Influence of Na^+ on DNA Reactions with Aromatic Epoxides and Diol Epoxides: Evidence That DNA Catalyzes the Formation of Benzo[*a*]pyrene and Benz[*a*]anthracene Adducts at Intercalation Sites

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Reactions of the benzo[*a*]pyrene (BP) and benz[*a*]anthracene (BA) metabolites, (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydro-BP (BPDE), (±)-*trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydro-BA (BADE), (±)-BP-4,5-oxide (BPO), and (±)-BA-5,6-oxide (BAO), were examined under pseudo-first-order conditions at varying Na^+ (2.0−100 mM) and native, calf thymus DNA (ctDNA) concentrations. In 0.2 mM ctDNA and 2.0 mM Na^+, at a pH of 7.3, most BPDE, BADE, BPO, and BAO (87−95%) undergo DNA catalyzed hydrolysis or rearrangement. For BPDE and BPO, overall, pseudo-first-order rate constants, k, in 2.0 mM Na^+ and 0.2 mM ctDNA are 21−72 times larger than values obtained without DNA. For BADE and BAO, the rate constants are less strongly influenced by DNA; k values in 0.2 mM ctDNA are only 9−12 times larger than values obtained without DNA. Kinetic data for BPDE, BPO, BADE, and BAO and DNA intercalation association constants (K_A) for BP and BA diols which are model compounds indicate that K_A values for BPDE and BPO in 2.0 mM Na^+ are 6.6−59 times larger than those of BADE and BAO. The greater DNA enhancement of rate constants for BPDE and BPO, versus BADE and BAO, correlates with the larger K_A values of the BP metabolites. DNA adducts, which account for less than 10% of the yields, also form. For BPDE in 0.20 mM ctDNA, k decreases 5.1 times as the Na^+ concentration increases from 2.0 to 100 mM. Nevertheless, the DNA adduct level remains constant over the range of Na^+ concentrations examined. These results provide evidence that, for BPDE in 0.20 mM DNA and 2.0 mM Na^+, ctDNA adduct formation follows a mechanism which is similar to that for DNA catalyzed hydrolysis. The pseudo-first-order rate constant for adduct formation, k_{Ad}, given approximately by $k_{Ad} \approx (k_{cat,Ad}K_A[DNA])/(1 + K_A[DNA])$, where $k_{cat,Ad}$ is a catalytic rate constant. For BADE, BPO, and BAO, the influence of varying DNA and Na^+ concentrations on k values is similar to that for BPDE, and provides evidence that the formation of adducts follows the same rate law.

Introduction

Covalent DNA modification by epoxide containing metabolites of polycyclic aromatic hydrocarbons (PAHs)[1] is important to carcinogenic mechanisms involving these molecules (*1*−*3*). Figure 1 shows structures of four epoxides, (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene (BPDE), (±)-benzo[*a*]pyrene 4,5-oxide (BPO), (±)-*trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydrobenz[*a*]anthracene (BADE), and (±)-benz[*a*]anthracene 5,6-oxide (BAO), which are derived from benzo[*a*]pyrene (BP) or benz[*a*]anthracene (BA). BPDE and BADE are bay region diol epoxides, while BPO and BAO are K region epoxides. Generally, bay region metabolites are more genotoxic and chemically reactive than K region metabolites (*3*−*7*). Investigation of PAH diol epoxide reactivities has led to the *bay region theory*. The theory relates the π electronic structure of diol epoxides to the reactivities of these metabolites toward biochemical targets, such as DNA (*3*). In this way, reactivity influences carcinogenic potency. A large number of factors affect genotoxic potency (*1*, *3*, *8*−*12*); nevertheless, predictions based on chemical reactivity often correlate with the mutagenic activities of PAH diol epoxides (*3*).

In aqueous solution with DNA, BPDE hydrolyzes to 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-BP (BP tetrol) and forms DNA adducts. These reactions are shown in Figure 2. Adduct formation occurs primarily at the 2-amino group of guanine (*1*). Hydrolysis is both spontaneous and catalyzed by DNA (*6*, *7*, *13*−*17*). The hydrolysis and adduct reactions of BPDE proceed via transition states with carbocationic character at the C-10 atom of BPDE (*3*).

At low BPDE concentrations, under pseudo-first-order conditions, the overall, pseudo-first order rate constant (k) has contributions (see following equation) from hydrolysis (k_{Hy}) and from reaction leading to DNA adducts (k_{Ad}) (*6*, *7*, *13*−*16*).

$$k = k_{Hy} + k_{Ad} \quad (1)$$

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[1] Abbreviations: BA, benz[*a*]anthracene; BA34D, *trans*-3,4-dihydroxy-3,4-dihydro-BA; BA56D, *cis*-5,6-dihydroxy-5,6-dihydro-BA; BADE, (±)-*trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydro-BA; BAO, (±)-BA-5,6-oxide; BA tetrol, 1,2,3,4-tetrahydroxy-1,2,3,4-tetrahydro-BA; BP, benzo[*a*]pyrene; BP45D, *trans*-4,5-dihydroxy-4,5-dihydro-BP; BP78D, *trans*-7,8-dihydroxy-7,8-dihydro-BP; BPDE, (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydro-BP; BPO, (±)-BP-4,5-oxide; BP tetrol, 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-BP; ctDNA, calf thymus DNA; 5-OH-BA, 5-hydroxy-BA; 6-OH-BA, 6-hydroxy-BA; 4-OH-BP, 4-hydroxy-BP; 5-OH-BP, 5-hydroxy-BP; PAH, polycyclic aromatic hydrocarbon; poly(A), poly(riboadenylic acid); poly(C), poly(ribocytidylic acid); poly(G), poly(riboguanylic acid); poly(I), poly(riboinosinic acid); poly(U), poly(ribouridylic acid).

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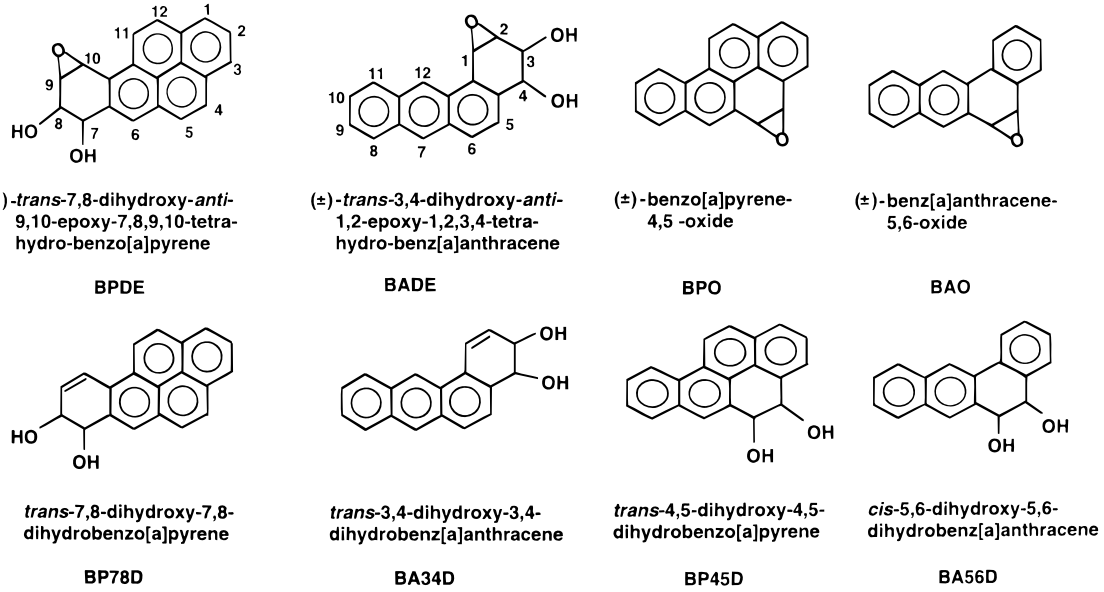

Figure 1. Structures of the bay region diol epoxides, BPDE and BADE, of the K region epoxides, BPO and BAO, and of the model compounds BP78D, BA34D, BP45D, and BA56D.

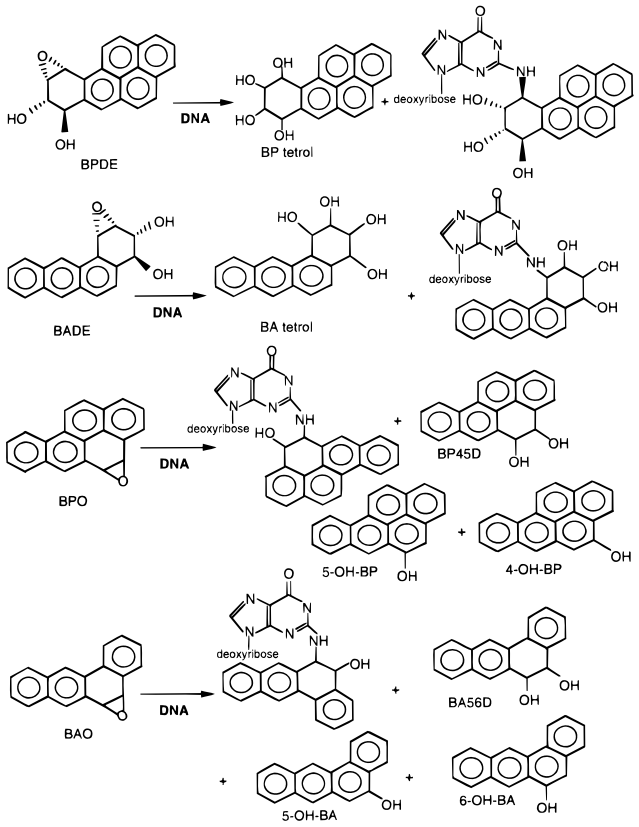

Figure 2. Major pathways for reactions of BPDE, BADE, BPO, and BAO in buffer with DNA. For BPO and BAO, adduct structures are based on preliminary results indicating that guanine is a major target site, and on the structures of the primary BPDE and BADE adducts. For BPO and BAO, the C-5 and C-6 adducts are shown, respectively. However, the formation of C-4 adducts of BPO and C-5 adducts of BAO cannot be ruled out. See refs 1, 19−21, 24, and 25.

