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MECHANISMS OF REACTION OF BENZO(*a*)PYRENE-7,8-DIOL-9,10-EPOXIDE WITH DNA IN AQUEOUS SOLUTIONS

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The physical and chemical reaction pathways of the metabolite model compound benzo(*a*)pyrene-7,8-diol-9,10-epoxide (BPDE) in aqueous (double-stranded) DNA solutions was investigated as a function of temperature (0–30 °C), pH (7.0–9.5), sodium chloride concentration (0–1.5M) and DNA concentration in order to clarify the relationships between the multiple reaction mechanisms of this diol epoxide in the presence of nucleic acids. The reaction pathways are (1) noncovalent intercalative complex formation with DNA, characterized by the equilibrium constant K, and X_b the fraction of molecules physically bound; (2) accelerated hydrolysis of BPDE bound to DNA; (3) covalent binding to DNA; and (4) hydrolysis of free BPDE (k_h). The DNA-induced hydrolysis of BPDE to tetraols and the covalent binding to DNA are parallel pseudo-first-order reactions. Following the rapid (millisecond time scale) noncovalent complex formation between BPDE and DNA, a much slower (~ minutes) H^+-dependent (either specific or general acid catalysis) formation of a DNA-bound triol carbonium ion (rate constant k_3) occurs. At pH 7.0 the activation energy of k_3 is 8.7 ± 0.9 kcal/mol, which is lower than the activation energy of hydrolysis of free BPDE in buffer solution (14.2 ± 0.7 kcal/mol), and which thus partially accounts for the acceleration of hydrolysis of BPDE upon complexation with DNA. The formation of the triol carbonium ion is followed by a rapid reaction with either water to form tetraols (rate constant k_T), or covalent binding to DNA (k_c). The fraction of BPDE molecules which undergo covalent binding is $f_{cov} = k_c/(k_c + k_T) = 0.10$ and is independent of the overall BPDE reaction rate constant $k = k_h(1 - X_b) + k_3 X_b$ if $X_b \to 1.0$, or is independent of X_b as long as $k_3 X_b \gg k_h(1 - X_b)$. Thus, at $X_b = 0.9$, f_{cov} is independent of pH (7.0–9.5) even though k exhibits a 70-fold variation in this pH range and $k \to k_h$ above pH 9 ($k_3 = k_h$). Similarly, f_{cov} is independent of temperature (0–30 °C), while k varies by a factor of approx. 3. In the range of 0–1.5 M NaCl, f_{cov} decreases from 0.10 to 0.04. These variations are attributed to a combination of salt-induced variations in the factors k_3, X_b and the ratio k_c/k_T.

1. Introduction

Polycyclic aromatic hydrocarbons are metabolically converted to a variety of oxygenated derivatives some of which are highly carcinogenic and mutagenic [1–7]. The metabolic pathways and biological activity of benzo(*a*)pyrene, which is a common environmental pollutant, have been most thoroughly investigated. The ultimate carcinogenic form of benzo(*a*)pyrene is believed to be a 'bay region' diol epoxide, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (BPDE), which bears the epoxide ring in the 9,10-position adjacent to the angular benzo ring (fig. 1), the bay region. Perturbational molecular orbital calculations indicate that bay-region diol epoxides are the most reactive of all the possible isomeric diol epoxides [5,8]. This exceptional reactivity is related to the relative ease of formation of triol carbonium ions (with the positive charge at the 10-position in

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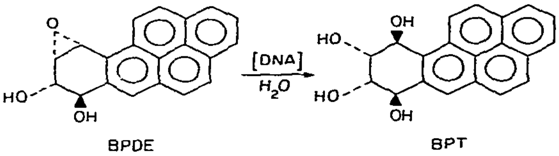

Fig. 1. Structural formulas of the dial epoxide (BPDE) and its tetraol (BPT) hydrolysis product (the *trans* stereoisomer is shown).

the case of BPDE) which can subsequently react with cellular nucleophiles. Thus, BPDE reacts with DNA in vivo and in vitro to form covalent bonds between the 10-position of BPDE and nucleotides. The dominant BPDE-DNA adduct involves the binding of BPDE to the exocyclic amino group of guanine [9–15]. The presence of such BPDE-guanine adducts has been correlated with the biological activity of BPDE in living cells [16–18].

We have extensively studied the reaction pathways of BPDE with DNA in aqueous solutions under controlled conditions. The major reaction pathway of BPDE with DNA in an aqueous environment is not covalent adduct formation, but the hydrolysis of BPDE to the tetraols BPT (fig. 1). This reaction is markedly accelerated in the presence of DNA and the hydrolysis rate constant k depends on the DNA concentration [19–23]. Under normal conditions (pH 7.0, 25 °C) hydrolysis to tetraols rather than covalent binding constitutes the major reaction pathway by a factor of approx. 10–20 [19]. The tetraols thus formed also form noncovalent complexes with DNA. A combination of equilibrium dialysis and fluorescence techniques has shown that there are two types of binding sites for BPT: an intercalation and an exterior binding site [24].The covalently bound pyrene residue resulting from the reaction of BPDE with DNA is located at an external binding site [25–27]. Therefore, the possibility was raised that initial noncovalent complex formation between BPDE and DNA gives rise to both an external and an intercalation binding site, and that the former undergoes a covalent binding reaction, while the latter results in hydrolysis and tetraol formation [22].

In order to clarify the relationship between the covalent binding reaction and hydrolysis of BPDE complexed with DNA, we have studied in detail the dependence of these two reactions on temperature and on pH [28] *, and the dependence of the covalent binding reaction on the DNA and NaCl concentrations. A simple model is proposed to explain these observations and the results suggest that the rate-determining step in both reactions is the formation of a triol carbonium ion [5,8,29–32] derived from BPDE noncovalently complexed with DNA.

