Higgens, N. P., Geballe, A. P., Snopek, T. J., Sugino, A., & Cozzarelli, N. R. (1977) *Nucleic Acids Res. 4*, 3175.
Kaufmann, G., & Littauer, U. Z. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3741.
Kornberg, A. (1980) *DNA Replication*, p 261, W. H. Freeman, San Francisco, CA.
Matsudaira, P. T., & Burgess, R. (1978) *Anal. Biochem. 87*, 386.
Modrich, P., Lehman, I. R., & Wang, J. C. (1972) *J. Biol. Chem. 247*, 6370.
Moseman-McCoy, M. I., Lubben, T. H., & Gumport, R. I. (1979) *Biochim. Biophys. Acta 562*, 149.
Neilson, T., Kofoid, E. C., & Ganoza, M. C. (1980) *Nucleic Acids Symp. Ser. No. 7*, 313.
Ohtsuka, E., Nishikawa, S., Markham, A. F., Tanaka, S., Miyake, T., Wakabayashi, T., Ikehara, M., & Sugiura, M. (1978) *Biochemistry 17*, 4894.
Ohtsuka, E., Uemura, H., Doi, T., Miyake, T., Nishikawa, S., & Ikehara, M. (1979) *Nucleic Acids Res. 6*, 443.
Ohtsuka, E., Miyake, T., Magao, K., Uemura, H., Nishikawa, S., Sugiura, M., & Ikehara, M. (1980) *Nucleic Acids Res. 8*, 601.
Sninsky, J. J., Last, J. A., & Gilham, P. T. (1976) *Nucleic Acids Res. 3*, 3157.
Stahl, D. A., Meyhack, B., & Pace, N. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5644.
Sugino, A., Snopek, T. J., & Cozzarelli, N. R. (1977) *J. Biol. Chem. 252*, 1732.
Uhlenbeck, O. C., & Cameron, V. (1977) *Nucleic Acids Res. 4*, 85.
Uhlenbeck, O. C., & Gumport, R. I. (1981) *Enzymes, 3rd Ed. 15*, 31.
Uhlenbeck, O. C., Baller, J., & Doty, P. (1970) *Nature (London) 225*, 508.

Noncovalent Binding of 7β,8α-Dihydroxy-9α,10α-epoxytetrahydrobenzo[*a*]pyrene to Deoxyribonucleic Acid and Its Catalytic Effect on the Hydrolysis of the Diol Epoxide to Tetrol†

Nicholas E. Geacintov,* Hiroko Yoshida, Victor Ibanez, and Ronald G. Harvey

ABSTRACT: In the presence of native DNA the hydrolysis of benzo[*a*]pyrene-7,8-diol 9,10-epoxide (BPDE) to tetrols (BPT) is markedly accelerated (by a factor of up to ~80 at 25 °C, pH 7.0, in 5 mM sodium cacodylate buffer solution). When stopped-flow kinetic techniques are utilized, it is shown that the pseudo-first-order hydrolysis rate constant k_H is smaller by a factor of ~3 in the presence of equivalent concentrations of denatured DNA, by a factor of 8–25 in the presence of nucleotides, and by a factor of 35–45 in the presence of nucleosides (depending on the nucleotide or nucleoside). In the presence of native DNA, k_H increases with increasing DNA concentration and reaches a limiting value of $k_H = 0.684 \pm 0.04\ s^{-1}$ at DNA concentrations in excess of $\sim 5 \times 10^{-4}$ M (expressed in concentration of nucleotides). A kinetic model based on (1) rapid formation of a noncovalent BPDE–DNA complex followed by (2) slower hydrolysis of BPDE to BPT at these binding sites is consistent with the experimental data. It is shown furthermore that the DNA concentration dependence of k_H and of noncovalent intercalative binding of BPDE to DNA is similar and that addition of magnesium ions (which is known to reduce intercalative binding of planar aromatic molecules to DNA) also reduces k_H. These results suggest, but do not necessarily prove, that the DNA binding sites at which the hydrolysis of BPDE (to BPT) is catalyzed are intercalative in nature.

Benzo[*a*]pyrene is a well-known polycyclic aromatic hydrocarbon carcinogen and environmental pollutant. The mechanisms of action of BaP[1] and of other polycyclic aromatic hydrocarbons in vivo have been extensively studied. These compounds are metabolically activated to reactive epoxide derivatives principally on the microsomes of the endoplasmic reticulum catalyzed by the mixed-function oxygenase enzymes. These epoxide derivatives subsequently react with cellular macromolecules, and it is widely believed that DNA is a critical target molecule in the initiation of chemically induced carcinogenesis. Among the more than 30 known metabolites of BaP, the major reactive derivative has been identified as BPDE (Gelboin, 1980; Harvey, 1981; Selkirk et al., 1976). This diol epoxide can react with DNA either in vivo or in vitro. A covalent adduct involving a chemical bond between the 10 position of the hydrocarbon and the exocyclic amino group of guanine is formed predominantly (Jeffrey et al., 1977;

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[1] Abbreviations: BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxytetrahydrobenzo[*a*]pyrene; BPT, 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene; BaP, benzo[*a*]pyrene; THF, tetrahydrofuran; dA, deoxyadenosine; dC, deoxycytidine; dG, deoxyguanosine; dT, thymidine; pdA, deoxyadenosine 5′-phosphate; pdC, deoxycytidine 5′-phosphate; pdG, deoxyguanosine 5′-phosphate; dpT, thymidine 5′-phosphate.

