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REACTIVITY AND BINDING OF BENZO(*a*)PYRENE DIOL EPOXIDE TO POLY(dG-dC)·(dG-dC) AND POLY(dG-m^{5}dC)·(dG-m^{5}dC) IN THE B AND Z FORMS

Khadija MOUSSAOUI [a], Nicholas E. GEACINTOV [a] and Ronald G. HARVEY [b]

[a] *Chemistry Department and Radiation and Solid State Laboratory, New York University, New York, NY 10003 and*
[b] *Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, IL 60637, U.S.A.*

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The physical and covalent binding of the carcinogen benzo(*a*)pyrene-7,8-diol-9,10-oxide (BaPDE) to poly(dG-dC)·(dG-dC) and poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B and Z forms were studied utilizing absorbance, fluorescence and linear dichroism techniques. In the case of poly(dG-dC)·(dG-dC) the decrease in the covalent binding of BaPDE with increasing NaCl concentration (0.1–4 M) as the B form is transformed to the Z form is attributed to the effects of high ionic strengths on the reactivity and physical binding of BaPDE to the polynucleotides; these effects tend to obscure differences in reactivities with the B and Z forms of the nucleic acids. In the case of poly(dG-m^{5}dC)·(dG-m^{5}dC) the B-to-Z transition is induced at low ionic strength (2 mM NaCl + 10 μM $Co(NH_3)_6Cl_3$) and the covalent binding is found to be 2–3-times lower to the Z form than to the B form. Physical binding of BaPDE by intercalation, which precedes the covalent binding reaction, is significantly lower in the Z form than in the B form, thus accounting, in part, for the lower covalent binding. The linear dichroism characteristics of BaPDE covalently bound to the Z and B forms of poly(dG-m^{5}dC)·(dG-m^{5}dC) are consistent with nonintercalative, probably external conformations of the aromatic pyrenyl residues.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) can be metabolically activated to powerful mutagens and carcinogens [1]. The ultimate biologically active forms of PAH compounds are the diol epoxide derivatives [2–4] which bind noncovalently and covalently to nucleic acids. The covalent binding of these reactive intermediates to nucleic acids is widely believed to be an important and critical event in mutation and in the initiation stages of tumorigenesis [1–3,5,6].

The mechanisms of reaction of PAH diol epoxides with DNA, the conformations and properties of the covalent adducts formed, and the relationships between these factors and the biological activities of these metabolites are subjects of intense current interest. The ultimate tumorigenic and mutagenic metabolite of the PAH compound benzo(*a*)pyrene is 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (BaPDE) and its reactions with DNA in vivo, as well as in model systems, have been extensively studied. It is known that BaPDE binds physically to double-stranded DNA, most likely by an intercalation mechanism [7,8], prior to reacting covalently with the nucleic acid bases. This covalent reaction occurs at the 10-position of BaPDE (fig. 1 inset) and preferentially involves the exocyclic amino group of guanine [4].

Sequence-dependent effects on the conformations and dynamic properties of DNA may be important factors in determining the reactivities of chemical carcinogens with nucleic acids. Since guanine is the primary target for BaPDE, studies of the interactions of this diol epoxide with the synthetic polynucleotides poly(dG-dC)·(dG-dC) and the 5-methylcytosine derivative poly(dG-

$m^5dC)\cdot(dG\text{-}m^5dC)$ are of interest. The influence of conformational effects on the reactivities can be investigated, since these polynucleotides are known to undergo salt concentration-dependent conformational changes from the B form (right-handed helix) to the Z form (left-handed helix) [9–13]. The existence of segments of left-handed helices in living cells has been demonstrated [14,15], and these phenomena may thus be important in vivo.

The possibility of studying the interactions of drugs and carcinogens with these nucleic acids in different conformations is receiving increasing attention. Intercalation of ethidium bromide [13,16], proflavine [16], actinomycin [16], daunomycin [17], and adriamycin [18] inhibits the B-to-Z transition. A similar effect is observed upon the covalent binding of the carcinogens aflatoxin B1 [19] and the *O*-acetyl derivative of 4-hydroxylaminoquinoline 1-oxide to poly(dG-dC)·(dG-dC) [20]. Sage and Leng [21] and Santella and co-workers [22,23] reported that the covalent binding of *N*-acetoxy-*N*-acetyl-2-aminofluorene (AAAF) favors a conformational change from the B form to the Z form, or to a Z-like form. The reactivities of some carcinogens with either the B or Z forms have also been investigated. Spodheim-Maurizot et al. [24] find that the B form is about twice as reactive as the Z form with respect to covalent binding with AAAF. A detailed investigation by Rio and Leng [25], however, indicates that the reactivity of this particular carcinogen does not depend on the conformation of the polymers; on the other hand, a related derivative, *N*-hydroxy-2-aminofluorene (N-OH-AF), binds covalently more extensively to poly(dG-dC)·(dG-dC) and $poly(dG\text{-}m^5dC)\cdot(dG\text{-}m^5dC)$ in the B forms than in the Z forms. Rio and Leng conclude that this preference of N-OH-AF for binding to these polynucleotides in the B forms is related to a lower efficiency of physical binding of this carcinogen to the Z forms of these polymers, assuming that the formation of such physical complexes precedes covalent binding.

In this work we have investigated the noncovalent and covalent binding of the PAH diol epoxide BaPDE to poly(dG-dC)·(dG-dC) and $poly(dG\text{-}m^5dC)\cdot(dG\text{-}m^5dC)$ in the B and Z forms utilizing absorbance, circular dichroism (CD) and linear dichroism (LD) spectroscopy techniques. In the case of poly(dG-dC)·(dG-dC), which is normally in the B form at low salt concentrations, the Z form was induced by increasing the NaCl concentration up to 4 M. In the case of $poly(dG\text{-}m^5dC)\cdot(dG\text{-}m^5dC)$ the B-to-Z transformation was induced at relatively low ionic strengths by utilizing $Co(NH_3)_6Cl_3$ [10,11,26].