BADE, like BPDE, forms DNA adducts and hydrolyzes (*18*). The hydrolysis product is 1,2,3,4-tetrahydroxy-1,2,3,4-tetrahydro-BA (BA tetrol). BPO and BAO, while less reactive than BPDE and BADE, also form DNA adducts (*19*−*21*). Like BPDE, BADE adducts form most favorably at the 2-amino group of guanine (*22*). For BPO and BAO, adduct structures have not been fully characterized. However, preliminary evidence indicates that significant reaction occurs at guanine, and, for BPO, it is likely that the 2-amino group of guanine is a major target site (*19*, *21*). For BAO, adduct yields in reactions with poly(G) are more than 5 times greater than yields with poly(U), poly(C), poly(A), or poly(I) (*20*). In addition to adduct reactions, BPO and BAO undergo DNA catalyzed hydrolysis to diols (4,5-dihydroxy-4,5-dihydro-BP and 5,6-dihydroxy-5,6-dihydro-BA) and rearrangement to phenols (*6*, *23*). For BPO and BAO, the following equation gives the overall, pseudo-first-order rate constant, where k_{Re} is the pseudo-first-order rate constant for rearrangement (*6*).

$$k = k_{Hy} + k_{Ad} + k_{Re} \qquad (2)$$

Without DNA, BPO and BAO have major reaction pathways leading to hydrolysis and rearrangement (*6*, *24*). While only one BPO rearrangement product, 4-hydroxy-BP (4-OH-BP), was reported in the DNA catalyzed rearrangement of BPO (*23*), results from later reactivity measurements (*25*) suggest that 5-hydroxy-BP is also formed. Analysis of relative yields of 5-hydroxy-BA (5-OH-BA) and 6-hydroxy-BA (6-OH-BA) formed via DNA catalyzed rearrangement of BAO has not been reported. However, in 1:9 dioxane−water with 0.1 M $NaClO_4$ at a pH of 4.3, BAO, without DNA, forms 5-OH-BA and 6-OH-BA in the ratio 2:1 (*24*). At pH 4.3, the BAO hydrolysis yield is 37% (*24*); however, at pH 7.1, almost all reaction proceeds via hydrolysis (*6*). Figure 2 shows products of BADE, BPO, and BAO reactions with DNA.

In buffer with DNA, BPDE hydrolysis accounts for approximately 90% of the total reaction (*6*). The majority of investigations indicate that the catalyzed hydrolysis in double-stranded DNA relies on the formation of intercalated complexes (*6*, *7*, *14*, *15*, *26*−*28*). Similarly, for BPDE hydrolysis in closed-circular, single-stranded DNA, current results provide evidence that catalysis occurs at local regions of the single-stranded DNA where base stacking occurs (*7*). Equation 3 gives the pseudo-first-order rate constant (k_{Hy}) for BPDE hydrolysis with DNA (*15*). Here, $k_{cat,Hy}$ is the rate constant for DNA catalyzed hydrolysis; $k_{0,Hy}$, the rate constant for spontaneous hydrolysis; $k_{H,Hy}$, the rate constant for H^+ catalyzed hydrolysis without DNA; and K_A, the intercalation association constant.

$$k_{Hy} = \frac{k_{0,Hy} + k_{H,Hy}[H^+] + k_{cat,Hy}K_A[DNA]}{1 + K_A[DNA]} \quad (3)$$

In eq 3, $k_{0,Hy}(1 + K_A[DNA])^{-1}$ is the contribution to k_{Hy} from spontaneous hydrolysis; $k_{H,Hy}[H^+](1 + K_A[DNA])^{-1}$, the contribution from specific acid catalyzed hydrolysis; and $k_{cat,Hy}K_A[DNA](1 + K_A[DNA])^{-1}$, the contribution from catalysis at intercalation sites. Earlier investigations indicate that DNA enhanced BPDE hydrolysis occurs via general acid catalysis involving phosphate or exocyclic amino groups on the bases (*2*, *13*, *27*).

Without DNA, at Na^+ concentrations below 100 mM, Na^+ has negligible influence on BPDE hydrolysis (*7*). However, with DNA, the pseudo-first-order rate constant for hydrolysis decreases as Na^+ concentrations increase in the range 2.0−100 mM. This is due to Na^+ induced decreases in the association constant, K_A, and catalytic rate constant, $k_{cat,Hy}$ (*7*, *15*). For BPDE at a pH of 7.3, an increase in the Na^+ concentration from 2.0 to 100 mM causes K_A and $k_{cat,Hy}$ to decrease 1.8−2.7 and 4.8−9.0 times, respectively (*7*).

Earlier investigations of intercalation association constants employed the diols *trans*-7,8-dihydroxy-7,8-dihydro-BP (BP78D), *trans*-4,5-dihydroxy-4,5-dihydro-BP (BP45D), *trans*-3,4-dihydroxy-3,4-dihydro-BA (BA34D), and *cis*-5,6-dihydroxy-5,6-dihydro-BA (BA56D) as model compounds of BPDE, BPO, BADE, and BAO, respectively (*6*, *7*, *29*, *30*). The parallel between the intercalation association constants of the models and of the corresponding epoxide containing metabolites is supported by the observation that the ratio of ctDNA association constants for BPDE versus BPO is similar to the ratio of association constants for BP78D versus BP45D (*6*). Figure 1 shows structures of the model compounds. A comparison of results from fluorescence quenching (*7*, *29*, *31*, *32*), UV absorption (*29*, *32*), linear dichroism (*33*), and dialysis binding measurements (*7*, *29*, *31*) demonstrates that, in the concentration range 10^{-6} to 10^{-7} M, virtually all reversible binding between BP and BA derivatives with native, double-stranded DNA occurs via intercalation (*7*, *29*, *31*).

Because DNA adduct formation is a minor pathway, the mechanism is not well understood (*2*, *13*, *14*, *16*, *17*). For reaction of BPDE with DNA, a decrease in pH causes an increase in the pseudo-first-order rate constant for hydrolysis. A proportional increase in the rate of covalent binding also occurs, with the net effect that covalent binding levels remain constant (*13*, *34*). This has led to speculation (*13*) that DNA catalyzes covalent adduct formation at intercalation sites, and, contrary to conclusions from other experiments, that catalyzed hydrolysis occurs at external sites. In contrast, it has also been speculated that hydrolysis of BPDE is catalyzed at intercalation sites, while covalent binding results from reaction at external sites (*17*). A third description invokes a mechanism in which hydrolysis and covalent adducts form in the same reversibly bound domains of DNA (*14*, *26*, *34*). The more recent development of a general theory (*16*) for mechanisms of DNA reaction with BPDE considers two possibilities. The first is that catalysis of either hydrolysis or covalent binding occurs in reversibly bound intercalation complexes. The second is that these reactions occur without complex formation. The results indicate that either possibility leads to equations for overall, pseudo-first-order rate constants and covalent binding fractions with the same DNA concentration dependence. Earlier theoretical and experimental investigations have not led to consensus concerning the relationship between reactions leading to BPDE hydrolysis and to DNA modification, nor to agreement concerning the role that intercalation plays in adduct reactions. A great deal remains to be elucidated regarding the reactions of hydrocarbon epoxides with DNA (*2*).

The goal here is to examine the relationship between BPDE, BADE, BPO, and BAO reactions leading to DNA adduct formation and reactions leading to hydrolysis or rearrangement. The BP and BA diol epoxides and epoxides examined were chosen because they exhibit a wide variation in reactivities, and in DNA intercalation association constants. The relationships between reactions leading to adduct formation, rearrangement, and hydrolysis in DNA were examined over Na^+ concentrations where the overall, pseudo-first-order rate constants of the diol epoxides and epoxides vary 2.7−15.4 times, and the model compound intercalation association constants vary 2.3−3.3 times.

Experimental Section

Fluorescence emission spectra were measured with a Perkin-Elmer 650-10 fluorescence spectrometer. Magic angle fluorescence lifetime measurements employed a Photochemical Research Associates Model 2000 nanosecond fluorescence spectrophotometer equipped with aberration-corrected optics. A Glan polarizer was mounted on the excitation side and a film polarizer on the emission side. Ultraviolet absorption spectra were measured with a Cary 17 spectrometer. Samples used in fluorescence experiments were contained in 1 cm cells.

Samples of BPDE, BADE, BPO, BAO, *trans*-7,8-dihydroxy-7,8-dihydro-BP (BP78D), *trans*-4,5-dihydroxy-4,5-dihydro-BP (BP45D), *trans*-3,4-dihydroxy-3,4-dihydro-BA (BA34D), and *cis*-5,6-dihydroxy-5,6-dihydro-BA (BA56D) were purchased from Chemsyn Science Laboratories (Lenexa, KS). 9,10-Dimethylanthracene was obtained from Sigma Chemical Co. (St. Louis, MO). Native calf thymus DNA (ctDNA) was purchased from Worthington (Freehold, NJ) and was found to have a hypochromicity of 35%. ctDNA concentrations are reported in terms of PO_4^- molarity calculated from an average base-pair molecular weight of 617.8. This is based on a calf thymus DNA composition which is 60% A-T base pairs (*31*). Concentrations have been corrected for the amounts of H_2O and Na^+ reported by the supplier for each DNA batch. From the DNA absorption spectrum, it was found that one A_{260} unit equals 1.5×10^{-4} M $[PO_4^-]$ (*31*). In control experiments, ctDNA obtained from the supplier was purified by using a phenol−chloroform procedure (*31*) which yielded an A_{260}/A_{280} ratio of 2.0. When Stern−Volmer quenching constants for 9,10-dimethylanthracene were compared in experiments using purified DNA and DNA directly from the supplier, the difference was less than 10%. Subsequent experiments were performed without repurification of the DNA. All experiments were carried out in 10 mM Tris·HCl and 1.0 mM EDTA buffer at pH 7.3. The temperature was maintained at 23 ± 0.5 °C with an Isotemp Circulator Model 210. **Caution:** *BPDE, BPO, BADE, BAO, BP78D, BP45D, BA34D, and BA56D are hazardous chemicals which were handled in accordance with NIH guidelines (35).*

