The Major, N^2-Gua Adduct of the (+)-*anti*-Benzo[*a*]pyrene Diol Epoxide Can Be Unstable in Double-Stranded DNA†

Elise E. Drouin,‡ Joseph Lech,§ and Edward L. Loechler*,‡

Department of Biology, Boston University, Boston, Massachusetts 02215, and Center for Advanced Food Technology, Cook College, Rutgers University, Rutgers, New Jersey 08903

Received June 1, 1994; Revised Manuscript Received November 28, 1994⊗

ABSTRACT: The mechanisms of mutagenesis by the (+)-*anti* diol epoxide of benzo[*a*]pyrene [(+)-*anti*-B[a]PDE] was investigated in *supF* of the *Escherichia coli* plasmid pUB3 [Rodriguez & Loechler (1993) *Biochemistry 32*, 1759]. pUB3 was reacted with (+)-*anti*-B[a]PDE, then either (1) transformed immediately into *E. coli* or (2) heated at 80 °C for 10 min prior to transformation—the latter to probe mechanism. Qualitatively, heating did not have a statistically significant effect on the mutagenic pattern, except at the major base substitution hot spot, G_{115}, in *supF*; principally, $G_{115} \rightarrow$ T mutations were obtained prior to heating, while after heating, $G_{115} \rightarrow$ A and $G_{115} \rightarrow$ C mutations became more prevalent. Quantitatively, heating caused an ~2-fold decrease in mutation frequency. Heating released a small fraction of adducts (~5%), and the chemistry and implications of this reaction are investigated herein. Although the major adduct of (+)-*anti*-B[a]PDE (formed at N^2-Gua) is generally regarded to be heat stable, it can be quite unstable in double-stranded (but not single-stranded) DNA at low [Mg^{2+}]. Heating releases the corresponding tetraols from (+)-*anti*-B[a]P−N^2-Gua in approximately the same ratio as for simple hydrolysis of (+)-*anti*-B[a]PDE itself. This and other results suggest that guanine remains in DNA when (+)-*anti*-B[a]P−N^2-Gua adducts are hydrolyzed. [No evidence for any other chemical change in (+)-*anti*-B[a]PDE adducts was found.] While no general acid/base or nucleophilic catalysis was observed, adduct hydrolysis was specific acid catalyzed down to pH ~5.6, where the pH−rate profile showed a break to a slope of ~0.0. This break probably indicates the pK_a of (+)-*anti*-B[a]P−N^2-Gua protonated at the N^2-position, which is higher than expected. If true, it suggests that this adduct can become conformationally strained in double-stranded DNA, thereby disrupting resonance between the N^2-position and the rest of the guanine moiety of the adduct and facilitating hydrolysis by raising the pK_a at N^2. Although heating causes adduct hydrolysis, various arguments suggest that hydrolysis is probably *not* the cause of either the quantitative or qualitative changes in mutagenesis. It is more likely that these mutagenic changes are the consequence of the fact that a single adduct (i.e., (+)-*anti*-B[a]P−N^2-Gua) can adopt multiple conformations in DNA with different mutagenic consequences.

We have been studying mutagenesis by benzo[*a*]pyrene (B[a]P)[1] (Benasutti *et al.*, 1988; Loechler, 1989, 1994; Loechler *et al.*, 1990; Mackay *et al.*, 1992; Rodriguez *et al.*, 1992; Rodriguez & Loechler, 1993a,b, 1995, Drouin & Loechler, 1993) as well as by dibenz[*a,j*]anthracene (Gill *et al.*, 1993a,b), which are both polycyclic aromatic hydrocarbons that belong to the family of bulky mutagens/carcinogens. B[a]P may be metabolized by a variety of pathways [e.g., Phillips *et al.* (1985), Marnett (1987), Cavalieri *et al.* (1990), and Devanesan *et al.* (1992)], but its corresponding (+)-*anti* diol epoxide [(+)-*anti*-B[a]PDE; Figure 1] appears to be one important carcinogenic metabolite in many cases [reviewed in Conney (1982), Phillips (1983), Singer and Grunberger (1983), and Harvey (1991)], where the major DNA adduct is (+)-*anti*-B[a]P−N^2-Gua (Cheng *et al.*, 1989; Sayer *et al.*, 1991).

In our work, an *Escherichia coli* plasmid pUB3 was reacted with (+)-*anti*-B[a]PDE *in vitro* [designated (+)-*anti*-B[a]PDE−pUB3] and subsequently either (1) transformed immediately into *E. coli* (ES87) cells (Rodriguez & Loechler, 1993a) or (2) heated at 80 °C for 10 min prior to transformation (Rodriguez & Loechler, 1993b). Our original intention was to probe the mechanism using mild heating to liberate labile adducts and to infer from the changes in mutagenic pattern what contribution, if any, these labile adducts made. The notion was that heating labile (e.g., *N*7-Gua) adducts would give AP sites, which might be less mutagenic if they were repaired efficiently. Initially, this model seemed plausible in that we saw ~5% of (+)-*anti*-B[a]PDE adducts being liberated by heating and an ~2-fold decrease in MF. However, other aspects of our findings, such as the fact that simple freeze-thawing also caused a 2-fold decrease but did not release a significant fraction of adducts, made us doubt that these effects were due to the liberation of labile adducts.

Qualitatively, heating did not affect mutagenesis, except at the major base substitution hot spot, G_{115}. Prior to heating,

† This work was supported by grants from the NIH (ES03775) and the American Cancer Society (CN-54).

* Author to whom correspondence should be addressed.

‡ Boston University.

§ Rutgers University.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1995.

[1] Abbreviations: B[a]P, benzo[*a*]pyrene; (+)-*anti*-B[a]PDE, (+)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*) (Figure 1); (+)-*anti*-B[a]P−N^2-Gua, the major adduct of (+)-*anti*-B[a]-PDE (Figure 1)—the trans addition adduct is implied unless stated otherwise; (+)-*anti*-B[a]P−*N*7-Gua, a putative adduct with (+)-*anti*-B[a]PDE bound at *N*7-Gua; AFB_1−*N*7-Gua, an adduct with aflatoxin B_1 bound at *N*7-Gua; (+)-*anti*-B[a]PDE−pUB3, pUB3 adducted with (+)-*anti*-B[a]PDE (Rodriguez & Loechler, 1993a,b); heated (+)-*anti*-B[a]PDE, (+)-*anti*-B[a]PDE−pUB3 heated for 10 min at 80 °C (pH 6.8); MF, mutation frequency; AP sites, apurinic/apurimidinic sites; TMS, trimethylsilyl; ds, double stranded.

0006-2960/95/0434-2251$09.00/0

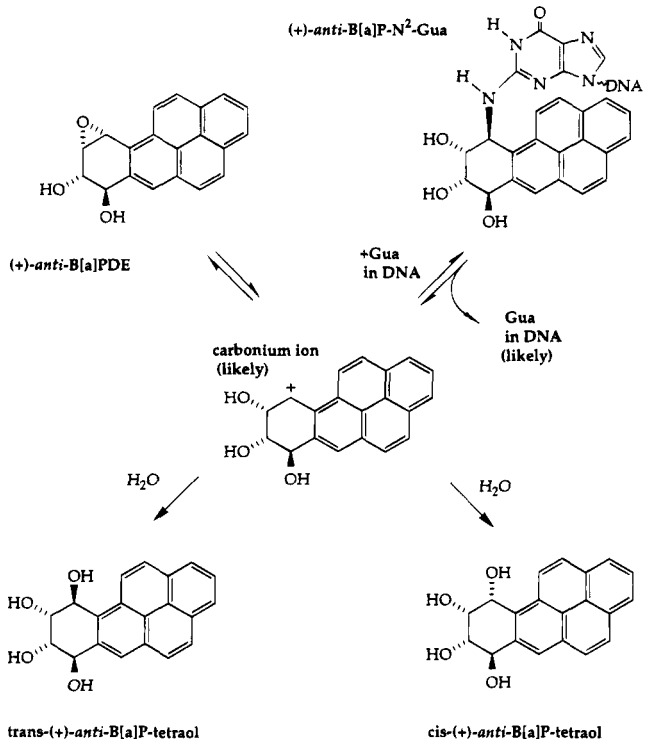

FIGURE 1: Structures and a proposed mechanism of (+)-*anti*-B[a]P-N^2-Gua hydrolysis to (+)-*anti*-B[a]P-tetraols. Hydrolysis is much faster in ds than ss DNA. The re-formation of guanine in DNA following adduct hydrolysis is likely, but not proven. The involvement of a C10-carbocation is also sensible but unproven.

G → T mutations (87%) dominated at G_{115} while after heating, G → T mutations (45%) were still prevalent, but there was a statistically significant increase in G → C (33%) and G → A (21%) mutations.

The quantitative changes in mutagenesis and the fact that the qualitative pattern of mutagenesis could change at one site following heating seemed significant to us, and several questions were raised by these findings. (1) What is the nature of the labile adduct? (2) Is there a link between the loss of a labile adduct and the ~2-fold decrease in MF? (3) Does the liberation of the labile adduct have any relationship to the changes in the mutational pattern at G_{115}?