2. Experimental section

2.1. Materials

The sources and preparation of racemic BPDE and native calf thymus DNA (hyperchromicity 38–40%) were the same as those described previously [20]. A 10^{-2} M stock solution of BPDE in tetrahydrofuran was prepared and small aliquots of this stock solution were added to the aqueous buffer solutions (sodium cacodylate).

2.2. Kinetics of hydrolysis of BPDE

A fluorescence method [33] was utilized to monitor the hydrolysis of BPDE (nonfluorescent) to the tetraols BPT (fluorescent). The pH of the solutions containing sodium cacodylate buffer (5 mM unless mentioned otherwise) was adjusted utilizing NaOH. The temperature of these solutions (contained in a 1 × 1 cm cuvette) was then allowed to reach equilibrium in the sample compartment of a Hitachi MPF-2A fluorometer whose temperature was maintained at the desired value by utilizing a Neslab RT8 circulating water bath. In some experiments involving rapid hydrolysis rates, a stopped-flow system was utilized [20,22]. In the experiments at lower temperatures the surface of the cuvette was continuously exposed to a stream of dry nitrogen gas to prevent the condensation of moisture. Aliquots of the BPDE/tetrahydrofuran stock solution were then added to

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* A preliminary account of this work was presented at the 73rd Annual Meeting of the Association of American Cancer Research, St. Louis, MO, April 1982.

the aqueous solution (the amount of organic solvent in the mixture did not exceed 0.2% by volume), and the fluorescence intensity was monitored as a function of time. During the time course of these experiments the temperature was constant to within ±0.1°C.

2.3. *Determination of extent of covalent binding of BPDE to DNA*

The solutions were prepared as described in section 2.2 and the reactions were allowed to proceed to completion (until the BPDE concentration reached zero). The DNA was then precipitated by adding 2.5 vols. of cold ethanol in the presence of 0.1 M NaCl. The precipitates were washed four to five times with ethanol and dried by flushing with nitrogen. The dried DNA was then redissolved in 5 mM sodium cacodylate buffer solution (pH 7.0) and dialysed five to six times at 4°C against the buffer solution to remove the remaining traces of tetraols. The amount of BPDE bound covalently to DNA was determined spectrophotophotometrically utilizing an extinction coefficient of 29000 M^{-1} cm^{-1} for the covalently bound pyrene chromophore at 345 nm [10].

3. Results

3.1. *pH dependence of the hydrolysis rate constant k*

It has been previously demonstrated that hydrolysis of BPDE in the presence of DNA is a pseudo-first-order process with rate constant *k* [20]. Observed values of *k* in the absence and presence of DNA are shown in fig. 2 at different pH values (at 25 ± 0.1°C). At a pH of 7.0 the values of *k* are about 30-times greater in the presence of 1.5×10^{-4} M DNA * than in the buffer solution. As the pH is increased, this difference gradually decreases and the catalytic effect of DNA disappears in the range pH 9–10 with the sodium cacodylate solutions utilized in this work.

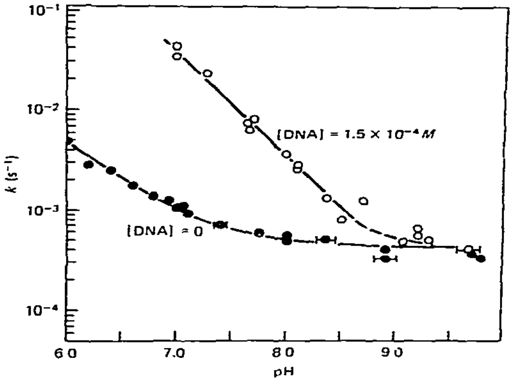

Fig. 2. The observed rate constant (k) for hydrolysis of BPDE to BPT in buffer solution (●) and in the presence of DNA (○) as a function of pH. (temperature, 25±0.1°C; initial BPDE concentration, 3.4×10^{-6} M; buffer, 5 mM sodium cacodylate).

3.2. *Temperature dependence of k*

The temperature dependence of the hydrolysis rate constant in the presence of DNA at two different DNA concentrations and in buffer solution is shown in fig. 3. In buffer solution the activation energy is $E_a = 14.2 \pm 0.7$ kcal/mol and that in the DNA solution is 8.7 ± 0.9 kcal/mol for both DNA concentrations.

3.3. *Covalent binding of BPDE to DNA*

The fraction, f_{cov}, of BPDE molecules initially present in the reaction mixture which react by binding covalently to DNA rather than by hydrolysis to tetraols has been determined as a function of pH and of temperature. The fraction f_{cov} remains constant, within experimental error, at all pH values and temperatures investigated (fig. 4), even though the kinetic rate constant of hydrolysis, and thus the lifetime of BPDE in solution, change by factors of as much as approx. 70.

3.4. *Kinetics of hydrolysis and of covalent binding*

Both of these reactions follow the same time course regardless of the pH or temperature (pH and temperature ranges as in fig. 4) at which the

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* The DNA concentrations mentioned throughout this work refer to equivalent molar concentrations of nucleotides.