2. Materials and methods

Racemic BaPDE was synthesized according to methods previously described [27]. Calf thymus DNA (38–40% hyperchromicity) was purchased from Worthington Chemicals (Freehold, NJ) and was treated as described previously [28]. The synthetic polynucleotides poly(dG-dC)·(dG-dC) and $poly(dG\text{-}m^5dC)\cdot(dG\text{-}m^5dC)$ were purchased from P&L Biochemicals (Milwaukee, WI). Some batches were treated with S_1 nuclease, proteinase K, and phenol extraction, followed by exhaustive dialysis against 5 mM sodium cacodylate buffer containing 50 μM EDTA; however, no differences in the CD, or relative absorbances A_{250}/A_{260} and A_{280}/A_{260} between treated and untreated batches were noted, and were in agreement with the standard published values [10,29]. The concentrations of the polynucleotides (expressed in terms of an equivalent concentration of nucleotides) were determined from the absorption maxima at 256 nm utilizing the following values of molar extinction coefficients: 8.4×10^3 M^{-1} cm^{-1} (poly(dG-dC)·(dG-dC)) and 7.0×10^3 M^{-1} cm^{-1} ($poly(dG\text{-}m^5dC)\cdot(dG\text{-}m^5dC)$).

Absorption spectra were determined utilizing a Perkin-Elmer 320 spectrophotometer, while the CD spectra were measured utilizing a Cary 60 spectrometer. Fluorescence measurements were made with a Spex Fluorolog spectrophotometer (Spex Industries, Metuchen, NJ). The electric LD spectra of the covalent adducts were determined with a specially constructed system [30] according to known principles [31]; the flow dichroism of the noncovalent adducts were determined utilizing a Couette flow cell [32], which is particularly suitable for measuring the LD spectra of nucleic acid solutions containing unstable PAH diol epoxides [7].

A stock solution of BaPDE in tetrahydrofuran was prepared. Small aliquots of this stock solution were added to the aqueous solutions (5 mM sodium cacodylate buffer and 50 μM EDTA) containing DNA or polynucleotides; the final concentration of tetrahydrofuran did not exceed 0.8%.

A number of experimental criteria are utilized to chacterize the B and Z forms of poly(dG-dC)·(dG-dC) and related polymers [12]. We have utilized for this purpose the ellipticity at 292 nm and the overall CD spectra, as well as absorption spectra, to establish whether these polynucleotides were in the B or Z forms. The kinetics of these transitions were followed by monitoring either the ellipticity at 292 nm, or the absorbance at 295 nm [16]. As reported first by Pohl and Jovin [9], the midpoint of the B-to-Z transition in poly(dG-dC)·(dG-dC) was found at approx. 2.5 M NaCl, as the salt concentration was increased from 0.1 to 4 M. In the case of poly(dG-m^{5}dC)·(dG-m^{5}dC) the Z form was induced by adding 10 μM $Co(NH_3)_6Cl_3$ [10] to a solution containing either 2 or 20 mM NaCl, and 5 mM sodium cacodylate buffer.

The rate of reaction of BaPDE in polynucleotide solutions was followed by utilizing a fluorescence method as previously described [28]. As the diol epoxides are converted to fluorescent tetraols (tetrahydrotetrahydroxybenzo(a)pyrene), the fluorescence yield $F(t)$ increases according to the expression $(1-\exp[-kt])$, where k is the pseudo-first-order rate constant of the reaction. When the reaction is complete the fluorescence yield approaches a constant value.

The covalent adducts were prepared by adding BaPDE to the polynucleotide or DNA solutions and allowing the reactions to go to completion at 23°C. Particularly at high salt concentrations, the reaction was carried out for approx. 12 h in order to allow the BaPDE crystals to redissolve, since in some reactions at high NaCl concentrations, the solubility of the diol epoxide is decreased (see below). The tetraols were then extracted 12 times with ether or, in some cases, by exhaustive dialysis against buffer. The level of covalent binding was estimated spectrophotometrically by measuring the absorbance of the pyrene residue in the 345–350 nm region [33]. The extent of covalent modification of the biopolymers is characterized by the ratio f_{cov}, which is equal to (mol BaPDE bound covalently)/(mol BaPDE initially added) [34].

3. Results and discussion

3.1. Binding to B and Z forms of poly(dG-dC)·(dG-dC)

In order to compare the levels of covalent binding of BaPDE to the B and Z forms of poly(dG-dC)·(dG-dC), it was first necessary to determine the relative solubilities of BaPDE at the different salt concentrations employed. At low ionic strengths (< 0.01) the solubility of BaPDE in aqueous solutions is only 10 μM [35]; however, as the NaCl concentration is increased, the solubility (as monitored by the absorbance of the diol epoxide at 344 nm) gradually decreases and is only 0.7 μM at 4 M salt concentration. In order to

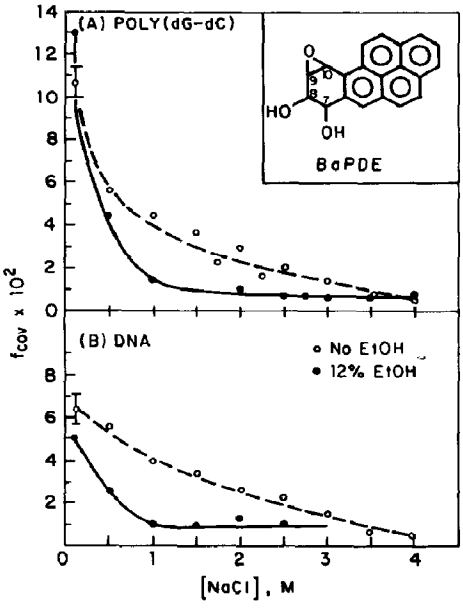

Fig. 1. Fraction of BaPDE molecules (f_{cov}) which bind covalently to (A) poly(dG-dC)·(dG-dC) and (B) calf thymus DNA as a function of NaCl concentration in the (●) presence and (○) absence of ethanol. Initial BaPDE concentration: 8.5×10^{-6} M, 0.1 mM nucleic acids, 5 mM sodium cacodylate buffer, pH 7.1, 23°C.

determine whether variations in the solubilities of BaPDE at different salt concentrations give rise to a distortion in the levels of covalent binding, the reactions were also performed in 15% ethanol. Under these conditions the amount of BaPDE (10 μM) dissolved in the solutions did not vary with the salt concentration. The addition of ethanol does not interfere, and in fact promotes the B-to-Z conversion [36]. A recent report suggests that water/ethanol mixtures are particularly suitable for the studies of complexation of ligands to the B and Z forms of poly(dG-dC)·(dG-dC) [37]. However, it should be kept in mind that PAH compounds in general [38], and BaPDE in particular [39], form much weaker physical association complexes with DNA than ionic ligands, and thus tend to dissociate in aqueous alcohol mixtures.

