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Biochemistry

Mechanism of the inhibition of mutagenicity of a benzo[*a*]pyrene 7,8-diol 9,10-epoxide by riboflavin 5′-phosphate

(ultimate carcinogen/Ames' *Salmonella typhimurium*/kinetics of epoxide hydrolysis/general acid catalysis/complex formation)

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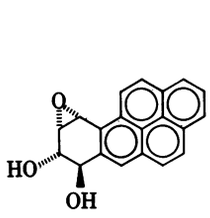
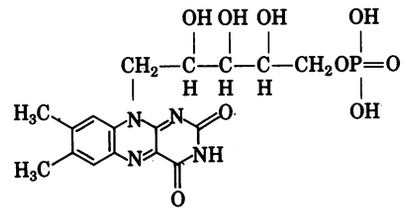
ABSTRACT **Riboflavin 5′-phosphate (flavin mononucleotide; FMN) inhibits the mutagenicity of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B[*a*]P diol epoxide), the only known ultimate carcinogenic metabolite of benzo[*a*]pyrene. Coincubation of 10, 25, and 50 nmol of FMN with strain TA100 of histidine-dependent *Salmonella typhimurium* inhibits the mutagenicity of 0.05 nmol of the diol epoxide by 50, 70, and 90%, respectively. Ribose 5-phosphate and riboflavin show no significant effects at comparable doses. Reaction of B[*a*]P diol epoxide with FMN in aqueous solution at neutral pH produces only tetraols, with no evidence for covalent adducts. At pH 7 the rate of hydrolysis of B[*a*]P diol epoxide in dioxane/water, 1:9 (vol/vol), at 25°C is increased more than 10-fold in the presence of 100 μM FMN. Spectrophotometric studies and quantitative rate data for the reaction of the diol epoxide with FMN indicate that a complex is formed between the diol epoxide and the flavin moiety of FMN (K_e = 1,400–3,400 M^{-1}) prior to general acid-catalyzed hydrolysis of the epoxide to tetraols by the phosphate monoanion of FMN. Comparable concentrations of ribose 5-phosphate and riboflavin do not significantly increase the rate of hydrolysis, although evidence for complex formation between riboflavin and the diol epoxide is observed. General acid-catalyzed hydrolysis of bay-region polycyclic hydrocarbon diol epoxides by compounds that have a high affinity for these ultimate carcinogens represents a potentially useful way of inhibiting their carcinogenic activity.**

Mutagenicity, metabolism, DNA binding, and tumorigenicity studies have indicated that the principal ultimate carcinogens of a dozen different polycyclic aromatic hydrocarbons are benzo-ring diol epoxides in which the epoxide moiety forms part of the bay-region of the hydrocarbon (1–4). Because polycyclic aromatic hydrocarbons are ubiquitous environmental carcinogens (5), we have initiated a search for agents that can block the adverse biological effects of their ultimate carcinogenic metabolites. Diol epoxides undergo spontaneous and acid-catalyzed hydrolysis to chemically unreactive and noncarcinogenic tetraols (6–10) and form nucleophilic addition products with compounds such as *p*-nitrothiophenol (6, 7). However, the rate of diol epoxide hydrolysis is not enhanced by epoxide hydrolase (11–14), and we have found (data not shown) that a variety of nucleophiles, including glutathione, L-methionine, *p*-nitrothiophenol, 1-naphthalenethiol, and DNA are not very effective in inhibiting the mutagenicity of bay-region diol epoxides of several hydrocarbons. In the present study, we report that riboflavin 5′-phosphate (flavin mononucleotide; FMN) markedly inhibits the mutagenicity of the ultimate carcinogen (10, 15) (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B[*a*]P diol epoxide), the predominant metabolically formed diastereomer (16, 17) in which the benzylic 7-hydroxyl group and the epoxide oxygen are *trans*. The probable mechanism of this inhibition involves complexation with the diol epoxide and catalysis of its hydrolysis to tetraols.



