying detectable Cotton effects are shown in Figure 4B (notice the reduced scale). Consistent with the absorbance observation, broad negative CD bands are observed for poly(dA-dC)·poly(dG-dT). A weak negative CD maximum around 351 nm is apparent in the poly(G) solution as a consequence of stacking interaction between the pyrene moiety and the guanine base. It is also interesting to note that negative CD maxima are detected for *anti*-BPDE in the Z-DNA solution at 343 and 327 nm, the same locations as those of absorbance. Although the order of magnitude smaller Cotton effects detected are, to be sure, partially due to the less extensive covalent binding of *anti*-BPDE to these polynucleotides (2–3 times less), it cannot account for the bulk of optical inactivity. For example, although the poly(dA)·poly(dT) solution is about half as effective as poly(dG)·poly(dC) in its chemical lesion to *anti*-BPDE, no detectable Cotton effects are observed. Thus, one is led to the conclusion that the covalent binding of *anti*-BPDE to these polynucleotides is less stereoselective than that to the G·C polymers.

To further distinguish the stereochemical binding of *anti*-BPDE to the two G-C polymers, difference CD spectra are obtained by subtracting the unmodified polynucleotide spectra from the corresponding *anti*-BPDE modified ones, and the results are presented in Figure 5 for the shorter wavelength region. Consistent with the dramatic difference in the pyrene spectral region, the difference CD spectra below 300 nm are quite distinct for the *anti*-BPDE bound to poly(dG-dC)·poly(dG-dC) and to poly(dG)·poly(dC).

Less Pronounced Stereoselectivity for syn-BPDE. The CD spectra of covalently bound *syn*-BPDE in poly(dG-dC)·poly(dG-dC) and poly(dG)·poly(dC) solutions are shown in Figure 6. Although the corresponding signs are the same as the *anti*-BPDE counterparts, their magnitudes are at least an order of magnitude smaller. Such 10-fold reduction most likely is

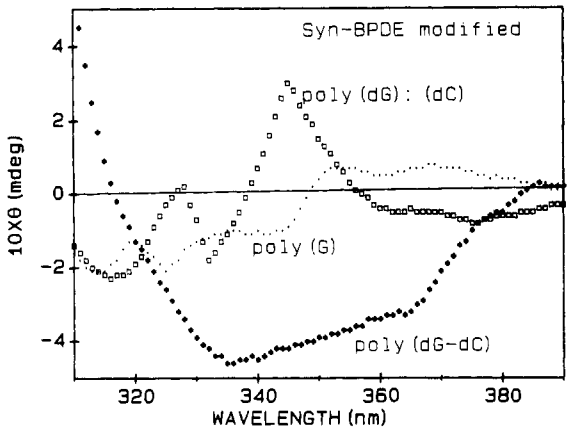

FIGURE 6: CD spectra in the pyrene spectral region of some *syn*-BPDE-modified polynucleotide solutions. 0.1 mM poly(dG)·poly(dC) (□); 0.1 mM poly(dG-dC)·poly(dG-dC) (+); 0.05 mM poly(G) (···). A 2-cm cell was used. Note that the ellipticity scale is 10 times smaller than in Figure 2A.

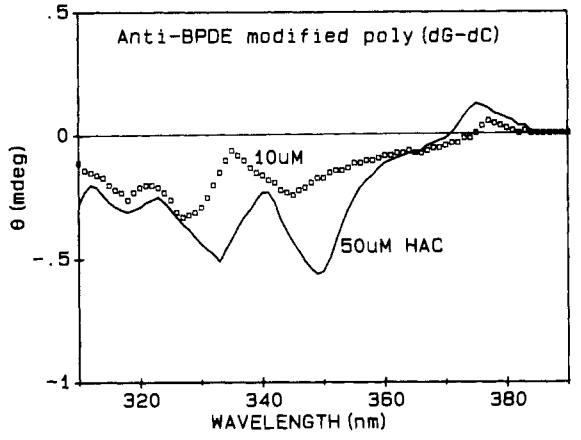

FIGURE 7: Pyrenyl CD spectra of *anti*-BPDE-modified (2.6%) 20 μM B-form poly(dG-dC)·poly(dG-dC) with 10 μM (□) and 50 μM (—) HAC added. Spectrum containing no HAC is almost identical with the 10 μM curve.