The levels of covalent binding f_{cov} for poly(dG-dC)·(dG-dC) and for calf thymus DNA as a function of the NaCl concentration are shown in fig. 1. Results are shown for both 12% ethanol and ethanol-free solutions. In these ethanol solutions the BaPDE reactions are completed within 1 h or less; the reactions were allowed to proceed to completion in all cases. In the presence of ethanol the decrease in f_{cov} is much sharper with increasing salt concentration than in the absence of alcohol. The level of covalent binding is also generally lower, even though the solubility of BaPDE is higher in the presence of alcohol. In alcohol-free solutions, the solubility of BaPDE is only slightly increased by the presence of the small amounts of nucleic acids present in our experiments at all salt concentrations tested (up to 4 M) (results not shown). At salt concentrations above 2.5 M the level of covalent modification is nearly the same in the absence as in the presence of 12% ethanol. Thus, the limited solubility of BPDE under these conditions does not seem to be an important factor in determining the levels of covalent binding. Presumably, the microcrystals of BPDE which are formed in the absence of ethanol at the high salt concentrations gradually redissolve during the 12 h reaction time employed in these particular experiments, as the concentration of BaPDE in solution is reduced by reaction.

The dependence of f_{cov} on the NaCl concentration is similar for calf thymus DNA and for poly(dG-dC)·(dG-dC) even though the latter undergoes the B-to-Z transition, while natural DNA does not. Unmodified poly(dG-dC)·(dG-dC) exhibits a sigmoidally shaped B-to-Z transition curve with a midpoint near 2.5 M as the NaCl concentration is increased. This sigmoidal curve is an indication of the cooperativity of the transition in which the B-to-Z equilibrium is increasingly shifted towards the left-handed form as the salt concentration is increased. The level of covalent binding, however, decreases smoothly as the salt concentration is increased. There is no discontinuity in f_{cov} in the 2–3 M NaCl concentration range in which the equilibrium shifts sharply from the B to the Z form. In fact, the behavior of f_{cov} as a function of salt concentration is similar for poly(dG-dC)·(dG-dC) and calf thymus DNA, in which the B-to-Z transition does not occur.

Both the formation of noncovalent complexes between BaPDE and DNA, as well as the reaction rate of the diol epoxide are reduced at high salt concentrations [34,38,40–43]. The lower yield of covalent products at the higher salt concentrations

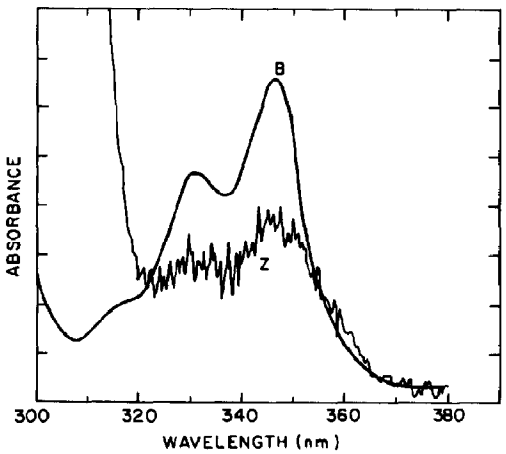

Fig. 2. Absorption spectra of covalent adducts obtained by the reaction of BaPDE with poly(dG-dC)·(dG-dC). B form: 0.1 M NaCl, 0.1 mM nucleotides, absorbance shown at 346 nm: 0.028 (f_{cov} = 0.11). Z form at 4 M NaCl (0.28 mM nucleotides), absorbance shown at 346 nm; 0.0035 (f_{cov} = 0.007). Other conditions as in legend to fig. 1. (In order to obtain the level of covalent binding shown for the Z form, two successive additions of BaPDE, 8.5×10^{-6} M each, were required.)

can thus be attributed to these salt-induced effects rather than to effects of the different conformations of poly(dG-dC)·(dG-dC).

The absorption spectra of the covalent adducts obtained with the B and Z forms of poly(dG-dC)·(dG-dC) are shown in fig. 2. In the case of the B form, the usual pyrene-like absorption spectrum with maxima at approx. 331 and 346 nm is observed. While the signal/noise ratio for the covalent BPDE-Z poly(dG-dC)·(dG-dC) adducts is considerably lower, the absorption spectrum appears to be similar to that obtained with the B form.

3.2. Binding to B and Z forms of poly(dG-m^{5}dC)·(dG-m^{5}dC)

The strong effect of high salt concentrations on the reactivities of BaPDE with poly(dG-dC)·(dG-dC) suggests that this is an unsuitable system for investigating effects of DNA conformation on reactivities with carcinogens such as BaPDE. Poly(dG-m^{5}dC)·(dG-m^{5}dC) undergoes the B-to-Z transition at much lower salt concentrations. The transition midpoints occur either at 0.7 M NaCl or 0.6 mM $MgCl_2$ (in the presence of 50 mM NaCl); the transition can be induced at even lower ionic strength in the presence of cobalt hexamine complex ions [10].