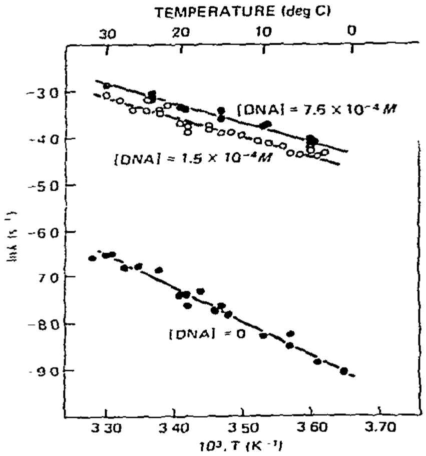

Fig. 3. Temperature dependence of the hydrolysis rate constant k as a function of temperature in buffer solution (lower set of points) and in the presence of DNA at two different concentrations at pH = 7.0. All other conditions are otherwise the same as in the legend to fig. 2. The experiments at the higher DNA concentration were performed with a stopped-flow apparatus.

experiments are performed. The overall concentration dependence of diol epoxide molecules decays exponentially (e^{-kt}), while the concentration of molecules bound covalently to DNA increases with time according to the expression ($1 - e^{-kt}$). A more detailed presentation of typical data may be found elsewhere [34]. These two processes thus constitute a set of parallel pseudo-first-order reactions. They are characterized by the same pH-dependent, and temperature-dependent, experimentally observed rate constant k in cacodylate buffer.

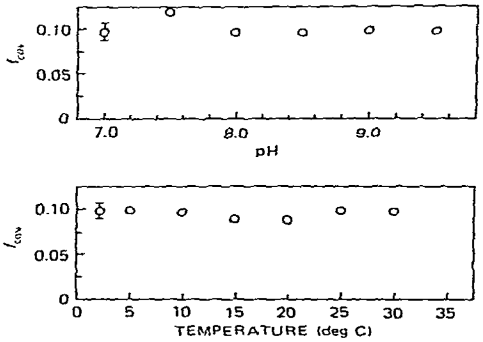

Fig. 4. Extent of covalent binding of BPDE to DNA (f_{cov}) as a function of pH at 25±0.1°C (top), and as a function of temperature at pH 7.0 (bottom). Reaction conditions: initial BPDE concentration, 3.4×10^{-6} M; DNA concentration, 7.5×10^{-4} M (top), 1.5×10^{-4} M (bottom). Top, Tris buffer (5 mM); bottom, 5 mM sodium cacodylate buffer.

3.5. Dependence of k and of covalent binding on the DNA concentration

It is by now well-recognized that the hydrolysis of BPDE to tetraols is greatly accelerated in the presence of DNA [20,23,35,36]. Using stopped-flow techniques [20] we have shown that k increases with increasing DNA concentration and reaches a limiting value of 0.068 s^{-1} at a DNA concentration of 10^{-3} M and at pH 7.0. The dependence of k on the concentration of DNA taken from ref. 20 is depicted schematically in fig. 5 by the continuous line.

In view of the parallel kinetics of hydrolysis and covalent binding, it was of interest to investigate the dependence of f_{cov} on the DNA concentration at a fixed ratio r defined by

$$r = \frac{[\mathrm{BPDE}]}{[\mathrm{DNA}]} \tag{1}$$

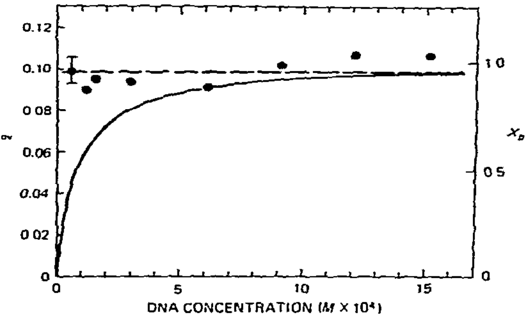

Fig. 5. DNA concentration dependence (expressed in concentration of nucleotoides) of the yield of covalent binding (f_{cov}) determined at a constant ratio $r = [\mathrm{BPDE}]/[\mathrm{DNA}] = 0.01$, where BPDE is the initial concentration of BPDE molecules; (———) DNA concentration dependence of k (the hydrolysis rate constant) and of X_b, the fraction of diol epoxide molecules bound to DNA noncovalently (intercalation) just after mixing the solutions (from ref. 20); 25±0.1°C, 5 mM sodium cacodylate buffer solution, pH 7.0.

where [BPDE] refers to the initial concentration of diol epoxide molecules.

With $r = 0.01$ at all DNA concentrations, it is found that f_{cov} is independent of [DNA] and is equal to 0.098 ± 0.007. This means that only approx. 10% of the diol epoxide molecules under these particular reaction conditions decay by covalent binding rather than by hydrolysis, at all DNA concentrations greater than 5×10^{-5} M.

This constancy in f_{cov} is observed only if DNA is present in excess (small r). If the experiments depicted in fig. 5 are performed not at constant r, but at a constant BPDE concentration, r will rise as the DNA concentration is decreased, giving rise to an apparent decrease in f_{cov}. We have found that this occurs when $r \gtrsim 0.1$, i.e., when there are less than five base-pairs available for binding per BPDE molecule. MacLeod et al. [37] have indeed observed a DNA concentration dependence of f_{cov}, since their reactions were apparently carried out under conditions such that r was greater than 0.11.

Therefore, in order to measure the intrinsic reactivities of diol epoxides with DNA, it is evident that the concentration of DNA should always be kept in excess ($r \ll 0.1$).

4. Discussion

We first discuss the pH dependence of the hydrolysis rate constant k. Subsequently, a kinetic model is postulated and the experimental results are discussed in terms of this model.

4.1. The pH dependence

It is of interest to compare the hydrolysis mechanisms of BPDE in DNA-free buffer solution, and in the same buffer solution, but with DNA added.

In buffer solution the hydrolysis rate constant can be represented by the following [30,31]:

$$k = k_o + k_H a_H + k_{HA}[HA] \qquad (2)$$

where a_H is the activity of H^+, k_H the rate constant for specific acid catalysis, [HA] the cacodylic acid concentration and k_{HA} the appropriate general acid catalysis rate constant; the term k_o represents the spontaneous (water-induced) rate constant. In the pH range investigated, general and specific base catalysis appear to be negligible and thus have not been included.The value of k_{HA} was determined by measuring k as a function of added concentration of cacodylic acid at pH = 7.0 in the range 0–30 mM. Utilizing the fact that the pK of cacodylic acid is 6.15 *, we find from the slopes of such plots (which are linear) that $k_{HA} = 0.71 \pm 0.02$ M^{-1} s^{-1} **.