B[*a*]P diol epoxide



Riboflavin-5′-phosphate

MATERIALS AND METHODS

Materials. Riboflavin, FMN, flavin adenine dinucleotide (FAD), and ribose 5-phosphate, obtained from Hoffmann–La Roche or Sigma, were used without further purification. Dioxane was distilled from sodium, and dimethyl sulfoxide was distilled from calcium hydride *in vacuo*. Concentrations of stock flavin solutions were determined by weight or from the absorbance at the three flavin λ_{max} values (18) in water. For kinetic and equilibrium experiments, solutions (800–900 μM) of riboflavin were prepared by heating (≈50°C) excess riboflavin with buffer solutions in dioxane/water, 1:9 (vol/vol), followed by cooling and filtration. The B[*a*]P diol epoxide (6, 7) and its enantiomers (19) were synthesized as described.

Mutagenesis Assays with Bacteria. Mutations to histidine-independent growth were assessed in strain TA100 of Ames' *Salmonella typhimurium* as described (14).

Kinetics of Diol Epoxide Hydrolysis. Rates of reaction were measured at 25°C in dioxane/water (1:9) made 0.1 M in $NaClO_4$. pH was maintained with 1 mM acetate or tris buffers where required. Reactions were followed at 346 nm with a Cary 219 spectrophotometer at a diol epoxide concentration of 4–20 μM.

Equilibrium Constants for Complex Formation. The effect of increasing concentrations of FMN or riboflavin on the absorbance of the diol epoxide at 315 nm was measured by addition of 30 μl of 900 μM diol epoxide in dioxane to 3 ml of flavin solutions containing 0.1 M $NaClO_4$, 1 mM tris buffer, and sufficient dioxane to give a final dioxane concentration of 10%. Absorbances were read against a blank containing an identical

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Abbreviations: B[*a*]P diol epoxide, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; FMN, riboflavin 5′ phosphate (flavin mononucleotide); FAD, flavin adenine dinucleotide.

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buffered flavin solution to which was added 30 μl of dioxane. For studies with FMN, 5-sec readings of the absorbance were taken beginning 15–30 sec after the solutions were mixed, and the observed absorbances were extrapolated to t_0, the time of mixing. For formation of a 1:1 complex, the relationship between flavin concentration and A_{obs}, the absorbance of the diol epoxide at t_0 in the presence of a given flavin concentration, is given by Eq. 1,

$$A_{obs} = A_0 - \{(\Delta A)(K_e)[\text{flavin}]/(1 + K_e[\text{flavin}])\}, \quad [1]$$

in which A_0 is the measured absorbance of the diol epoxide in the absence of flavin and ΔA is the total absorbance change ($A_0 - A_{obs}$) that would be observed in the limiting case of 100% complex formation. Values of K_e and ΔA were calculated from the data by the use of a curve-fitting program (20).

Product Analysis. High-performance liquid chromatography was carried out on a Spectra Physics model 8000 liquid chromatograph with a Du Pont Zorbax ODS column (6.2 × 250 mm) eluted with a linear gradient of 0–100% methanol in water over 50 min at a flow rate of 1.5 ml/min. Products were quantified by integration of the area of the peaks (measured at 254 nm) corresponding to the tetraols (7, 12) formed from the diol epoxide, relative to an internal standard of *p*-nitrobenzyl alcohol.

RESULTS

The dose-dependent inhibition of the mutagenicity of B[*a*]P diol epoxide by FMN and FAD is shown in Fig. 1. Addition of 10 nmol of FMN to the bacteria prior to the addition of 0.05 nmol of the diol epoxide inhibited its mutagenicity by 50%, and 100 nmol of FMN essentially abolished mutagenic activity. FAD also inhibited the mutagenic activity of the diol epoxide, although 5–7 times more of this flavin was needed to achieve a comparable degree of inhibition. Neither riboflavin nor ribose 5-phosphate had any significant antimutagenic effect at comparable doses (Fig. 1); thus, both a phosphoester and the isoalloxazine (flavin) moiety are required for activity. Neither FMN nor FAD had any discernable effects on the growth of the bacteria, the spontaneous mutation frequency in the absence of added diol epoxide, or the mutagenic activity of several structurally unrelated mutagens. Therefore, the antimutagenic effects of FMN and FAD appear attributable to a specific effect on the diol epoxide.