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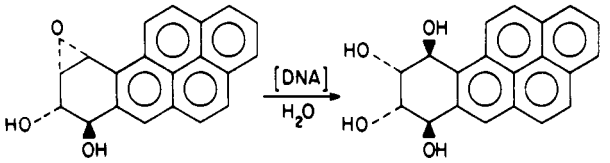

BPDE BPT

FIGURE 1: Structural formula of the diol epoxide (BPDE) and its tetrol (BPT) hydrolysis product (the trans stereoisomer is shown).

Weinstein et al., 1976); other minor adducts have also been detected (King et al., 1976; Jeffrey et al., 1977; Meehan et al., 1977; Koreeda et al., 1978; Kakefuda et al., 1978; Osborne et al., 1981). However, the major reaction pathway of BPDE with DNA in an aqueous environment is not covalent adduct formation but is the DNA-catalyzed hydrolysis of BPDE to tetrols; typically, at 25 °C, at pH 7.0, and in 5 mM cacodylate buffer solution, only 5–10% of the total BPDE added to calf thymus DNA solutions is found to bind covalently to DNA. Most of the diol epoxide is hydrolyzed (Figure 1), a reaction which is markedly accelerated in the presence of DNA (Geacintov et al., 1980, 1981). This catalytic effect of DNA on the hydrolysis of BPDE has also been noted by Kootstra et al. (1980).

The hydrolysis of BPDE in aqueous, buffered solutions and at different values of pH has been studied previously by many workers (Keller et al., 1976; Thakker et al., 1976; Yagi et al., 1977; Yang et al., 1977a,b; Whalen et al., 1979). In aqueous solutions BPDE is known to undergo a hydrolysis by specific and general acid catalysis, and thus the hydrolysis rate constant depends strongly on the pH and on the nature of the buffer (Keller et al., 1976; Yang et al., 1977b; Whalen et al., 1979). The products of the hydrolysis of BPDE are tetrols, which result from the stereoselective cis and trans addition of water, a reaction which involves an intermediate benzylic carbonium ion at the C-10 position of BPDE (Yang et al., 1977b; Keller et al., 1976; Yang et al., 1977).

Both the diol epoxide (Geacintov et al., 1981) and the tetrols (Ibanez et al., 1980) bind noncovalently to DNA by an intercalation mechanism. In these intercalation complexes the pyrene ring system is sandwiched between two adjacent base pairs of the DNA, a conformation which is characterized by a red shift in the maximum from 343 (in buffer) to 353–354 nm in the intercalation complex. The fluorescence of BPT appears to be completely quenched at these binding sites. The covalently bound molecules display only a small red shift upon covalent adduct formation (from 343 to 345–346 nm), and the pyrene ring system is believed to reside in an external binding site (Geacintov et al., 1978, 1980; Prusik et al., 1979; Prusik & Geacintov, 1979; Lefkowitz et al., 1979). It has been proposed that covalent adduct formation is preceded by noncovalent binding of BPDE to DNA (Meehan & Straub, 1979), either at an external binding site (Ibanez et al., 1980) or at an intercalation site; in the latter case, a rearrangement must occur before covalent bond formation occurs since the conformations, absorption, and fluorescence properties of the physical, noncovalent BPT intercalation complexes and of the covalently bound adducts are quite different (Geacintov et al., 1981).

In this work, stopped-flow kinetic methods are utilized to measure the pseudo-first-order hydrolysis rate constant k_H as a function of DNA concentration. The DNA concentration dependence of k_H is rationalized in terms of a simple kinetic model which involves the formation of a noncovalent BPDE–DNA complex, followed by hydrolysis of BPDE to its tetrol at this binding site. In accordance with such a model, the rate constant k_H is significantly larger in the presence of native DNA than in the presence of magnesium ions, denatured DNA, or at equivalent concentrations of nucleotides or nucleosides. Furthermore, it is shown that the DNA concentration dependence of k_H is within experimental error equivalent to the noncovalent intercalation binding isotherm of BPDE–DNA complexes.