The levels of covalent binding of BaPDE in the B (0.1 M NaCl) and Z forms (50 mM NaCl, 2 mM $MgCl_2$) are compared in fig. 3. In the case of the B form the absorption spectrum displays the usual maxima at 332 and 346 nm, which is similar to the absorption spectra of covalent adducts obtained with calf thymus DNA [30]. In the case of the Z form, the level of covalent binding is nearly 5-times smaller. The absorption spectrum is structureless which is usually an indication of a physical heterogeneity of binding sites. If the Z form is induced by increasing the salt concentration to 4 M NaCl rather than by adding $MgCl_2$, f_{cov} is reduced still further, to about 0.03 (table 1).

The level of covalent binding of BaPDE is higher in the case of poly(dG-m^{5}dC)·(dG-m^{5}dC) than with poly(dG-dC)·(dG-dC) at all of the different salt concentrations investigated (table 1).

The absorption spectra of the covalent adducts

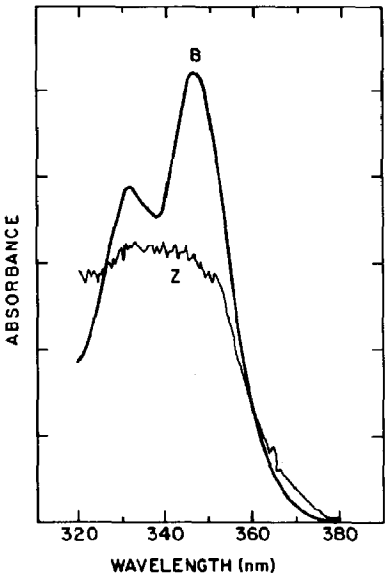

Fig. 3. Absorption spectra of covalent adducts obtained by reacting BaPDE with poly(dG-m^{5}dC)·(dG-m^{5}dC), 0.1 mM nucleotide concentration. B form: 0.1 M NaCl, absorbance shown at 346 nm; 0.044 (f_{cov} = 0.18). Z form: 50 mM NaCl + 2 mM $MgCl_2$, absorbance shown at 346 nm: 0.011 (f_{cov} = 0.04). Other reaction conditions as in legend to fig. 1; no ethanol.

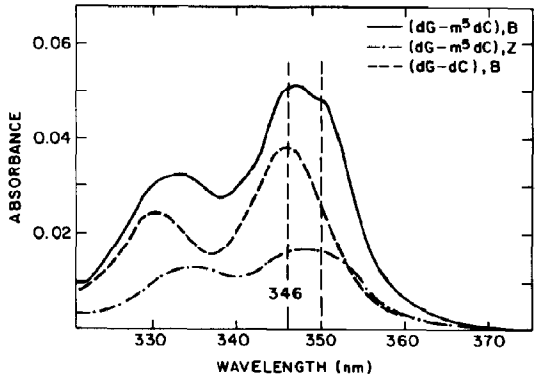

Fig. 4. Quantitative comparison of spectra of covalent adducts obtained by reacting BaPDE with poly(dG-dC)·(dG-dC) (— — —) and poly(dG-m^{5}dC)·(dG-m^{5}dC) (————), both in 20 mM NaCl and in the B forms, and poly(dG-m^{5}dC)·(dG-m^{5}dC) in the Z form (–·–·–) in 20 mM NaCl + 10^{-5} M $Co(NH_3)_6Cl_3$. All nucleotide concentrations, 0.15 mM; initial BaPDE concentrations, 8.5×10^{-6} M; all other conditions as in legend to fig. 1 (no ethanol).

Table 1

Summary of effects of salt concentrations on covalent binding expressed in terms of f_{cov} (BaPDE molecules bound)/(total BaPDE molecules present at the beginning of the reaction)

All error bars (except for the two smallest values of f_{cov}): ±0.01.

[NaCl]	Other ions	DNA	Poly(dG-dC)	Poly(dG-m^{5}dC)
2 mM	–	0.08 (B)	0.15 (B)	0.20 (B)
2 mM	10 μM $Co(NH_3)_6^{3+}$	0.08 (B)	–	0.08 (Z)
20 mM	–	0.08 (B)	0.17 (B)	0.20 (B)
50 mM	2 mM $MgCl_2$	–	–	0.04 (Z)
100 mM	–	0.06 (B)	0.11 (B)	0.18 (B)
4 M	–	< 0.01 (B)	< 0.01 (Z)	0.03 (Z)

obtained upon reacting BaPDE with poly(dG-dC)·(dG-dC) and poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B form, and poly(dG-m^{5}dC)·(dG-m^{5}dC) in the Z form induced by cobalt hexamine ions at low ionic strengths, are shown in fig. 4. The CD and absorption spectra of the same poly(dG-m^{5}dC)·(dG-m^{5}dC) preparations just before the additions of BaPDE are shown in fig. 5, confirming that the polynucleotides were either in the B or Z forms. After the covalent binding reactions and after extraction of the noncovalently bound tetraols,

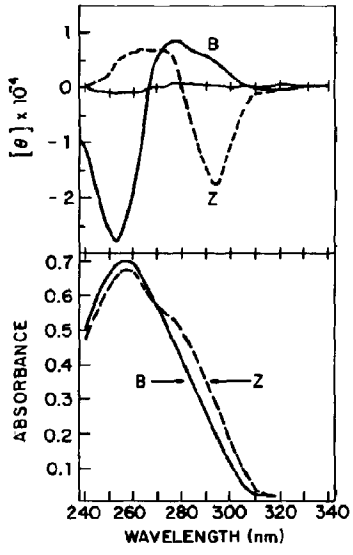

Fig. 5. Typical CD (top) and absorption spectra (bottom) of 0.1 mM poly(dG-m^{5}dC)·(dG-m^{5}dC) samples in the B and Z forms before adding BaPDE; other conditions same as in the legend to fig. 4.

only minor changes in the CD spectra were detected; while the CD spectra were qualitatively similar before and after reaction, a uniform shift of these spectra (~ 25%) with respect to the baselines in the positive direction were noted after the reactions (results not shown).

