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Hydrolysis of benzo[*a*]pyrene diol epoxide and its covalent binding to DNA proceed through similar rate-determining steps

(kinetics/carcinogens/binding mechanisms/carcinogen–DNA adducts/DNA-catalyzed solvolysis)

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ABSTRACT **The mutagenic and carcinogenic metabolite of benzo[*a*]pyrene, (7*R*,8*S*)-dihydroxy-(9*R*,10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, undergoes two major reactions in the presence of DNA: (*i*) hydrolysis and (*ii*) covalent binding. We report that hydrolysis and covalent binding are specific and general acid-catalyzed reactions with the same or similar rate-determining steps. To account for the similarity of rate-determining steps in covalent binding and hydrolysis we propose and test two models. In each model, the rate-determining step results in formation of a carbonium ion, which serves as a precursor for both tetrol and adduct. In model *A* the carbonium ion is partitioned between two domains (1 and 2), while in model *B* there is only one domain. Measurements of pseudo-first-order rate constants, product ratios, and rate ratios support model *A*, while kinetic results are inconsistent with model *B*. Domain 1 most likely represents activated benzo[*a*]pyrenes that are intercalated into DNA, while domain 2 hydrocarbons are physically bound to the outside of the DNA helix.**

Chemical carcinogens are thought to initiate tumors by inducing mutations (1, 2). Many environmental pollutants are precarcinogens requiring metabolic activation prior to exhibiting biological activity. Benzo[*a*]pyrene belongs to this class of precarcinogen and is metabolically activated to a potent mutagenic and carcinogenic form, (7*R*,8*S*)-dihydroxy-(9*R*,10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)*anti*-BPDE] (3–8). The epoxide undergoes covalent binding to DNA *in vivo* and *in vitro*, with the primary site of attachment the exocyclic amino group of guanine (6, 9–11). Reaction at the guanine site is stereoselective both *in vivo* (6) and *in vitro* (9, 12) and results from asymmetries in the secondary structure of DNA (12). Minor adducts between racemic *anti*-BPDE [(±)*anti*-BPDE] and adenine (9, 10, 13), cytosine (9, 10), and the N-7 position of guanine (14) have been reported.

(±)*anti*-BPDE is unstable in aqueous media and readily undergoes hydrolysis to form isomeric 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrenes (tetrols). Hydrolysis of (±)*anti*-BPDE and the *syn* diastereomer (in which the 7-hydroxyl group is *cis* to the epoxide) occurs by general acid catalysis (15–17). Furthermore, product analyses and ionic strength effects have led to similar proposals for the mechanism of (±)*anti*-BPDE hydrolysis (16, 17). This mechanism involves a rapid equilibrium between general acid catalyst and (±)*anti*-BPDE, followed by proton transfer and carbonium ion formation in the rate-determining step (rds).

DNA catalyzes hydrolysis of (±)*anti*-BPDE (18, 19), and in a recent study the acid dependence of this reaction was investigated at pH 6.5–7.5 (20). The results indicated that hydrolysis in the presence of DNA was also an acid-catalyzed process (20). A model has been proposed for DNA-catalyzed hydrolysis and covalent binding in which a carbonium ion formed in the rds serves as a common intermediate for both reactions (21). The rate of DNA-catalyzed hydrolysis may be important to the total level of covalent adduct obtained and, thus, to the genesis of tumors induced by chemical carcinogens. We have, therefore, investigated the mechanisms of these reactions in an *in vitro* model system utilizing calf thymus DNA. Our results suggest that a carbonium ion is formed in the rds for each process, but the activated intermediates for hydrolysis and covalent binding are formed in different domains. Kinetic results support our model that carbonium ion precursors to covalent adducts are derived from physically intercalated hydrocarbons, while tetrols are formed from carbonium ions generated on the outside of the DNA helix.

MATERIALS AND METHODS

Synthesis. (±)*anti*-BPDE was synthesized as previously described (10, 12). The preparation of ^{3}H-labeled (±)*anti*-BPDE has also been reported (10).

Chemicals. Calf thymus DNA was obtained from Sigma. All other chemicals were obtained from commercial sources and were reagent quality or higher grade purity.

Hydrolysis Kinetics. Hydrolysis of (±)*anti*-BPDE was followed spectrophotometrically (15–17), both at an absorption band of the hydrocarbon (345.5 nm) and at the long-wavelength red-shifted transition representing the BPDE–DNA intercalation complex (centered at 353 nm) (22, 23). First-order plots resulted in straight lines, indicating that hydrolysis was measured under pseudo-first-order conditions. Lines were fitted by least-squares analysis and the resulting slopes were used to determine pseudo-first-order rate constants. Replicate values for rate constants were within 5%.