Materials and Methods

BPDE was synthesized at the University of Chicago according to methods discussed by Harvey & Fu (1978). The calf thymus DNA (Worthington Biochemicals, Freehold, NJ) was dissolved in 5 mM sodium cacodylate buffer, 0.1 M NaCl, and 3 mM ethylenediaminetetraacetate and extensively dialyzed against 5 mM sodium cacodylate buffer; the hyperchromicity of this DNA was 40%. A stock solution of BPDE in THF (10^{-2} M) was made up fresh for a given series of experiments. Aliquots of this solution were added to DNA solutions (in 5 mM sodium cacodylate buffer, pH 7.0); the final THF concentration was about 0.2% by volume.

The nucleosides and nucleotides were obtained from different sources: dA, dC, and pdC from Sigma (St. Louis, MO), dG and pdA from Calbiochem (San Diego, CA), pdG from Schwarz Biochemicals (Mount Vernon, NY), pT from Schwarz/Mann (Orangeburg, NY), and dT from Boehringer-Mannheim, Gmbh (West Germany). All solutions were prepared by dissolving the solid directly in 5 mM cacodylate buffer, pH 7.0, and filtering on Millipore filters. Concentrations were determined spectrophotometrically by using the published extinction coefficients (Sober, 1970). Other reagents were of purest grade and were used without further purification. The sodium cacodylate buffer solutions were prepared from deionized glass-distilled water.

The kinetics of BPDE hydrolysis were followed by the enhancement of the fluorescence intensity after BPDE and buffer, nucleoside, nucleotide, or DNA solutions were mixed. These experiments were performed either in an Aminco-Morrow fluorescence stopped-flow instrument for hydrolysis reactions that were comparatively fast or in a Hitachi MPF-2A spectrofluorometer. Excitation was at 344 nm, and emission was observed at 400 nm. The temperature of the solutions was maintained at 25 ± 1 °C.

Results

Native DNA. The hydrolysis of BPDE to its tetrols in the presence or absence of DNA can be conveniently followed by fluorescence methods (Kootstra et al., 1979, 1980; Geacintov et al., 1980). BPDE is nonfluorescent (or has a negligible fluorescence yield), while the tetrol BPT displays the normal pyrene-like fluorescence. Thus, the fluorescence yield of a solution containing BPDE increases with time as the diol epoxide is converted to BPT. Examples of this type of behavior in cacodylate buffer and DNA–cacodylate buffer solutions at several different DNA concentrations are shown in Figure 2. Because BPDE is unstable, especially in the presence of traces of water, a given BPDE sample may be partially hydrolyzed at the beginning of an experiment; the initial fluorescence is usually nonzero and varies from experiment to experiment depending on the BPT content; this variable initial fluorescence level has been subtracted from each of the five curves in Figure 2. These experimental results were obtained with the stopped-flow system.

The time-dependent increase of the fluorescence due to the hydrolysis of the diol epoxide to tetrol is expected to follow the relationship

$$F(t) = F_M(1 - e^{-k_H t}) \quad (1)$$

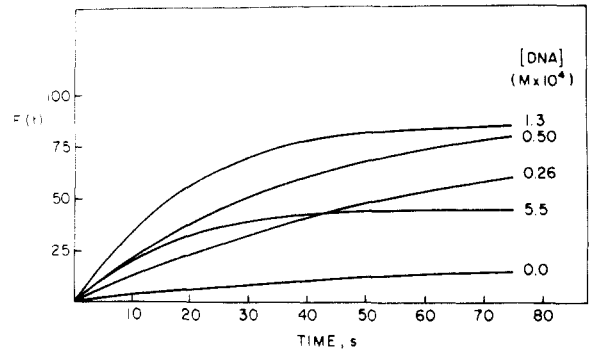

FIGURE 2: Time course of the fluorescence yield due to tetrols which are formed when the diol epoxide BPDE is added (at time = 0 s) to solutions of different DNA concentrations (expressed in concentrations of nucleotides), pH 7.0, 25 °C, in 5 mM cacodylate buffer. The relative (maximum) fluorescence yields (F_M) at equilibrium at the different DNA concentrations were as follows: (a) 100 in buffer, (DNA) = 0, (b) 96 at (DNA) = 0.26×10^{-4} M, (c) 91 at (DNA) = 0.50×10^{-4} M, (d) 87 at (DNA) = 1.3×10^{-4} M, and (e) 43 at (DNA) = 5.5×10^{-4} M.