A chemical basis for this inhibition of mutagenesis is suggested by the effect of FMN on the rate of disappearance of the diol epoxide in dioxane/water (1:9) at ionic strength 0.1 M ($NaClO_4$) at 25°C. In the absence of FMN, the diol epoxide undergoes both hydronium-ion-catalyzed and pH-independent hydrolysis to give tetraol products (7, 8); at pH 6.95, the diol epoxide had a half-life of 39 min, in good agreement with reported kinetic constants (8). Low concentrations of FMN markedly accelerated the disappearance of the diol epoxide; for example, the half-life of the epoxide was decreased to ≈3 min at this pH in the presence of 120 μM FMN. At FMN concentrations from 50 to 480 μM, good pseudo-first-order kinetics were observed for diol epoxide disappearance through at least three half-lives.

Products of the reaction of the diol epoxide (30–40 μM) with 200 μM FMN at pH ≈7 (where more than 94% of the reaction proceeds by the FMN-dependent pathway) were analyzed by high-performance liquid chromatography. The same total amount of tetraol products was found in the reaction with FMN and in the control reaction of an identical sample of the diol epoxide in 1 mM perchloric acid, a reaction medium that is known (8) to give 100% acid hydrolysis to tetraols. Furthermore, the ratio of *cis* to *trans* opening of the oxirane ring in the reaction with FMN at pH 7 is 6:94, identical within experimental error to that observed for hydrolysis of this epoxide catalyzed by hydronium ion (8) or dihydrogen phosphate monoanion (9) and very different from the ratio of ≈1:1 for uncatalyzed hydrolysis at neutral pH (8). These product studies indicate that the observed rate acceleration of diol epoxide disappearance in the presence of FMN is caused by catalysis of epoxide hydrolysis rather than by formation of a stable covalent FMN adduct.

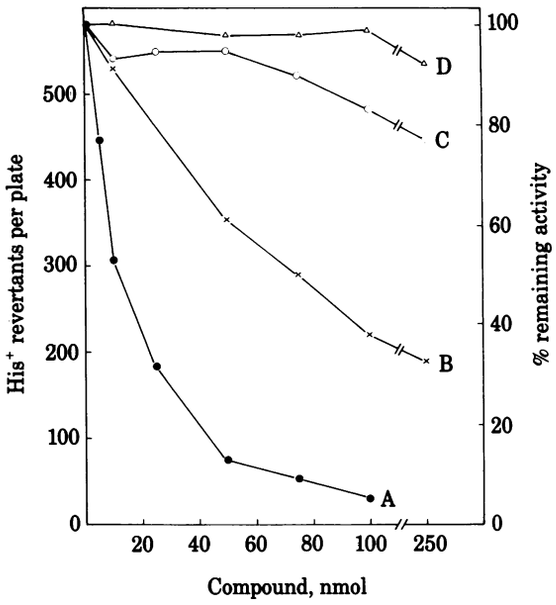


FIG. 1. Effect of FMN (curve A), FAD (curve B), riboflavin (curve C), and ribose 5-phosphate (curve D) on the mutagenicity of B[*a*]P diol epoxide in strain TA100 of *Salmonella typhimurium*. Bacteria (2×10^8) were incubated with the indicated amounts of the various compounds in 0.5 ml of phosphate-buffered saline (pH 7.0) for 1 min at 37°C prior to a 5-min exposure to 0.05 nmol of the diol epoxide (added in 15 μl of dimethyl sulfoxide). Spontaneous mutation frequencies of 90 His^+ revertants per plate have been subtracted from the data, which represent the mean of three replicate determinations.

