Vol. 101, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1981 Pages 317-325

THE BINDING OF BENZO[a]PYRENE-7,8-DIHYDRODIOL TO BACTERIOPHAGE ϕX174 DNA

Wen-Tah Hsu[††], Ronald G. Harvey[††], and Samuel B. Weiss[*†]
[*]The Franklin McLean Memorial Research Institute,
[†]The Departments of Biochemistry and Microbiology, and
[††]The Ben May Laboratory for Cancer Research,

The University of Chicago,
Chicago, Illinois 60637.

Received June 1, 1981

SUMMARY

As described in our previous study (Hsu et al. [1979] Biochem. Biophys. Res. Commun. 87, 416), the trans-7,8-dihydrodiol metabolite of benzo[a]pyrene selectively binds and inactivates single-stranded ϕX174 DNA, whereas the infectivity of double-stranded ϕX DNA (RF) is not significantly affected by the dihydrodiol. Anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide, on the other hand, inhibits the infectivity of single-and double-stranded ϕX DNAs with equal efficiency. The nature of the dihydrodiol and diolepoxide interaction with ϕX DNA was examined by agarose gel electrophoresis and Sephadex LH20 chromatography. The results indicate that, although both benzo[a]pyrene metabolites complex with ϕX174 DNA, no stable covalently bound adducts are detected with the dihydrodiol under conditions which permit demonstration of diolepoxide adducts.

INTRODUCTION

We previously reported that certain dihydrodiols of polycyclic aromatic hydrocarbons (PAH) inhibit the replication of bacteriophage single-stranded ϕX174 DNA (1). Our study, in which we used labeled trans-benzo[a]pyrene-7,8-dihydrodiol (BPD) and anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), indicated that the dihydrodiol preferentially reacts with single-stranded DNA, whereas the diolepoxide reacts equally well with single- and double-stranded DNAs, and with RNA.

In animal tissues, PAH-dihydrodiols are normal metabolites of PAH oxidation, and they have been shown to be precursors for diolepoxide formation (2). The finding that certain diols can bind to DNA and inhibit its biochemical expression raises the important question whether these PAH intermediates are directly involved in chemical carcinogenesis.

0006-291X/81/130317-09$01.00/0

BPDE reacts covalently with nucleic acids, forming adducts primarily with guanylate residues (3-5); covalent linkage with other bases has also been identified (6,7). The chemical nature of the binding between BPD and DNA is not known. In this study, we compared certain properties of ϕX174 DNA complexes formed with BPD and BPDE and concluded that the binding of BPD is noncovalent.

MATERIALS AND METHODS

Preparation of Phage and Viral DNA, and Infectivity Assay. Unlabeled and labeled bacteriophage ϕX174am3 were prepared as reported previously (8). Labeled phage was prepared from phage-infected Escherichia coli C by addition of [^{3}H]-guanosine or [^{3}H] thymidine to the medium several minutes after infection. The ϕX174 DNA isolated from guanosine-labeled phage contained 80% of the total radioactivity in deoxyguanosine; the remainder was in deoxyadenosine. For the preparation of RF (replicative form, circular duplex) ϕX DNA, chloramphenicol (50 μg/ml final concentration) was added to infected cells 10 minutes after infection (MOI of 14), and rotary shaking was continued for 1 hour at 37°. After harvesting of cells by centrifugation, RF DNA was isolated according to the procedure of Lovett et al. (9). The purified RF DNA preparation contained both supercoiled (RF1) and open-circled (RF2) forms. Infectivity of viral DNA was assayed as described previously (10). Phage DNA was incubated with E. coli spheroplasts for 12 minutes at 30° and plated on agar with E. coli HF4714 as indicator. Plaques were counted after incubation over night at 31°. The values for percent inhibition of the viral plaques formed are based on average numbers obtained from triplicate plates.

Binding Reaction for PAH Derivatives. Single-stranded or RF ϕX174 DNAs (100 μg/ml), in 5 mM EDTA-10 mM Tris · HCl, pH 7.5, were mixed with 0.05 volume of dimethylformamide containing different amounts of dissolved BPD or BPDE, to give a desired concentration of the BP metabolites. After incubation for 15 minuts at 30°, the reaction mixture was adjusted to contain 0.7% potassium acetate, pH 5.4, and the DNA was precipitated with ethanol. After standing over night at -20°, the precipitate was sedimented by centrifugation and washed successively with acetone, ethyl acetate, and ether, unless otherwise specified. The PAH-exposed DNA treated in the above manner was dried and dissolved in an appropriate buffer for further use.