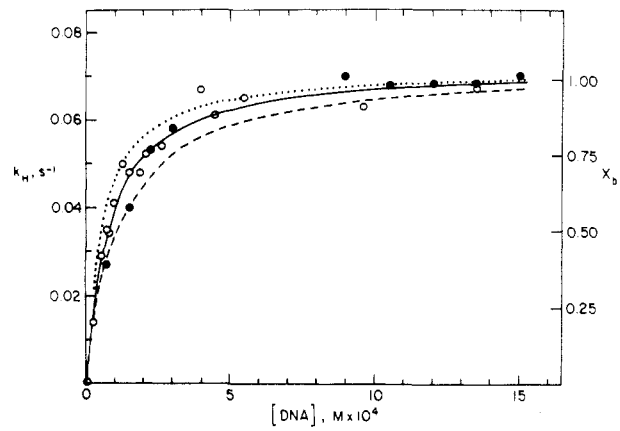

FIGURE 3: Dependence of the relative hydrolysis rate constant (k_H) of diol epoxide to tetrol (○) and of the fraction of diol epoxide molecules (X_b) intercalated (noncovalently) into DNA (●). The smooth lines are representations of eq 20 utilizing different values of the (intercalation) association constant K: (---) 8250 M^{-1}, (—) 11 900 M^{-1}, and (···) 16 500 M^{-1}.

where $F(t)$ is the instantaneous fluorescence yield, F_M is the maximum fluorescence yield attained when all of the BPDE is hydrolyzed, k_H is the hydrolysis rate constant, and t is the time. The hydrolysis rate constant is obtained by plotting ln $[F_M[F_M - F(t)]^{-1}]$ as a function of time. When the data in Figure 2 are utilized, straight lines are obtained demonstrating the validity of eq 1, and the k_H values are obtained from the slopes of such plots.

The limiting values of the fluorescence F_M, at the different DNA concentrations, are given in the legend of Figure 2. It is evident that F_M decreases with increasing DNA concentration, a phenomenon which has been previously noted (Ibanez et al., 1980). The fluorescence quantum yield of intercalated BPT molecules is ~0 (Ibanez et al., 1980), and as the DNA concentration is increased, a larger fraction of BPT molecules are bound to DNA, thus leading to a lower fluorescence yield.

The dependence of k_H on the DNA concentration is shown in Figure 3. It resembles a typical binding isotherm and reaches a limiting value of 0.068 ± 0.04 s^{-1} at high DNA concentration. Shown on the same graph is the fraction (X_b) of the total BPDE molecules which are bound to DNA by the intercalation mechanism. The values of X_b are obtained by a stopped-flow absorbance method in which the optical density at 353 nm (which is due to intercalated diol epoxide molecules) is measured as a function of DNA concentration (Geacintov et al., 1981).

Denatured DNA. In the presence of denatured DNA, the fluorescence–hydrolysis curves also obey pseudo-first-order kinetics. The k_H values at a given DNA concentration for native and heat-denatured DNA are compared in Table I. The hydrolysis rate constant is reduced by a factor of 3 when the DNA is denatured but is still about 10 times larger than that in buffer solution. This result shows that the secondary structure of the DNA is an important parameter in determining the magnitude of k_H.

Effect of Magnesium Ions. In the presence of magnesium ions (and of other cations), the repulsive effects between adjacent phosphate groups on the DNA backbone are reduced (Gilbert & Claverie, 1968), and the extent of binding of aromatic dye molecules (Chan & McCarter, 1970) is decreased. Accordingly (V. Ibanez and N. E. Geacintov, unpublished results) the extent of intercalative binding (as determined by the absorbance at 353 nm) of BPDE into DNA is reduced in the presence of Mg^{2+} ions. The effect of magnesium ions on k_H at a given DNA concentration is shown in Table I. At a magnesium ion concentration of 1 mM, k_H is reduced by a factor of about 15 from its value in the absence of Mg^{2+} at the same DNA concentration.

Nucleosides and Nucleotides. The nucleosides have only a negligible effect on k_H (Table II). However, most of the nucleotides have a significant effect on the value of the hydrolysis rate constant. Since the only difference between the nucleosides and nucleotides is the presence of phosphate groups in the latter, it is likely that the catalytic effect of the nucleotides can in part be attributed to the phosphate groups (Keller et al., 1976; Whalen et al., 1979). However, since there are differences in the k_H values for the different nucleotides, other effects, possibly noncovalent complex formation, may also play a role. A further investigation of these effects was beyond the scope of this work. These results, however, show that the effect of DNA on k_H is much larger than that exerted by the individual nucleotides. Thus once more these results show that the structure of the DNA is a crucial parameter in catalyzing the hydrolysis of BPDE. Thus, pdA is the most effective nucleotide and gives the largest k_H value; however, this value is about 10 times less than that in the presence of native DNA.

Discussion

The dependence of k_H on the DNA concentration can be rationalized in terms of a model in which (1) the BPDE forms a noncovalent complex with DNA and (2) the BPDE is hydrolyzed to tetrols at this binding site. The formation of a DNA complex prior to hydrolysis is strongly suggested by the effects of magnesium ions, native vs. denatured DNA, and nucleotides, as reported in Tables I and II. This model can be represented by

$$\text{BPDE} + \text{DNA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{BPDE}\cdots\text{DNA}] \xrightarrow{k_3} \text{tetrols} \quad (2)$$

The following symbols will be utilized in the equations below in order to simplify the notation in what follows: (B) is the free diol epoxide concentration, (C) is the concentration of bound BPDE molecules, (D) is the DNA concentration, and (T) is the total tetrol concentration.