the result of decreased stereoselectivity of this metabolite rather than the consequence of reduced binding as *anti*-BPDE forms only about twice the amount of covalent adducts as *syn*-BPDE in these polynucleotide solutions. The broadness of the CD spectra is consistent with the notion of significant contribution from the intercalated species as indicated by the prominence of the 10-nm red-shifted absorption bands (Figure 4). Little or no pyrenyl CD is detected for adducts with other polynucleotides (including the Z form), and only poly(G) is shown for comparison. Although the much reduced Cotton effects due to the *syn*-BPDE adducts make the CD measuremetns less reliable, the gross spectral features in the pyrene spectral region do indicate oppositely signed CD for the homopolymers vs. alternating G·C polymers.

Effect of Salt on the anti-BPDE-Modified Poly(dG-dC)·Poly(dG-dC). To see if the *anti*-BPDE adduct environment in the Z DNA solution is similar to that of the B form and to investigate the effect of *anti*-BPDE covalent binding on the B–Z interconversions, CD measurements for the *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) are made under B (0 and 10 μM HAC) and Z (50 μM HAC) conditions with the results shown in Figures 7 and 8 for the pyrenyl and DNA spectral regions, respectively.

CD spectra in the presence of 0 and 10 μM HAC are almost identical, and thus, only the later is shown. Intensity enhancement of the pyrenyl CD is apparent in the 50 μM HAC

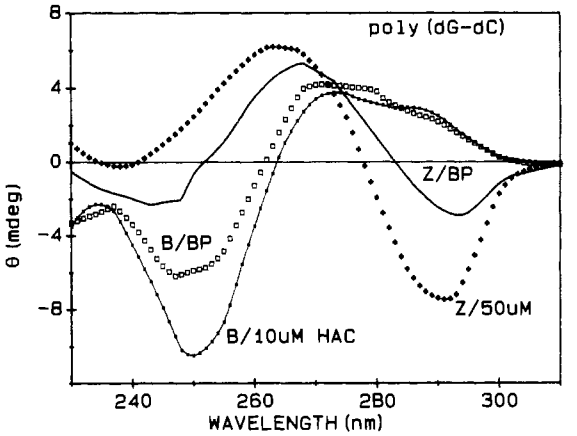

FIGURE 8: Comparison of CD spectra in the DNA region for 20 μM poly(dG-dC)·poly(dG-dC) containing 10 μM HAC (B form) and 50 μM HAC (Z form), 2.6% *anti*-BPDE-modified B-poly(dG-dC)·poly(dG-dC) with 10 μM HAC (B/BP), and 50 μM HAC (Z/BP) added.

solution with the concomitant 4–5 nm spectral red shift from the B condition. Progressive red shift and intensity enhancement are also observed with the NaCl titration (results not shown). The maximum spectral red shift and intensity enhancement are attained before the conversion to the Z form, suggesting that these effects are the consequence of increased ionic strength rather than the gross conformational changes of DNA. These observations are consistent with increased stacking interactions at high salt, most likely the consequence of intercalation.

As is expected, the presence of 50 μM HAC in the unmodified poly(dG-dC)·poly(dG-dC) solution results in a near complete inversion of the CD spectrum in the DNA region (Behe & Felsenfeld, 1982). The consequence of 2.6% *anti*-BPDE modification, however, is to inhibit such Z conversion in 50 μM HAC solution by about 50% as judged from the intensity change at 293 nm (see Figure 8). This is consistent with the intercalative enhancement at high salt and the fact that intercalative binding stabilizes B conformation.