One model ruled out was that a labile (e.g., B[a]P-*N*7-Gua) adduct causes one pattern of mutagenesis at G_{115} prior to heating but is hydrolyzed to an AP site, which gives the pattern following heating (Drouin & Loechler, 1993). Herein, we investigate chemical reactions other than AP site formation. We show that (+)-*anti*-B[a]P-N^2-Gua can be unstable in double-stranded DNA and is hydrolyzed to (+)-*anti*-B[a]P-tetraols, which should leave guanine intact in DNA (model in Figure 1).

MATERIALS AND METHODS

(+)-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydro[1,3-^{3}H]benzo[*a*]pyrene (*anti*) was purchased from Chemsyn Science Laboratories (catalogue number R702H; lot CSL-92-356-33-22 (1101.4 mCi/mmol)]. (+)-*anti*-B[a]PDE was handled as described previously [Benasutti *et al.* (1988), including working under yellow lights with it, its adducts, and (+)-*anti*-B[a]P-tetraols]. Aquasol-2 and [γ-^{32}P]dATP were obtained from New England Nuclear. DNase I (type II-S) and phosphodiesterase I (type VII) were obtained from Sigma Chemical Company, while all other enzymes were from New England Biolabs. All materials not explicitly mentioned were of the manufacturers' highest grade purity. Strains and plasmids were as described elsewhere (Rodriguez *et al.*, 1992).

HPLC Analysis of Heated (+)-anti-B[a]PDE−pUB3 Adducts. Approximately 70 μg of pUB3 randomly adducted with [^{3}H]-(+)-*anti*-B[a]PDE to give ~41 adducts/plasmid and ethanol precipitated (Rodriguez & Loechler, 1993a), which had been stored at −80 °C following adduction, was brought to 300 μL with 100 mM HEPES and 1 mM EDTA, pH 6.8, and extracted with 3 vols of water-saturated 1-butanol and then 2 vols of diethyl ether in order to remove any remaining (+)-*anti*-B[a]PDE hydrolysis products (Cheng *et al.*, 1989). Thereafter, the adducted DNA samples were divided into six aliquots, and each was brought up to a final volume of 200 μL with 100 mM HEPES and 1 mM EDTA, pH 6.8. To prevent evaporation during heating, 200 μL of light mineral oil (Sigma) was placed on top of each sample. Samples were then heated at 70 °C for either 0, 2, 4, 8, 24, or 48 h. At appropriate times, samples were removed and transferred to new tubes to remove the mineral oil. The original tube was rinsed with 100 μL of 100 mM HEPES and 1 mM EDTA, pH 6.8, and this was also transferred to the new tube.

When indicated, the samples were enzymatically digested to deoxynucleosides using DNase I, phosphodiesterase I, and calf-intestinal alkaline phosphatase essentially as described (Benassutti et al., 1988; final volume 500 μL). Digested deoxynucleosides were chromatographed by HPLC (Pruess-Schwartz *et al.*, 1986). Aquasol-2 (New England Nuclear) was used as the scintillation cocktail.

The following experiment was performed to ensure that no quantitatively significant adducts were lost during storage of (+)-*anti*-B[a]PDE (at −80 °C). After adduction (1 h at room temperature), (+)-*anti*-B[a]PDE−pUB3 was ethanol precipitated (~1.25 h at 0 or 4 °C), extracted with 3 vols of water-saturated 1-butanol and then 2 vols of diethyl ether (~5 min at room temperature), immediately heated, and then analyzed as described above.

Mass Spectral Analysis of (+)-anti-B[a]P-tetraols. Mass spectral analyses were conducted essentially following the procedures for (+)-*anti*-B[a]P-tetraols of Taghizadeh and Skipper (1994), which involves trimethylsilyl (TMS) derivatization of the alcoholic functionalities. Details are available upon request.

Tritiated material released from [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 (~19μg) by heating was purified by HPLC (see above), except the DNA was not enzymatically digested to ensure that the species eluting at 41 min (Figure 2b) was not contaminated with (+)-*anti*-B[a]P-N^2-Gua, which elutes ~6 min later. (+)-*anti*-B[a]P-tetraol standards were obtained by hydrolyzing (+)-*anti*-B[a]PDE in TE (10 mM Tris·HCl and 1 mM EDTA, pH 8.0) overnight at room temperature and then purifying by HPLC as described above.

Each tetra-TMS-derivatized (+)-*anti*-B[a]P-tetraol sample gave a molecular ion at *m/z* 608 and fragment ions at *m/z* 518, which corresponds to the loss of one trimethylsilanol group [M-TMSOH]$^+$, as well as at *m/z* 404, which corresponds to the loss of [M-TMSOCHCHOTMS]$^+$, where the underlined carbon atoms correspond to two of the four derivatized positions in the original (+)-*anti*-B[a]PDE moiety. A peak at *m/z* 191 corresponds to [TMSOCHOTMS]$^+$, which is a typical rearrangement fragment of persilyated molecules (Chizhov *et al.*, 1967). The ratio of heights of

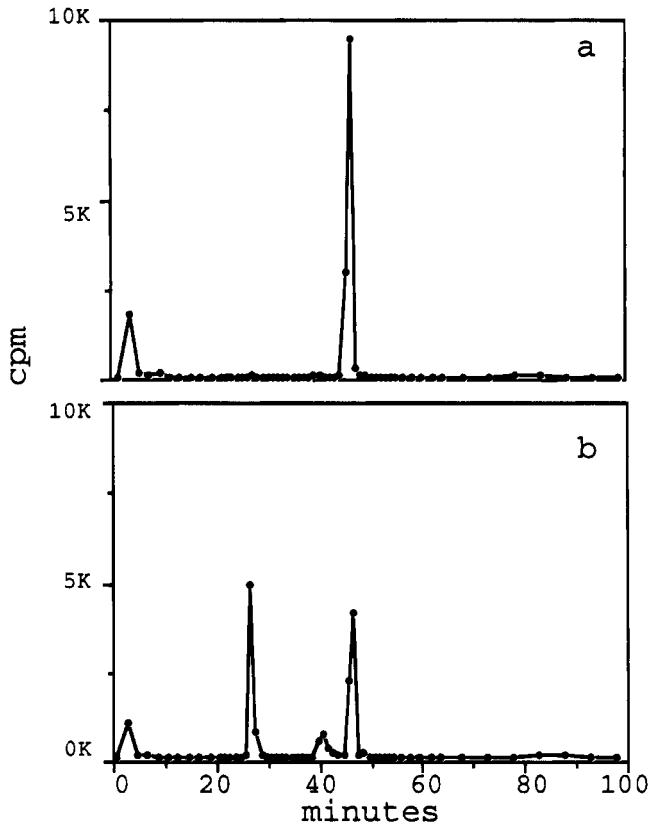

FIGURE 2: Reverse-phase HPLC chromatograms of unheated [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 digested to nucleosides (panel a) and [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 heated for 24 h before digestion to nucleosides (panel b). Purified [^{3}H]-*trans*-(+)-*anti*-B[a]P−N^2-Gua eluted at ~47 min, purified *trans*-[^{3}H]-(+)-*anti*-B[a]P-tetraol eluted at ~28 min, and purified *cis*-[^{3}H]-(+)-*anti*-B[a]P-tetraol eluted at ~41 min (data not shown). Heating was at 70 °C in 100 mM HEPES and 1 mM EDTA, pH 6.8. Fractions of varying amounts were collected for 98 min: 2-mL fractions from 0 to 21 min; 1-mL fractions from 22 to 53 min; 2-mL fractions from 54 to 63 min; and 5-mL fractions from 64 to 98 min.

the peaks at m/z = 608:518:404:191 was as follows. *trans*-(+)-*anti*-B[a]P-tetraol (6:8:80:100) as reported in Takahashi *et al.* (1979) is comparable to that for the peak at 28 min from the hydrolysis of (+)-*anti*-B[a]P−N^2-Gua (15:9:80:100) and from the peak at 28 min from the hydrolysis of (+)-*anti*-B[a]PDE itself (16:7:76:100). *cis*-(+)-*anti*-B[a]P-tetraol (5:NA:100:40) as reported in Takahashi *et al.* (1979) is comparable to that for the peak at 41 min from the hydrolysis of (+)-*anti*-B[a]P−N^2-Gua (10:<2:100:46) and from the peak at 41 min from the hydrolysis of (+)-*anti*-B[a]P-tetraols (11:<2:100:39). This confirms the assignments.

Heating an Adducted Single-Stranded Oligonucleotide. The single-stranded oligonucleotide 5′-GCGGCCAAAG-3′ with a single (+)-*anti*-B[a]P−N^2-Gua adduct at G^3 (underlined) was a generous gift from N. Geacintov at New York University. Adducted (~6 ng) and unadducted (~5 ng) oligonucleotide samples in 80 μL of 100 mM HEPES and 1 mM EDTA, pH 6.8, were divided in half, and 40 μL of light mineral oil was placed on top of each sample (see above). An aliquot of each sample was incubated at 17 or 70 °C for 24 h. Afterwards, all samples were 5′-radiolabeled by mixing 1 pmol of oligonucleotide with 10 mM $MgCl_2$, 2.5 mM DTT, 15 units of T4 polynucleotide kinase, 4 pmol of ATP, and 6.8 pmol of [γ-^{32}P]dATP (in a total volume of 55 μL) at 37 °C for 20 min. The reactions were terminated by adding 1 μM EDTA, pH 8.0, and all oligonucleotides were analyzed using a 17% denaturing acrylamide gel.