Covalent Binding Assays. Covalent binding of (±)*anti*-BPDE was measured by using a ^{3}H-labeled hydrocarbon. Samples contained the same components and same concentration as used for the hydrolysis measurements. Reactions were stopped by precipitation of the DNA, and unreacted hydrocarbons were removed by solvent extraction. Rate constants were determined in a manner analogous to the procedure described for the hydrolysis of (±)*anti*-BPDE. Covalent binding assays were routinely run in triplicate and replicate values varied <5%.

Instruments. Radioactivity was measured on a Beckman LS-9000. Spectrophotometric equipment used in these studies has been described (22).

RESULTS

Acid-Catalyzed Hydrolysis and Covalent Binding. Rates of (±)*anti*-BPDE hydrolysis in the presence of DNA and rates of covalent binding are plotted as a function of pH in Fig. 1. The rates were measured in three buffer systems: Tris, sodi-

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Abbreviations: (+)*anti*-BPDE, (7*R*,8*S*)-dihydroxy-(9*R*,10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (±)*anti*-BPDE, racemic *anti*-BPDE; tetrols, isomeric 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrenes; rds, rate-determining step(s).

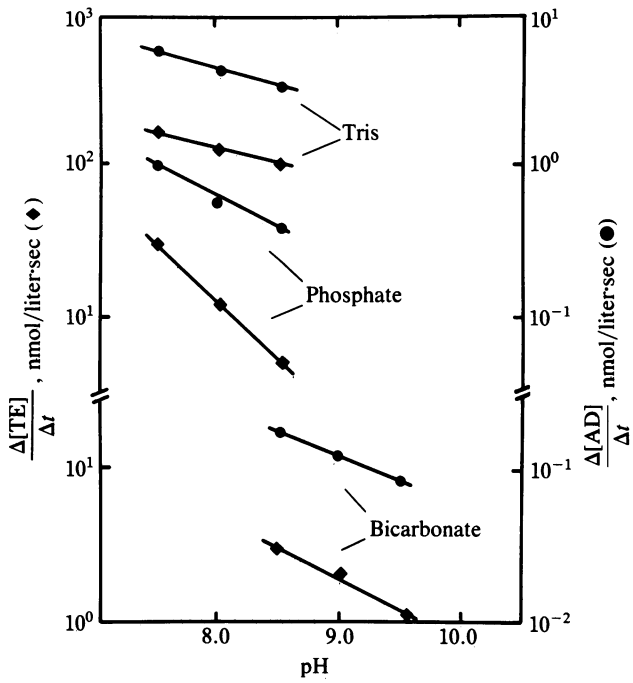

FIG. 1. Initial rates of hydrolysis ($\Delta[TE]/\Delta t$) and initial rates of covalent binding ($\Delta[AD]/\Delta t$) are plotted on logarithmic scales versus pH. To determine initial rates of hydrolysis, assays were carried out for <¼ of one (±)*anti*-BPDE half-life. The same protocol was used to determine initial rates of covalent binding. Rates were measured in the indicated buffers over the pH range 7.5–9.5. Covalent binding and hydrolysis assays were carried out at 21 ± 0.5°C on samples containing 0.01 M buffer, 10% (vol/vol) acetone, and calf thymus DNA at 1 mg/ml. Reactions were started with 10 μl of tetrahydrofuran, containing 10 nmol of (±)*anti*-BPDE, per ml of assay mixture.

um phosphate, and sodium bicarbonate. Rates of DNA-catalyzed hydrolysis and covalent binding were fastest with Tris, intermediate with sodium phosphate, and slowest with sodium bicarbonate. These differences are readily evaluated at pH 8.5, where rates were measured in all three buffer systems. The rates of DNA-catalyzed hydrolysis of (±)*anti*-BPDE were 99, 5.4, and 2.96 nmol/liter·sec, while covalent binding rates were 3.25, 0.38, and 0.18 nmol/liter·sec for Tris, sodium phosphate, and sodium bicarbonate, respectively. The rates of covalent binding and DNA-catalyzed hydrolysis are inversely proportional to pH. These reactions are, therefore, acid-catalyzed processes. Since both reactions are acid catalyzed they must possess the same or similar rds.