Difference in the Kinetics of BPDE Hydrolysis

Preliminary studies on the BPDE hydrolysis rate in various polynucleotide solutions have also been carried out. The interesting finding is the distinctly different rate dependence exhibited by *anti*- vs. *syn*-BPDE on the polynucleotide composition and sequence in solutions. In contrast to *anti*-BPDE, the hydrolysis rate of *syn*-BPDE is virtually independent of the presence of polynucleotides (for time greater than 20 s). This may have its origin in the more efficient hydrolysis at neutral pH due to the fact that the 9,10-epoxide is hydrogen bonded to the C-7 benzylic hydroxyl.

It is known that the rate of *anti*-BPDE hydrolysis is markedly accelerated by the presence of DNA in the solution (Geacintov et al., 1980). This is confirmed by the appearance of semilog kinetic plots (not shown) for the polynucleotide solutions all curving below the buffer line. It is apparent that multiple processes (such as uncatalyzed and DNA-catalyzed hydrolyses, covalent bond formation, etc.) are involved upon addition of *anti*-BPDE to DNA solutions as evidenced by the curvatures in the plots. These curves, however, are unusual in that slight convexities appear in most cases. The origin of such anomaly most likely is due to dimer formation as a consequence of high [*anti*-BPDE]/[DNA] ratio of 0.25 used in our experiments. Data of detailed kinetic studies supported by other techniques will be presented elsewhere, and thus, only qualitative results are indicated here.

Our evidence indicates that the rate of *anti*-BPDE hydrolysis depends not only on the composition but also on the base sequence of polynucleotide in the solution. For example, it is noteworthy that poly(dA-dT)·poly(dA-dT) enhances hydrolysis more than poly(dA)·poly(dT) and the initial rate of *anti*-BPDE hydrolysis is slightly faster in poly(dG-dC)·poly(dG-dC) than in poly(dG)·poly(dC) solution; the initial hydrolysis rates are faster in the solutions containing G·C polymers than those of A·T polymers. The very slow rate of hydrolysis of *anti*-BPDE in Z DNA solution containing 3.6 M NaCl is also observed which is consistent with the strong salt concentration dependence observed by others (Michaud et al., 1983; Geacintov et al., 1984a).

Discussion

The following simple correlations of spectra of purified adducts from the literature and their potential contributions to our CD spectra of polymers containing such adducts are quite tentative in view of the absence of detailed HPLC analysis of adducts in this work and the sensitivity of CD spectra to possible conformational changes due to adduct formation, although we believe the conformational effect is minor for the G·C polymers as judged by the gross CD spectra and studies by others on denaturation of poly(dG-dC)·poly(dG-dC) upon *anti*-BPDE binding (Kakefuda & Yamamoto, 1978).

The most interesting finding in the present study is the strikingly different stereochemical binding toward *anti*-BPDE as exhibited by the alternating polynucleotide poly(dG-dC)·poly(dG-dC) in contrast to the homopolymer poly(dG)·poly(dC). This is evidenced by the oppositely signed CD spectra in the pyrene spectral region (Figure 4A) as well as distinctive difference spectra in the shorter wavelength region (Figure 5). The positive CD maxima at 280 and 250 nm and the negative maxima at 344 and 328 nm exhibited by *anti*-BPDE-modified poly(dG-dC)·poly(dG-dC) are consistent with spectrum of the predominant adduct in poly(G) (Moore et al., 1977) and natural DNA (Jeffrey et al., 1977) which has been identified as N2–dG trans addition at C10 of (+)-*anti*-BPDE. The difference spectrum below 300 nm for the *anti*-BPDE-modified poly(dG)·poly(dC) does not appear to conform with any reported CD on diastereomeric *anti*-BPDE–guanosine adducts (Moore et al., 1977), suggesting combined contribution from more than one stereomeric adduct. Indeed, the positive 348-nm band (Figure 4A) most likely is due to contribution from the cis addition at C10 of (+)-*anti*-BPDE by N2–dG. Although the positive 348-nm CD is also in line with the trans addition of the (−) enantiomer, the overwhelming preference for the (+) isomer at the guanine sites of native DNA (Pulkrabek et al., 1979; Osborne et al., 1981; Meehan & Straub, 1979) makes this explanation less likely. The appearance of a negative maximum at 328 nm and that of a positive maximum around 250 nm probably also indicate significant contribution from the trans addition to (+)-*anti*-BPDE.