Heating Double-Stranded and Single-Stranded (+)-anti-B[a]PDE−pUB3 To Determine (+)-anti-B[a]PDE Adduct Stability. Approximately 3 μg of [^{3}H]-*anti*-B[a]PDE−pUB3 containing ~38 adducts/plasmid in 100 mM HEPES and 1 mM EDTA, pH 6.8, were extracted with water-saturated 1-butanol and diethyl ether as described above to remove any (+)-*anti*-tetraols and then divided into three aliquots. To each aliquot, $MgCl_2$ was added to 10 mM; then each was treated with either (i) 150 units of *Hin*dIII, (ii) 150 units of heat-inactivated *Hin*dIII (heated at 100 °C for 10 min), or (iii) no enzyme and then placed at 37 °C for 90 min. Samples were drop-dialyzed (Marusyk & Sargeant, 1980) at room temperature against 100 mM HEPES and 1 mM EDTA, pH 6.8, for 60 min. After dialysis, each sample was brought up to 500 μL with sterile distilled water, heated at 100 °C for 1 min (to denature linear DNA—supercoiled DNA remains double stranded [data not shown]), and then placed immediately on ice. Subsequently, each 500-μL sample was divided into five aliquots, and buffer was added until the final concentration was 100 mM HEPES and 1 mM EDTA, pH 6.8 in a final volume of 200 μL. Samples were heated at 70 °C for 0, 2, 4, 8, and 24 h with 200 μL of mineral oil placed on top (see above). At appropriate times, samples were removed and transferred to new tubes (see above). To determine the fractions of hydrolyzed adducts, samples were extracted with 3 vols of water-saturated 1-butanol, which removed hydrolyzed adducts leaving unhydrolyzed adducts in the aqueous phase. $MgCl_2$ (10 mM) and 4 units of DNase I were added to the aqueous phase (inconsistent and changing results were obtained unless the DNA was digested), and it was allowed to incubate overnight at room temperature. (Hydrolysis of adducts under these conditions was insignificant.) The following day, the fraction of tritium-containing material in the 1-butanol phase versus the aqueous phase was used to determine the fraction of adducts liberated. All samples were made 73% 1-butanol, 0.3 mM $MgCl_2$, 24 mM HEPES, and 0.24 mM EDTA so there would be no difference in solutes or solvent that might affect counting efficiency.

Determining Rates of (+)-anti-B[a]PDE Adduct Hydrolysis. Semi-log plots for release of adducts from (+)-*anti*-B[a]PDE−pUB3 were not linear with time (Results) as would be expected for a simple first-order process. Rather than using only the tangent at short times to estimate the initial rate, the reaction was treated as a second-order process according to

$$([1/\mathrm{Fr}] - 1) = k_{\mathrm{rel}}t \qquad (1)$$

where Fr is the fraction of adducts remaining in DNA and k_{rel} is an apparent second-order rate constant. k_{rel} is an observed second-order rate constant with units of min^{-1} and is not corrected for DNA or adduct concentration to give a true second-order rate constant with units of M^{-1} min^{-1}. Treating the data as a second-order process gave excellent straight lines (data not shown). Equation 1 provides only a minor correction for curvature, and no significant difference resulted if no correction was applied and a simple first-order process was assumed. Linear first-order plots can be obtained by assuming a non-zero end point for the reaction; e.g., a straight line is obtained for the data in Figure 3 if t_{infinity} = 36% is assumed.

Determining the Rate of (+)-anti-B[a]PDE Adduct Hydrolysis in Double-Stranded Copolymers. The rate of adduct

Table 1: Effect of Heating on [^{3}H]-(+)-*anti*-B[a]PDE Adduct Profile

time[a]	counts injected[b]	(+)-*anti*-B[a]P-N^2-Gua[c]	(+)-*anti*-B[a]P-tetraol 1[d]	(+)-*anti*-B[a]P-tetraol 2[e]	other counts[f]	total counts[g]	% released[h]	% recovery[i]
0	16.5[j]	12.8	0.09	0.14	2.3	15.4	1.8	93
2	16.0	11.6	0.94	0.57	2.3	15.4	11	97
4	16.2	11.0	1.5	0.55	1.8	14.9	16	92
8	16.6	9.8	2.7	0.86	1.8	15.2	27	91
24	15.9	6.6	5.6	1.7	1.3	15.2	53	96
48	15.6	5.0	6.3	1.8	1.4	14.5	62	93

[a] [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 was heated in 100 mM HEPES and 1 mM EDTA, pH 6.8, for 0, 2, 4, 8, 24, and 48 h. [b] Total counts (cpm) of digested [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 injected onto column for reverse-phase HPLC analysis as determined by counting 10% of the sample before injection. [c] Total counts (cpm) of [^{3}H]-*anti*-B[a]P−N^2-Gua isolated, which eluted in the peak at ~47 min (Figure 2a). [d] Total counts (cpm) of [^{3}H]-*trans*-(+)-*anti*-B[a]P-tetraol isolated, which eluted in the peak at ~28 min (Figure 2b). [e] Total counts (cpm) of [^{3}H]-*cis*-(+)-*anti*-B[a]P-tetraol isolated, which eluted in the peak at ~41 min (Figure 2b). [f] Not all counts could be attributed to (+)-*anti*-B[a]P−N^2-Gua or (+)-*anti*-B[a]P-tetraols. A small fraction of counts (between 8 and 14%) eluted at ~4 min (Figure 2) and probably represent a water-soluble contaminant of (+)-*anti*-B[a]PDE itself (see text). Also, a small fraction of counts (0.9−1.7%) eluted in the range between 80 and 100 min, which could include (+)-*anti*-B[a]P−*N*7-Ade (Cheng *et al.*, 1989). [g] The total amount of tritium (cpm) recovered from the column, which is the sum of the tritium in columns 3−6. [h] *trans*- and *cis*-(+)-*anti*-B[a]P-tetraols]/[*trans*- and *cis*-(+)-*anti*-B[a]P-tetraols + (+)-*anti*-B[a]P−N^2-Gua] × 100%. [i] Percent recovery of counts off the column as determined by total counts divided by counts injected. [j] All samples were analyzed for tritium (cpm) using Aquasol-2 as a cocktail with a Beckman scintillation counter (Materials and Methods). The values reported equal cpm × 10^{-3}.

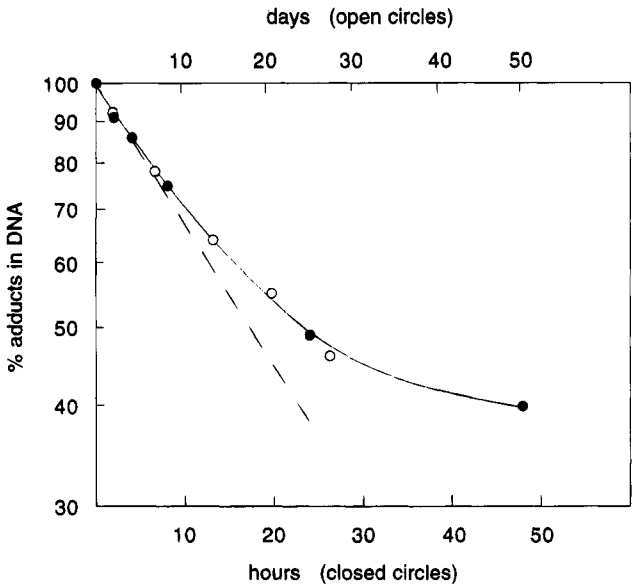

FIGURE 3: Semi-log plot of the loss of adducts (as a percent) from (+)-*anti*-B[a]PDE−pUB3 upon heating (100 mM HEPES and 1 mM EDTA, pH 6.8) at 70 °C (closed circles relative to scale on the lower abscissa) and at 37 °C (open circles relative to scale on the upper abscissa). A single smooth curve was drawn through both data sets. The relationship between the scales for the two abscissas was determined by comparing the time it took to hydrolyze a particular percentage of adducts from (+)-*anti*-B[a]PDE−pUB3 at 70 vs 37 °C. An excellent linear relationship was observed, which implies that the curvatures were similar for both data sets and from which it was determined that the hydrolysis reaction is 25.6-fold faster at 70 °C than at 37 °C. The data at 70 °C were from Table 1 using the HPLC method, while the data at 37 °C used the 1-butanol extraction procedure (Materials and Methods).

hydrolysis in double-stranded DNA copolymers was determined using 1-butanol extractions as described above for (+)-*anti*-B[a]P−pUB3. The four copolymers were used as follows: poly(dG)·poly(dC), poly(dA-dG)·poly(dC-dT), poly(dC-dG)·poly(dG-dC), and poly(dT-dG)·poly(dC-dA) (Pharmacia), which were randomly adducted to give ~0.086, 0.035, 0.039, and 0.011 adducts/bp, respectively.