There are significant differences in the levels of binding and in the absorption spectra of BaPDE bound covalently to poly(dG-dC)·(dG-dC) and its methylated derivative in the B forms. The value of f_{cov} is 15% in the case of poly(dG-dC)·(dG-dC) and is approx. 20% in the case of poly(dG-m^{5}dC)·(dG-m^{5}dC). Furthermore, in the latter case, the absorption maxima are red-shifted (336 and 348 nm), and a shoulder is apparent at 350–352 nm. These differences in the levels of covalent binding can be attributed to the presence of the methyl group at the 5-position of cytosine since, otherwise, the structures of the two polymers are very similar [15].

In contrast to the results obtained with the Z form of poly(dG-dC)·(dG-dC) at high NaCl concentrations, and poly(dG-m^{5}dC)·(dG-m^{5}dC) in 2 mM $MgCl_2$ + 50 mM NaCl, a structured pyrenyl absorption band is obtained in the case of the binding of BaPDE to the Z form of poly(dG-m^{5}dC)·(dG-m^{5}dC) at low ionic strengths (fig. 4). The value of f_{cov} (0.08 ± 0.01, for the Z form induced by cobalt complex ions) is only 2–3-times smaller in the case of the Z form than the B form under identical conditions (table 1). In order to verify that these differences in covalent binding are not due to the presence of cobalt ions, the same binding experiments were performed with calf thymus DNA at 2 mM NaCl with and without

10 μM $Co(NH_3)_6Cl_3$; it was found that f_{cov} is approx. 0.08 under these conditions and independent of the presence or absence of the polyvalent metal ions (table 1).

3.3. LD and conformations of covalent adducts

In LD techniques the DNA can be oriented either in a flow gradient or by the application of electric field pulses to the DNA solutions. The average orientations of the transition moments of the carcinogens bound to the polymers relative to those of the nucleic acid bases can be probed utilizing polarized light. Experimentally, the LD depends on the wavelength (λ) and is defined by:

$$\Delta A(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda) \quad (1)$$

where $A_{\parallel}(\lambda)$ and $A_{\perp}(\lambda)$ are the respective absorbances of the sample measured with the polarization vector of the light beam oriented parallel or perpendicular with respect to the direction of the applied electric field, or flow direction. For a single oriented chromophore, the $\Delta A(\lambda)$ signal corresponding to one particular electronic transition has the same shape as the absorption band, but can be either positive or negative in sign. When more than one absorption band with differently oriented transition moments overlap one another, $\Delta A(\lambda)$ can exhibit more complex behavior [30,44].

In this work we have utilized LD techniques as a qualitative tool for probing the conformations of the pyrenyl chromophore relative to the orientation of the DNA bases. Since the transition moments of the nucleic acids lie within the planes of the bases, and since the axis of the helix tends to align itself parallel to either the applied electric field or flow directions, $\Delta A(\lambda)$ is negative in sign within the nucleic acid absorption band below 300 nm. The 310–360 nm transition moment of the pyrenyl ring system is oriented within the plane and along the long axis of the pyrenyl chromophore. Consequently, intercalation-like conformations of this aromatic residue in which the plane of the pyrene ring system tends to be parallel to the planes of the nucleic acid bases, also give rise to a negative $\Delta A(\lambda)$ signal in the 310–360 nm region. External conformations in which the pyrenyl long axis is oriented closer to the axis of the helix tend to give rise to positive LD spectra [30].

The LD spectra of covalent adducts derived from the covalent binding of BaPDE to the B and Z forms of poly(dG-m^{5}dC)·(dG-m^{5}dC) for samples oriented by electric field pulses are displayed in fig. 6. Analogous results are obtained in the case of flow orientation (results not shown). The $\Delta A(\lambda)$ spectra for both samples are positive in sign and resemble the absorption spectra (fig. 4) in approx. 320–360 nm region. Below 310 nm, the negative ΔA signal due to the nucleic acid bases dominates the LD.

The LD spectra of the adducts can be interpreted in terms of the two different classes of binding sites previously identified for adducts obtained when different metabolite model compounds derived from benzo(*a*)pyrene or benzo(*e*)pyrene bind to DNA [44]. Site I is characterized by a negative LD spectrum and a 10–12 nm shift in the absorption maxima of the pyrene ring system, and the

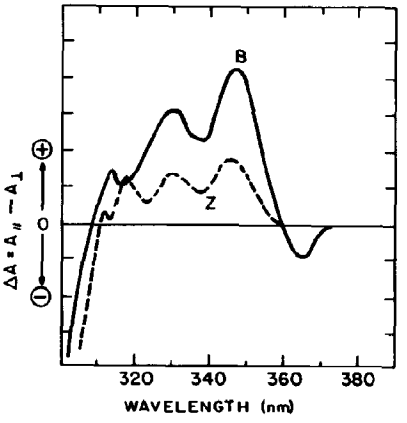

Fig. 6. Electric LD spectra of covalent adducts obtained by reacting BaPDE with poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B and Z forms (the latter induced by 10^{-5} M $Co(NH_3)_6Cl_3$) in 2 mM NaCl, 0.1 mM nucleotide concentrations; other reaction conditions as in legend to fig. 1 (no ethanol). Conditions of measurements of LD spectra: 0°C; applied electric field, 1800 V cm^{-1}; pulse duration, 1.5 ms. Absorbances of samples at 346 nm: 0.047 (B), 0.040(Z), prepared as described in legend to fig. 4 (two additions of 8.5×10^{-6} M BaPDE). Value of f_{cov}: 0.19 (B form), 0.080 (Z form).

absorption maximum is located at 352–354 nm. These characteristics are consistent with a conformation in which the pyrenyl chromophore is at least partially stacked with the nucleic acid bases. Site II is characterized by a positive LD spectrum and only a small (2–3 nm) red shift in the absorption bands, and the maximum occurs at 346 nm. These properties suggest an external type of conformation in which the pyrene ring system is not oriented parallel to the bases.