In order to introduce anions, as well as cations, which occur under physiological conditions, Na^+ concentrations were adjusted by addition of NaCl (*36*). While the present experiments focus on the effects which Na^+ has on DNA catalysis, earlier investigations indicate that Cl^- also influences reactions of diol epoxides and epoxides (*25*, *36*, *37*, *38*). For BPDE reactions with ctDNA, Cl^- enhances the formation of *cis* opened hydrolysis products and adducts, at the expense of *trans* opened products, through the formation of chlorohydrin intermediates (*36*, *37*). However, with ctDNA, a significant decrease in the formation of *trans* products (*36*) has only only been observed at much higher Cl^- concentrations (1 M) than the concentrations employed here, which never exceed 100 mM. In reactions of the (+) and (−) enantiomers of BPDE with poly(G) at a Cl^-

concentration (250 mM) which is 2.5 or more times greater than the Cl^- concentrations employed in the present experiments, the yield of *trans* opened BPDE adducts (*36*) is less than 15% smaller than the yield without Cl^-. Similarly, in 3 mM ctDNA at a pH of 7.0, the percentage of BPDE hydrolysis resulting in *trans* products decreases only slightly, from 93% to 91%, as the NaCl concentration increases from 0 to 100 mM. With DNA, the influence of Cl^- on overall, pseudo-first-order rate constants, *k*, has not been observed. Without DNA, significant effects of NaCl on *k* values for PAH epoxide hydrolysis and rearrangement reactions have, again, only been observed (*37*, *38*) at high Cl^- concentrations (0.5−1 M). For BPDE and BPO, without DNA, at the low buffer concentrations used in the present experiments, *k* values measured in 100 mM Cl^- are the same as those measured without Cl^- (*7*). These observations strongly indicate that the effects of the secondary chlorohydrin mechanism on the results presented here are negligible.

Overall, Pseudo-First-Order Rate Constants. All reactions were carried out under pseudo-first-order conditions with concentrations of diol epoxides and epoxides in the range 10^{-6} to 10^{-7} M. Overall, pseudo-first-order rate constants of BPDE, BADE, BPO, and BAO were obtained by measuring the increase in fluorescence intensity which occurs as products are formed. The epoxides have negligible fluorescence intensities compared to the DNA adducts or to the hydrolysis and rearrangement products (*6*, *7*, *18*). Excitation wavelengths used to monitor BPDE, BADE, BPO, and BAO reactions were 350, 365, 330, and 310 nm, respectively. The emission wavelengths were 404, 410, 388, and 365 nm. Each reaction was initiated by injecting 20 μL of a THF solution containing BPDE, BADE, BPO, or BAO into 1.0 mL of buffer with or without ctDNA (*6*, *7*). Overall, pseudo-first-order rate constants are equal to minus the slopes of least-squares linear fits of plots of $\ln([I_{max} - I(t)]/[I_{max} - I_0])$ versus *t*. Here, I_{max} is the maximum fluorescence intensity measured after the reaction was completed, $I(t)$ is the intensity at time *t*, and I_0 is the intensity when the reaction was initiated. In the present experiments, the fluorescence emission intensities, extrapolated to the time at which the reactions were initiated, were less than 8% of the intensities observed at the completion of the reactions. For the experiments reported here, the overall, pseudo-first-order rate constants for BPDE and BPO, with and without ctDNA, differ by factors of 1.4−2.4 from values reported earlier (*7*). These differences are due to the improved temperature control which was employed in the current experiments.

Stern−Volmer Quenching Constants. Stern−Volmer plots were obtained from measurements of fluorescence quenching caused by ctDNA. Quenching measurements were carried out on BP78D, BA34D, BP45D, and BA56D which are model compounds for BPDE, BADE, BPO, and BAO, respectively. Hydrocarbon concentrations were in the range 10^{-6} to 10^{-7} M. Excitation and emission wavelengths used for each model compound were the same as those used to measure the overall, pseudo-first-order rate constants of the corresponding diol epoxide or epoxide. Quenching constants, K_{SV}, were obtained from Stern−Volmer plots and are equal to the slopes of least-squares fits to plots of the ratio I_0/I versus the ctDNA concentration. Here, I_0 corresponds to the fluorescence emission intensity of the model compound without ctDNA, and *I* corresponds to the intensity with DNA. For BP78D, BA34D, BP45D, and BA56D, the slopes of the Stern−Volmer plots (K_{SV}) are equal to association constants (K_A) for intercalation into DNA (*6*, *7*, *30*).

Fluorescence Lifetimes. Fluorescence lifetime measurements of BP78D, BP45D, BA34D, and BA56D were carried out at the same excitation and emission wavelengths used in the fluorescence quenching measurements. The quenching of the diol fluorescence by ctDNA required that, in measurements with DNA, a subtraction procedure be employed to correct for scattered light. In this procedure, the decay profile of a ctDNA blank was subtracted from the profile measured for a diol with DNA (*7*, *29*). Lifetime measurements with ctDNA were carried out at DNA concentrations of 0.10, 0.20, 0.50, and 0.50 mM for BP78D, BP45D, BA34D, and BA56D, respectively. The more weakly quenched diols (BP45D, BA34D, and BA56D) were examined at higher ctDNA concentrations than BP78D, which is strongly quenched. Analysis of the lifetime data was carried out using a least-squares deconvolution method (*6*, *7*).

Adduct and Hydrolysis Yields. BPDE, BADE, BPO, and BAO adduct yields were determined for reactions in 0.20 mM ctDNA at varying Na^+ concentrations. BPO and BAO hydrolysis yields were also determined under these conditions, and for reactions in buffer without ctDNA. The reactions were run under the same conditions as the kinetics experiments. Reactions in ctDNA were initiated by adding 20 μL of BPDE, BADE, or BPO in THF, or 20 μL of BAO in 95% ethanol, to a conical tube containing 0.20 mM ctDNA in 1.0 mL of buffer. For the analysis of BPO and BAO hydrolysis yields without DNA, the buffer conditions were the same as for reactions with DNA. The separation of ctDNA adducts employed a method similar to that described previously (*6*). After the reactions were completed, samples containing DNA adducts were extracted 3 times with 3.0, 2.0, and 2.0 mL of buffer saturated ethyl acetate.[2] This removed the hydrolysis and rearrangement products from the adducts, which remained in the aqueous phase. Ethyl acetate dissolved in the buffer was removed by placing the sample in boiling water for 1 min. The covalently bound adducts were released by HCl hydrolysis for 1.5 h in sealed tubes. For BPDE and BADE adduct samples in buffer, the hydrolysis was carried out by adding 50 μL of 1 M HCl and heating to 93 °C. For BPO and BAO adduct analysis, 20 μL of HCl was added and the samples were heated to 89 °C. By carrying out one set of control experiments in which the reaction times were varied, and a second set of control experiments in which standard samples of BP tetrol, BA tetrol, BP45D, and BA56D were subjected to the hydrolysis conditions, it was determined that the hydrolysis reaction conditions, which were employed, resulted in complete adduct hydrolysis without decomposition of the hydrolysis products (*6*). After cooling, the pH of the solutions containing the hydrolyzed adducts was adjusted to 4.5 by adding 2.0 M NaOH. Adducts from BPDE and BADE were analyzed as BP tetrol and BA tetrol; adducts from BPO and BAO were analyzed as 4,5-dihydroxy-4,5-dihydro-BP and 5,6-dihydroxy-5,6-dihydro-BA.

(A) Determination of BPDE and BADE Adduct Yields. The observation that reactions of BPDE and BADE, in buffer without DNA, lead exclusively to BP tetrol and BA tetrol was exploited in the preparation of standards for determining BPDE and BADE adduct yields. The percentage of the initial BPDE or BADE reactant which formed DNA adducts was quantified with a standard BP tetrol or BA tetrol solution prepared from the same solution of diol epoxide in THF which was employed to prepare the adducts. The standards were obtained by hydrolyzing 20 μL of the THF solution containing BPDE or BADE in 1.0 mL of buffer without DNA. The standards were treated in the same manner as the samples. To each standard, 0.5 mL of ethyl acetate was added and then removed by placing in boiling water. Each standard was also acidified with HCl, heated, and neutralized with NaOH in the same manner as the sample. To measure the percentage of diol epoxide which formed adducts in reactions with ctDNA, the fluorescence intensity of the BP tetrol or BA tetrol obtained via hydrolysis of the adducts was compared to that of the corresponding standard at the same emission and excitation wavelengths employed in the BPDE or BADE kinetics measurements.

(B) Determination of BPO and BAO Adduct and Hydrolysis Yields. The analysis of BPO and BAO adduct yields was also carried out using standards of the products formed in the adduct hydrolysis reactions. However, because BPO and BAO, in buffer without DNA, not only hydrolyze to diols but can also rearrange to phenols, the method used to prepare diol standards for analysis of BPO and BAO adducts was different from that used to prepare tetrol standards. The percentage of the epoxide which formed ctDNA adducts was calculated from the number of moles of reactant epoxide, which was determined from UV absorption measurements of the initial epoxide con-

[2] Products from reactions of BPO and BAO in buffer without ctDNA were also extracted in this manner. The hydrolysis products were then analyzed in ethyl acetate.