General Acid Catalysis. The rate of covalent binding of (±)*anti*-BPDE to DNA is a linear function of bicarbonate (at pH 8.5) and phosphate concentration (at pH 7.5) in the range of 0.02–0.2 M (data not shown). Thus, covalent binding is a general acid-catalyzed reaction. Hydrolysis of (±)*anti*-BPDE in the absence of DNA is also a general acid-catalyzed reaction (15–17). We have also measured hydrolysis rates in the presence of DNA as a function of sodium phosphate concentration (pH 7.5) and find a linear response in rates through the range 0.02–0.2 M (data not shown). Thus, hydrolysis in the presence of DNA is also a general acid-catalyzed reaction.

Sensitivity of Hydrolysis and Covalent Binding to Buffer Concentration. The slopes of the buffer concentration–hydrolysis and covalent binding rate curves are a measure of the sensitivity of these two processes to general acid catalysis. The slope of the hydrolysis–buffer concentration curve was much larger (>10 fold) than the corresponding value obtained from the covalent binding–buffer concentration curve. Thus, hydrolysis is much more sensitive to buffer concentration than covalent binding is.

Model for Common rds. We have developed two models to explain the common rds in covalent binding and hydrolysis. These models are summarized in Fig. 2. We postulate a mechanism for covalent binding and hydrolysis in the presence of DNA, similar to that proposed for hydrolysis of the hydrocarbon in the absence of DNA (16, 17). Thus, the first two steps in both models are the same: (*i*) a rapid formation of [BPDE–HA] complex (in which HA represents a general acid) and (*ii*) carbonium ion formation in the rds to form $BPDEH^+$. In model *A*, the $BPDEH^+$ is partitioned between two domains (see *Discussion*). Due to the short lifetime of the carbonium ion species the partitioning probably occurs before proton transfer. Since the rate of formation of

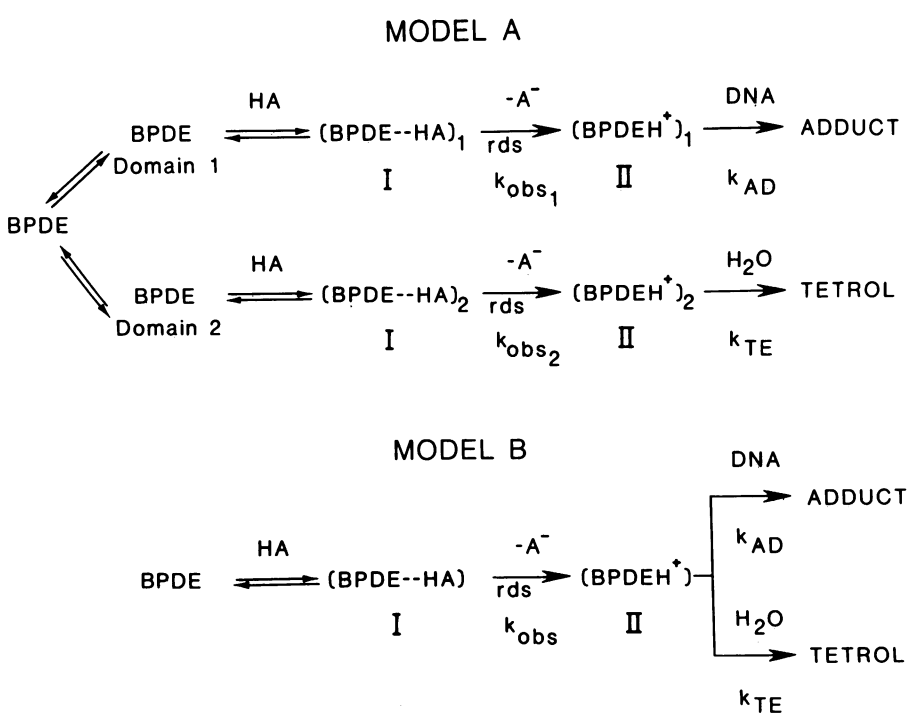

FIG. 2. Models for the acid-catalyzed hydrolysis and covalent binding of (±)*anti*-BPDE to DNA. Model *A* describes the acid-catalyzed formation of carbonium ions in similar rds for hydrolysis and covalent binding in two domains (1 and 2). Model *B* represents carbonium ion formation in the rds for both reactions in the same domain. See text for more details.