RESULTS

Does (+)-anti-B[a]PDE Form Labile Adducts? pUB3 adducted with [^{3}H]-(+)-*anti*-B[a]PDE to give ~41 adducts/plasmid (stored at −80 °C) was heated at 70 °C for 0, 2, 4, 8, 24, and 48 h, then enzymatically digested to nucleosides, and analyzed by reverse-phase HPLC. (Unless noted otherwise, heating was always in 100 mM HEPES and 1 mM EDTA, pH 6.8). Figure 2a shows that *un*heated (+)-*anti*-B[a]PDE−pUB3 subjected to this procedure gave one major adduct peak at 47 min, which is the same as purified (+)-*anti*-B[a]P−N^2-Gua (data not shown). When [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 was heated at 70 °C for 24 h, digested to nucleosides, and analyzed by reverse-phase HPLC, two new radioactive peaks at 28 and 41 min appeared (Figure 2b). These two new peaks eluted at the same times as purified *trans*- and *cis*-(+)-*anti*-B[a]P-tetraols (data not shown), respectively. The material from both peaks was isolated, and this assignment was confirmed by mass spectrometry (Materials and Methods; Takahashi *et al.*, 1979; Taghizadeh & Skipper, 1994). The formation of (+)-*anti*-B[a]P-tetraols can be quantitatively attributed to the loss of (+)-*anti*-B[a]P−N^2-Gua (Table 1), which demonstrates that the former was derived from the latter.

The material eluting at 4 min in the unheated and heated (+)-*anti*-B[a]PDE−pUB3 samples (Figure 2) represents ~15% and ~9% of the total counts injected on the column, respectively. It is not tritiated water (data not shown). A water-soluble contaminant of (+)-*anti*-B[a]PDE at ~0.2% would be sufficient to give a peak of this size. The latter seems likely given that this peak (1) appeared when (+)-*anti*-B[a]PDE was hydrolyzed in the absence of DNA; (2) appeared when (+)-*anti*-B[a]PDE−pUB3 was not digested to deoxynucleoside adducts (data not shown); and (3) was not observed in purified (+)-*anti*-B[a]P−N^2-Gua or purified (+)-*anti*-B[a]P-tetraols (data not shown). This peak was ignored in the assessment of potential (+)-*anti*-B[a]PDE adducts.

The recovery of material as assessed by radioactivity for all samples of unheated and heated (+)-*anti*-B[a]PDE−pUB3 was always >90%, the average being ~94% (Table 1). When purified (+)-*anti*-B[a]P−N^2-Gua or purified (+)-*anti*-B[a]P-tetraols were injected under identical conditions, the percent recovery was ~93% in each case. These results demonstrated that both (+)-*anti*-B[a]P−N^2-Gua and (+)-*anti*-B[a]P-tetraols could be quantitatively recovered and accurately analyzed by our methods.

No quantitatively significant material (<~3%)[2] could have been missing from our HPLC analysis, unless an unstable

adduct was lost during (+)-*anti*-B[a]PDE−pUB3 storage at −80 °C. To assess this, a similar experiment was performed except that the analysis was initiated immediately after adduction/cleanup, so that no material could have been lost or unaccounted for. Heating for 0 and 24 h gave essentially the same results as in Table 1 (e.g., ~52% of adducts hydrolyzed to tetraols after heating at 70 °C for 24 h), and recovery of tritium—indicative of total (+)-*anti*-B[a]PDE adduct—was 94% and 97%, respectively (data not shown). This makes it extremely unlikely that any quantitatively significant adduct(s) was(were) missing from the analysis unless its instability is unprecedented. Furthermore, one would have to propose that the hydrolysis of this putative adduct leaves something in DNA other than an AP site based upon our previous work (Drouin & Loechler, 1993). This makes the existence of such an entity unlikely.

Figure 3 shows a semi-log plot for hydrolysis at 70 °C (data from Table 1) and 37 °C. A straight line is expected for a first-order reaction; however, curvature (similar at both 70 and 37 °C) is apparent, suggesting some subtlety or anomaly in the reaction kinetics. (This is addressed in the Discussion.) Nevertheless, $t_{1/2}$ ~ 24 and ~600 h, respectively, for (+)-*anti*-B[a]P−N^2-Gua hydrolysis could be interpolated. Rate constants were estimated following a simple correction for curvature (see Materials and Methods).

Stability of a Single (+)-anti-B[a]P−N^2-Gua Adduct in a Single-Stranded Oligonucleotide. The results in Figure 2 and Table 1 were unexpected because (+)-*anti*-B[a]P−N^2-Gua is generally regarded to be stable both as the nucleoside adduct and in single-stranded oligonucleotides as reported by Benasutti *et al.* (1988) and Mao *et al.* (1992). To reaffirm the latter under identical conditions, a single-stranded oligonucleotide containing a single (+)-*anti*-B[a]P−N^2-Gua adduct was heated for 24 h at 70 °C, 5′-^{32}P-radiolabeled, and then analyzed on a denaturing polyacrylamide gel. There were no observable differences between the heated vs the unheated adducted oligonucleotide (Figure 4; lanes 3 vs 4, respectively). If heating had hydrolyzed any of the (+)-*anti*-B[a]P−N^2-Gua adducts in the oligonucleotide a new band would have been expected to migrate at or near the unadducted oligonucleotide (Figure 4, lanes 1 and 2), which was not the case.

Effects of Heating (+)-anti-B[a]PDE Adducts in Double-Stranded vs Single-Stranded DNA. The apparent conflict between the instability of (+)-*anti*-B[a]P−N^2-Gua in pUB3 compared to that in an oligonucleotide could be reconciled if (+)-*anti*-B[a]P−N^2-Gua adducts were more labile in double-stranded than single-stranded DNA. To investigate this, [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 was made single-stranded by cleaving with *Hin*dIII (to give linear DNA), heated at 100 °C for 1 min (to denature the strands), and then quickly cooled on ice. Following subsequent heating

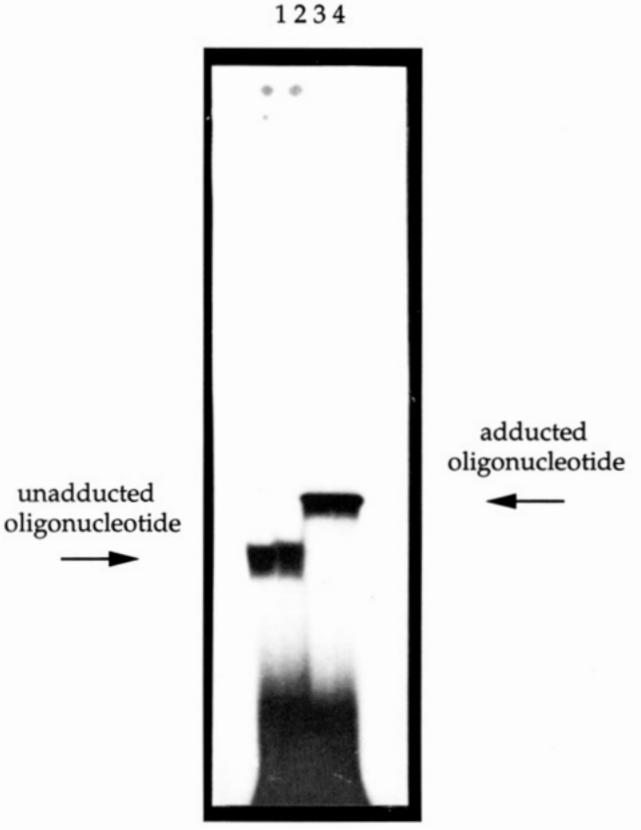

FIGURE 4: Stability of a (+)-*anti*-B[a]P−N^2-Gua adduct in a single-stranded oligonucleotide. 5′-GCGGCCAAAG-3′ containing a single (+)-*anti*-B[a]P−N^2-Gua adduct at the underlined guanine was heated at 70 °C for 24 h (100 mM HEPES and 1 mM EDTA, pH 6.8), 5′-[^{32}P]-radiolabeled, and then analyzed on a 17% denaturing polyacrylamide gel. The autoradiogram shows the unheated and heated unadducted oligonucleotides (lanes 1 and 2, respectively), and the unheated and heated adducted oligonucleotides (lanes 3 and 4, respectively).

at 70 °C for 0, 2, 4, 8, and 24 h, hydrolyzed adducts (i.e., (+)-*anti*-B[a]P-tetraols) were extracted into water-saturated 1-butanol (Cheng *et al.*, 1989), which leaves unhydrolyzed adducts in the aqueous phase attached to plasmid DNA. The fraction of tritium-containing material in the 1-butanol phase (and therefore labile adducts) was determined. (+)-*anti*-B[a]-PDE adducts in single-stranded pUB3 were relatively stable with, for example, only ~8% of the adducts being liberated after heating at 70 °C for 24 h. In comparison, ~8% of the adducts in double-stranded (+)-*anti*-B[a]PDE−pUB3 were released after heating at 70 °C for only ~1 h, and ~60% of the adducts were released after 24 h. To ensure that this difference in stability was not due to the presence of the enzyme *Hin*dIII, (+)-*anti*-B[a]P−pUB3 was mock-cleaved with heat-inactivated *Hin*dIII (Materials and Methods) and subjected to the same protocol; the results were comparable to double-stranded pUB3 (data not shown). The 1-butanol extraction procedure gave results virtually identical to the HPLC analysis procedure (Table 1), indicating that this simpler procedure for quantitating the fraction of (+)-*anti*-B[a]P-tetraols released was reliable.