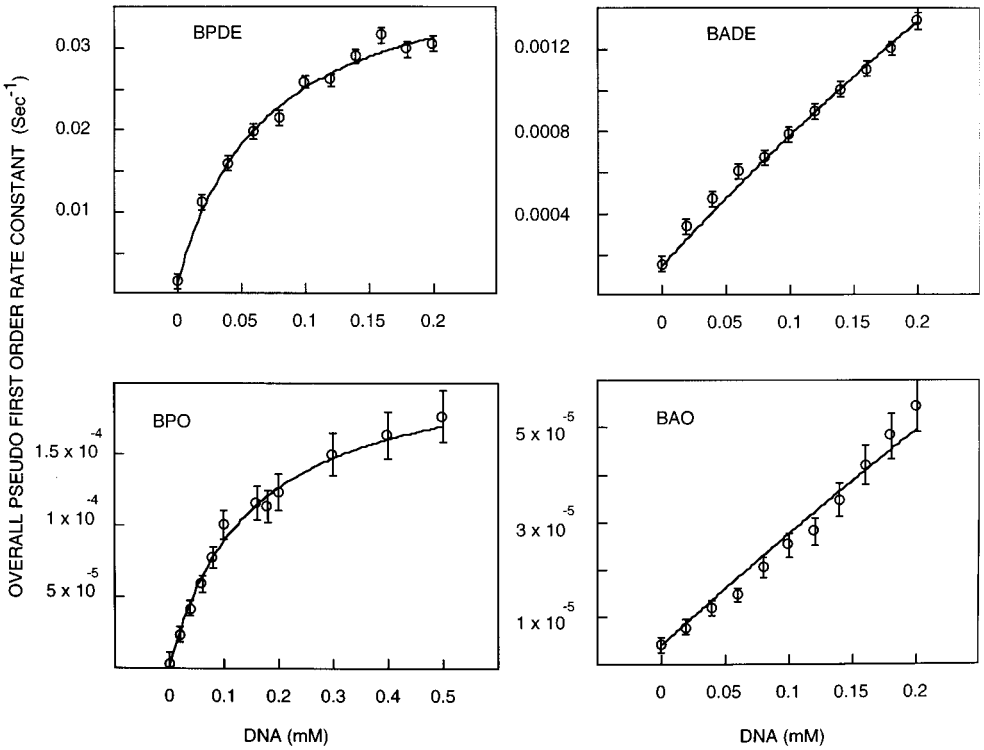

Figure 3. Overall, pseudo-first-order rate constants, k, of BPDE, BADE, BPO, and BAO versus ctDNA concentration. Solid lines show least-squares fits to the rate constants obtained using eq 4. For BPDE and BPO, values of k_{cat} and K_A were optimized to obtain the calculated curves. For BADE and BAO, values of K_A were based on association constants for the model compounds BA34D and BA56D, respectively, and values of k_{cat} were optimized to obtain the calculated curves. See text.

centration,[3] and from the number of moles of diol formed after hydrolysis of the resultant DNA adducts. Determination of the diol concentration resulting from hydrolysis of BPO or BAO adducts was carried out by comparing the fluorescence intensity of the hydrolysis product with that of a BP45D or BA56D standard solution in buffer, for which the concentration was determined by UV absorption.[3] The fluorescence intensities of the adduct hydrolysis products and the standard solutions were compared at the same excitation and emission wavelengths used in the epoxide kinetic measurements. Like the tetrol standard solutions, the BP45D and BA56D standards were treated in a manner identical to the diols formed by hydrolysis of the ctDNA adducts.

The percentage of BPO or BAO which formed hydrolysis products in reactions with or without ctDNA was determined by analyzing the reaction products contained in the ethyl acetate extract obtained after the reactions in buffer were completed. The percentage of BPO or BAO which hydrolyzed was determined by comparing the fluorescence intensity of the hydrolysis products with that of a BP45D or BA56D standard which was prepared in ethyl acetate.[3]

Results

Figure 3 shows plots of the overall, pseudo-first-order rate constants (k) for BPDE, BADE, BPO, and BAO at varying ctDNA concentrations. Without DNA, the ordering of the rate constants is BPO ($(1.71 \pm 0.2) \times 10^{-6}$ s^{-1}) < BAO ($(4.15 \pm 0.3) \times 10^{-6}$ s^{-1}) < BADE ($(1.47 \pm 0.2) \times 10^{-4}$ s^{-1}) < BPDE ($(1.44 \pm 0.2) \times 10^{-3}$ s^{-1}). With ctDNA, the data in Figure 3 are similar for BPDE, BPO, BADE, and BAO. As the ctDNA concentration increases, values of k for all of the epoxides and diol epoxides increase. However, the results in Figure 3 also point out small differences between the behavior of BPDE and BPO versus BADE and BAO. For example, the results for BPDE and BPO show evidence of saturation kinetics. At the higher ctDNA concentrations, the increases in the rate constants of BPDE and BPO, caused by increases in ctDNA concentration, become small. In contrast, the results for BADE and BAO do not exhibit evidence of saturation over the range of ctDNA concentrations examined. For BADE and BAO, the rates at which k increases with increasing ctDNA concentration are nearly constant. Furthermore, the results in Figure 3 demonstrate that, when compared to rate constants without DNA, ctDNA causes a greater increase in k values of BPDE and BPO than of BADE and BAO. In 0.20 mM ctDNA, the rate constants of BPDE and BPO are 21.2 and 71.9 times larger than rate constants without DNA. For BADE and BAO in 0.20 mM ctDNA, the rate constants are only 9.1 and 12.0 times larger than rate constants without DNA. The results in Figure 3 also demonstrate that the more effective way in which DNA enhances the rate constants of the BP metabolites causes the ordering of the overall, pseudo-first-order rate constants obtained with ctDNA to differ from that obtained without DNA. Without DNA, the rate constant of BAO is larger than that of BPO; in 0.2 mM ctDNA, the rate constant of BPO ($(1.23 \pm 0.1) \times 10^{-4}$ s^{-1}) is larger than that of BAO ($(5.00 \pm 0.6) \times 10^{-5}$ s^{-1}).

Results from earlier investigations (*6*, *7*, *29*, *31*) have shown that Stern−Volmer quenching constants (K_{SV}) obtained from measurements of the fluorescence quenching of BP and BA derivatives by double-stranded DNA are equal to intercalation association constants (K_A) when the fluorescence lifetimes of the metabolites measured, with and without DNA, are equal. This occurs if the

[3] Extinction coefficients, in M L^{-1} cm^{-1}, for BPO ($\epsilon_{276} = 118\,537$) in THF, and for BAO ($\epsilon_{270} = 54\,577$), P45D ($\epsilon_{273} = 90\,950$), and BA56D ($\epsilon_{267} = 43\,284$) in 95% ethanol, were obtained from Chemsyn Science Laboratories product data sheets. To prepare standard solutions of BP45D and BA56D in ethyl acetate or in buffer, 20 μL of a known concentration of the diol in 95% ethanol was added to 1 mL of ethyl acetate or buffer. In the quantification of the BPDE, BADE, BPO, and BAO adducts, and of the BPO and BAO hydrolysis yields, the extinction coefficients and the fluorescence quantum yields of corresponding *cis* and *trans* tetrols, and *cis* and *trans* diols, were taken to be equal.

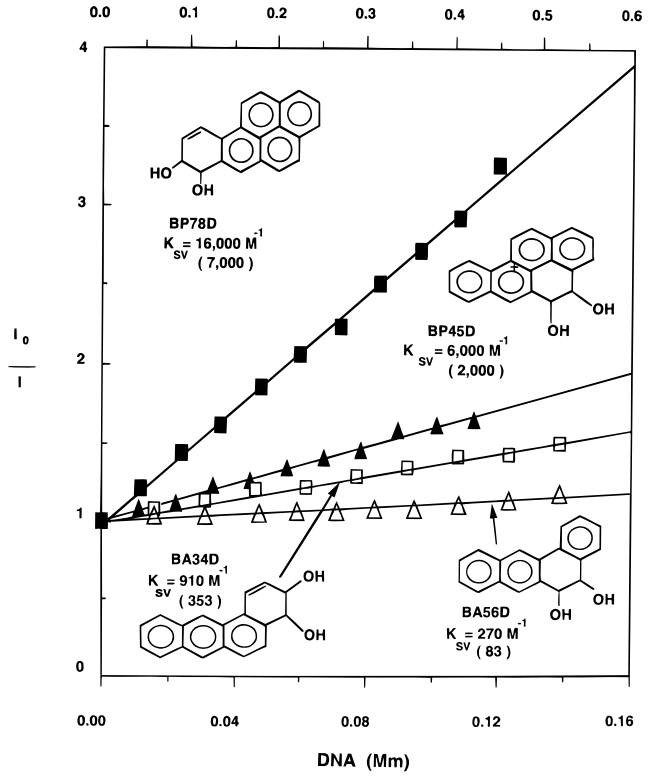

Figure 4. Stern−Volmer plots for the ctDNA quenching of BP78D (closed squares), BP45D (closed triangles), BA34D (open squares), and BA56D (open triangles) in 2.0 mM Na^+. Excitation wavelengths were 350, 365, 330, and 310 nm, respectively. Emission wavelengths were 404, 410, 388, and 365 nm. DNA concentrations are given on the bottom scale for BP78D and BP45D, and on the top scale for BA34D and BA56D. Stern−Volmer quenching constants obtained from least-squares fits of the data are given without parentheses. Stern−Volmer quenching constants measured in 100 mM Na^+ are given in parentheses.