$BPDEH^+$ is the rds, tetrol and adduct formation will be proportional to (±)*anti*-BPDE disappearance,

$$\frac{d[AD]}{dt} \propto -\frac{d[BPDE]}{dt} \quad [1]$$

and

$$\frac{d[TE]}{dt} \propto -\frac{d[BPDE]}{dt}, \quad [2]$$

in which [AD] and [TE] = adduct and tetrol concentration, respectively, and [BPDE] = (±)*anti*-BPDE concentration. The proportionality constants for these processes will differ for models *A* and *B*. As a corollary to Eqs. 1 and 2, the rate of hydrolysis and adduct formation must also be proportional to each other,

$$\frac{d[AD]}{dt} \propto \frac{d[TE]}{dt}. \quad [3]$$

If hydrolysis and covalent adduct formation are pseudo-first-order rate processes, then the ratio of their rates of formation will be equal to the ratio of their product concentrations. For model *A* this is expressed as

$$\frac{\frac{d[TE]}{dt}}{\frac{d[AD]}{dt}} = \frac{k'_{TE}[BPDEH^+]_2}{k'_{AD}[BPDEH^+]_1} = \frac{[TE]}{[AD]}, \quad [4]$$

in which k'_{TE} and k'_{AD} = pseudo-first-order rate constants for tetrol and adduct formation, respectively, and $[BPDEH^+]$ = the concentration of protonated (±)*anti*-BPDE. For model *B* the corresponding expression is

$$\frac{\frac{d[TE]}{dt}}{\frac{d[AD]}{dt}} = \frac{k'_{TE}}{k'_{AD}} = \frac{[TE]}{[AD]}. \quad [5]$$

Pseudo-First-Order Rate Plots. Tetrol formation is pseudo-first-order due to the large excess of solvent present. Covalent adduct formation is also pseudo-first-order, due to the presence of DNA containing an excess number of binding sites. Pseudo-first-order rate plots of hydrolysis and covalent binding are presented in Fig. 3. Covalent binding and hydrolysis of (±)*anti*-BPDE in the presence of DNA were carried out in bicarbonate buffer (pH 8.5), and the fraction of reactant converted to product is plotted on a logarithmic scale versus time (Fig. 3*a*). Measurements were also made in sodium phosphate buffer (pH 7.5) and in Tris (pH 8.5), and the curves describing hydrolysis and covalent binding are presented in Fig. 3*b*. From the data, we have calculated the pseudo-first-order rate constants for hydrolysis (k'_{TE}) and covalent binding (k'_{AD}).

Testing for Model *A* or *B*. We have found that the ratio of k'_{TE} to k'_{AD} is nearly equal to 1 under several experimental conditions. Utilizing this result, we have calculated the value of product and rate ratios for models *A* and *B*, and these results are presented in Table 1. For model *B* if $k'_{TE}/k'_{AD} = 1$, the product ratio must also be equal to 1. However, we have observed a tetrol-to-adduct product ratio of ≈20 (see Table 1), which is clearly inconsistent with model *B*. In model *A*, if the ratio of k'_{TE} to k'_{AD} is equal to 1, the rate ratios can take on any value consistent with the ratio of $[BPDEH^+]_2/[BPDEH^+]_1$. Thus, the results of product and rate ratio measurements are fully consistent with model *A*.