Effects of Mg^{2+}, Buffer, and pH on (+)-anti-B[a]PDE Adduct Stability. Initially to analyze the stability of (+)-*anti*-B[a]PDE adducts in ds pUB3, samples were heated at 80 °C because this was the temperature used in the heating step in our mutagenesis studies (Rodriguez & Loechler, 1993b). Subsequently, we determined that ds (+)-*anti*-B[a]-PDE−pUB3 denatured at 80 °C with a $t_{1/2}$ of ~45 min (data

[2] Aside from *trans*-(+)-*anti*-B[a]P−N^2-Gua, no other HPLC peak indicative of adducts exceeded ~2% in our HPLC analysis. Other minor species include *cis*-(+)-*anti*-B[a]P−N^2-Gua and *trans*-(+)-*anti*-B[a]P−N^6-Ade, which have been reported to account for ~1% and ~4% of adducts, respectively (Cheng *et al.*, 1989). Based upon the tritium that appears in the entire HPLC analysis, we can quantitatively account for 94% of the tritium injected (Table 1). Although this suggests that ~6% of our material is unaccounted for, we found that the injection of purified *trans*-(+)-*anti*-B[a]P−N^2-Gua, *trans*-(+)-*anti*-B[a]P-tetraol or *cis*-(+)-*anti*-B[a]P-tetraol gave recoveries of ~93%. From this finding, we infer that HPLC recovery is not 100% and that in fact virtually all material we injected appears in a peak and is accounted for.

Table 2: Percent (+)-*anti*-B[a]PDE Adducts Hydrolyzed after Heating [^{3}H]-(+)-*anti*-B[a]PDE−pUB at 70 °C under Various Conditions

time[a]	B[a]P−pUB3[b] (~41 adducts/plasmid)	B[a]P−pUB3[c] (~10 adducts/plasmid)	B[a]P−pUB3 plus tetraols[c,d]	single-stranded[c,e] B[a]P−pUB3	10 mM $MgCl_2$[c,f]	10 mM $MgSO_4$[c,f]	20 mM KCl[c,f]	20 mM NaCl[c,f]
0	1.8[g] (± 0.25)[i]	1.0 (± 0.21)		0.76[h]	0.15[h]			
2	11 (± 1.3)	8.7 (± 0.045)		2.2[h]	1.3[h]			
4	16 (± 1.6)	15 (± 0.57)		2.4[h]	1.6[h]			
8	27 (± 0.64)	25 (± 0.71)		4.0[h]	1.7[h]			
24	53 (± 1.4)	53 (± 1.1)	51 (± 0.85)	6.7 (± 0.71)	2.9 (± 4.9)	3.6 (± 2.1)	43[h]	42[h]
48	62 (± 0.71)		65[h]					

[a] All [^{3}H]-(+)-*anti*-B[a]PDE−pUB3 samples were heated in 100 mM HEPES and 1 mM EDTA, pH 6.8, at 70 °C for 0, 2, 4, 8, 24, and 48 h (except where indicated). [b] The fraction of adducts hydrolyzed was determined by reverse-phase HPLC (Table 1). [c] The fraction of adducts hydrolyzed was determined by the 1-butanol extraction procedure (Materials and Methods). [d] Purified *trans*- and *cis*-(+)-*anti*-B[a]P-tetraols were added to (+)-*anti*-B[a]PDE−pUB3 to mimic the hydrolysis of ~50% of the adducts, which is the same as heating for 24 h at 70 °C. [e] (+)-*anti*-B[a]PDE−pUB3 was made single stranded by linearizing adducted pUB3 with *Hind*III and heating at 100 °C for 1 min to denature strands (Materials and Methods). [f] (+)-*anti*-B[a]PDE−pUB3 was heated in the presence of either 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM KCl, or 20 mM NaCl. [g] The percent of (+)-*anti*-B[a]PDE adducts hydrolyzed from (+)-*anti*-B[a]PDE−pUB3. [h] Single determinations made it impossible to determine standard deviations. [i] The numbers in parentheses represent the standard deviation.

not shown) causing the hydrolysis reaction to stop. To eliminate this complication, conditions were sought where adduct hydrolysis but not denaturation occurred. Both heating at 70 °C and the addition of Mg^{2+} prevented denaturation of double-stranded (+)-*anti*-B[a]P−pUB3 (data not shown). However, the addition of Mg^{2+} unexpectedly also stabilized (+)-*anti*-B[a]PDE adducts; e.g., ~50%, ~20%, and ~2% of the adducts were hydrolyzed with 0, 1, or 10 mM $MgCl_2$, respectively, after hydrolysis for 24 h (additional data in Table 2). Similar results with $MgSO_4$ (Table 2) suggested that this effect is cation dependent. Monovalent cations stabilized to a lesser extent (Table 2).

(+)-*anti*-B[a]PDE−pUB3 was heated at 70 °C for 24 h at various pH values (4.5−8.2), in various buffers (all with 1 mM EDTA), at various concentrations, and all maintained at 125 mM ionic strength with KCl (Table 3). Increasing buffer concentration did not affect the rate of the reaction, which rules out both general acid/base and nucleophilic catalysis. Hydrolysis is specific acid catalyzed (Figure 5).

Effect of DNA Sequence Content on Hydrolysis of (+)-anti-B[a]PDE Adducts. To investigate the effect of sequence context on hydrolysis, four copolymers [poly(dG)·poly(dC), poly(dA-dG)·poly(dC-dT), poly(dC-dG)·poly(dG-dC), and poly(dT-dG)·poly(dC-dA)] were adducted with (+)-*anti*-B[a]-PDE and heated for 0, 1, 2, 4, 8, and 24 h at 70 °C. The (+)-*anti*-B[a]PDE adducts in poly(dG)·poly(dC) and poly-(dA-dG)·poly(dC-dT) were relatively stable; ~13% and ~7.2%, respectively, of the adducts were released after heating for 24 h (Figure 6). In contrast, the adducts in poly-(dCdG)·poly(dGdC) and poly(dT-dG)·poly(dC-dA) were relatively unstable with ~68% and ~44%, respectively, of the adducts being released after 24 h (Figure 6). Heating at 70 °C for 24 h did not significantly denature any of these DNA molecules based upon an analysis of these species in agarose gels (data not shown). A cursory analysis of each copolymer by reverse-phase HPLC as in Figure 2 revealed that (+)-*anti*-B[a]P−N^2-Gua predominated in adduction and that (+)-*anti*-B[a]P-tetraols were released by heating.

Table 3: Effect of Buffer Concentration and pH on Rate of (+)-*anti*-B[a]PDE Adduct Hydrolysis

	pH	15 mM[a]	30 mM	45 mM
HEPES[b]	8.2	0.71[c] (± 0.56)[d]	1.7 (± 0.49)	3.0 (± 0.57)
(pK_a = 7.5)	7.5	5.6 (± 0.49)	5.9 (± 0.85)	5.9 (± 0.57)
	6.8	20 (± 1.4)	21 (± 0.71)	20 (± 1.4)
imidazole	7.8	4.6 (± 0.28)	4.6 (± 0.21)	4.1 (± 0.42)
(pK_a = 7.1)	7.1	23 (± 2.1)	19 (± 0.71)	22 (± 5.7)
	6.4	64 (± 0.071)	58 (± 8.5)	57 (± 3.5)
phosphate	7.5	2.3 (± 0.28)	4.0 (± 1.6)	4.0 (± 0.071)
(pK_a = 6.8)	6.8	7.8 (± 0.21)	9.5 (± 0.071)	9.5 (± 0.071)
	6.1	31 (± 3.5)	32 (± 0.71)	33 (± 1.4)
TE[e] (pK_a = 8.1)	8.0	24 (± 1.4)		
TES (pK_a = 7.5)	8.2			2.1[f]
BES (pK_a = 7.2)	7.9			3.2[f]
MOPS (pK_a = 7.2)	7.9			1.5[f]
PIPES (pK_a = 6.8)	7.5			3.2[f]
MES (pK_d = 6.2)	6.9			8.3[f]
	5.8	76 (40)[g] (± 1.4)		
	5.5	65 (23)[g] (± 1.4)		
methoxyamine	5.0	79 (62, 33)[h]		
(pK_a = 4.7)		(± 2.1)		
	4.5	70 (56, 35)[i]		
		(± 0.7)		

[a] Buffer concentrations. [b] All buffers included 1 mM EDTA and were maintained at 125 mM ionic strength with KCl. [c] Percentage of [^{3}H]-(+)-*anti*-B[a]PDE adducts hydrolyzed after heating (+)-*anti*-B[a]PDE−pUB3 at 70 °C for 24 h (as determined by 1-butanol extraction procedure; Materials and Methods). [d] The numbers in parentheses represent the standard deviation. [e] TE was 10 mM Tris and 1 mM EDTA with no KCl. As a result, the relative fraction of (+)-*anti*-B[a]PDE adducts hydrolyzed was higher because monovalent cations stabilize (+)-*anti*-B[a]PDE adducts, although not as efficiently as divalent cations. [f] Single determinations made it impossible to determine standard deviations. [g] 40% and 23% of these adducts were hydrolyzed after 5 h at 70 °C and pH 5.8 and 5.5, respectively. k_{rel} calculated for the 5- and 24-h time points at each pH value agree closely, indicating that the values are reliable and that eq 1 is reasonable (see Materials and Methods). [h] 62% and 33% of these adducts were hydrolyzed after 5 and 2 h, respectively, at 70 °C and pH 5.0. k_{rel} was calculated using the 5- and 2-h time points, which agreed closely; the 24-h time point gave a value for k_{rel} ~2-fold lower. [i] 56% and 35% of these adducts were hydrolyzed after 5 and 2 h, respectively, at 70 °C and pH 4.5. k_{rel} was calculated using the 5- and 2-h time points, which agreed closely; the 24-h time point gave a value for k_{rel} ~2-fold lower.