The quantities k_o and k_H were also determined from the data in fig. 2. Using the known pK_a value of cacodylic acid, the HA concentration was calculated at each experimental pH value. Then, since the experimental values of k are known, the quantity $k - k_{HA}[HA] = k_o + k_H a_H$ was calculated and plotted vs. a_H according to eq. 2. The data can be well represented by a straight line with an intercept of $k_o = (4.4 \pm 0.2) \times 10^{-4}$ s^{-1}, and slope $k_H = (2.1 \pm 0.2) \times 10^3$ M^{-1} s^{-1}. Utilizing eq. 2, the solid line in fig. 2 ([DNA] = 0) has been calculated using these constants, and superimposed on the experimental points. A good representation of the acid- and buffer-catalyzed hydrolysis processes is thus obtained. Whalen et al. [38] have previously obtained $k_o = (5.4 \pm 0.8) \times 10^{-4}$ s^{-1} in water, and $k_H = (1.4 \pm 0.2) \times 10^3$ M^{-1} s^{-1}. Our values are slightly different, but the overall agreement can be considered to be satisfactory.

In the presence of DNA, however, the hydrolysis rate constant increases in magnitude for pH <

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* We remeasured the pK_a of cacodylic acid under our own reaction conditions (5 mM concentration) and obtained a pK_a of 6.1, which is sufficiently close to the published handbook value of 6.15.

** Experiments of determining k as a function of cacodylic acid concentration (0–30 mM) were performed in water as well as in 0.1 M $NaClO_4$ solution to maintain the ionic strength constant; in the latter case the ionic strength varied by not more than 10%, while without the added salt the ionic strength varied by a factor of as much as 6 as the cacodylic acid concentration was varied. A plot of k vs. the cacodylic acid concentration is linear and the values of k_{HA} can be determined from the slopes. Thus, within experimental error, the slopes of the lines with and without added $NaClO_4$ were the same. Thus, no effect of ionic strength on the hydrolysis rate of BPDE catalyzed by cacodylic acid was detected (see ref. 56). The reasons for selecting 0.1 M $NaClO_4$ to maintain the ionic strength constant are also discussed in ref. 56.

9.0 and, in the range pH 7.0–8.5, the experimental points (open circles in fig. 2) can be superimposed on a straight line whose slope is −1.0 as shown in fig. 2. Above pH 9, the values of k are the same with and without DNA. The hydrolysis rate constant is thus represented by the equation:

$$k(\mathrm{DNA}) = k'_{\mathrm{H}} a_{\mathrm{H}} + k_{\mathrm{o}} \qquad (3)$$

where k'_{H} is the hydronium ion catalysis rate constant for BPDE complexed with DNA. For the DNA concentration of 1.5×10^{-4} M utilized in the experiments of fig. 2, we find that $k'_{\mathrm{H}} = (3.6 \pm 0.4) \times 10^{5}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. Michaud et al. [23] have recently studied the salt concentration and pH dependence of the DNA-catalyzed hydrolysis of BPDE; a similar pH dependence was observed and the value of k'_{H} was reported to be 8.55×10^{4} $\mathrm{M}^{-1}\ \mathrm{s}^{-1}$ under somewhat different experimental conditions from ours.

The ratio of the hydronium ion-catalyzed constants in the presence and absence of DNA is $k'_{\mathrm{H}}/k_{\mathrm{H}} = 180$. This enhancement for complexed BPDE may be due to a direct attachment of the hydronium ion to the epoxide oxygen thus catalyzing the formation of a benzylic carbocation in a rate-determining step [30]. The local environment of BPDE complexed to DNA may facilitate this process for reasons that are not yet clear. However, general acid catalysis would also give the same pH dependence. Thus, Michaud et al. [23] suggest that another, kinetically equivalent mechanism involving general acid catalysis by a protonated phosphodiester group of the DNA is possible. Such a mechanism in general acid-catalyzed hydrolysis of several different epoxides by unionized phosphoric acid, even at pH values about 4 units above the pK_{a} of phosphoric acid, have been reported [39,40].

4.2. Kinetic equations

It is well established that BPDE forms noncovalent complexes with DNA immediately (within less than a few milliseconds) upon mixing [22]. If $r \ll 1$, and if the DNA concentration is in excess of 10^{-3} M, more than 95% of the diol epoxide molecules are bound to the DNA. The ensuing hydrolysis reaction occurs on time scales of minutes and appears to take place at these DNA-binding sites [20].

The constancy of the fraction of BPDE molecules which bind covalently to DNA (fig. 4), as well as the parallel behavior of the kinetics of the hydrolysis and the covalent binding reactions, suggest that both reactions may proceed via a kinetically common intermediate. The rate-determining step in both reactions is the formation of this intermediate which is both temperature and pH dependent. Based on previous work on the mechanisms of hydrolysis of BPDE in aqueous solutions [30,31,41–46] and the chemical reactivities of aromatic epoxide derivatives in general [5,29,32,47,48], this reactive intermediate is likely