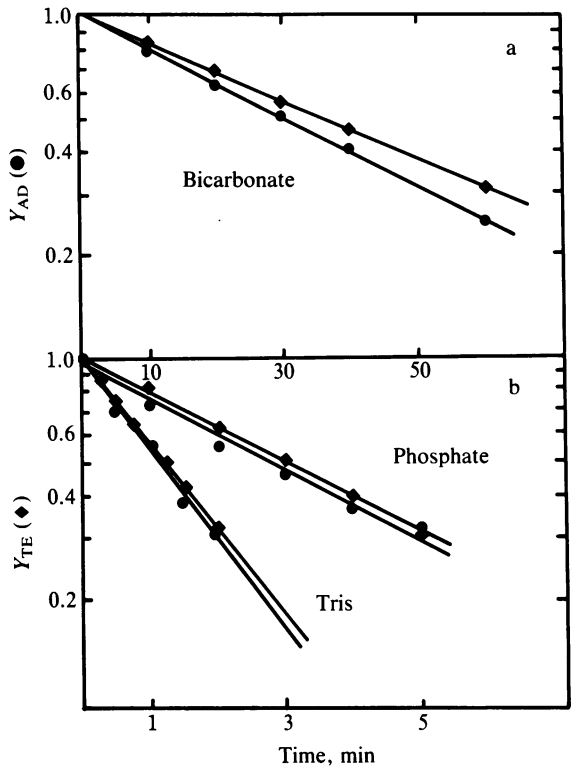

FIG. 3. Pseudo-first-order plots of (±)*anti*-BPDE covalent binding and DNA-catalyzed hydrolysis. The fractions of reactants converted to covalent adduct (Y_{AD}) or the fractions of reactants converted to tetrol (Y_{TE}) are plotted on a logarithmic scale against time. The amount of tetrol formed was calculated as the difference between the total amount of (±)*anti*-BPDE reacted (measured spectrophotometrically) and the amount that resulted in the formation of covalent adducts. Reverse-phase high-performance liquid chromatography of typical assays detected only tetrols and the expected small amounts of stable covalent adducts reported previously (10, 12). Slopes were used to calculate pseudo-first-order rate constants (k'_{AD} and k'_{TE} for adduct and tetrol, respectively). Points are experimental, lines are derived from least-squares analyses. Reactions took place in bicarbonate (*a*) or phosphate and Tris (*b*).

DISCUSSION

DNA-catalyzed hydrolysis of (±)*anti*-BPDE has been reported to undergo a significant rate decrease with increasing

Table 1. Kinetic parameters used to distinguish models *A* and *B*

Parameter	Values		
	Observed	Predicted	
		Model *A*	Model *B*
k'_{TE}/k'_{AD}*	≈1	1	1
Rate ratio†	≈20	$1 \times \frac{[BPDEH^+]_2‡}{[BPDEH^+]_1}$	1
Product ratio§	≈20	$1 \times \frac{[BPDEH^+]_2‡}{[BPDEH^+]_1}$	1

*The ratio of pseudo-first-order hydrolysis and covalent binding rate constants.
†Hydrolysis to covalent binding rate ratios.
‡See under *Models for Common rds* in *Results*.
§Ratio of tetrol to covalent adducts.

salt concentration (20). On the basis of these results, it was proposed that hydrolysis occurred by a general acid-catalyzed mechanism (with hydronium ion or DNA phosphodiester groups as proton donors) and that a physically intercalated BPDE was an intermediate in the process. The salt effect was interpreted as electrostatic destabilization in the binding of a charged transition intermediate to the polyanionic DNA (20). Our results confirm the salt effect on BPDE hydrolysis. However, several considerations are inconsistent with the proposed physical intercalation–hydrolysis mechanism (20). (*i*) Intercalation and carbonium ion reaction rates do not support the proposal that a charged transition intermediate physically binds to DNA. Although $BPDEH^+$ generation in the rds is slow ($k \approx 10^{-2}$ sec^{-1}), nucleophilic attack of the carbonium ion, once formed, is much faster than physical intercalation. Alkylation of amines by a benzyl cation in organic solvent occurs with a rate constant of $>10^9$ liter/mol·sec (24). The lifetime of a carbonium ion in polar medium (on the outside of DNA) would also be on the same order, since stabilization due to a polar environment would be offset somewhat by the presence of water as the nucleophile. We have measured intercalation rates by temperature-jump relaxation methods and find rate constants of $\approx 10^3$–10^4 liter/mol·sec (unpublished results). These rates indicate that carbonium ions will react with nucleophiles before any significant physical binding can take place. Thus, $BPDEH^+$ generated on the outside of DNA will react with the nearest nucleophile (water), while those generated inside will react primarily with nucleophilic sites in the biopolymer. (*ii*) Model building studies indicate that protonation of intercalated (±)*anti*-BPDE by phosphodiester groups in DNA is unlikely because it would require significant distortion of the helix. We have reported a superhelical DNA unwinding angle for intercalated (±)*anti*-BPDE of 13° (22), which suggests that this complex results in relatively minor distortion of DNA. These results do not rule out phosphate participation in the domain 2 solvolysis reaction. (*iii*) Hydrolysis of a significant fraction of intercalated hydrocarbon is not supported by the two-domain model reported here.

One study has dealt with both covalent binding and DNA-catalyzed hydrolysis of BPDE (21). Each reaction was first-order with respect to acid concentration and the ratio of covalent adducts to solvolysis products was constant as a function of pH (21). These results suggested that the two reactions share the same rds. It was proposed that protonation of BPDE occurred only after physical intercalation of the hydrocarbon into DNA, which is equivalent to model *B* presented in Fig. 2. We also obtain similar results in our pH studies; however, product ratios did vary slightly. Salt and buffer concentrations had more pronounced differential effects on these reactions. Presumably, the smaller proton can more readily reach both domains 1 and 2, while steric factors probably restrict the bulkier molecules to the outside of the DNA helix. Thus, protonations of (±)*anti*-BPDE in the rds for covalent binding and hydrolysis, although similar reactions, are not identical since they occur in different domains.