The free BPDE molecules can also undergo hydrolysis directly (rate constant k_h) without catalysis by DNA:

$$\text{B} \xrightarrow{k_h} \text{T} \quad (3)$$

Table I: Pseudo-First-Order Hydrolysis Rate Constant (k_H) of BPDE to Tetrols under Different Conditions[a]

solution	k_H (s^{-1}) × 10^3
native DNA	45 ± 4
denatured[b] DNA	14 ± 1
native DNA + 1 mM Mg^{2+}	2.9 ± 0.2
buffer only	0.88 ± 0.05

[a] DNA concentration: 1.5×10^{-4} M. [b] The native DNA sample was denatured by heating at 95 °C for 15 min and subsequent rapid cooling in an ice bath.

Table II: Pseudo-First-Order Hydrolysis Rate Constant (k_H) of BPDE to Tetrols in the Presence of Nucleosides and Nucleotides (1.5×10^{-4} M)

	k_H (s^{-1}) × 10^3
nucleosides	
dG, dT, dC	1.3 ± 0.1
dA	1.0 ± 0.1
nucleotides	
pdA	5.8 ± 0.3
pdG	4.8 ± 0.3
pdT	1.8 ± 0.2
pdC	2.8 ± 0.2
buffer only	0.88 ± 0.05
DNA (1.5×10^{-4} M)	45 ± 4

The rate equations describing the time-dependent concentrations of (B) and (C) are

$$\frac{d(B)}{dt} = -[k_h + k_1(D)](B) + k_2(C) \quad (4)$$

$$\frac{d(C)}{dt} = k_1(D)(B) - (k_2 + k_3)(C) \quad (5)$$

In writing these equations, it has been assumed that the concentration of DNA binding sites is much greater than the concentration of diol epoxide. This is the case in most of our experiments since the ratio (D)/(B) ≥ 10. Furthermore, these equations are valid only if the hydrolysis rate is independent of the initial diol epoxide concentration. We have verified that this is the case in the diol epoxide concentration range of interest [(B) = (1–7) × 10^{-6} M].

The solutions of the coupled differential eq 4 and 5 are well-known (Bernasconi, 1976) and are given by

$$B(t) = a_1 e^{-\lambda_1 t} + a_2 e^{-\lambda_2 t} \quad (6)$$

$$C(t) = a_3 e^{-\lambda_1 t} + a_4 e^{-\lambda_2 t} \quad (7)$$

The coefficients a_1–a_4 can be determined from the initial conditions at $t = 0$, when the diol epoxide and DNA solutions are initially mixed. Under conditions in which the equilibrium between B and D is established rapidly, before any significant hydrolysis of the diol epoxide occurs, these constants are given by

$$\lambda_1 = k_h + k_2 + k_3 + k_1(D) \quad (8)$$

$$\lambda_2 = \frac{k_h k_3 + k_2 k_h + k_3 k_1(D)}{k_h + k_2 + k_3 + k_1(D)} \quad (9)$$

It can be shown that these solutions are valid as long as $k_1(D)$, $k_2 \gg k_3$, k_h.

Experimentally, it is known that the formation of noncovalent (intercalation) complexes is faster than the resolution time of the stopped-flow apparatus (Geacintov et al., 1981), which is about 5 ms. Furthermore, from the limiting value of k_H at high DNA concentrations, $k_3 = 0.068\ s^{-1} \ll k_1(D) \lesssim 200\ s^{-1}$, while $k_3 \gg k_h$ throughout most of the DNA concentration range studied. While the values of k_2 are not established, for acridine dyes the values of k_2 are known to be of the order of 1 ms or less (Li & Crothers, 1968; Geacintov et al., 1981). We therefore assume that eq 8 and 9 are reasonable approximations for λ_1 and λ_2. Leaving out some of the smaller rate constants we obtain

$$\lambda_1 \approx k_2 + k_1(D) \quad (10)$$

$$\lambda_2 \approx \frac{k_h}{1 + K'(D)} + k_3 \frac{K'(D)}{1 + K'(D)} \quad (11)$$

where

$$K' = \frac{k_1}{k_2 + k_3 + k_h} \approx \frac{k_1}{k_2 + k_3} \quad (12)$$

It follows that $K' \leq K$, where K is the equilibrium association constant for the formation of the noncovalent diol epoxide–DNA complex (C):

$$K = \frac{k_1}{k_2} \quad (13)$$

From the condition $k_2, k_1(D) \gg k_3, k_h$, it also follows that $\lambda_1 \gg \lambda_2$. Thus, the time dependences of (B) and of (C) reduce to