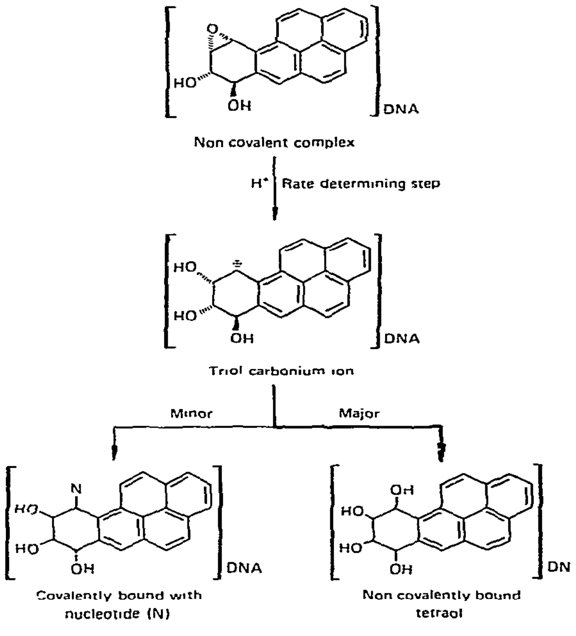
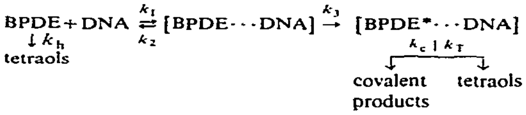


Fig. 6. Proposed reaction scheme for BPDE noncovalently bound to DNA (intercalation complex). Formation of a triol carbonium ion constitutes the rate-determining step. For the sake of simplicity, the stereochemical properties of the reaction products are not indicated.

to be a benzylic triol carbocation derived from the oxirane ring opening of BPDE bound noncovalently to DNA. The structure of a protonated triol carbonium ion, and the suggested reaction scheme in general, are depicted in fig. 6.

A more quantitative picture of this kinetic model is useful in order to allow a better insight into the different factors which govern noncovalent binding of BPDE to DNA, the hydrolysis, and covalent binding reaction of the complexed diol epoxide molecules.

The simplest kinetic scheme which encompasses all of the above considerations is the following:



$$\begin{array}{ccccc} \mathrm{BPDE+DNA} & \underset{k_2}{\overset{k_1}{\rightleftarrows}} & [\mathrm{BPDE\cdots DNA}] & \xrightarrow{k_3} & [\mathrm{BPDE^{*}\cdots DNA}] \\ \downarrow k_{\mathrm{h}} & & & & k_{\mathrm{c}} \downarrow k_{\mathrm{T}} \\ \text{tetraols} & & & & \text{covalent products} \quad \text{tetraols} \end{array} \tag{4}$$

It is assumed that the activated complex (rate constant of formation k_3) decays irreversibly either by reacting with a nearby nucleic acid base, or by the formation of tetraols. In order to simplify the notation, the following abbreviations will be adopted:[BPDE] = [B], the BPDE$\cdots$DNA noncovalent complex will be denoted by C, and the activated comples by C*, and the DNA concentration is replaced by [D].

The rate equations describing the time dependence of [B], [C] and [C*] are:

$$\frac{\mathrm{d}[\mathrm{B}]}{\mathrm{d}t} = -\{k_{\mathrm{h}} + k_1[\mathrm{D}](1 - yr_{\mathrm{c}})\}[\mathrm{B}] + k_2[\mathrm{C}] \tag{5}$$

$$\frac{\mathrm{d}[\mathrm{C}]}{\mathrm{d}t} = k_1[\mathrm{B}][\mathrm{D}](1 - yr_{\mathrm{c}}) - (k_2 + k_3)[\mathrm{C}] \tag{6}$$

$$\frac{\mathrm{d}[\mathrm{C}^*]}{\mathrm{d}t} = k_3[\mathrm{C}] - (k_{\mathrm{c}} + k_{\mathrm{T}})[\mathrm{C}^*] \tag{7}$$

where $r_{\mathrm{c}} = [\mathrm{C}]/[\mathrm{D}]$, and y is the number of bases which become unavailable to physical binding by a second BPDE molecule, after a first one has bound noncovalently. The first two coupled equations can be solved separately, thus obtaining the quantities $[\mathrm{B}(t)]$ and $[\mathrm{C}(t)]$.

Since C* is presumed to be highly reactive, its concentration is likely to be very small ($k_{\mathrm{c}}, k_{\mathrm{T}} \gg k_3$). As usual in kinetic problems of this type, under these conditions the stationary-state approximation $\mathrm{d}[\mathrm{C}^*]/\mathrm{d}t = 0$ can be applied, giving:

$$[\mathrm{C}^*] \approx \frac{k_3}{k_{\mathrm{c}} + k_{\mathrm{T}}}[\mathrm{C}] \tag{8}$$

The coupled equations, eqs. 5 and 6, can be easily solved in the case where $r_{\mathrm{c}} \ll 1$. Since in our work $r \ll 0.1$, the r_{c} terms in these equations can be neglected.

We have previously shown [20] that the condition

$$k_1[\mathrm{D}], k_2 \gg k_3, k_{\mathrm{h}} \tag{9}$$

is valid for noncovalent complex formation between BPDE and DNA. This means that the formation and dissociation of the complexes are much more rapid than the decay of BPDE either within the complex or in the aqueous solution outside of the DNA macromolecule. When the condition, eq. 9, is fulfilled, both of the species (B and C) decay according to first-order kinetics with the same rate constant [20]:

$$[\mathrm{C}(t)] = [\mathrm{C}_0]e^{-\lambda t}$$

$$[\mathrm{B}(t)] = [\mathrm{B}_0]e^{-\lambda t} \tag{10}$$

where the quantities $[\mathrm{B}_0]$ and $[\mathrm{C}_0]$ are the initial concentrations of free and DNA-bound noncovalent diol epoxide molecules respectively. These two quantities are related to one another by the equilibrium constant K:

$$K = \frac{k_1}{k_2} = \frac{[\mathrm{C}_0]}{[\mathrm{B}_0][\mathrm{D}]} \tag{11}$$