Investigation of the effects of pH and FMN concentration on the observed pseudo-first-order rate constants (k_{obs}) for hydrolysis of the diol epoxide (Fig. 2*A*) and comparison of the reaction of FMN with that of ribose 5-phosphate (Fig. 2*B*), a model for the sugar phosphate moiety of FMN, indicate the following significant features of the catalysis by FMN: (*i*) ≈100 times as much ribose 5-phosphate as FMN is required to give comparable rate accelerations at similar pH values; (*ii*) at any given concentration of FMN or ribose 5-phosphate, k_{obs} increases as the pH decreases; and (*iii*) the rate constants show a nonlinear dependence on the concentration of FMN but are linearly related to the concentration of ribose 5-phosphate, even at concentrations up to 20 mM. Thus, the catalysis by ribose 5-phosphate follows a simple rate law:

$$k_{obs} = k_u + k_{RP^-}[\text{R-5-P}]_{tot}(f^-), \quad [2]$$

in which k_u is the observed rate constant for hydrolysis in the absence of ribose 5-phosphate, k_{RP^-} is a pH-independent rate constant for hydrolysis catalyzed by ribose 5-phosphate monoanion ($pK_a = 6.51$), and f^- is the fraction of ribose 5-phosphate present as the monoanion. This catalysis is completely analogous to the previously observed general acid catalysis of diol epoxide hydrolysis by inorganic dihydrogen phosphate monoanion (9). The rate constant, k_{RP^-}, of 0.8 M^{-1} sec^{-1} for catalysis by the ribose 5-phosphate monoanion is similar to the value of 0.5 $M^{-1}sec^{-1}$ for dihydrogen phosphate. In contrast, the sec-

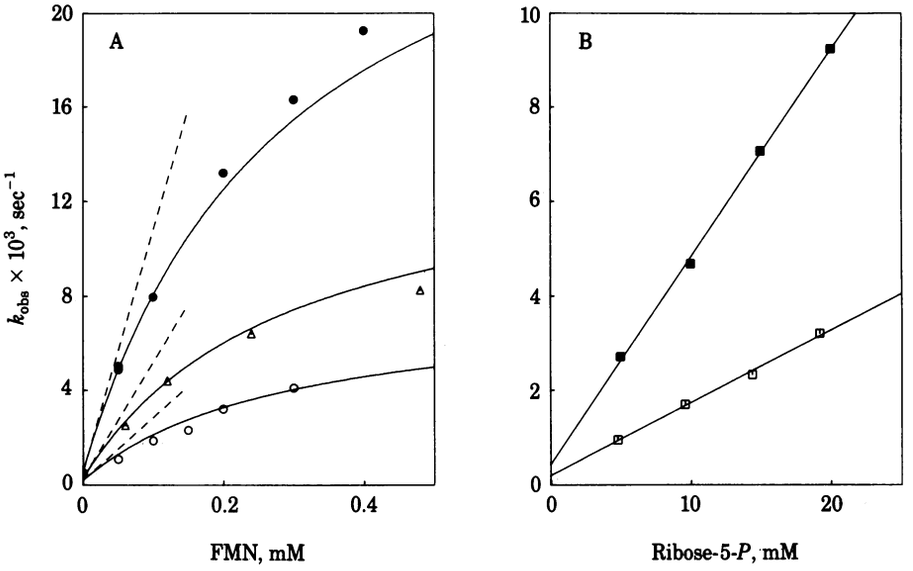
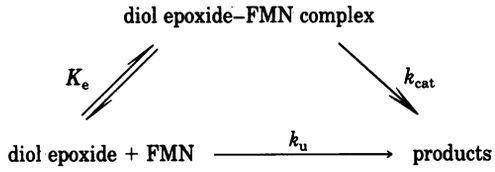


FIG. 2. Dependence of the observed pseudo-first-order rate constants for hydrolysis of (±)-B[*a*]P diol epoxide on the total concentration of FMN (*A*) or ribose 5-phosphate (*B*) at various pH values in dioxane/water, 1:9 (vol/vol), at 25°C. In *A*, pH values are 6.45 (●), 6.94 (△), and 7.3 (○); solid lines are theoretical curves based on Eqs. 3 and 4 and the average values of K_e and k_{FMN^-} given in the text. The broken lines show the initial slopes, $K_e k_{FMN^-}(f^-)$ for FMN, which are analogous to the second-order rate constants, $k_{RP^-}(f^-)$ for ribose 5-phosphate given by the slopes of the solid lines in *B*, where pH values are 6.43 (■) and 7.08 (□).

ond-order rate constants for catalysis by low concentrations of FMN (initial slopes of Fig. 2*A*) are ≈250 times greater than those for ribose 5-phosphate at comparable pH values.