$$B(t) = B_0 e^{-\lambda_2 t} \quad (14)$$

$$C(t) = C_0 e^{-\lambda_2 t} \quad (15)$$

where B_0 and C_0 are the initial (equilibrium) values of the free and complexed diol epoxide concentrations, respectively, which are obtained immediately (within ~5 ms) upon mixing of the two solutions in the stopped-flow experiments. These initial concentrations are related to the equilibrium constant by

$$K = \frac{(C_0)}{(B_0)(D)} \quad (16)$$

The appearance of products (tetrols) is equal to the total rate of disappearance of BPDE:

$$\frac{dT}{dt} = -\frac{d}{dt}[B(t) + C(t)] \quad (17)$$

Upon substitution of eq 14 and 15 and integration, we obtain

$$T(t) = (B_0 + C_0)(1 - e^{-\lambda_2 t}) \quad (18)$$

Thus the maximum amount of tetrol obtained as $t \rightarrow \infty$ is just $B_0 + C_0$, which is the total amount of diol epoxide initially present. This equation has the same form as the empirical relation describing the fluorescence yield as a function of time (eq 1). Equations 18 and 1 are related simply by assuming that the fluorescence yield at any given time is proportional to the concentration of tetrol molecules [$F_M \propto (B_0 + C_0)$]. The overall hydrolysis rate constant is thus

$$\lambda_2 = k_H \approx k_3 \frac{K'(D)}{1 + K'(D)} \quad (19)$$

From Figure 3 it is evident that X_b follows, within experimental error, the dependence of k_H on the DNA concentration. The simplest expression for X_b is

$$X_b = \frac{K(D)}{1 + K(D)} \quad (20)$$

When eq 19 and 20 are compared, it appears that the functional dependence of k_H and of X_b on the DNA concentration should be the same, as long as $K' \approx K$. Since, within

experimental error, this appears to be the case, we conclude that $K \approx K'$ or that $k_2 \gg k_3 \simeq 0.07\ s^{-1}$.

Fits of eq 19 or 20 using three different values of K are shown in Figure 3. The best fit is obtained with a value of $K = 12000\ M^{-1}$; when values of $K = 8300\ M^{-1}$ or $K = 16500\ M^{-1}$ are utilized, the calculated curves appear on opposite sides of most of the experimental points. Thus, the most likely value of the association constant is $K = (1.2 \pm 0.2) \times 10^4\ M^{-1}$. This value is somewhat higher than the one previously estimated ($K \sim 8300\ M^{-1}$) from the diol epoxide–DNA binding curves alone (Geacintov et al., 1981).

Conclusions

The similarity between the DNA concentration dependence of the formation of noncovalent diol epoxide intercalation complexes and of the DNA-catalyzed hydrolysis rate constant suggests, but does not prove, that these two effects are related. The effect of magnesium ions which are known to reduce the intercalative binding of aromatic molecules to DNA, and also reduce the hydrolysis rate of BPDE, also favors this hypothesis. Furthermore, this catalytic effect strongly depends on the secondary structure of the DNA rather than on its chemical composition. The catalytic effects of DNA on the hydrolysis of the diol epoxide to tetrols may be therefore due to the preliminary formation of a noncovalent benzo[*a*]pyrene diol epoxide–DNA complex in which the intermediate benzylic carbonium ion at the C-10 position is stabilized (relative to free aqueous solvent), favoring the nucleophilic attack by a water molecule (Yang et al., 1977a,b).

If the DNA-catalyzed hydrolysis (rather than covalent binding) occurs indeed at intercalation binding sites, it may be possible to explain why the characteristics of the covalent benzo[*a*]pyrene diol epoxide–DNA adducts are so different from intercalation-type complexes and the reason for which such covalent intercalation complexes are not formed. While certain physical properties of covalent BPDE–DNA adducts have been taken as evidence for intercalation-type structures (Drinkwater et al., 1978; Hogan et al., 1981), comparisons of the absorption, orientation, and fluorescence properties of these covalent adducts are strikingly different from those displayed by intercalated pyrene-like chromophores (Geacintov et al., 1978, 1980, 1981; Prusik et al., 1979; Prusik & Geacintov, 1979). In addition, the zero-field magnetic resonance properties of the pyrenyl chromophore triplet state in the covalent complexes also indicate that the pyrene residue is completely exposed to the aqueous solvent medium (Lefkowitz et al., 1979; Lefkowitz & Brenner, 1981). These results can be interpreted in terms of an outside binding model in which the pyrene residue resides in the minor groove of the DNA (Beland, 1978).

Acknowledgments

The technical assistance of M. Benjamin is gratefully acknowledged. We thank Professors R. B. Murphy and P. Coleman for the loan of the stopped-flow apparatus and acknowledge stimulating discussions with Professor M. Pope.

References

Beland, F. A. (1978) *Chem.-Biol. Interact. 22*, 329–339.

Bernasconi, C. F. (1976) *Relaxation Kinetics*, pp 98–112, Academic Press, New York.