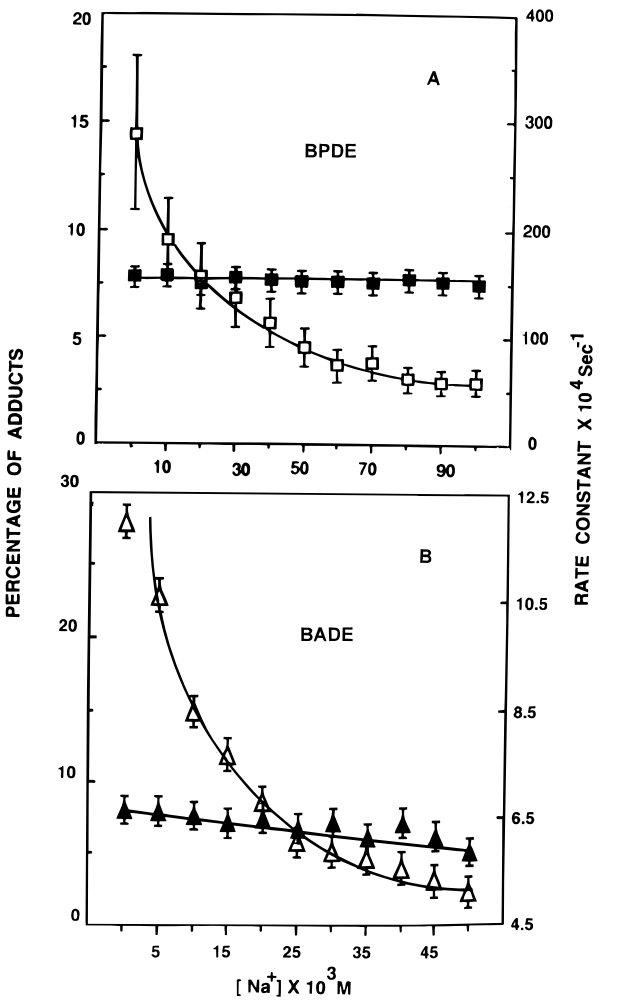

Figure 5. (A) Overall, pseudo-first-order rate constants of BPDE (open squares) and percentage of BPDE resulting in ctDNA adducts (closed squares) at varying Na^+ concentrations. (B) Overall, pseudo-first-order rate constants of BADE (open triangles) and percentage of BADE forming ctDNA adducts (closed triangles) at varying Na^+ concentrations. Adduct yields are given on the left-hand scales. Overall, pseudo-first-order rate constants are given on the right-hand scales. The ctDNA concentration was 0.2 mM.

fluorescence quenching is static, and if the fluorescence quantum yields of the intercalated BP or BA derivatives are negligible compared to those of the unbound molecules (*29*, *31*). Figure 4 shows Stern−Volmer plots, measured in 2.0 mM Na^+, for the fluorescence quenching of the model compounds, BP78D, BP45D, BA34D, and BA56D, by ctDNA. The figure shows the ratio of fluorescence intensity measured without DNA (I_0) over the intensity measured with ctDNA (I) as a function of DNA concentration. In addition to Stern−Volmer quenching constants measured in 2.0 mM Na^+, the figure also shows Stern−Volmer quenching constants measured in 100 mM Na^+.

Measurements of the influence of ctDNA on the fluorescence lifetimes of the diol epoxide and epoxide model compounds were carried out in order to determine whether, for these molecules, K_{SV} equals K_A under the conditions at which the kinetic experiments were carried out. Fluorescence decay profiles of the BADE and BPDE model compounds, BA34D and BP78D, were measured in 10 mM Tris·HCl and 1.0 mM EDTA buffer at pH 7.3, with and without ctDNA. For BP78D, BP45D, BA34D, and BA56D, the ctDNA concentrations used were 0.1, 0.2, 0.5, and 0.5 mM, respectively. Fluorescence lifetimes were obtained by fitting the decay profiles with a single-exponential decay law. Within experimental uncertainty, the fluorescence lifetimes are unchanged when ctDNA is added. For BP78D, BP45D, BA34D, and BA56D, fluorescence lifetimes without DNA are 25.1, 20.4, 7.37, and 44.1 ns, respectively. The χ^2 values are 1.01, 1.08, 1.02, and 1.11. With ctDNA, the fluorescence lifetimes are 24.2, 19.8, 7.36, and 43.9 ns; and the χ^2 values are 0.98, 1.10, 0.97, and 1.16. Results from fluorescence lifetime measurements support the conclusion that the fluorescence quenching shown in Figure 4 is static; that the fluorescence quantum yields of the diols bound to ctDNA are negligible; and that K_{SV} equals K_A.

Figure 3 indicates that the reactivities of BPDE and BPO are more effectively enhanced by ctDNA than are the reactivities of BADE and BAO. Figure 4 indicates that the intercalation association constants of the BPDE and BPO model compounds are larger than those of the BADE and BAO model compounds. In 2.0 mM Na^+, the intercalation association constants of BP78D and BP45D are 6.6−59 times larger than the association constants of BA34D and BA56D. The data in Figure 4 also indicates that an increase in the Na^+ concentration from 2.0 to 100 mM is accompanied by a 2.3−3.3-fold decrease in the K_A values of the model compounds. These data are consistent with previously reported results (*7*, *14*, *15*, *28*, *39*).

Figure 5 shows overall, pseudo-first-order rate constants of BPDE and BADE in ctDNA, and the percentages of these diol epoxides which form ctDNA adducts at varying Na^+ concentrations. Panel A of Figure 5 shows results for BPDE. In Panel A, *k* decreases 5.1

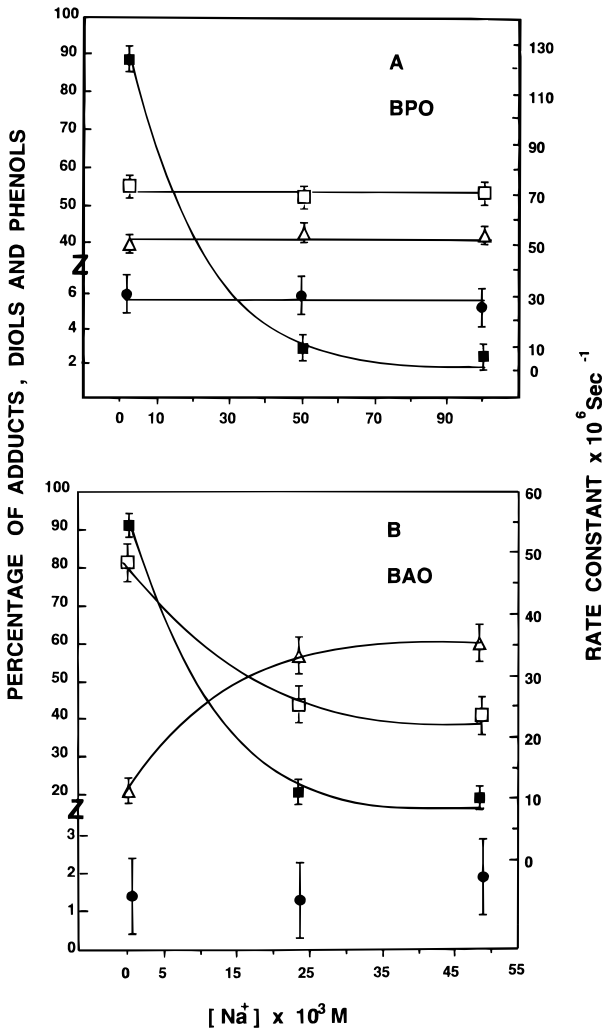

Figure 6. Overall, pseudo-first-order rate constants (closed squares) and percentage yields of ctDNA adducts (closed circles), and hydrolysis (open triangles) and rearrangement products (open squares) in 0.2 mM ctDNA at varying Na^+ concentrations. Panels A and B show results for BPO and BAO, respectively. Adduct, hydrolysis, and rearrangement yields are given on the left-hand scales. Overall, pseudo-first-order rate constants are given on the right-hand scales. Yields for rearrangement products were obtained by subtraction using results from the determination of adduct and hydrolysis yields.

times as the Na^+ concentration increases from 2.0 to 100 mM. Earlier experiments indicated that varying the Na^+ concentration, over the range examined in Figure 5, has no influence on the values of k for BPDE and BPO measured without DNA (*7*).

The decrease in the value of k for BPDE, observed in Figure 5, is due to decreases in the intercalation association constant, K_A, and in the catalytic rate constant for hydrolysis, $k_{cat,Hy}$, which occur as the Na^+ concentration increases (*7*, *14*, *15*). In contrast to the strong dependence of k on the Na^+ concentration, the BPDE adduct yield remains almost constant. The adduct yield changes less than 1.2 times. Panel B shows similar results for BADE, at Na^+ concentrations between 2.0 and 50 mM. Here, the value of k decreases 2.7 times as the Na^+ concentration increases, while the adduct yield changes less than 1.4 times.

Figure 6 contains overall, pseudo-first-order rate constants for BPO and BAO at Na^+ concentrations between 2.0 and 100 mM, and 2.0 and 50 mM, respectively. Figure 6 also shows yields for adduct formation, hydrolysis, and rearrangement. For BPO, the results in panel A are like those for BPDE and BADE in Figure 5. As the rate constant decreases 15.4 times with an increase in Na^+ concentration from 2 to 100 mM, the adduct yield varies no more than 1.1 times. For BPO, the hydrolysis and rearrangement yields are also nearly constant. Both vary by only 1.2 times. For BAO, the results are different from BPO. Here the low adduct yields (~2%), and the large relative errors (±150%) make it impossible to determine whether the adduct yield remains constant. However, for BAO, quantitative analysis of the 5,6-dihydroxy-5,6-dihydro-BA formed indicates that the hydrolysis and rearrangement yields change. As the Na^+ concentration increases from 2.0 to 50 mM, the hydrolysis yield increases from 18% to 56%, and the rearrangement yield decreases from 80% to 42%.

Discussion

The results for BPDE in Figure 5, like results from an earlier investigation of the pH dependence of BPDE kinetics (*13*), demonstrate that the rate of adduct formation is changing in a manner which parallels changes in the rate of hydrolysis. The observed parallel kinetics, coupled with the finding that, under the conditions employed, most of the BPDE reaction (82−88%)[4] occurs via ctDNA catalyzed hydrolysis, support the earlier suggestion that BPDE adduct formation is autocatalyzed (*13*). For BADE and BPO, the results in Figures 5 and 6 are similar to those for BPDE. Again, as the Na^+ concentration increases, and the overall, pseudo-first-order rate constants decrease 2.7 and 15.4 times, the product distributions remain almost unchanged. For BAO, the behavior of the adduct yield is uncertain; however, the hydrolysis yield increases and the rearrangement yield decreases as the Na^+ concentration increases.

With ctDNA, the available reaction pathways are different for BPDE and BADE, which form adducts and hydrolyze, than for BPO and BAO, which form adducts, hydrolyze, and also rearrange. However, in all cases adduct formation is a minor pathway, and the most important contribution to the overall, pseudo-first-order rate constants (k), given in Figures 5 and 6, is due to catalyzed hydrolysis or rearrangement. The results in Figure 3 indicate that the k values of the BP metabolites, BPDE and BPO, are more strongly influenced by ctDNA than those of the BA metabolites, BADE and BAO. The results in Figure 4 indicate that intercalation association constants, K_A, for the BPDE and BPO model compounds (BP78D and BP45D, respectively) are significantly larger than association constants for the corresponding BADE and BAO model compounds (BA34D and BA56D). Together, the results in Figures 3 and 4 provide qualitative evidence that the catalytic contribution to k increases as K_A of the diol epoxides and epoxides increases.