The magnitude of the catalytic effect of FMN relative to ribose 5-phosphate and the nonlinear dependence of the rate constants for FMN catalysis on concentration strongly suggest complexation of the diol epoxide with the isoalloxazine moiety of FMN prior to hydrolysis of the epoxide catalyzed by the phosphate group of FMN (Scheme I). In the scheme, K_e is the



Scheme I

equilibrium constant for formation of the diol epoxide–FMN complex, k_u is the observed pseudo-first-order rate constant for hydrolysis in the absence of FMN, and k_{cat} is a pH-dependent pseudo-first-order rate constant for the breakdown of the diol epoxide–FMN complex. The rate law for this mechanism, given by Eq. 3,

$$k_{obs} = (k_u + K_e k_{cat}[FMN]_{tot})/(1 + K_e[FMN]_{tot}), \quad [3]$$

predicts that at low FMN concentrations, k_{obs} will increase linearly with increasing FMN concentration because the denominator will be essentially unity. At higher concentrations of FMN, the rate equation predicts that k_{obs} will become independent of FMN concentration and, as complete conversion to the diol epoxide–FMN complex is approached, k_{obs} will eventually equal k_{cat}. Values of the limiting rate constant (k_{cat}), determined from the kinetic data shown in Fig. 2*A*, increase with decreasing pH, consistent with general acid catalysis within the complex by the monoanion of FMN ($pK_a = 6.46$). Thus, the pH-dependent rate constant, k_{cat}, is related to the pH-independent rate constant, k_{FMN^-}, for catalysis by the monoanion of FMN by Eq. 4,

$$k_{cat}[FMN]_{tot} = k_{FMN^-}[FMN]_{tot}(f^-), \quad [4]$$

in which f^- represents the fraction of FMN present as the monoanion at a given pH. The average values for K_e and k_{FMN^-}, calculated from the experimental data shown in Fig. 2*A* by double reciprocal plots or by a computer program for curve-fitting (20), are 3,400 ± 800 M^{-1} and 0.06 ± 0.01 sec^{-1}, respectively.

Additional evidence for the formation of a diol epoxide–FMN complex is provided by the observation that there is an FMN-dependent decrease (extrapolated to time zero) in the absorbance of the diol epoxide at 315 nm when ≈10 μM diol epoxide is added to solutions of FMN (Fig. 3). Determination of the K_e from this absorbance decrease gave a value of 1,400 M^{-1}. A similar spectrophotometric determination of K_e for riboflavin gave a curve (Fig. 3) that was superimposable upon the FMN curve, with K_e = 1,700 M^{-1}. Because spectrophotometric and kinetic measurements were possible only with relatively low concentrations of FMN and riboflavin, the values of K_e are approximate, and no significance is attributed to the difference between these spectrophotometric values and the higher value (≈3,400 M^{-1}) determined from the kinetic data of Fig. 2*A*.

Also consistent with complex formation between the isoalloxazine moiety and the diol epoxide is the observation that 300 μM riboflavin inhibits the hydrolysis of the diol epoxide in dioxane/water (1:9) at pH 6.55 by ≈40–45% relative to solvent alone. This observation indicates that interaction between the isoalloxazine and the aromatic diol epoxide provides no catalytic advantage *per se* and that catalysis by FMN must result only from the enforced proximity of the phosphate group of FMN to the oxirane ring in the FMN–diol epoxide complex.

Catalysis by FMN does not involve significant enantiomeric selectivity for the diol epoxide, despite the asymmetric nature of the ribityl moiety in FMN. At pH 6.42 in the presence of 40–320 μM FMN, (+)-B[*a*]P diol epoxide reacted only ≈15% faster than its (−)-enantiomer. This very small difference in rates is consistent with our observation of good pseudo-first-order kinetics for the reaction of the racemic compound.