Chan, L. M., & McCarter, J. A. (1970) *Biochim. Biophys. Acta 204*, 252–254.

Drinkwater, N. R., Miller, J. A., Miller, E. C., & Yang, N. C. (1978) *Cancer Res. 38*, 3247–3255.

Geacintov, N. E., Gagliano, A. G., Ivanovic, V., & Weinstein, I. B. (1978) *Biochemistry 17*, 5256–5262.

Geacintov, N. E., Ibanez, V., Gagliano, A. G., Yoshida, H., & Harvey, R. G. (1980) *Biochem. Biophys. Res. Commun. 92*, 1335–1342.

Geacintov, N. E., Yoshida, H., Ibanez, V., & Harvey, R. G. (1981) *Biochem. Biophys. Res. Commun. 100*, 1569–1577.

Gelboin, H. V. (1980) *Physiol. Rev. 60*, 1107–1166.

Gilbert, M., & Claverie, P. (1968) *J. Theor. Biol. 18*, 330–349.

Harvey, R. G. (1981) *Acc. Chem. Res. 14*, 218–226.

Harvey, R. G., & Fu, P. P. (1978) in *Polycyclic Hydrocarbons and Cancer* (Gelboin, H. V., & Ts'o, P. O. P., Eds.) Vol. 1, pp 133–165, Academic Press, New York.

Hogan, M. E., Dattagupta, N., & Whitlock, J. P. (1981) *J. Biol. Chem. 256*, 4504–4513.

Ibanez, V., Geacintov, N. E., Gagliano, A. G., Brandimarte, S., & Harvey, R. G. (1980) *J. Am. Chem. Soc. 102*, 5661–5666.

Jeffrey, A. M., Weinstein, A. B., Jennette, K. W., Grzeskowiak, K., Nakanishi, K., Harvey, R. G., Autrup, H., & Harris, C. (1977) *Nature (London) 269*, 348–350.

Kakefuda, T., & Yamamoto, H. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 415–419.

Keller, J. W., Heidelberger, C., Beland, F. A., & Harvey, R. G. (1976) *J. Am. Chem. Soc. 98*, 8276–8277.

King, H. W. S., Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2679–2681.

Kootstra, A., Slaga, T. J., & Olins, D. E. (1979) in *Polynuclear Aromatic Hydrocarbons* (Jones, P. W., & Lebers, P., Eds.) pp 819–834, Ann Arbor Scientific Publishing Co., Ann Arbor, MI.

Kootstra, A., Haas, B. L., & Slaga, T. J. (1980) *Biochem. Biophys. Res. Commun. 94*, 1432–1438.

Koreeda, J., Moore, P. D., Wislocki, P. G., Levin, W., Conney, A. H., Agi, H., & Jerina, D. M. (1978) *Science (Washington, D.C.) 199*, 778–781.

Lefkowitz, S. M., & Brenner, H. C. (1981) *J. Am. Chem. Soc. 103*, 5257–5259.

Lefkowitz, S. M., Brenner, H. C., Astorian, D. G., & Clarke, R. H. (1979) *FEBS Lett. 105*, 77–80.

Li, H. J., & Crothers, D. M. (1969) *J. Mol. Biol. 39*, 461–477.

Meehan, T., & Straub, K. (1979) *Nature (London) 277*, 410–412.

Meehan, T., Straub, K., & Calvin, M. (1977) *Nature (London) 269*, 725–727.

Osborne, M. R., Jacobs, S., Harvey, R. G., & Brookes, P. (1981) *Carcinogenesis (N.Y.) 2*, 553–558.

Prusik, T., & Geacintov, N. E. (1979) *Biochem. Biophys. Res. Commun. 88*, 782–790.

Prusik, T., Geacintov, N. E., Tobiasz, C., Ivanovic, V., & Weinstein, I. B. (1979) *Photochem. Photobiol. 29*, 2223–2232.

Selkirk, J. K., Yang, S. K., & Gelboin, H. V. (1976) *Carcinog.—Compr. Surv. 1*, 153–169.

Sober, H. A. (1970) *Handbook of Biochemistry and Molecular Biology*, p G-8, Chemical Rubber Publishing Co., Columbus, OH.

Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., & Jerina, D. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3381–3385.

Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Katanishi, K. (1976) *Science (Washington, D.C.) 193*, 592–595.

Whalen, D. L., Ross, A. M., Montemarano, J. A., Thakker,

D. R., Yagi, H., & Jerina, D. M. (1979) J. Am. Chem. Soc. 101, 5086–5088.
Yagi, H., Thakker, D. R., Hernandez, O., Koreeda, M., & Jerina, D. M. (1977) J. Am. Chem. Soc. 99, 1604–1611.
Yang, S. K., McCourt, D. W., Gelboin, H. V., Miller, J. R., & Roller, P. P. (1977a) J. Am. Chem. Soc. 99, 5124–5130.
Yang, S. K., McCourt, D. W., & Gelboin, H. V. (1977b) J. Am. Chem. Soc. 99, 5130–5134.