The absence of significant (−) enantiomer contribution to the poly(dG)·poly(dC) binding has been confirmed by our recent studies using optical isomers (results not shown). This is evidenced by the low level modification and weak Cotton effects resulting from the (−)-*anti*-BPDE binding as well as the fact that spectral features exhibited by the (+)-*anti*-BPDE lesion are almost identical with those of racemic modification presented here. The enantiomeric results will be detailed elsewhere.

The absence of detectable pyrenyl CD spectra for the *anti*-BPDE-modified poly(dA-dT)·poly(dA-dT) and poly(dA)·poly(dT) is also consistent with the lack of stereoselectivity at the dA sites of natural DNA toward *anti*-BPDE (Pulkrabek et al., 1979; Meehan & Straub, 1979). Although the extent of *anti*-BPDE modification of poly(G) is greater than that of poly(dG-dC)·poly(dG-dC) (see Table I), much weaker CD is detected in the pyrene spectral region, suggesting reduced stereoselectivity. This is consistent with the observation that diastereomers are formed in approximately equal amounts in the poly(G) system (Meehan et al., 1977).

It is interesting to note that the lower *anti*-BPDE modification of poly(dA-dT)·poly(dA-dT) as compared to poly(dA)·poly(dT) is consistent with the dominant intercalative physical binding of the former toward BPDE model compounds (Chen, 1983, 1984; Yang et al., 1983) and the slightly accelerated rate of hydrolysis of these sites, thus giving credence to our earlier speculation on the effect of intercalation at the A·T sites on the *anti*-BPDE detoxification. The significant modification of poly(dA)·poly(dT) at the dA sites may be the consequence of the slower hydrolysis rate of *anti*-BPDE in this polynucleotide solution due to nonintercalation. It is to be noted, however, others studying with native DNA (Geacintov et al., 1984a; Meehan & Bond, 1984) have shown that hydrolysis and covalent binding exhibit parallel pseudo-first-order reactions and the rates of hydrolysis are not important in determining the levels of covalent binding. It may be that the kinetic behavior of hydrolysis and covalent binding at the dG sites is quite different from that of dA sites. Further studies are needed to sort out these questions.

Extensive studies on the binding of benzo[*a*]pyrene metabolite model compounds to natural DNA have led Geacintov et al. (1982) to classify the covalent adducts according to type I (intercalative-like) and type II (external). Such classification has been used by Undeman et al. (1983) to characterize the adducts derived from DNA reaction with *anti*- and *syn*-BPDE. Type II exhibits characteristic absorption and fluorescence excitation maxima at 316, 330, and 345 nm while those of site I are about 7-nm red shifted. In *anti*-BPDE–DNA, the type II component dominates and the type I component amounts to less than 10%. In *syn*-BPDE–DNA, approximately 35% of the adduct is of type II and approximately 65% of type I. Upon denaturation, both *anti*-BPDE–DNA and *syn*-BPDE–DNA give a single-stranded complex (Type III) with light absorption and fluorescence excitation maxima at 316, 332, and 351 nm. The chromophores are probably sandwiched between the DNA bases.