In earlier investigations of reactions of BPDE, BPO, and BAO in DNA, Na^+ and Mg^{2+} induced decreases in k have been reported (*6*, *7*, *13*−*15*, *28*). For BPDE, these decreases arise from reductions in both the association constant, K_A, and the catalytic rate constant for hydrolysis, which occur as the Na^+ or Mg^{2+} concentration increases (*7*, *15*). The similar way in which Na^+ reduces the overall, pseudo-first-order rate constants of BPDE,

[4] These values were obtained from calculations using eqs 2 and 3, and earlier results from Tables I and IV of ref 7 indicating that as the Na^+ concentration increases from 2.0 to 100 mM, K_A decreases 1.8−2.7 times, and $k_{cat,Hy}$ decreases 4.8−9.0 times. In Table IV of ref 7 there is a misprint in the heading of the third column. The correct heading is $(k_0 + k_H[H^+])/(1 + K_A[DNA])$.

Table 1. Rate Constants for Hydrolysis, Rearrangement, and ctDNA Adduct Formation for Reactions of Benzo[*a*]pyrene and Benz[*a*]anthracene Diol Epoxides and Epoxides[a]

	[DNA] = 0.0 mM[b]			[DNA] = 0.20 mM			
	$k_{i,Hy}$[c,d]	$k_{i,Re}$[d,e]	K_A[f]	k[d,g]	$k_{cat,Hy}$[d,h]	$k_{cat,Re}$[d,i]	$k_{cat,Ad}$[d,j]
BPDE	$(1.44 \pm 0.2) \times 10^{-3}$		14500 ± 1500 (16000 ± 2000)	$(3.05 \pm 0.1) \times 10^{-2}$	$(3.75 \pm 0.3) \times 10^{-2}$		$(4.17 \pm 0.3) \times 10^{-3}$
BADE	$(1.47 \pm 0.2) \times 10^{-4}$		820 ± 100 (910 ± 240)	$(1.34 \pm 0.2) \times 10^{-3}$	$(9.33 \pm 0.7) \times 10^{-3}$		$(7.02 \pm 0.5) \times 10^{-4}$
BAO	$(3.53 \pm 0.6) \times 10^{-6}$	$(6.2 \pm 1) \times 10^{-7}$	320 ± 64 (270 ± 30)	$(5.00 \pm 0.6) \times 10^{-5}$	$(1.39 \pm 0.1) \times 10^{-4}$	$(6.23 \pm 0.6) \times 10^{-4}$	$(1.08 \pm 0.1) \times 10^{-5}$
BPO	$(9.6 \pm 1.8) \times 10^{-7}$	$(7.5 \pm 1) \times 10^{-7}$	7100 ± 1400 (6000 ± 800)	$(1.23 \pm 0.1) \times 10^{-4}$	$(8.74 \pm 0.9) \times 10^{-5}$	$(1.21 \pm 0.1) \times 10^{-4}$	$(1.10 \pm 0.1) \times 10^{-5}$

[a] In all experiments the Na^+ concentration was 2.0 mM. Reactions were carried out in 10 mM Tris·HCl and 1.0 mM EDTA buffer at pH 7.3. [b] For reactions without ctDNA, overall, pseudo-first-order rate constants are equal to the sum of rate constant for hydrolysis plus rearrangement. [c] Pseudo-first-order rate constant for hydrolysis, measured in buffer without ctDNA. [d] In units of s^{-1}. [e] Pseudo-first-order rate constant for rearrangement to phenols, measured in buffer without ctDNA. [f] Association constants of diol epoxides and epoxides given without parentheses. Association constants of corresponding model compounds BP78D, BP45D, BA34D, and BA56D given in parentheses. In units of M^{-1}. [g] Overall, pseudo-first-order rate constants measured in ctDNA. [h] Rate constant for ctDNA catalyzed hydrolysis. [i] Rate constant for ctDNA catalyzed rearrangement. [j] Rate constant for ctDNA catalyzed adduct formation.

BADE, BPO, and BAO provides evidence that these two factors are also important for understanding the influence of Na^+ on reactions of BADE, BPO, and BAO in ctDNA.

The manner in which ctDNA enhances the overall, pseudo-first-order rate constants, k, of all of the epoxides and diol epoxides examined, and the manner in which values of k, for all of these epoxides and diol epoxides, decrease as the Na^+ concentration increases, points to basic similarities between the reactions of BPDE, BADE, BPO, and BAO in ctDNA. For BPDE, BADE, and BPO, the finding that the product distributions remain nearly unchanged, as the Na^+ concentration increases, also provides evidence of similar mechanisms. These results support the conclusion that, for BPDE, BADE, BPO, and BAO, k, in eqs 1 and 2, is given by eq 4, which has the same form (*2*) as that of the pseudo-first-order rate constant for BPDE hydrolysis in eq 3. In eq 4, k_i represents the contribution to k from reactions which are not DNA catalyzed.

$$k = \frac{k_i + k_{cat}K_A[DNA]}{1 + K_A[DNA]} \quad (4)$$

For BPDE and BADE, k_i has components associated with adduct formation and hydrolysis.

$$k_i = k_{i,Ad} + k_{i,Hy} \quad (5)$$

For BPO and BAO, k_i has components associated with adduct formation, hydrolysis, and rearrangement.

$$k_i = k_{i,Ad} + k_{i,Hy} + k_{i,Re} \quad (6)$$

For BPDE and BAO, $k_{i,Hy}$ equals $k_{0,Hy} + k_{H,Hy}[H^+]$ (*15*, *24*). For BAO, $k_{i,Re}$ (equal[5] to $k_{0,Re} + k_{H,Re}[H^+]$) has the same general form as $k_{i,Hy}$ (*24*). It is likely that $k_{i,Hy}$ for BADE and BPO and $k_{i,Re}$ for BPO also have the same form. In eqs 5 and 6, $k_{i,Ad}$ equals k_2[DNA], where k_2 is the second-order rate constant for reaction which does not proceed through formation of a reversibly bound DNA complex. Table 1 lists values of $k_{i,Hy}$ and $k_{i,Re}$ obtained from overall, pseudo-first-order rate constants and from hydrolysis product yields measured without DNA.[6]

[5] Here, $k_{0,Re}$ is a rate constant for spontaneous rearrangement and $k_{H,Re}$ is a rate constant for H^+ catalyzed rearrangement without DNA.

[6] For BPDE and BADE, $k_{i,Hy}$ equals the overall, pseudo-first-order rate constant measured without DNA. For BPO and BAO, values of $k_{i,Hy}$ were obtained by multiplying k by the percent yield of hydrolysis products measured without DNA. Values of $k_{i,Re}$ were obtained by subtraction.

Under conditions where most diol epoxide or epoxide reacts via DNA catalysis ($k_i \ll k_{cat}K_A$[DNA]), the overall, pseudo-first-order rate constant in eq 4 is approximately given by

$$k \approx \frac{k_{cat}K_A[DNA]}{1 + K_A[DNA]} \quad (7)$$

For BPDE and BADE, k_{cat} can be written in the form

$$k_{cat} = k_{cat,Ad} + k_{cat,Hy} \quad (8)$$

For BPO and BAO,

$$k_{cat} = k_{cat,Ad} + k_{cat,Hy} + k_{cat,Re} \quad (9)$$

Under conditions where eq 7 is valid,

$$k_{cat,P} = k_{cat}Y_P \quad (10)$$

Here, $k_{cat,P}$ is either $k_{cat,Ad}$, $k_{cat,Hy}$, or $k_{cat,Re}$, the catalytic rate constant for adduct formation, hydrolysis, or rearrangement, respectively, and Y_P is the corresponding percent yield of adducts, or hydrolysis or rearrangement products obtained from reaction in ctDNA.

Under conditions where eq 7 is valid, eqs 1 or 2, and 8 or 9, indicate that

$$k_P \approx \frac{k_{cat,P}K_A[DNA]}{1 + K_A[DNA]} \quad (11)$$

where k_P is either k_{Ad}, k_{Hy}, or k_{Re}, the pseudo-first-order rate constant for adduct formation, hydrolysis, or rearrangement. Equations 7, 8 or 9, and 10 are able to describe the invariance in the product distributions observed in Figures 5 and 6 for reactions of BPDE, BADE, and BPO, even though $k_{cat,P}$ and K_A are changing significantly, if the relative contribution of $k_{cat,P}$ to k_{cat} is nearly constant over the range of Na^+ concentrations examined. This relationship is expressed by the equation:

$$k_{cat,P}/k_{cat} \approx \text{constant} \quad (12)$$

In Figures 5 and 6, the BPDE, BADE, and BPO product distributions, which remain nearly constant as the rate constants change, agree with product distributions that are described by eqs 1 or 2, 7, 8 or 9, 11, and 12.

While these equations provide a simple description of epoxide and diol epoxide kinetics that accounts for the BPDE, BADE, and BPO results in Figures 5 and 6, it is

important to note the limitations. They apply only when DNA catalysis dominates. The finding, in Figure 5, that a measurable decrease occurs in the BADE adduct yield, over the range of Na^+ concentrations examined, suggests that noncatalyzed hydrolysis is not entirely negligible at high Na^+ concentrations. As the Na^+ concentration increases and DNA catalyzed reactions account for a smaller fraction of the total reaction, eqs 7 and 11 become less accurate. This is especially apparent in the BAO results of Figure 6. For BAO, which has the smallest DNA intercalation association constant, the fraction of total reaction that is DNA catalyzed in 50 mM Na^+ is smaller than the fractions of BADE, BPDE, and BPO reactions that are DNA catalyzed at the highest Na^+ concentrations examined in Figures 5 and 6. For BAO, the relative increase in hydrolysis versus rearrangement yields which occurs as the Na^+ concentration increases from 2.0 to 50 mM reflects a reduction in the fraction of reaction which is DNA catalyzed. At 2.0 mM Na^+, most of the reaction undergoes general acid catalysis involving DNA, and the product distribution reflects the favored rearrangement pathway which is observed at low pH (*24*). In 50 mM Na^+, a significantly smaller fraction of the reaction proceeds via ctDNA catalysis. This is accompanied by an increase in the hydrolysis yield, which in buffer without DNA at pH 7.3 accounts for 85% of the total product.

ctDNA Association Constants (K_A) of BPDE and BPO, and ctDNA Catalytic Rate Constants for Hydrolysis ($k_{cat,Hy}$), Rearrangement ($k_{cat,Re}$), and Adduct Formation ($k_{cat,Ad}$). For BPDE, BPO, BADE, and BAO, Table 1 lists intercalation association constants (K_A) in 2.0 mM Na^+, and overall, pseudo-first-order rate constants, k, measured in 2.0 mM Na^+ and 0.2 mM ctDNA. The table gives catalytic rate constants ($k_{cat,Hy}$ and $k_{cat,Ad}$) for hydrolysis and adduct formation. For BPO and BAO, the table also lists catalytic rate constants ($k_{cat,Re}$) for rearrangement.