DISCUSSION

When ds-(+)-*anti*-B[a]PDE−pUB3 was heated, (+)-*anti*-B[a]P-tetraols were hydrolyzed from (+)-*anti*-B[a]P−N^2-Gua adducts. It is worth noting that the rate of this reaction is not insignificant, being only an order of magnitude slower at 37 °C than the rate of hydrolysis of AFB_1−N^7-Gua (Groopman *et al.*, 1980) and ~3−4-fold slower than the hydrolysis of *N*7-methylguanine (Lawley & Brookes, 1963). In fact at pH 6.0, the rate of (+)-*anti*-B[a]P−N^2-Gua and AFB_1−*N*7-Gua hydrolysis are comparable. Adducts from other polycyclic aromatic hydrocarbons, which might naively be expected to be stable, have also been noted to be unstable (Dipple *et al.*, 1985).

We attempted two extensive lines of investigation to confirm that guanine remains in DNA following tetraol liberation from (+)-*anti*-B[a]P−N^2-Gua (Figure 1), but both

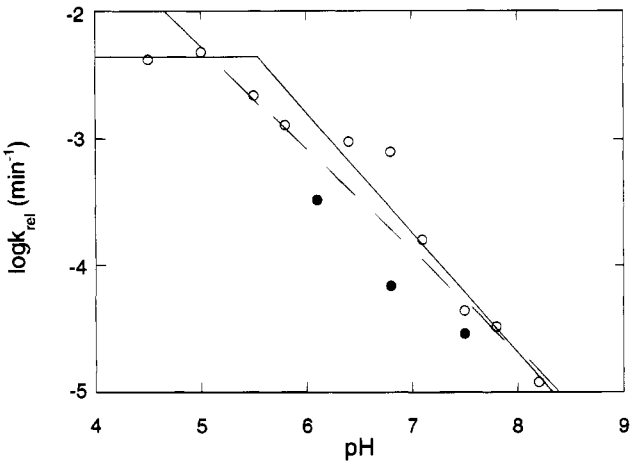

FIGURE 5: pH−rate profile of (+)-*anti*-B[a]PDE adduct hydrolysis (Materials and Methods). (+)-*anti*-B[a]PDE−pUB3 was heated at 70 °C for 24 h at various pH values (5.5−8.2) and in various buffers (HEPES, imidazole, phosphate, TES, BES, MOPS, PIPES, MES, and methoxyamine; all with 1 mM EDTA), and maintained at 125 mM ionic strength with KCl (opened circles). The data is derived from Table 3. (TE data were excluded because they were not at 125 mM.) In all cases, the value with the lowest buffer concentration was used. In the case of methoxyamine buffer (pH 4.5 and 5.0), the average of the 2- and 5-h time points was used to determine k_{rel}, which gave similar rate constants that were ~2-fold greater than k_{rel} determined from the data for 24 h of hydrolysis (Table 3). The values obtained with phosphate buffers (closed circles) lie below the line probably because of a specific salt effect. k_{rel} is in min^{-1} as explained in Materials and Methods. The dashed line (slope ~0.86) is the best straight line through the open circles, while the solid lines are of slope 0.0 and 1.0 assuming specific acid catalysis with a species with pK_a ~ 5.6.

were unsuccessful.[3,4] However, the re-formation of guanine (Figure 1) seems most likely based on the following. Both *trans*- and *cis*-tetraols were obtained from the hydrolysis of

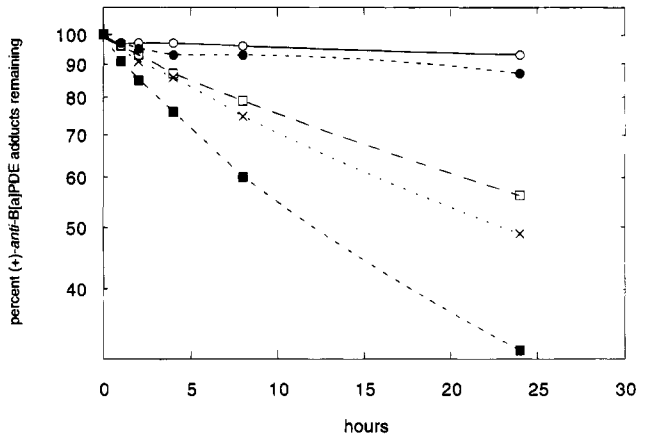

FIGURE 6: Comparison of the stability of (+)-*anti*-B[a]PDE adducts in double-stranded DNA copolymers of defined sequence context. Adducted DNA copolymers were heated at 70 °C (100 mM HEPES and 1 mM EDTA, pH 6.8) for 0, 1, 2, 4, 8, and 24 h, and the fraction of adducts hydrolyzed was determined by the procedure involving 1-butanol extractions (Materials and Methods). (○) poly(dA-dG)·poly(dC-dT); (●) poly(dG)·poly(dC); (□) poly(dT-dG)·poly(dC-dA); (■) poly(dC-dG)·(poly(dG-dC); (×) (+)-*anti*-B[a]PDE−pUB3 (from Table 1).

(+)-*anti*-B[a]PDE itself, which argues for an S_N1 reaction mechanism via the corresponding C10-carbocation [e.g., see Geacintov (1986)]. Cis addition is disfavored sterically because of the stereochemistry of the hydroxyl group at C9 of (+)-*anti*-B[a]PDE. The ratio of *trans*/*cis*-tetraols from (+)-*anti*-B[a]P−N^2-Gua hydrolysis (~3.4; from the data for 24 and 48 h of hydrolysis in Table 1 and other data) is very similar to hydrolysis of (+)-*anti*-B[a]PDE itself (~2.4) (data not shown). This argues that a similar carbocation intermediate forms during adduct hydrolysis, especially because an S_N2 mechanism should lead to a preponderance of the *cis*-tetraol. The only sensible mechanism, and the one suggested by the principle of microscopic reversibility, is simple S_N1 breakdown of (+)-*anti*-B[a]P−N^2-Gua via direct reversal of the adduction step, which would leave guanine in DNA. Other types of hydrolysis chemistry should give products other than a tetraol; e.g., hydrolysis via a tetrahedral intermediate at C2 would liberate (+)-*anti*-B[a]P−aminotriol. Finally, we know that hydrolysis does not leave an AP site in DNA most of the time, because when ~5% of the adducts are released by heating, no more than ~0.6% are converted to AP sites (Drouin & Loechler, 1993).

We found no evidence during HPLC or mass spectrometric analysis that the (+)-*anti*-B[a]P−N^2-Gua adducts that remain in DNA during the heating process were altered, and no peaks by HPLC other than (+)-*anti*-B[a]P-tetraols were observed even after heating for 48 h.