Our studies indicate a predominant type II binding of *anti*-BPDE for all polynucleotides although the adducts for the G·C-containing polymers exhibit a 2–3-nm red shift from the A·T-containing polynucleotides. The lack of observable CD spectra for the covalent adducts derived from the A·T polymers is consistent with the fact that these adducts are different from those of site II observed in G·C-rich polymers. The covalent modifications by *syn*-BPDE, however, are distinctly different with the G·C polymers now exhibiting important contribution from type I as evidenced by the appearance of the 352-nm maxima, but the A·T polynucleotides are still predominantly of type II binding. Significant site I contribution observed in *syn*-BPDE-modified DNA mentioned earlier is thus consistent with its guanine specificity. Both *anti*-BPDE and *syn*-BPDE bind to poly(G) as mixture of types I (or III) and II, with only the type I (or III) of *anti*-BPDE binding showing significant pyrenyl CD. The appearance of the 350–352-nm band (8–10-nm red shift from the corresponding tetraols) suggest that the interaction between the pyrene moiety and the guanine base is stronger in poly(G) than in the guanine-containing duplex polynucleotides (only 2–3-nm

shift) for the *anti*-BPDE adducts. Various studies have indicated that both the aromatic rings (MacLeod et al., 1982; Gagliano et al., 1982; Geacintov et al., 1982) and the hydroxyl groups in *anti*-BPDE (Kinoshita et al., 1982) can influence the stereochemistry of its reaction with DNA. Our work presented here further indicates that the base sequence can also play an important role.

Another interesting observation is that the hexaamminecobalt-induced Z-form poly(dG-dC)·poly(dG-dC) suffers significant modification by both *anti*-BPDE and *syn*-BPDE. The reduced modification on the Z form as compared to the B form may be the consequence of the fact that now the reactive 2-amino group of guanine is residing in the deep groove (Wang et al., 1979), thus making it slightly less accessible for the ligand attack and/or reduced reactivity as a result of increased ionic strength due to the presence of 50 μM HAC (Michaud et al., 1983; Geacintov et al., 1984a). The pyrenyl spectral characteristics, however, are very similar to those of poly(dA)·poly(dT) but not to those of B-form poly(dG-dC)·poly(dG-dC), suggesting nonintercalative binding as little interactions with the bases are apparent. A significant amount of lesion to phosphate groups of buffer (Koreeda et al., 1976) and DNA backbone (Gamper et al., 1977) in both poly(dA)·poly(dT) and Z DNA solutions may be the culprit for such similarity since their primary and secondary structures are entirely different. If binding to the buffer phosphate (10 mM) is indeed important, it will also help explain the unusually high level of modification suffered by the two A·T polymers, the exhibited external binding characteristics, and their lack of detectable CD. The lower level of modification on poly(dA-dT)·poly(dA-dT) when compared to its homopolymer may thus be rationalized on the intercalative ability of the alternating polymer in reducing the phosphate encounters by the *anti*-BPDE molecules.

A rather unexpected result on salt titration of *anti*-BPDE-modified poly(dG-dC)·poly(dG-dC) is that the "externally" bound *anti*-BPDE appears to become intercalated at high-salt condition as evidenced by 4–5-nm red shifts, most likely to avoid the high concentration of cation clustering around the DNA phosphate backbones. This suggests that the energy barrier between the intercalative and external sites may not be as high as one assumes and renders the covalent binding model at the intercalative site (Meehan & Straub, 1979) with subsequent out-sliding of pyrenyl moiety to be quite plausible.

Since the completion of this work, Geacintov et al. (1984b,c) have published linear dichroism (LD) results on *anti*-BPDE enantiomer adducts studies with calf thymus DNA. The LD and absorption spectra of covalent DNA complexes have been interpreted in terms of a superposition of two types of binding sites (I and II). Site II adducts are dominant (~90%) in the covalent complexes derived from the (+) enantiomer but account for only about 50% of the adducts in the case of the (−) enantiomer. The orientation of site II complexes is different by about 20° in the adducts derived from the binding of the (+) and the (−) enantiomers to DNA, the long axis of the pyrene chromophore being oriented more parallel to the axis of the DNA helix in the case of (+) enantiomer. Comparison with the product analysis of Brookes & Osborne (1982) led to the suggestion of possible identification of sites I with O6–dG and N6–dA adducts. The absence of 352-nm absorbance and CD maxima in the covalent complexes of *anti*-BPDE and the two A·T polymers in our studies may thus suggest that either the N6–dA adduct does not contribute to site I formation of the binding of *anti*-BPDE to adenine in these two polymers is very different from the random-sequence native DNA. The interpretation, however, is complicated by the possible dominant contribution of buffer phosphate binding as mentioned earlier.