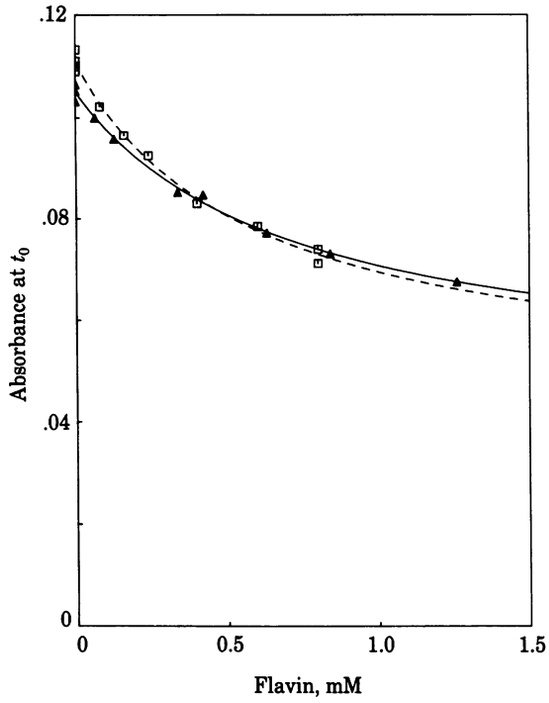


FIG. 3. Absorbance changes at 315 nm (extrapolated to the time of mixing) of (±)-B[*a*]P diol epoxide in the presence of riboflavin (□) at pH 7.45–7.61 or FMN (▲) at pH 7.49–7.55 in dioxane/water, 1:9 (vol/vol), at 25°C. The lines are theoretical curves based on Eq. 1 with K_e = 1,740 M^{-1} and ΔA = 0.065 for riboflavin (---) and with K_e = 1,435 M^{-1} and ΔA = 0.058 for FMN (——).

DISCUSSION

FMN effectively protects *Salmonella typhimurium* in a dose-dependent manner from the mutagenic activity of B[*a*]P diol epoxide, the principal ultimate carcinogen of benzo[*a*]pyrene. Lack of any evidence for covalent adduct formation between the diol epoxide and FMN, quantitative kinetic and spectral data, and comparative studies between FMN, riboflavin, and ribose 5-phosphate indicate that FMN is complexing with the diol epoxide and catalyzing its hydrolysis to tetraols.

The formation of a complex between benzo[*a*]pyrene and FMN in methanol has been reported (21), and inhibition of a flavoenzyme by derivatives of benz[*a*]anthracene has been ascribed to complexation between the hydrocarbons and the flavin moiety (22). The quenching of riboflavin fluorescence by several polycyclic aromatic hydrocarbons has been ascribed to complexation, with equilibrium constants of the order of 20–100 M^{-1} in ethanol (23). Formation of analogous complexes between pyrene and the 5′-monophosphates of guanosine, cytidine, adenosine, and thymidine, with equilibrium constants of 13–52 M^{-1} in 1:19 methanol/water, has also been observed (24).

That a complex between FMN and the diol epoxide can have both (*i*) a favorable stacking interaction between the polycyclic aromatic hydrocarbon and the isoalloxazine ring and (*ii*) a proper orientation of the phosphate group for general acid catalysis is indicated by the computer-generated (25) molecular models shown in Fig. 4. The structures of several mixed crystals of flavin derivatives and aromatic compounds are known (26–28). A representative structure (27), the mixed crystal of 10-propylisoalloxazine and naphthalene-2,3-diol, was used as a model for the stacking interaction between FMN and the diol epoxide. In the structure shown, the aromatic moieties (3.44 Å apart) were overlapped in one of the several possible ways that are consistent with the stacking in this crystal. The conformation of the ribityl phosphate side chain was optimized so that a hydrogen on the phosphate group is located near one of the lone pairs of electrons on the oxirane oxygen (29) and is at a reasonable distance (2.1 Å) and angle (O—H···O = 174°) for hydrogen bonding to this oxygen; in this optimization, as many unfavorable eclipsing interactions involving the side chain as possible were avoided.