The LD spectrum of BaPDE covalently bound to poly(dG-dC)·(dG-dC) adduct (B form) is positive in sign throughout the absorption band of the pyrene ring system (310–370 nm); both the absorption and ΔA maxima occur at 346 nm. Similar results are obtained with calf thymus DNA [30,44] and these adducts are of the site II type. The LD of the covalent adducts obtained with poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B form is also mostly positive in sign and is thus of the site II type; however, a small negative contribution is also observed near approx. 360 nm which indicates that some site I adducts are also present (fig. 6). The presence of such site I adducts can also be discerned in the absorption spectra of the adducts (fig. 4), since a shoulder appears in the absorption spectra at 350–354 nm in the case of the dG-m^{5}dC adduct (which is not observed in the case of the dG-dC polymer). The absorption and LD spectra are thus qualitatively in agreement with one another and a more detailed analysis will be presented elsewhere (in preparation).

The LD spectra of the covalent adducts obtained with the methylated derivative in the Z form are positive in sign throughout the entire spectral interval and are thus consistent with a site II type of conformation as well.

3.4. LD spectra of noncovalent BaPDE-poly(dG-m^{5}dC)·(dG-m^{5}dC) adducts

In contrast to the covalently bound adducts, the noncovalent complexes between BaPDE and DNA which are formed prior to covalent binding [34,39,42,43] are intercalative in nature and give rise to negative $\Delta A(\lambda)$ signals in the approx. 310–360 nm region of the spectrum [7]. The negative LD of the physically bound diol epoxide molecules decreases with increasing time after adding BaPDE to the DNA solutions because of chemical reactions generating tetraols and covalent adducts; the latter give rise to a positive contribution to the LD (fig. 6); thus the negative LD due to the intercalated BaPDE molecules decreases as the reaction time increases [7].

In order to elucidate the underlying reasons for the lower reactivity of the Z form, we have utilized the LD technique to compare the relative levels of noncovalent binding of BaPDE to the two different forms of poly(dG-m^{5}dC)·(dG-m^{5}dC) just after adding BaPDE to a DNA solution and prior to the covalent binding reaction. Immediately after mixing, when the concentration of diol epoxide molecules is maximal, the largest negative LD (site I) signal due to intercalated molecules should be observable. Under our experimental conditions (~ 0.10–0.15 mM concentration of nucleotides, 0.0086

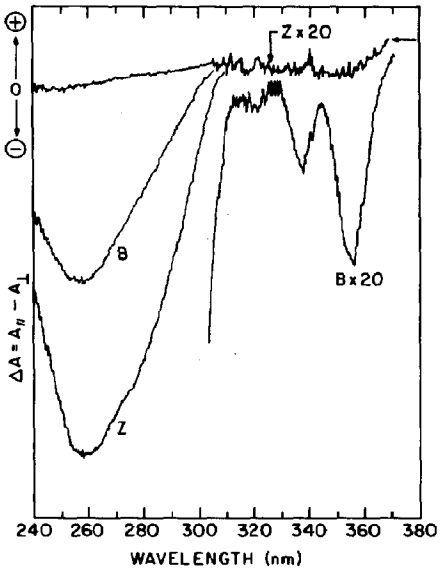

Fig. 7. Flow LD spectra of reaction mixtures containing 0.1 mM poly(dG-m^{5}dC)·(dG-m^{5}dC) in B and Z forms, 8.5×10^{-6} M BaPDE. The spectral scan requires 15 s and was started 28 s after mixing in order to minimize decomposition of BaPDE. LD spectra below approx 310 nm in the nucleic acid absorption region were measured at a lower sensitivity setting just before adding BaPDE. Concentration of NaCl, 2 mM; pH 7.7, 23°C; to induce the Z form, 10^{-5} M $Co(NH_3)_6Cl_3$ was added to the solution.

mM BaPDE), a substantial fraction of the diol epoxide molecules are not complexed to the polynucleotides. In the case of DNA, the equilibrium association constant is of the order of approx. 10^4 M^{-1} under similar conditions of ionic strength, so that less than 50% of the molecules are physically bound to the DNA [34]. Under these conditions, the LD method is particularly suitable for detecting DNA-bound ligands, since only those molecules which are complexed with the polymer contribute to the ΔA signal, while free molecules do not [7].

The flow LD spectra of unmodified poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B and Z forms in the 240–300 nm region are shown in fig. 7 (left-hand side). These spectra were obtained with the same sample just before and just after adding 10 μM $Co(NH_3)_6Cl_3$, and before the addition of BaPDE. Both of these LD spectra are negative in sign and resemble the inverted absorption spectra $A(\lambda)$ of these same solutions. In fact, the reduced LD, $\Delta A(\lambda)/A(\lambda)$, is found to be constant (within $\pm 5\%$) in the wavelength range of 250–290 nm. Similar behavior is observed with natural DNA [30,45]. This behavior is indicative of either a single transition moment, or overlapping transition moments which have the same orientations in this wavelength range [31]. The bulge in the absorption spectrum which appears in the approx. 270–290 nm range upon conversion of the B form to the Z form is also observed in the ΔA spectrum.

The ΔA signal measured at 257 nm is about 1.8–1.9-times higher for the Z form than for the B form, suggesting that the Z form is stiffer and less flexible, and is thus better oriented in the flow gradient than the B form. This interpretation is consistent with dynamic and static light-scattering studies which suggest a large increase in the chain stiffness upon the salt-induced conversion of poly(dG-dC)·(dG-dC) from the B to Z form [46].

The LD spectrum within the absorption band of BaPDE, measured within 30–45 s after adding the diol epoxide to the B form of poly(dG-m^{5}dC)·(dG-m^{5}dC), exhibits the negative ΔA bands at 339 and 355 nm which are characteristic of intercalated BaPDE [7]. The ΔA spectrum is not quite proportional to the absorption spectrum of intercalated BaPDE molecules [39,43] due to the formation of covalent adducts, even during the short time required to perform the measurements (45 s). The covalent adducts, which contribute a positive ΔA signal, distort the negative LD signal due to intercalated diol epoxide molecules, especially below 350 nm. From the kinetic data given in fig. 8, it can be calculated that about 60% of the BaPDE molecules have already reacted at the end of the LD scan, 45 s after mixing.