Purification and Characterization of Cytoplasmic Protamine Messenger Ribonucleoprotein Particles from Rainbow Trout Testis Cells†

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ABSTRACT: Poly(A)-containing protamine messenger ribonucleoprotein particles [poly(A+) pmRNP particles] have been isolated from the polysomal and free cytoplasmic subcellular fractions of trout testis cells by a two-step isolation procedure. Ethylenediaminetetraacetic acid (EDTA) treated particles from both cytoplasmic fractions were first fractionated by sucrose gradient centrifugation and the putative pmRNP particles localized by utilizing ^{3}H-labeled protamine complementary DNA (pcDNA) probes. In addition, particles present in these fractions were characterized by their translational activity in the heterologous, rabbit reticulocyte cell-free system and the protein components of crude mRNP complexes analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoesis. The final purification step involved affinity chromatography of pooled gradient fractions on oligo(dT)–cellulose from which intact pmRNP could be eluted with distilled water at 40 °C. Highly purified particles from both polysomal and free cytoplasmic fractions prepared by this procedure had buoyant densities of 1.35–1.37 g/cm^3 in CsCl or a protein content of approximately 82%. Particles isolated from EDTA-dissociated polysomes were actively translated in vitro, while their free cytoplasmic counterparts were not. High salt washed pmRNP particles or the RNA extracted from pmRNP preparations, however, directed the synthesis of trout protamines in this system. A model of the activation of stored pmRNP particles in vitro and in vivo is presented.

Spermatogenesis in rainbow trout (*Salmo gairdnerii*) testes is characterized by several well-defined events which occur during the terminal differentiation of germ cells to highly specialized spermatozoa. The most striking change in morphology and molecular composition of developing sperm cells, however, occurs during the final phase of sperm cell maturation (spermiogenesis) with the synthesis of a set of highly basic sperm-specific nuclear proteins: the protamines (Ling et al., 1971). Both the protamines and the messenger RNAs coding for these polypeptides have been well characterized biochemically (Gedamu & Dixon, 1976a,b), and a complementary cDNA [specific for protamine messenger RNA (pmRNA) sequences] has been synthesized (Iatrou & Dixon, 1977; Iatrou et al., 1978).

A major question which is central to an understanding of protamine gene expression is how the synthesis of protamine is triggered precisely at the spermatid stage of cell differentiation at a time when the transcriptional activity (Marushige & Dixon, 1969) and diversity of cytoplasmic mRNA sequences in the tissue were rapidly declining (Levy W. & Dixon, 1977). A ^{3}H-labeled protamine cDNA probe has been particularly useful in this regard for elucidating the distribution of such sequences in fractionated testis cells (Iatrou et al., 1978). These studies indicated that pmRNA synthesis began early in sperm cell development (about the primary spermatocyte stage) well before protamine synthesis in these cells. These data were consistent with, although they did not prove, a hypothesis in which newly transcribed pmRNA sequences were stored in the cytoplasm until the spermatid cell stage, at which time they moved to the polysomes and the synthesis of protamines on diribosomal complexes was initiated.

In many other systems, the processing, transport, and translation of mRNA in vivo are thought to involve the packaging of mRNA into specific ribonucleoprotein complexes (Neissing & Sekeris, 1971; Irwin et al., 1975; Beyer et al., 1977; Jenkins et al., 1978; Brunel & Lelay, 1979; Bag & Sells, 1979a,b; Rose et al., 1979; Liautard et al., 1976). A previous report from this laboratory (Gedamu et al., 1977) indicated the pmRNA might also be complexed with proteins in the cytoplasm of developing sperm cells; however, there was little information on the precise nature of these complexes or whether the translational control of protamine synthesis in vivo might be exerted through storage and subsequent activation of these cytoplasmic particles. In the present study, high specific activity [^{3}H]poly(U) and [^{3}H]pcDNA probes were employed to detect these complexes and thus aid in their isolation and analysis. Poly(A+) pmRNA containing RNP complexes from the steady-state free cytoplasmic and polysomal subcellular compartments of testes tissue have been characterized by this means. Data are presented indicating that the control of protamine synthesis involves the activation of stored protamine messenger ribonucleoprotein (pmRNP) particles by processes which can be mimicked in vitro by treatment of purified free cytoplasmic pmRNP with high ionic strengths.

Materials and Methods

Oligo(dT)–cellulose (T_3) was purchased from Collaborative Research Inc. RNase A was from Worthington Biochemical Corp., S1 nuclease was from Miles Laboratories, and AMV reverse transcriptase was a generous gift of Dr. J. Beard, Division of Cancer Cause and Prevention, National Cancer Institute. [^{3}H]dCTP of specific activity 23 Ci/mmol, [^{3}H]-

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0006-2960/82/0421-1869$01.25/0