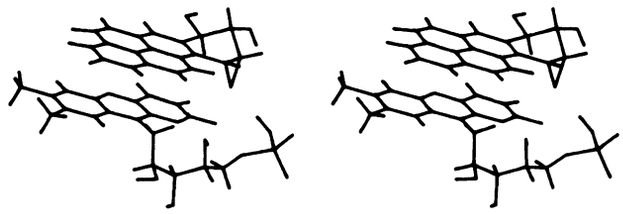


FIG. 4. Stereodrawing of a possible structure for the diol epoxide–FMN complex. The drawing may be viewed using a stereoviewer such as that sold by the Hubbard Scientific Co., Northbrook, IL.

Insight into the interrelationships between complex formation and catalysis is provided by comparison of the effects of FMN, riboflavin, and ribose 5-phosphate on the rate of hydrolysis of the diol epoxide. Riboflavin, which lacks only the catalytic phosphate group of FMN, is about as effective as FMN in complexation with the diol epoxide, and such complexation leads to inhibition of diol epoxide hydrolysis. Thus, complexation of the aromatic moiety of the diol epoxide with the isoalloxazine ring system actually provides a catalytic disadvantage that is overcome by enforced proximity of the phosphate and oxirane groups so that rate acceleration is observed in the catalytically active FMN complex. At low FMN concentrations, this effect of bringing together catalyst and substrate molecules from dilute solution by favorable equilibrium complexation provides a substantial rate acceleration. The initial slopes (broken lines) of the FMN catalysis curves in Fig. 2*A* correspond to an apparent second-order rate constant, $K_e k_{FMN^-}$, of 204 M^{-1} sec^{-1}—about 250 times greater than the corresponding value of k_{RP^-} for ribose 5-phosphate, which does not form any detectable complex with the diol epoxide. However, at ≈0.1 M, the kinetic advantage of FMN relative to ribose 5-phosphate is predicted to disappear because the pseudo-first-order rate constant for reaction with 0.1 M ribose 5-phosphate becomes equivalent to the limiting rate constant for the reaction of the FMN–diol epoxide complex. An alternative statement of this fact is that the "effective molarity" of the catalytic phosphate group *within the FMN–diol epoxide complex* is about 0.1 M.

Factors that may contribute to a suboptimal level of catalysis by FMN are (*i*) formation of "nonproductive" complexes in which the sugar phosphate group cannot rotate into proximity with the epoxide, (*ii*) other "nonproductive" geometries of the complex or complexes in which water cannot approach the intermediate carbocation in the proper orientation, and (*iii*) unfavorable charge-transfer interactions with the flavin moiety (a possible charge-transfer acceptor) that may destabilize the carbocation-like transition state relative to the neutral reactant. The observed inhibition of hydrolysis of the diol epoxide by riboflavin is consistent with either or both of factors *ii* and *iii*.

The results of the present study raise the question of the role the flavin nucleotides play, if any, in protecting animals or man from the adverse biological consequences of exposure to polycyclic hydrocarbons. Liver contains the highest level of flavin nucleotides and FAD predominates over FMN by several-fold in this and other organs (30). Most of the FMN and FAD is tightly associated with enzyme protein (31, 32), and excess levels of flavin nucleotides are rapidly dephosphorylated by phos-

phatases (33, 34). Thus, in the presence of a carefully regulated homeostatic system, it would appear difficult to obtain tissue or blood levels of the flavin nucleotides higher than those normally seen with an adequate dietary intake of riboflavin. Interestingly, a riboflavin-deficient diet results in a significantly higher incidence of skin papillomas in mice treated with 7,12-dimethylbenz[*a*]anthracene (35). Whether alterations in FMN and FAD levels can affect the toxicity of polycyclic hydrocarbons in animals is unknown. Clearly, acid-catalyzed hydrolysis of polycyclic hydrocarbon diol epoxides by chemicals that also have a high affinity for these ultimate carcinogens represents a potentially important way of preventing the adverse effects of polycyclic aromatic hydrocarbons.

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