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Registry No. (±)-*anti*-BPDE, 58917-67-2; poly(G), 25191-14-4; poly(dG-dC)·poly(dG-dC), 36786-90-0; poly(dG)·poly(dC), 25512-84-9; poly(dA)·poly(dT), 24939-09-1; poly(dA-dT)·poly(dA-dT), 26966-61-0; poly(dA-dC)·poly(dG-dT), 55684-99-6; (±)-*syn*-BPDE, 58917-91-2.

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Secondary Structure of a 345-Base RNA Fragment Covering the S8/S15 Protein Binding Domain of *Escherichia coli* 16S Ribosomal RNA†

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ABSTRACT: A technique for isolating defined fragments of a large RNA has been developed and applied to a ribosomal RNA. A section of the *Escherichia coli rrn*B cistron corresponding to the S8/S15 protein binding domain of 16S ribosomal RNA was cloned into a single-stranded DNA phage; after hybridization of the phage DNA with 16S RNA and digestion with T_1 ribonuclease, the protected RNA was separated from the DNA under denaturing conditions to yield a 345-base RNA fragment with unique ends (bases 525–869 in the 16S sequence). The secondary structure of this fragment was determined by mapping the cleavage sites of enzymes specific for single-stranded or double-helical RNA. The fragment structure is almost identical with that proposed for the corresponding region of intact 16S RNA on the basis of phylogenetic comparisons [Woese, C. R., Gutell, R., Gupta, R., & Noller, H. (1983) *Microbiol. Rev. 47*, 621–669]. We conclude that this section of RNA constitutes an independently folding domain that may be studied in isolation from the rest of the 16S RNA. The structure mapping experiments have indicated several interesting features in the RNA structure. (i) The largest bulge loop in the molecule (20 bases) contains specific tertiary structure. (ii) A region of long-range secondary structure, pairing bases about 200 residues apart in the sequence, can hydrogen bond in two different mutually exclusive schemes. Both appear to exist simultaneously in the RNA fragment under our conditions. (iii) The long-range secondary structure and one adjacent helix melt between 37 and 60 °C in the absence of Mg^{2+}, while the rest of the structure is quite stable.

Much of our understanding of RNA secondary and tertiary structure comes from extensive physical studies of tRNA. Most other cellular RNAs are at least an order of magnitude larger and have been much less amenable to structural studies. However, in the last few years an abundance of sequence information has made it feasible to search for RNA secondary structures by comparing sequences of the same RNA specie from different organisms: as the sequence varies, pairs of compensating base changes are observed, which preserve the base pairing of some potential helices (Noller, 1980; Noller & Woese, 1981). This "phylogenetic" approach has been extensively applied to ribosomal RNAs, and there is now substantial agreement on the secondary structures of these molecules (Woese et al., 1983; Maly & Brimacombe, 1983).

The availability of plausible secondary structures has been a major advance in the study of large RNA molecules; however, detailed studies of tertiary structures, conformational transitions, and protein–RNA interactions in RNAs of >1000 bases are still intimidating to contemplate. We have considered the possibility that large RNA molecules are organized into independently folding domains which may be studied individually. For the best studied of the larger RNAs, the *Escherichia coli* 16S ribosomal RNA, it has been suggested that the RNA is organized into three major structural domains that each bind specific sets of the ribosomal proteins and fold (to a first approximation) independently (Zimmermann, 1974). Many fragments of ribosomal RNAs do retain their ability

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