Values of K_A, for BPO and BPDE, which are large (7100−14 500 M^{-1}), have been obtained using a different procedure from that used to evaluate values of K_A for BAO and BADE, which are small (320−820 M^{-1}). At ctDNA and Na^+ concentrations of 0.2 and 2.0 mM, respectively, 58% of BPO and 74% of BPDE are reversibly intercalated. For BPDE and BPO, K_A and k_{cat} were determined using eq 4 and the results in Figure 3 which show how the overall, pseudo-first-order rate constants, k, change as the ctDNA concentration increases. For BPDE and BPO, values of K_A and k_{cat} in Table 1 were obtained from a least-squares optimized fit of the data in Figure 3 with calculated values of k obtained from eq 4. In the fitting procedure, k_i is equal to the overall, pseudo-first-order rate constant, measured without DNA, and to the sum of $k_{i,Hy}$ and $k_{i,Re}$ values listed in Table 1. Values of K_A and k_{cat} are variable parameters.[7] For BPDE and BPO, values of $k_{cat,Ad}$, $k_{cat,Hy}$, and $k_{cat,Re}$, listed in Table 1, were calculated from values of k_{cat} obtained from the least-squares analysis of the data in Figure 3. This calculation was carried out using eq 10 and results from measurements of product yields in 0.20 mM ctDNA and 2.0 mM Na^+.[8]

Values of K_A, $k_{cat,Hy}$, $k_{cat,Re}$, and $k_{cat,Ad}$ for BADE and BAO. The small values of K_A for the model compounds BA34D and BA56D, and the results in Figure 3, indicating that the plots of the overall, pseudo-first-order rate constants of BADE and BAO show no evidence of saturation over the range of ctDNA concentrations examined, support the conclusion that the association constants of BADE and BAO are small. For this reason, reliable values of K_A for BADE and BAO cannot be obtained from the kinetic data of Figure 3.[9] Instead, values of K_A for these molecules were calculated using eq 13, association constants of the model compounds BA34D, BA56D, BP78D, and BP45D, and K_A values of BPDE and BPO listed in Table 1.

$$K_A = K_{A,m} R_m \tag{13}$$

In evaluating K_A for BADE (BAO), $K_{A,m}$ is the association constant of the model compound BA34D (BA56D), and R_m is the ratio of the association constant of BPDE (BPO) to that of BP78D (BP45D). A comparison of the K_A values listed in Table 1 indicates that the intercalation association constant of BPDE is 18 times larger than that of BADE, and the association constant of BPO is 22 times larger than that of BAO. The K_A values of the bay region diol epoxides, BPDE and BADE, are 2.0 and 2.6 times larger than those of the corresponding K region epoxides, BPO and BAO.

For BADE and BAO, k_{cat} was obtained from a least-squares fit of the kinetic data in Figure 3. However, in determining k_{cat} for BADE and BAO, values of K_A were calculated from eq 13, and only k_{cat} was varied during the fitting process. Values of $k_{cat,Ad}$, $k_{cat,Hy}$, and $k_{cat,Re}$, which are listed in Table 1, were calculated from eq 10 using values of k_{cat} obtained from the least-squares fitting procedure, and product distributions measured in 0.20 mM ctDNA and 2.0 mM Na^+.

Comparison of BPDE, BADE, BPO, and BAO Reactions. As the product distributions in Figures 5 and 6 indicate, most reaction involves DNA catalyzed hydrolysis or rearrangement which, in 0.20 mM ctDNA and 2.0 mM Na^+, accounts for 89%, 95%, 87%, and 90% of the total reaction of BPDE, BPO, BADE, and BAO, respectively.[10] For BPDE and BPO in 100 mM Na^+ and 0.2 mM ctDNA, DNA catalyzed hydrolysis or rearrangement accounts for 82% and 77% of the total reaction; for BADE and BAO in 50 mM Na^+ and 0.20 mM ctDNA, catalyzed hydrolysis or rearrangement accounts for 68% and 55% of the total reaction.[10]

The results also demonstrate that the overall, pseudo-first-order rate constants ($k = k_{Hy,i} + k_{Re,i}$) obtained without DNA increase in the order BPDE > BADE > BAO > BPO. In earlier work, values of k were employed

[7] In the least-squares fitting procedure for BPDE and BPO, as well as in a similar procedure used for BADE and BAO, $k_{i,Ad}$, the rate constant for adduct formation which does not proceed through a ctDNA complex, was taken to be negligible when compared to other contributions to the overall, pseudo-first-order rate constant, k (see eqs 4−6). This is supported by the finding that, for all of the BP and BA epoxides and diol epoxides, the total adduct yield is less than 10%, coupled with the observation that for the BPDE, BADE, and BAO reactions, which have the greatest adduct yields, adduct formation parallels the hydrolysis and rearrangement reactions which occur primarily via ctDNA catalyzed pathways. See text and footnotes 10 and 11.

[8] The validity of this approach depends on the accuracy of eq 7 which, in turn, relies on the relationship $k_{cat}K_A[DNA] \gg k_i$. For BPO, BPDE, BAO, and BADE in 0.2 mM ctDNA and 2.0 mM Na^+, the results in Table 1 indicate that $(k_{cat}K_A[DNA])/k_i$ equals 182, 84, 12, and 11.

[9] For BADE and BAO, it is not currently possible to carry out kinetic measurements at sufficiently high ctDNA concentrations to achieve saturation kinetics. For BAO in 2.0 mM Na^+, a ctDNA concentration of 8.9 mM is required to achieve the 74% reversible binding level that occurs for BPDE in 0.20 mM ctDNA. The smaller BADE and BAO association constants give rise to large uncertainty in the K_A values obtained when the least-squares fitting procedure, which was used for BPDE and BPO, is applied to the BADE and BAO kinetic results in Figure 3. This procedure requires k values near saturation in order to yield accurate K_A values.

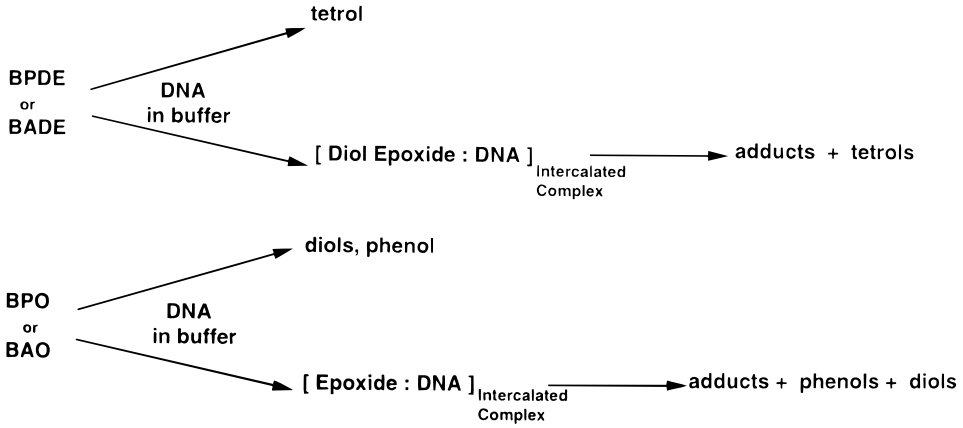

Figure 7. Mechanisms for major reaction pathways of BPDE, BPO, BADE, and BAO in buffer with ctDNA.

as markers of the inherent reactivities of diol epoxides (*40*). The ordering of k values measured without ctDNA agrees with earlier data indicating that BPDE is more reactive than BADE, BAO, or BPO (*2*, *6*, *7*, *15*, *18*, *26*, *40*). Without DNA, the overall, pseudo-first-order rate constants of the bay region diol epoxides, BPDE and BADE, are 842 and 35 times larger than rate constants for the corresponding K region epoxides, BPO and BAO. More importantly, the results in Table 1 indicate that the ordering of the values of k, measured without ctDNA, is the same as the ordering of the values of k_{cat}, the catalytic rate constants for hydrolysis, rearrangement, and adduct formation. The catalytic rate constants ($k_{cat} = k_{cat,Hy} + k_{cat,Ad}$) for BPDE ($(4.17 \pm 0.3) \times 10^{-2}$) and for BADE ($(1.00 \pm 0.1) \times 10^{-2}$) are 190 and 13 times larger than the rate constants ($k_{cat} = k_{cat,Hy} + k_{cat,Re} + k_{cat,Ad}$) for BPO ($(2.19 \pm 0.1) \times 10^{-4}$) and BAO ($(7.73 \pm 0.3) \times 10^{-4}$). For BPDE and BADE, the catalytic rate constants for adduct formation alone, $k_{cat,Ad}$, are 379 and 65 times larger than values for the corresponding K region epoxides. This observation indicates that the ordering of reactivities, at ctDNA catalytic sites where reaction occurs 9−72 times faster than in buffer, is the same as the ordering in buffer without DNA and supports the *bay region theory*.