In contrast to the result obtained with the B form, an LD signal is not observed when BaPDE is added to the Z form of poly(dG-m^{5}dC)·(dG-m^{5}dC) under the same conditions (fig. 7). This negative result is obtained in spite of the fact that the observation of an LD signal due to BaPDE bound physically to the Z form should be favored for two reasons: (1) the reaction of BaPDE is much slower in the presence of the Z form than in the presence of B form DNA (fig. 8), and (2) the Z form exhibits a larger LD signal than the B form and thus tends to be better oriented in the flow field. On the basis of the noise level and the magnitude of the signal at approx. 355 nm observed in the case of the B form, our results

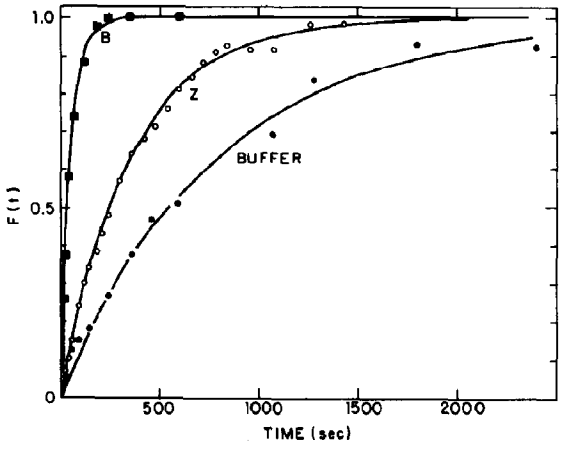

Fig. 8. Normalized (to unity) time dependence of the fluorescence intensities of reaction mixtures of 8.5×10^{-6} M BaPDE, 0.15 mM poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B form (■) and Z form (○), and in 5 mM sodium cacodylate buffer solution (●); 20 mM NaCl, pH 7.1, 23°C, in the case of the Z form the solution contained 10^{-5} M $Co(NH_3)_6Cl_3$. The solid traces are plots of the equation $F(t) = (1 - \exp[-kt])$. Values of k: 19×10^{-3} (B), 2.8×10^{-3} (Z), 1.2×10^{-3} s^{-1} (buffer).

suggest that ΔA for BaPDE physically bound to the Z form at this wavelength is at least 40-times smaller than in the case of the B form.

The absence of an LD signal in the case of BaPDE/Z poly(dG-m^{5}dC)·(dG-m^{5}dC) solutions indicates that the formation of physical intercalation complexes with the Z form is markedly inhibited. Of course, there are special orientations (the 'magic' angle of ~ 55° with respect to the direction of the flow) for which the LD signal is expected to approach zero [31]. However, a more likely explanation is that physical intercalation of BaPDE into poly(dG-m^{5}dC)·(dG-m^{5}dC) in the Z form is considerably weaker than in the case of the B form, since the physical binding of ethidium bromide [13], daunomycin [17], and adriamycin [18] to Z DNA is also known to be lower than in the case of the B form.

3.5. Differences in reaction kinetics of BaPDE in the presence of B and Z poly(dG-m^{5}dC)·(dG-m^{5}dC)

Under the appropriate conditions of pH and salt concentration, the formation of physical BaPDE-DNA complexes can lead to a significant acceleration of the reaction rate of the diol epoxide [34,40]. The pseudo-first-order reaction rate constant k is a function of the fraction of diol epoxide molecules which are intercalated into DNA [28]. Therefore, the increase in k in the presence of DNA over its value in buffer solution in the absence of DNA can thus also serve as an indication of physical binding.

The course of the reactions of BaPDE in sodium cacodylate buffer alone, and in the presence of poly(dG-m^{5}dC)·(dG-m^{5}dC) in the B and Z forms is shown in fig. 8. As tetraol molecules are formed, the fluorescence intensity increases in an exponential manner. In buffer solution $k = 0.001\ s^{-1}$, but its value is larger by a factor of approx. 16 in the presence of the polynucleotide in the B form. However, when the polymer is in the Z form, the reaction rate of BaPDE is increased by a factor of approx. 2 only. These results are also in agreement with the conclusions drawn from the LD data that the extent of physical binding of BaPDE is lower in the case of the Z form than in the case of the B form of poly(dG-m^{5}dC)·(dG-m^{5}dC).

3.6. The B-to-Z transition in modified polymers

The transition from the B form to the Z form upon abruptly changing the NaCl concentration was monitored by following either changes in the CD spectra or the absorbance at 295 nm. The kinetics of the B-to-Z transition depend on the chain length and are thus batch-dependent [16]. Under our conditions, unmodified 0.28 mM poly(dG-dC)·(dG-dC) (5 mM sodium cacodylate solution, pH 7.1, 23°C, salt concentration change from 0.1 to 4 M), the initial rate constant of transformation was $k = (720\ s)^{-1}$. In the case of poly(dG-m^{5}dC)·(dG-m^{5}dC) under similar conditions (except that the solution composition was 2 mM NaCl, and the transformation was produced by adding 10 μM $Co(NH_3)_6Cl_3$), the rate of the transition was considerably faster with a rate constant $k = 270\ s^{-1}$.