The rate constant λ can be identified with the experimentally observed hydrolysis rate constant k and its value is [20].

$$k = \lambda = \frac{k_{\mathrm{h}}}{1 + K'[\mathrm{D}]} + k_3\frac{K[\mathrm{D}]}{1 + K'[\mathrm{D}]} \tag{12}$$

The first term on the right-hand side represents the rate constant of hydrolysis of uncomplexed BPDE; the second term describes the decay of complexed molecules, and is the rate-determining step of the formation of tetraols and covalent products when DNA is present in excess. The constant K' is given by:

$$K' = \frac{k_1}{k_1 + k_2 + k_3} \approx K \tag{13}$$

We have shown experimentally [20] that $K' \approx K$, signifying that $k_1, k_2 \gg k_3, k_h$ in accord with the approximation of eq. 9.

4.3. *Characteristics of covalent binding*

The rate of appearance of covalent products (cov) is given by

$$\frac{d[\mathrm{cov}]}{dt} = k_c[\mathrm{C}^*] \tag{14}$$

The concentration of covalently bound BPDE molecules at any time t after the start of the reaction is

$$[\mathrm{cov}]_t = \left(\frac{k_c}{k_c + k_T}\right)\left(\frac{k_3}{k}\right)[\mathrm{C}_0](1 - e^{-kt}) \tag{15}$$

This equation is in accord with the experimentally observed kinetics of the covalent binding [34], and the fact that the observed pseudo-first-order rate constat is equal to the hydrolysis rate constant k.

We define the fraction X_b of diol epoxide molecules which are complexed noncovalently to DNA:

$$X_b = \frac{[\mathrm{C}]}{[\mathrm{B_T}]} = \frac{K[\mathrm{D}]}{1 + K[\mathrm{D}]} \tag{16}$$

Where $[\mathrm{B_T}] = [\mathrm{B_0}] + [\mathrm{C_0}]$ is the total concentration of diol epoxide molecules which are initially present. The fraction of BPDE molecules which at the end of the reaction ($t \to \infty$) become bound covalently to DNA can be calculated from eq. 12, 13, 15 and 16, and is equal to the quantity f_{cov} (see fig. 4):

$$\begin{aligned} f_{\mathrm{cov}} &= \left(\frac{k_c}{k_c + k_T}\right)\left(\frac{k_3 X_b}{k}\right) \\ &= k_3\left(\frac{k_c}{k_c + k_T}\right)\left(\frac{K[\mathrm{D}]}{k_h + k_3 K[\mathrm{D}]}\right) \end{aligned} \tag{17}$$

This equation explains the lack of a dependence of f_{cov} on the DNA concentration (fig. 5). Since $k_h \approx 10^{-3}\ \mathrm{s}^{-1}$, $k_3 = 0.068\ \mathrm{s}^{-1}$, and $K = 12000\ \mathrm{M}^{-1}$ at pH 7 [20], for all DNA concentrations $[\mathrm{D}] \geqslant 10^{-5}$ M, eq. 14 reduces to the DNA-independent quantity:

$$f_{\mathrm{cov}} \approx \frac{k_c}{k_c + k_T} \tag{18}$$

in agreement with the experimental results. It should be emphasized that this relationship is valid only if DNA is present in excess, and the hydrolysis of free BPDE in the solvent medium is a negligible decay channel compared to hydrolysis of BPDE at the DNA-binding sites.

Thus, while the intercalation binding isotherm X_b (eq. 16, fig. 5) and the experimentally measured hydrolysis rate k increase strongly in the DNA concentration range 5×10^{-5}–10^{-3} M, f_{cov} remains constant within experimental error. This fact suggests that changes in the equilibrium constant K, brought about by changes in the solvent composition, do not effect the values of f_{cov}, provided $k_3 X_b \gg k_h(1 - X_b)$.

For example, we have shown that addition of ethanol to a rection mixture (BPDE, DNA, 5 mM sodium cacolylate buffer) decreases k and X_b significantly, but f_{cov} remains unchanged [34]. Because of dynamic equilibrium (large k_1 and k_2), even if $X_b \ll 1.0$ all BPDE molecules will have a chance to reside at a DNA-binding site and react there with a rate constant k_3, which is the rate-determining step for both hydrolysis and covalent binding. Thus, f_{cov} is independent of X_b upon addition of ethanol as long as decay channels of BPDE in the free solution can be neglected ($k_h \ll k_1\mathrm{D}, k_3$).

4.4. *The pH and temperature dependence*

We have experimentally verified that X_b, the fraction of noncovalently intercalated BPDE molecules, and the equilibrium constant K do not change significantly with temperature nor as a function of pH in the pH range studied. The decreases in the hydrolysis rate constant k with decreasing temperature or increasing pH thus cannot be attributed to variations in K, nor in X_b.

In fig. 3, the slope of the lines drawn through the experimental points of k as a function of $1/T$

at the two different DNA concentrations are the same within experimental error. At the lower DNA concentration $X_b = 0.7$, while at the higher DNA concentration $X_b = 0.9$, and the dependence of k on the equilibrium constant K, and thus X_b, disappears. Since $k_3 \gg k_h$ in these experiments, these results indicate that the activation energy $E_a = 8.7 \pm 0.9$ kcal/mol pertains to the rate-determining step characterized by k_3. In the buffer solution the hydrolysis mechanism is a composite one consisting of three terms (eq. 2), and it is not possible to attribute the observed $E_1 = 14.2 \pm 0.7$ kcal/mol to any one of these mechanisms. It is of interest to note, however, that there is a significant lowering of the effective activation energies upon binding of BPDE to DNA which, at least in part, accounts for the enhancement of the hydrolysis rate in the presence of DNA.