Effect of pH, Buffer, Ionic Strength, and Mg^{2+} on Adduct Stability. No evidence was obtained for general acid/base or nucleophilic catalysis of (+)-*anti*-B[a]PDE adducts (Table 3), while the pH−rate profile (Figure 5) indicated specific acid catalysis. The best straight line through the data in Figure 5 has a slope of ~0.86 with considerable scatter, while a slope of 1.0 is required for specific acid catalysis. The explanation for this discrepancy may be complications from the fact that the kinetics are not perfectly first order (Materials and Methods) and/or specific salt effects. However, a chemically more reasonable possibility is that there is a break in the pH−rate profile at pH ~5.6 (Figure 5), which would indicate the pK_a of a critical group in the hydrolysis. This seems more sensible based on the fact that the rate of

[3] A set of oligonucleotides, 5′-G[1]CG[2]G[3]CCAAAG[4]-3′ (synthesized at New York University in the laboratory of N. Geacintov) contained a single (+)-*anti*-B[a]P−N^2-Gua adduct at one of the guanines denoted 2, 3 , or 4. Each adducted oligonucleotide was annealed to either 5′-CTTTGGCCGC-3′ or 5′-GCCGCCTTTG-3′ and ligated, which would have required blunt-end or sticky-end ligation, respectively. The only samples that gave a significant amount of double-stranded concatomers were the unadducted oligonucleotide (control) annealed to either complementary strand and the oligonucleotide adducted at G[4] annealed to the complementary strand that resulted in sticky-end ligation. (Attempts to improve yield were unsuccessful.) The G[4]-adducted oligonucleotide, which had been 5′-^{32}P-radiolabeled before annealing, was heated at 55 °C (heating at higher temperature resulted in denaturation) for 24 h. Both adducted and unadducted (control) samples were digested to nucleotides with spleen phosphodiesterase, which leaves 3′-monophosphates and thereby transfers the radioactive phosphate to the 3′-hydroxyl of the adducted G[4], and analyzed by reverse-phase HPLC. (+)-*anti*-B[a]P−N^2-Gua as the 3′-monodeoxynucleotide was the only peak observed without or with heating; e.g., no 3′-dGMP was observed as expected based upon Figure 1. Furthermore, the adducted/ligated/heated sample was also digested back to decamers with *Eae*I (recognition site: 5′-CGGCCA-3′), and polyacrylamide gel electrophoresis revealed adducted but no de-adducted decamers. Both results indicate no hydrolysis occurred.

[4] pUB3 was randomly adducted with (+)-*anti*-B[a]PDE. Because at best only ~5% of the guanines per plasmid were adducted, any guanine liberated from an adduct would be masked by unadducted guanines (~95%) remaining in the plasmid. To circumvent this, (+)-*anti*-B[a]PDE−pUB3 was treated with nitrous acid to deaminate 90% of guanines to xanthine before heating at 45 or 55 °C for 48 h. [(+)-*anti*-B[a]P−N^2-Gua is a secondary amine and does not deaminate.] Only ~0.9% and ~1.9% of the adducts were hydrolyzed, respectively, compared to ~52% when nitrous acid was excluded. Nitrous acid deamination of guanine, adenine, and cytosine was probably sufficiently extensive to enhance single-strand character, which inhibits adduct hydrolysis.

hydrolysis varied >10-fold when the pH changed by 1.4 units using HEPES or imidazole as buffers at relatively high pH, while the rate of hydrolysis varied ≪2-fold when the pH changed by 1.3 units using MES and methoxyamine buffers at lower pH (Table 3).

No normal constituent of DNA has a pK_a of ~5.6. It seems sensible to attribute this pK_a to N^2 in (+)-*anti*-B[a]P−N^2-Gua, because protonation here would facilitate hydrolysis. This is higher than the analogous pK_a in unadducted guanine. If the B[a]P moiety forced the C2−N^2 adduct bond of (+)-*anti*-B[a]P−N^2-Gua to rotate such that the sp^2 lone-pair orbital at N^2 could not hybridize with the π-orbitals in the aromatic rings of the guanine moiety, then the N^2-position would be more like an alkylamine and have a higher than normal pK_a. [This effect is discussed by Loechler (1991).] Furthermore, hydrolysis might be faster in ds DNA than in ss DNA if the B[a]P moiety of the adduct were more constrained in the minor groove of ds DNA, such that the sp^2 orbital at N^2 was less able to resonate.

There may be a connection between specific acid catalysis of hydrolysis and inhibition of hydrolysis by Mg^{2+} ions. It seems conceivable that the local pH is lower in the vicinity of the polyanionic phosphates of DNA in the absence of Mg^{2+} ions. Mg^{2+} may also cause a structural change. Attributing the faster hydrolysis in ds vs ss DNA to the greater polyanionic character of ds DNA seems unlikely because of the results with the copolymers, which all have equal polyanionic character and yet differ in their rate of adduct hydrolysis (Figure 6).

It is only because we chose to heat in EDTA with no Mg^{2+}, which are conditions commonly used to store plasmid DNA, that we observed (+)-*anti*-B[a]P−N^2-Gua instability. Under normal circumstances, including in cells where divalent ion concentration is high, hydrolysis of this adduct should be relatively slow and inconsequential biologically. Nevertheless, for the purposes of understanding mutagenic mechanisms, this reaction may prove to be revealing.

Nonlinear First-Order Plots and Effect of Sequence Context on Rate Constant of (+)-anti-B[a]PDE Adduct Stability. First-order plots of the hydrolysis of (+)-*anti*-B[a]P−N^2-Gua from random sequence DNA (i.e., pUB3) appeared to be curved (solid line in Figure 3) when compared to the initial rate (dashed line in Figure 3). This effect is reproducible (e.g., see standard deviations in Table 2). Several trivial explanations for this anomalous kinetic behavior were ruled out based on results in Table 3, including curvature due to (1) a decrease with time in the number of adducts per plasmid; (2) the accumulation of tetraols with time; (3) a true second-order reaction; and (4) an upward drift in pH during hydrolysis.[5] Both (5) curvature because of the loss of supercoiling of the plasmid with time of heating and (6) the slow accumulation of ss DNA during hydrolysis also seem unlikely.[6]

Sequence context affected the rate of hydrolysis of (+)-*anti*-B[a]PDE adducts (Figure 6), which is an attractive hypothesis to explain the curvature in random DNA sequences (Figure 3). However, the hydrolysis curves in Figure 6 with the copolymers, in which sequence context is invariant, were also curved. Furthermore, the shape of this curvature is very similar for (+)-*anti*-B[a]PDE adducts in pUB3 in comparison to poly(dT-dG)·poly(dC-dA) and poly(dC-dG)·poly(dG-dC) based on plots like Figure 3 (data not shown). This suggests that the effect of sequence context on hydrolysis rate is unlikely to be the main reason that adduct hydrolysis in (+)-*anti*-B[a]PDE−pUB3 slowed down with time. A slow reaction of DNA (e.g., cytosine deamination), which might change adduct conformation, might also be important. In conclusion, we can offer no definitive explanation for the curvature in the kinetics of adduct hydrolysis with time.

What Adducts Are Responsible for (+)-anti-B[a]PDE Mutagenesis? The existence of an *N*7-Gua adduct of (±)-*anti*-B[a]PDE had been inferred from a variety of circumstantial evidence, including the presence of a labile adduct (Osborne *et al.*, 1978; King *et al.*, 1979) and the formation of strand breaks following piperidine hydrolysis of (±)-*anti*-B[a]PDE-adducted DNA (Sage & Haseltine, 1984; Lobanenkov *et al.*, 1986) as well as the pure (+)-*anti*-B[a]PDE (Rill & Marsch, 1990). Some work has suggested that the formation of B[a]P−*N*7-Gua adducts from (+)-*anti*-B[a]PDE was minimal (Osborne *et al.*, 1981; Osborne & Merrifield, 1985; Devanasen *et al.*, 1992); however, these studies did not rule out the possibility that a quantitatively significant adduct was formed but lost during analysis. Because we have quantitatively accounted for all of the radioactivity associated with (+)-*anti*-B[a]PDE adducts, we can conclude that ~95% of all adducts are (+)-*anti*-B[a]P−N^2-Gua, and no quantitatively significant adducts (<~3%)[2] can be missing from our analysis, including an *N*7-Gua adduct. The absence of *N*7-Gua adducts is also consistent with the observation that labile adducts of (+)-*anti*-B[a]PDE do not generate a significant fraction of AP sites *in vitro* (Drouin & Loechler, 1993) or *in vivo* (Moran & Ebisuzaki, 1991). *N*7-Gua adducts are formed from other diol epoxides of benzo[*a*]pyrene [e.g., MacLeod *et al.* (1994)].

In toto, these results suggest that (+)-*anti*-B[a]P−N^2-Gua is the only quantitatively significant adduct formed at G:C base pairs. Both *trans*- and *cis*-(+)-*anti*-B[a]P−N^2-Gua adducts form, but the latter is quantitatively insignificant (Cheng *et al.*, 1989; Sayer *et al.*, 1991). This makes it likely that the mutations observed at guanines are principally due to *trans*-(+)-*anti*-B[a]P−N^2-Gua. Using site-specific means, we previously showed that this adduct can induce G → T mutations (Mackay *et al.*, 1992) and more recently G → A

[5] (Reason 1) The rate constant of hydrolysis was similar for (+)-*anti*-B[a]P−pUB3 initially with 41 versus 10 adducts/plasmid (Table 2), which rules out curvature due to a decrease with time in the number of adducts per plasmid of hydrolysis. (Reason 2) Hydrolyzed (+)-*anti*-B[a]P-tetraols, which acumulate with time, might interact with DNA to decrease the rate of hydrolysis of the remaining adducts. The fraction of adducts hydrolyzed after heating for 24 and 48 h at 70 °C was not affected by the addition of purified (+)-*anti*-B[a]P-tetraols (Table 2), which rules out this explanation. (Reason 3) The rate of adduct hydrolysis did not depend on the initial concentration of DNA (data not shown), and the data in reason 1 argue against hydrolysis being adduct concentration dependent. These results show that the hydrolysis reaction is not a true second-order process in DNA and/or adduct concentration. (Reason 4) The pH was identical before and after heating (data not shown); thus, curvature could not be due to an increase in pH during the reaction, which would decelerate hydrolysis based on Figure 5.