While the ordering of the catalytic rate constants, k_{cat}, reflects inherent reactivity, as indicated by the ordering of k values, measured without DNA, the results in Table 1 demonstrate that reversible intercalation significantly influences the rate of epoxide and diol epoxide reactions in ctDNA. This is illustrated by a comparison of BPO and BAO overall, pseudo-first-order rate constants, for which the ordering is reversed with and without DNA. Although the value of k_{cat} for BAO is larger than that for BPO, the value of k for BPO in ctDNA is larger than that for BAO because the intercalation association constant of BPO (7100 M^{-1}) is 22 times larger than that for BAO. These observations point out that differences occurring between the intercalation association constants of BP and BA epoxides can significantly alter the relative rates of reactions occurring with DNA.

For BPDE, BADE, BAO, and BPO, the similar influence that DNA and Na^+ have on the overall, pseudo-first-order rate constants, together with the finding that, for BPDE, BADE, and BPO, the ratios of the pseudo-first-order rate constants for adduct formation, hydrolysis, and rearrangement are constant under conditions where the absolute values of the rate constants are rapidly changing, provides evidence that a common catalytic mechanism leads to all of the products which have been examined. In ctDNA, the similarities between reactions resulting in BPDE adduct formation and BPDE hydrolysis, and the similarity between the BPDE reactions and the reactions of BADE, BPO, and BAO, provide evidence that, for all of these epoxides and diol epoxides, the most important reaction mechanisms are shown in Figure 7. Of the two major reaction routes shown in the figure, one involving direct reactions and the second involving catalysis at ctDNA intercalation sites, the latter is, by far, the most important for hydrolysis and rearrangement reactions in 0.2 mM ctDNA and 2.0 mM Na^+. For BPDE in 0.2 mM ctDNA at Na^+ between 100 and 2.0 mM, ctDNA catalyzed hydrolysis accounts for 82−89% of all reaction.[10] Because of the manner in which the yield for adduct formation tracks the yields for hydrolysis and rearrangement, under conditions where the rate constants and the intercalation association constants vary significantly, it is likely that catalysis at intercalation sites represents the most important route through which adduct formation occurs. Interestingly, the important role that the initial formation of intercalated complexes plays is consistent with early speculation about the mechanism of BPDE adduct formation (*41*, *42*).

For BADE and BAO, the smaller association constants and the greater variation in product yields over the range of Na^+ concentrations examined indicate that at high Na^+ concentrations noncatalyzed hydrolysis and rearrangement become significant. However, for all of the epoxides and diol epoxides, the data in Table 1 indicate that, in 0.2 mM ctDNA and 2.0 mM Na^+, 90−99% of all hydrolysis and rearrangement occurs via DNA catalysis.[11] These

[10] In 2.0 mM Na^+ and 0.2 mM ctDNA, values of the percent reaction which occurs via ctDNA catalyzed hydrolysis and rearrangement were calculated by using eqs 1 or 2, 4, 5 or 6, and 8 or 9; values of k, $k_{i,Hy}$, $k_{i,Re}$, $k_{cat,Hy}$, $k_{cat,Re}$, $k_{cat,Ad}$, and K_A from Table 1; and the simplifying approximation that $k_{i,Ad}$ contributes negligibly to k (see footnote 7). For BPDE and BPO in 100 mM Na^+, and for BADE and BAO in 50 mM Na^+, values of the percent reaction which occurs via ctDNA catalyzed hydrolysis and rearrangement were obtained in the same manner, using adduct and hydrolysis yields, k values, and model compound K_A values measured in 100 and 50 mM Na^+. For BPDE and BPO in 100 mM Na^+, k values of $(6.03 \pm 0.6) \times 10^{-3}$ and $(6.00 \pm 0.6) \times 10^{-6}$ s^{-1} and K_A values of 6300 and 2400 M^{-1} were used in the calculations. For BADE and BAO in 50 mM Na^+, k values of $(5.11 \pm 0.6) \times 10^{-4}$ and $(1.02 \pm 0.1) \times 10^{-5}$ s^{-1} and K_A values of 300 and 177 M^{-1} were used. Values of $k_{cat,Hy}$ and $k_{cat,Re}$ in 100 and 50 mM Na^+ were calculated from eqs 4 and 10. For BPDE and BPO in 100 mM Na^+, and BADE and BAO in 50 mM Na^+, the calculated $k_{cat,Hy}$ values were $(8.9 \pm 1) \times 10^{-3}$, $(6.3 \pm 0.3) \times 10^{-6}$, $(6.3 \pm 0.5) \times 10^{-3}$, and $(1.0 \pm 0.1) \times 10^{-4}$ s^{-1}. For BPO in 100 mM Na^+ and BAO in 50 mM Na^+, the $k_{cat,Re}$ values were $(8.0 \pm 3) \times 10^{-6}$ and $(7.2 \pm 3) \times 10^{-5}$ s^{-1}.

[11] The percentages of hydrolysis and rearrangement which are ctDNA catalyzed were obtained using a method analogous to that used in determining the percentage of total reaction which occurs via ctDNA catalyzed hydrolysis and rearrangement. See footnote 10.

results provide evidence that, under the same conditions, similarly high percentages of adducts are also formed via DNA catalysis.

While the present experiments provide a qualitative description of the reaction mechanisms examined, the need remains for a full characterization of the transition states associated with the formation of DNA adducts from BP and BA epoxides and diol epoxides. Of particular interest will be information which provides clues relating reversibly bound intercalated conformations, the importance of which is indicated by binding and kinetic data, to the nonintercalated equilibrium conformations associated with covalently bound adducts (*43*, *44*). One explanation is that reactions occur via "loose" transition states involving reactants which are approaching or have recently exited a reversibly bound intercalation site. Another possibility is that the conformational flexibility of covalently modified DNA and the reaction exothermicity result in a change in adduct conformation from initial high energy intercalated geometries to more favorable external geometries. Further investigation will be required to test the validity of these or of other possibilities which have not yet been considered.

Nevertheless, recognition that DNA catalysis and reversible intercalation influence the covalent modification of ctDNA by BPDE, BADE, BPO, and BAO leads to an understanding of the complexity of factors influencing the biochemical activity of aromatic DNA alkylating agents. In biochemical environments which provide additional reaction pathways, that have not been examined here, DNA modification, which relies on catalysis at intercalation sites may influence adduct yields by enhancing adduct formation at the expense of DNA independent detoxification pathways. These include glutathione *S*-transferase catalyzed formation of glutathione conjugates (*8*) and epoxide hydrolase catalyzed hydrolysis (*9*−*11*). In this way, the catalytic role which DNA plays in enhancing adduct formation may be important to mutagenic and carcinogenic mechanisms of BP and BA metabolites which rely on covalent modification of DNA.

Conclusions

In summary, this investigation leads to a description of the relationship between reversible binding which occurs at DNA intercalation sites and DNA catalyzed reactions of BPDE, BADE, BPO, and BAO which result in covalent DNA modification. The main conclusions are as follows:

1. For BPDE in 0.2 mM ctDNA and at Na^+ concentrations between 2.0 and 100 mM, the kinetics for the minor pathway resulting in adducts closely parallels the kinetics for the most important pathway, which results in hydrolysis, which accounts for 82−89% of all reaction, and which occurs via catalysis at ctDNA intercalation sites. The reactions of BPDE, BADE, and BPO resulting in DNA adducts behave similarly. In all cases, Na^+ induced decreases in the pseudo-first-order rate constant for DNA adduct formation parallel decreases in pseudo-first-order rate constants for major pathways leading to hydrolysis and rearrangement. Over ranges of increasing Na^+ concentration, in which the overall, pseudo-first-order rate constants of BPDE, BADE, and BPO decrease 2.7−15.4 times, the adduct yields vary only by factors of 1.2, 1.4, and 1.1, respectively.

2. There is significant variation in the association constants of different BP and BA epoxides and diol epoxides. In 2.0 mM Na^+, the association constants of the BP metabolites, BPDE and BPO, are 18 and 22 times larger than the association constants of the corresponding BA metabolites, BADE and BAO.

3. In 2.0 mM Na^+ and 0.2 mM ctDNA, the ordering of catalytic rate constants (k_{cat}) for reactions is BPDE > BADE > BAO > BPO. This is the same ordering as that of the inherent reactivities indicated by overall, pseudo-first-order rate constants, k, measured without DNA. However, for reactions in ctDNA, the ordering of the k values is influenced not only by reactivity but also by intercalation association constants. Comparisons of pseudo-first-order rate constants for hydrolysis and rearrangement in buffer without DNA ($k_{i,Hy}$ and $k_{i,Re}$) and of catalytic rate constants (k_{cat}) indicate that BPO is less reactive than BAO. However, in ctDNA, the larger intercalation association constant of BPO gives rise to an overall, pseudo-first-order rate constant for reactions leading to hydrolysis, rearrangement, and adduct formation which is 2.3 times larger than that of BAO.

4. The similar catalytic influence of ctDNA on the hydrolysis of BPDE, BADE, BPO, and BAO, and on the rearrangement reactions of BPO and BAO, coupled with the invariance in the BPDE, BADE, and BPO adduct yields, which occurs at varying Na^+ concentrations, provide evidence that, in DNA at low Na^+ concentrations, all reactions are dominated by DNA catalysis. For each pathway, the contribution to the overall, pseudo-first-order rate constant is given approximately by eq 11.

5. For BAO, which has the smallest DNA intercalation association constant, and for which DNA catalysis is least efficient, increasing the Na^+ concentration from 2.0 to 50 mM, in reactions with DNA, causes an increase in the hydrolysis yield and a decrease in the rearrangement yield. This is consistent with the kinetic data in Table 1, which indicate that, for BAO at the highest Na^+ concentration examined, the percentage of the total reaction which proceeds via DNA catalysis is approximately 55%. The smaller role that DNA catalysis plays at high Na^+ concentrations results in product distributions which are more similar to that occurring in buffer without DNA.

6. For BPDE, BADE, BPO, and BAO in 0.2 mM ctDNA and 2.0 mM Na^+, the percentages of the hydrolysis and rearrangement reactions which undergo catalysis at DNA intercalation sites are between 90% and 99%. Under the same conditions, it is likely that similarly high percentages of reactions resulting in adducts proceed in the same manner.

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