The kinetics of the B-to-Z transition were also determined in the case of poly(dG-dC)·(dG-dC) in which about 1% of the bases were modified by

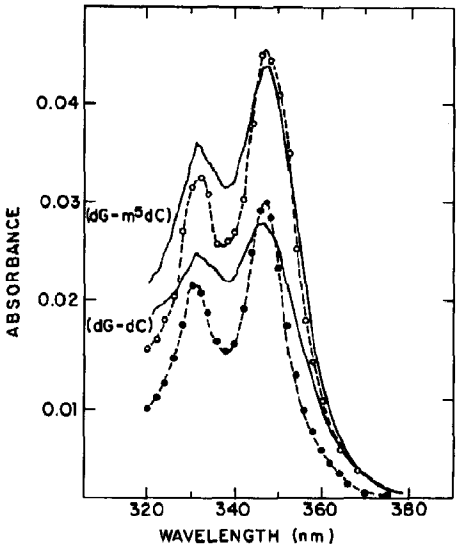

Fig. 9. Spectra of covalent adducts derived from the binding of BaPDE to poly(dG-m^{5}dC)·(dG-m^{5}dC) and poly(dG-dC)·(dG-dC) in B forms (0.1 M NaCl); (O--O) poly(dG-m^{5}dC)·(dG-m^{5}dC); (●-●) poly(dG-dC)·(dG-dC). (————) Spectra after transformation to Z forms (as verified by CD and absorbance spectra) by adding 4 M NaCl.

the covalent binding of BaPDE. Within experimental error, the rate constant was the same as for the unmodified polymer, at least at this low level of modification.

In another experiment, 0.1 M NaCl solutions of poly(dG-dC)·(dG-dC) and poly(dG-m^{5}dC)·(dG-m^{5}dC) previously modified covalently with BaPDE (about 1% of the bases modified), were transformed to the Z form by abruptly changing the salt concentration to 4 M. The CD spectra of the polymers before and after the conversion corresponded to those of the B and Z forms of the unmodified polymers, respectively (data not shown). However, only small changes in the absorption spectra of the covalently bound BaPDE chromophores are observed as is shown in fig. 9. The vibrational absorption bands of the pyrenyl chromophore at 332 and 346 nm appear to overlap one another to a somewhat greater extent in the Z form than in the B form, indicating at most small differences in the local environment of the pyrenyl chromphore. Direct covalent modification of the Z form of either polymer at these high salt concentrations, particularly in the case of poly(dG-m^{5}dC)·(dG-m^{5}dC), gives rise to broad and relatively unstructured spectra. Thus, the local conformational changes near the binding sites of BaPDE appear to be small, and it is likely that B-to-Z transitions are inhibited in the vicinity of the covalent binding sites. Such effects due to other covalently bound carcinogens have been previously reported [19,20]. Because of the low level of modification (~ 1%), such a local inhibition of the B-to-Z transition may not be evident as changes in the overall CD and absorption spectra of the polymer below 300 nm.

3.7. Factors influencing the relative reactivities of the B and Z forms

The level of covalent binding of carcinogens with DNA may depend on a number of different factors. Pullman and co-workers [47,48] considered in detail the effects of steric accessibilities and electrostastic potential minima of the various reactive sites in right-handed B and left-handed Z DNA. A third factor, especially in the case of PAH diol epoxides, is the formation of physical complexes which precedes the covalent binding reaction. The structures and steric properties of these noncovalent diol epoxide-DNA microcomplexes play a major role in determining the level of covalent binding [49].

The level of covalent binding of BaPDE is 2–3-times lower in the case of the Z form than for the B form of poly(dG-m^{5}dC)·(dG-m^{5}dC). This difference in the binding levels is thus not as large as that observed by Rio and Leng [25] in the case of the reaction of N-OH-AAF with poly(dG-br^{5}dC)·(dG-br^{5}dC).

The major site of attack of BaPDE is the exocyclic amino group of guanine; the electrostatic potential at this site is similar in the B and Z forms of poly(dG-dC)·(dG-dC), while the steric accessibility is slightly larger in the case of the Z form [47,48]. Assuming that these considerations can be extended to the methylated form of the polymer, it appears, as discussed earlier for N-OH-AAF [25], that these two factors do not play a major role in the covalent binding of BaPDE to these polynucleotides. Instead, our experimental data suggest that the physical binding of BaPDE to Z DNA is much less efficient than in the case of the B form. Therefore, the formation of prereaction noncovalent intercalative complexes are of primary importance in determining the reactivity and covalent binding of BaPDE with B and Z DNA.

The decreased ability of BaPDE to bind physically to Z DNA by intercalation can be rationalized by considering the dynamic properties of the nucleic acids [25]. Studies of the exchange rates of protons involved in hydrogen bonding between base-pairs indicate that the exchange rates are considerably slower when poly(dG-dC)·(dG-dC) is in the Z form [50,51]. Analogous observations have been reported for the methylated polymer [25]. If it is assumed that the protons can exchange only when the double helix is in an open state, then such open states are approx. 50-times less likely to occur in the Z form than in the B form [52]. Since intercalation, or similar forms of physical binding of carcinogens to DNA, involve a separation of adjacent base-pairs, this type of physical binding should be inhibited in the case of the Z-form, as is indeed observed experimentally.

4. Conclusions

In determining the relative reactivities of PAH diol epoxide carcinogens with nucleic acids in the B and Z forms, the effects of ionic strength are of primary importance. At high salt concentrations, physical complex formation, the rates of reaction, and fractions of molecules which undergo the covalent binding reaction are greatly diminished. Miller [49] has suggested that at high ionic strengths there is a competition for binding sites between protons needed for acid hydrolysis of BaPDE and other positive ions in the most negative region of the DNA, i.e. near the N2 position of guanine; this effect may account for the diminished reactivity of BaPDE at high salt concentrations. For all of these reasons the effects of polymer conformations on the covalent binding of BaPDE to the polynucleotides in the B and Z forms can be obscured.

In the absence of these salt effects, the lower level of covalent binding of BaPDE to the Z form relative to the B form of poly(dG-m^5dC)·(dG-m^5dC) can be primarily attributed to a reduced physical, intercalative binding of this carcinogen to the Z form. These results further demonstrate that the physical intercalative binding of PAH diol epoxides is an important factor in determining the level of covalent binding to nucleic acids. However, physical intercalation and the level of covalent binding are not necessarily directly proportional to one another as has been described earlier [34,39]. Thus, the fact that physical intercalative binding to the Z form is at least 40-times lower, while the level of covalent binding is smaller by a factor of 2–3 only, can be accounted for. Finally, our results suggest that DNA segments in vivo in the B form rather than the Z form may be the preferred sites of attack of polycyclic aromatic diol epoxide carcinogens.

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