If a common intermediate existed for both covalent binding and hydrolysis, the ratio of pseudo-first-order rate constants for both of these processes must be equal to the ratio of their products. Since we found widely different values for these ratios, we propose a two-domain model for covalent binding and hydrolysis. For this model the product and rate ratios need not necessarily be the same. Domain 1 results from physically intercalated BPDE. Carbonium ion formation in this domain results in covalent adduct formation. Domain 2 is occupied by physically bound hydrocarbons on the outside of the DNA helix. Protonation in this domain results in solvolysis products. Several lines of evidence support this model. (*i*) Covalent binding is slightly more efficient than hydrolysis ($k'_{AD} > k'_{TE}$). Formation of a carbonium ion may be more favorable (lower energy of activation) in a physical intercalation complex compared to the relatively free hydrocarbons bound to the outside of the DNA helix. (*ii*) Domain 2 is much more sensitive to buffer concentration than domain 1 (which may be due to relative accessibility). (*iii*) DNA-catalyzed hydrolysis has been shown to be highly sensitive to salt concentration (20). We also find that hydrolysis is sensitive to salt, and this sensitivity is much greater than for covalent binding (unpublished results). These results can also be explained by the relative accessibility of domain 2. This analysis is supported by the report that physical binding of proflavin to the outside of the DNA helix is much more sensitive to salt concentration than is intercalation of proflavin (25). This is presumably due to strong electrostatic interactions between salt and DNA phosphates on the outside of the helix. The proflavin results (25) also support the interpretation of the buffer concentration experiment discussed under point *ii*. (*iv*) The ratio of products for covalent binding and hydrolysis and pseudo-first-order rate constants strongly support the two-domain model. Binding evidence (see below) supports physically intercalated hydrocarbons in domain 1, while the requirements for DNA-catalyzed hydrolysis support outside bound hydrocarbons in domain 2.

Several observations support our previously proposed physical intercalation–covalent binding mechanism (12, 22). (*i*) We have found that intercalation is faster than alkylation (see above). This is a necessary but not sufficient condition for the mechanism. (*ii*) Alkylation targets are highly specific in DNA—e.g., guanine bases and exocyclic amino groups (9, 10). (*iii*) Covalent adduct formation with double-stranded DNA is stereoselective for (+)*anti*-BPDE (12). (*iv*) Intercalation levels correlate with covalent binding levels under a variety of conditions (unpublished results). (*v*) Kinetic data (reported here) suggest that covalent binding occurs in a different domain than hydrolysis. Our physical intercalation–covalent binding model does not predict the conformation of the adduct after the alkylation step. Several conflicting reports have appeared on this issue (26–29) and separate experiments will be needed to resolve it.

It has been suggested that enhancing the solvolysis rate of (±)*anti*-BPDE might protect an organism from the deleterious effects of epoxide-bearing chemical carcinogens (30). Our results show that increasing solvolysis rates under some conditions (e.g., with pH) also causes a proportional increase in covalent binding rates, with the net effect that covalent binding levels remain about the same. To protect cells from activated carcinogens, agents must be used that (*i*) differentially act on the rates of hydrolysis and covalent binding (e.g., salt or buffer concentration) or (*ii*) reduce intercalation levels. The reported inhibition of (±)*anti*-BPDE mutagenicity by riboflavin 5′-phosphate has been attributed to its catalytic effect on hydrolysis rates (30), but it probably involves, in addition, a reduction in DNA intercalation levels.

The results of our study demonstrate that both hydrolysis and covalent binding follow a similar general acid-catalyzed mechanism. Since DNA markedly catalyzes hydrolysis of (±)*anti*-BPDE (18, 19), an intriguing outcome of this report is that the biopolymer must also catalyze, or more aptly autocatalyze, formation of covalent adducts with itself. This is a consequence of the DNA-catalyzed formation of $BPDEH^+$ in the rds of both hydrolysis and covalent binding. Thus, the physical properties of DNA are, in part, responsible for the potent mutagenic (and possibly carcinogenic) effects of intercalating polycyclic aromatic hydrocarbons. A significant fraction of the cancers that occur in developed countries can most easily be prevented by reducing exposure to chemical carcinogens. This is not always possible and, therefore, alterations in the processes of carcinogen activation, covalent binding, or solvolysis may offer useful approaches to arresting oncogenesis.

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