[6] (Reason 5) The fact that copolymers, which are not supercoiled, also showed curvature (Figure 6) suggests that curvature in Figure 3 could not be due to loss of supercoils in (+)-*anti*-B[a]PDE−pUB3 by nicking during hydrolysis [(+)-*anti*-B[a]PDE−pUB3 is ~50% each open circular and linear after 24 h at 70 °C]. (Reason 6) Curvature could be explained if (+)-*anti*-B[a]PDE−pUB3 were being converted from double-stranded to single-stranded DNA with time. Such an effect would have to be independent of adduct concentration based on reason 1. If pUB3 was becoming single-stranded, then it should do so relatively faster at 70 °C than at 37 °C, and thus, hydrolysis would be expected to slow down preferentially at 70 °C, which is not the case (Figure 3).

and G → C as well (Jelinsky and Loechler, unpublished observations). Thus, (+)-*anti*-B[a]P−N^2-Gua can account for all base substitutions, and there is no need to invoke a heat-induced adduct degradation reaction in order to account for any mutations at G:C base pairs.

Implications of Adduct Hydrolysis for Quantitative and Qualitative Changes in Mutagenesis. Heating caused a ~2-fold decrease in MF from (+)-*anti*-B[a]PDE−pUB3 (Introduction; Rodriguez & Loechler, 1993b). As discussed above, the hydrolysis of (+)-*anti*-B[a]P−N^2-Gua probably leaves guanine in DNA (Figure 1), a process that would be expected to decrease MF. However, this is unlikely to be the cause of the ~2-fold decrease in MF, because only ~5% of total (+)-*anti*-B[a]PDE adducts were liberated by the heating procedure used in our mutagenesis study (Rodriguez & Loechler, 1993b). Furthermore, a ~2-fold decrease in MF was also caused by simply freeze-thawing (+)-*anti*-B[a]-PDE−pUB3, which liberated virtually no adducts (<~1%; Rodriguez & Loechler, 1993b). Some of our data suggest that a conformational change from a more to a less mutagenic conformation of a (+)-*anti*-B[a]PDE adduct is more likely to be responsible for the ~2-fold decrease in MF brought about by heating and freeze-thawing (Rodriguez & Loechler, 1995), although this remains an open question.

The mutagenic pattern at G_{115} (the major base substitution hot spot) changed from principally G → T prior to heating to G → T, A, and C when (+)-*anti*-B[a]PDE−pUB3 was heated prior to transformation (Introduction; Rodriguez & Loechler, 1993b). It is impossible to attribute this change to chemistry depicted in Figure 1. The major caveats to this are as follows: (1) the work described herein was with bulk (+)-*anti*-B[a]PDE adducts in pUB3 and not with an adduct at G_{115}, which could behave differently; and (2) there could be a minor chemical reaction that is responsible. These concerns are being investigated, but seem unlikely.

ACKNOWLEDGMENT

We thank Nicholas Geacintov and his colleagues for providing adducted oligonucleotides. We gratefully acknowledge the Cancer Research Program of the National Institutes of Health, Division of Cancer Cause and Prevention, Bethesda MD, for providing (+)-*anti*-B[a]PDE.

REFERENCES

Benasutti, M., Ezzedine, Z. D., & Loecher, E. L. (1988) *Chem. Res. Toxicol. 1*, 160−168.
Cavalieri, E. L., Rogan, E. G., Devanesan, P. D., Cremonesi, P., Cerny, R. L., Gross, M. L., & Bodell, W. J. (1990) *Biochemistry 29*, 4820−4827.
Cheng, S. C., Hilton, B. D., Roman, J. M., & Dipple, A. (1989) *Chem. Res. Toxicol. 2*, 334−340.
Chizhov, O. S., Molodtsov, N. V., & Kochetkov, N. K. (1967) *Carbohydr. Res. 4*, 273−276.
Conney, A. H. (1982) *Cancer Res. 42*, 4875−4917.
Devanesan, P. D., RamaKrishna, N. V. S., Todorovic, R., Rogan, E. G., Cavalieri, E. L., Jeong, H., Jankowiak, R., & Small, G. J. (1992) *Chem. Res. Toxicol. 5*, 302−309.
Dipple, A., Moschel, R. C., Pigott, M. A., & Tondeur, Y. (1985) *Biochemistry 24*, 2291−2298.
Drouin, E. E., & Loechler, E. L. (1993) *Biochemistry 32*, 6555−6562.
Geacintov, N. E. (1986) *Carcinogenesis 7*, 759−766.
Gill, R. D., Rodriguez, H., Cortez, C., Harvey, R. G., Loechler, E. L., & DiGiovanni, J. (1993a) *Mol. Carcinog. 8*, 145−154.
Gill, R. D., Min, Z., Cortez, C., Harvey, R. G., Loechler, E. L., & DiGiovanni, J. (1993b) *Chemical Res. Toxicol. 6*, 681−689.
Groopman, J. D., Croy, R. G., & Wogan, G. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5445−5449.
Harvey, R. G. (1991) *Polycyclic aromatic hydrocarbons: Chemistry and cancer*, Cambridge University Press, Cambridge, U.K.
King, H. W. S., Osborn M. E., & Brookes, P. (1979) *Chem.-Biol. Interact. 24*, 345−353.
Lawley, P. D., & Brookes, P. (1963) *Biochem. J. 89*, 127−138.
Lobanenkov, V. V., Plumb, M., Goodwin, G. H., & Grover, P. L. (1986) *Carcinogenesis 7*, 1689−1695.
Loechler, E. L. (1989) *Biopolymers 28*, 909−927.
Loechler, E. L. (1991) *Methods Enzymol. 203*, 458−476.
Loechler, E. L. (1994) in *The Toxicology of Aflatoxins: Human health, Veterinary, and Agricultural Significance* (Eaton, D. L., & Groopman, J. D., Eds.) pp 149−178, Academic Press Inc., Orlando, FL.
Loechler, E. L., Benasutti, M., Basu, A. K., Green, C. L., & Essigmann, J. M. (1990) in *Progress in Clinical and Biological Research, Vol. 340A, Mutation and the Environment, Part A1, Basic Mechanisms* (Mendelsohn, M. L., & Albertini, R. J., Eds.) pp 51−60, Wiley-Liss, New York.
Mackay, W., Benasutti, M., Drouin, E., & Loechler, E. L. (1992) *Carcinogenesis 13*, 1415−1425.
MacLeod, M. C., Evans, F. E., Lay, J., Chiarelli, P., Geacintov, N. E., Powell, K. L., Daylomg, A., Luna, E., & Harvey, R. G. (1994) *Biochemistry 33*, 2977−2987.
Mao, B., Margulis, L. A., Li, B., Ibanez, V., Lee, H., Harvey, R. G., & Geacintov, N. E. (1992) *Chem. Res. Toxicol. 5*, 773−778.
Marnett, L. J. (1987) *Carcinogenesis 8*, 1365−1373.
Marusyk, R., & Sargent, A. (1980) *Anal. Biochem. 105*, 403−404.
Moran, M. F., & Ebisuzaki, K. (1991) *Mutat. Res. 262*, 79−84.
Osborne, M. R., & Merrifield, K. (1985) *Chem.-Biol. Interact. 53*, 183−195.
Osborne, M. R., Harvey, R. G., & Brooks, P. (1978) *Chem.-Biol. Interact. 20*, 123−130.
Osborne, M. R., Jacobs, S., Harvey, R. G., & Brooks, P. (1981) *Carcinogenesis 2*, 553−558.
Phillips, D. H. (1983) *Nature 303*, 468−472.
Phillips, D. H., Hewer, A., & Grover, P. L. (1985) *Cancer Res. 45*, 4167−4174.
Pruess-Schwartz, D., Baird, W. M., Nikbakht, A., Merrick, B. A., & Selkirk, J. K. (1986) *Cancer Res. 46*, 2697−2702.
Rill, R. L., & Marsch, G. A. (1990) *Biochemistry 29*, 6050−6058.
Rodriguez, H., & Loechler, E. L. (1993a) *Carcinogenesis 14*, 373−383.
Rodriguez, H., & Loechler, E. L. (1993b) *Biochemistry 32*, 1759−1769.
Rodriguez, H., & Loechler, E. L. (1995) *Mutat. Res. 326*, 29−37.
Rodriguez, H., Bhat, U. P., Snow, E. T., & Loechler, E. L. (1992) *Mutat. Res. 270*, 219−231.
Sage, E., & Haseltine, W. A. (1984) *J. Biol. Chem. 259*, 11098−11102.
Sayer, J. M., Chadha, A., Agarwal, H. S. K., Yeh, H. J. C., Yagi, H., & Jerina, D. M. (1991) *J. Org. Chem. 56*, 20−29.
Singer, B., & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.
Taghizadeh, K., & Skipper, P. L. (1994) *Methods Enzymol. 231*, 668−674.
Takahashi, G., Kinoshita, K., Hashimoto, K., & Yasuhira, K. (1979) *Cancer Res. 39*, 1814−1818.

BI941200O