Throughout the temperature range investigated (figs. 3 and 4), $k_h \ll k$, f_{cov} reduces to eq. 18. Therefore, under these conditions, the fraction of BPDE molecules initially present which bind covalently to DNA is independent of X_b, the fraction which is noncovalently bound to DNA at any instant of time. This is supported by data in fig. 4 (bottom) which shows that f_{cov} is independent of temperature at $X_b = 0.7$, and by the fact that the same f_{cov} values are obtained when $X_b = 0.9$ (data not shown). At $X_b = 0.7$, only 70% of all BPDE molecules present are bound noncovalently to DNA at any instant of time. However, using the values of k_3 and k_h (pH 7) given under eq. 17, it can be calculated that greater than 99% of the BPDE molecules undergo reaction while bound noncovalently to the DNA, rather than as free molecules in the solution.

Because of the dynamic equilibrium in eq. 4 with $k_1, k_2 \gg k_3, k_h$ there is a rapid exchange of BPDE molecules between the binding sites and the outside solution. As long as the rate constant of hydrolysis for free BPDE is lower than the rate of reaction at DNA-binding sites, f_{cov} is independent of X_b.

Similar conclusions can be reached regarding the pH dependence of k (fig. 2) and of f_{cov} (fig. 4, top). However, as the pH is raised, $k \to k_h$ in the range pH 9.0–9.5; under these conditions, $k_3 = k_h$ for all values of X_b, while eq. 17 reduces to

$$f_{cov} = \left(\frac{k_c}{k_c + k_T} \right) X_b \tag{19}$$

Thus, at pH values for which $k \gg k_n$, f_{cov} obeys eq. 18 and gradually changes to eq. 19 as $k \to k_h$ at the higher pH values. However, in the presence of an excess of DNA, when $X_b \to 1$, f_{cov} is predicted to be independent of the values of k, even when $k = k_h \approx k_0$. Thus, provided X_b is independent of pH (which we have experimentally verified in the range pH 7–9.5), f_{cov} is also independent of pH (fig. 4, top), within the experimental error of ±8%.

4.5. Effects of ionic strength on f_{cov} and on k

Additions of small amounts of Mg^{2+} [20,35] and Na^+ can bring about dramatic decreases in

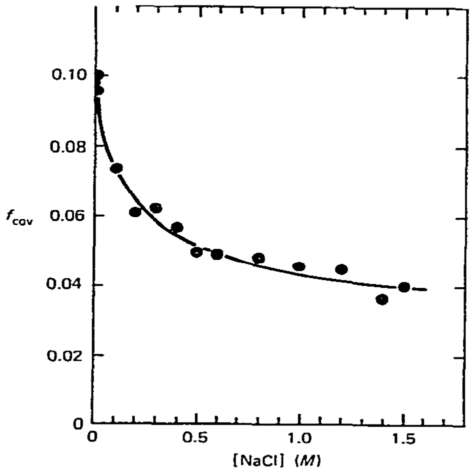


Fig. 7. Effect of NaCl concentration on the yield of covalent binding products (f_{cov}), determined at 25.0±0.1°C, pH 7.0, 5 mM sodium cacodylate buffer solution, [DNA] = 7.5×10^{-4} M, initial [BPDE] = 1.7×10^{-5} M. Note: the BPDE solubility under these conditions decreases with increasing salt concentration, however, this effect does not affect f_{cov} as long as the reaction time is at least 12–24 h. This was demonstreated by carying out similar reactions in 15% ethanol: the solubility is unaffected by NaCl under these conditions, and identical results to those shown here are obtained.

Table 1

Effect of NaCl concentration on hydrolysis (k, k_3), noncovalent binding (X_b), and covalent binding (f_{cov}) of BPDE in DNA (7.5×10^{-4} M) solution of pH 7.0, 25 °C, 5 mM sodium cacodylate buffer

[NaCl] (M)	k (s^{-1})($\times 10^3$)	k_3 (s^{-1})($\times 10^3$)	X_b	$(k_3 X_b)/k$ *	f_{cov}
0	67 ± 3	74 ± 3	0.90 ± 0.02	0.99	0.10
0.5	3.5 ± 0.3	5.2 ± 0.3	0.52 ± 0.02	0.86	0.050
1.0	1.5 ± 0.2	2.0 ± 0.2	0.45 ± 0.02	0.62	0.046
1.5	1.1 ± 0.2	1.3 ± 0.2	0.42 ± 0.03	0.49	0.040

* Fraction of BPDE molecules which undergo reaction while complexed to DNA.

the hydrolysis rate constant k. Addition of cations is known to decrease intercalaction by aromatic dye molecules [49], of BPDE and of similar molecules [35,50]. It has also been reported that Mg^{2+} and Na^+ reduce the level of covalent binding of BPDE to DNA [37,51]. For example, Gamper et al. [51] have studied the NaCl concentration dependence of the covalent binding of BPDE to viral DNA; however, their experiments were performed at high relative concentrations of BPDE ($r = 0.6$). In order to explore further the relationships between f_{cov}, k and X_b we investigated the effect of NaCl on these variables at pH 7 and in the presence of an excess of DNA ($r = 0.022$).

The effect of NaCl concentration on f_{cov} is shown in fig. 4. In contrast to the behavior of the efficiency of covalent binding as a function of pH, temperature, and ethanol concentration [34], there is a significant decrease in f_{cov} as the salt concentration is increased. In view of the dependence of f_{cov} on $k_3 X_b$ and k (eq. 17), it is necessary to examine the behavior of these parameters at different NaCl concentrations (table 1).

As reported by Michaud et al. [23], k decreases with increasing salt concentration by a factor of as much as 70. However, X_b, the fraction of BPDE molecules bound noncovalently to DNA (as measured according to previously described absorption techniques [20,22]), decreases by a factor of only 2–3 in the same range of salt concentrations. At this particular DNA concentration (7.5×10^{-4} M), X_b is a relatively weak function of the non-covalent equilibrium constant K. Using eq. 16 and $K = 12000 \pm 2000$ m M^{-1} [20] for [NaCl] = 0, values of $K = 1400 \pm 250$, 1100 ± 200 and 750 ± 150 M^{-1} at 0.5, 1.0 and 1.5 M NaCl, respectively, can be calculated. Michaud et al. [23] found a value of 2400 M^{-1} under different conditions of ionic strength (0.1 M NaCl and 1 mM sodium cacodylate buffer concentration) which is consistent with our data. Such decreases in K in the presence of an excess of positive cations can be attributed to a reduction in the repulsive forces between neighboring negatively charged phosphate groups, resulting in a tighter winding of the helix and a decrease in the base-pair separation [52]. It is therefore reasonable to assume that insertion of a BPDE molecule between adjacent base-pairs becomes energetically more difficult, thus leading to a lowering in the values of K.

The values of $k_h \approx 10^{-3}$ s^{-1} are independent of ionic strength within experimental error. Thus, k_3 can be calculated utilizing eq. 12 and the data in table 1; as reported elsewhere [23], we also find that k_3 decreases more dramatically with increasing NaCl concentration than either K or X_b. Thus, the major effect of increasing ionic strength on the reaction rate constant k is due to variations in k_3 rather than on the fraction of molecules X_b which are intercalated.

The factor $k_3 X_b / k$, the fraction of BPDE molecules which undergo reaction while bound noncovalently to DNA, is tabulated at various NaCl concentrations in table 1. This fraction is greater than 99% at zero NaCl concentration, but decreases to 40% at 1.5 M NaCl. According to eq. 17, f_{cov} is proportional to variations in $k_3 X_b / k$ only if the reaction branching ratio $k_c/(k_c + k_T)$ remains independent of ionic strength. By comparing the last two columns on the right in table 1 it is evident that the NaCl-induced variations in these two quantities are different from one another. Thus, utilizing eq. 17 and the data in table 1, it is evident that the branching ratio decreases from 0.1

at zero NaCl concentration to values between approx. 0.06 and 0.08 at the higher salt concentrations. We thus conclude that the decrease in f_{cov} with increasing ionic strength is induced in part by a drop in the fraction of molecules which undergo reactions at DNA-binding sites, and in part to decreases in the branching ratio $k_c/(k_c + k_T)$. The exact dependence of this ratio as a function of ionic strength is presently being investigated in detail and will be reported elsewhere. The effects of Na^+ on the branching ratio are small as compared to the effect on the rate of formation of the triol carbonium ion (k_3). The effect of positive metal ions on the latter effects was considered by Miller et al. [53] from a theoretical point of view: counterions, upon binding to DNA, may displace H^+ or other acids which otherwise induce carbonium ion formation by general acid catalysis, thus decreasing the value of k_3. Variations in k_T/k_c, on the other hand, are likely to be due to changes in the microstructure of the triol carbonium ion-DNA transition complex and/or to variations in the electrostatic free energy change on the binding of the charged transition state complex to DNA [23].

5. Conclusions and summary

Experimental results [20,22] and the covalent binding data of Meehan and Straub [54], as well as the theoretical considerations by Lin et al. [55] and Miller et al. [53], indicate that a noncovalent intercalation type of complex is formed rapidly upon mixing BPDE into a DNA solution. The three reaction pathways of BPDE in the presence of DNA are thus (1) hydrolysis to tetraols of free uncomplexed BPDE, (2) hydrolysis at DNA-binding sites, and (3) covalent binding of BPDE at DNA-binding sites. Pathways 2 and 3 are parallel pseudo-first-order reactions which appear to involve a common intermediate. This intermediate is most likely a triol carbonium ion whose rate of formation (rate constant k_3) is greatly accelerated upon binding of BPDE to DNA at pH 7, but decreases with decreasing pH, temperature and ionic strength. The rate of addition of water molecules to this carbocation is about 10-times faster than the rate of covalent binding to the DNA bases, and is independent of pH and temperature, but decreases by 20 ± 10% in the presence of NaCl.

The fraction of molecules undergoing the covalent binding reaction (f_{cov}) is directly proportional to $k_3 X_b / k$, which is the fraction of BPDE molecules which undergo reaction at DNA-binding sites rather than in solution, where X_b is the fraction of molecules bound physically to DNA at any instant of time. Thus, f_{cov} is independent of X_b as long as $k_3 X_b \gg k$, and is independent of k_3 and k, provided $X_b \rightarrow 1$, since under these conditions $k = k_h(1 - X_b) + k_3 X_b \approx k_3 X_b$.

In conclusion, it is evident that alterations in the intercalative, physical binding of BPDE to DNA induced by metal ions [37,51,53], and the concomitant decreases in covalent binding, cannot be used as evidence supporting the hypothesis that intercalation (X_b) is or is not an important preliminary step in the covalent binding reaction. The covalent binding efficiency depends on the branching ratio $k_3 X_b / k$. Thus, changes in X_b do not necessarily entail a change in the efficiency of covalent binding.

The noncovalent BPDE-DNA adducts which are initially formed are believed to be intercalation complexes [20,22,35,50]. On the other hand, the covalent BPDE-DNA adducts which are formed subsequently, display the characteristics of external binding site complexes [25–27]. The covalent binding reaction may thus involve externally physically complexed diol epoxide molecule which may be present as minor components [24], or a local conformational transformation at the binding site during or after the covalent binding reaction. These subjects will be discussed in more detail in a future publication.

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