Sephadex LH20 Chromatography. Chromatography on Sephadex LH20 was performed by the method of Baird and Brookes (11). A column (1.2 cm x 65 cm) was packed with Sephadex LH20 and equilibrated with 30% methanol in 2 mM NH_4HCO_3, pH 8.4. The material to be chromatographed was added to the column and eluted stepwise first with the above equilibration medium, then with 100% methanol. Fractions (300 drops) were collected with PE-160 polyethylene tubing used as an outlet.

Materials. [CH_2-^{3}H] Thymidine (6 Ci/mM) and [8-^{3}H] guanosine (5.4 Ci/mM) were purchased from ICN Pharmaceuticals, Inc., Irvine, CA. [^{3}H] BPDE (specific activity, 517 cpm/pmole) and [^{3}H] BPD (specific activity, 489 cpm/pmole) were synthesized under NCI Contract CP-33387 by Midwest Research Institute, Kansas City, MO. Unlabeled BPDE and BPD were synthesized according to the procedures described previously (2a). Sephadex LH20 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Agarose (SeaKem) was manufactured by FMC Corp., Rockland, ME. DNase 1 from bovine pancreas, Grade 1 (2000 units/mg), and alkaline phosphatase from calf intestine, Grade 1 (512 units/mg), were purchased from Boehringer Mannheim Biochemicals, Indianapois, IN. Nuclease S1 from Aspergillus

oryzae, Type III (344,000 units/mg) was from Sigma Chemical Co., St. Louis, MO. Snake venom phosphodiesterase (26.76 units/mg) was obtained from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

To determine the extent of [^{3}H] BPD binding to nucleic acid, we had previously employed an assay procedure which involved ethanol precipitation of the nucleic acid in the binding reaction, followed by acetone washing of the precipitate. Table 1 shows that, when single-stranded DNA is reacted with [^{3}H] BPD, the above washing procedure (Procedure I) is as effective in removing unreacted labeled diol as Procedure II (which includes two additional washings with ethyl acetate and ether) when the concentration of BPD in the reaction mixture is 0.5 μg per 0.1 ml or less. At higher concentrations of BPD, Procedure II gives fewer counts associated with the DNA precipitate than does Procedure I, suggesting that the additional washings are necessary for removal of unbound diol. Table 1 also shows that, with either wash procedure and for each level of diol employed, the binding of BPD to double-stranded ϕX DNA (RF) is much less than its binding to single-stranded ϕX DNA, in agreement with our earlier findings (1). Both washing procedures give essentially the same binding results at each concentration of diol used.

The inactivation of the infectious capacity of single- and double-stranded ϕX DNAs by BPD and BPDE was determined at different concentrations of the BP metabolites in the binding reaction (Fig. 1). Whereas BPDE inhibits the infectivity of both ϕX DNA forms with nearly equal efficiency, BPD selectively inactivates single-stranded ϕX DNA and has little effect on the infectivity of RF DNA. These results are consistent with the observation that BPD binds preferentially to single-stranded ϕX DNA. However, even at relatively high BPD concentrations, inactivation of ϕX174 DNA does not approach the almost complete inhibition obtained with BPDE.

The stability of the complex formed between BPD and ϕX174 DNA was examined by electrophoresis and chromatography on LH20 Sephadex. Agarose gel electrophoresis of the washed complex formed with [^{3}H] BPD indicated that most of the radioactivity migrated coincident with unreacted ϕX DNA and with BPDE-alkylated

Table 1. Comparison of Two Washing Procedures for the Removal of Unreacted BPD from Viral DNA in the Binding Reaction

DNA	Concentration of [^{3}H] BPD (μg/0.1 ml)	Counts Recovered After Washing	
		Procedure I (cpm)	Procedure II (cpm)
SS ϕX DNA	0.25	4,340	4,492
SS ϕX DNA	0.50	8,844	8,640
SS ϕX DNA	5.00	26,512	12,867
RF ϕX DNA	0.25	657	304
RF ϕX DNA	0.50	1,180	1,071
RF ϕX DNA	5.00	2,453	2,589

The binding mixture (0.1 ml) contained 10 μg of single-stranded (SS) or duplex (RF) ϕX174 DNA and [^{3}H] BPD in the amounts indicated above. After incubation for 15 min at 30°, ethanol was added to the binding mixture and the DNA precipitate was collected. In Washing Procedure I, the precipitate was washed once with acetone. In Procedure II, the precipitate was washed successively with acetone, ethyl acetate, and ether, as described in "Methods". After washing, the DNA was dissolved in 1 ml of 1 mM EDTA-10 mM Tris · HCl (pH 7.5), and 0.5-ml samples were counted for their radioactivity content.

ϕX DNA (Fig. 2). Chromatography on LH20 Sephadex showed that the majority of the labeled [^{3}H] BPD appeared in the void volume, as was the case with unreacted and BPDE-alkylated ϕX DNA (Fig. 3). Although these experiments indicate the presence of small amounts of unbound BP derivatives in the washed DNA complex, the results

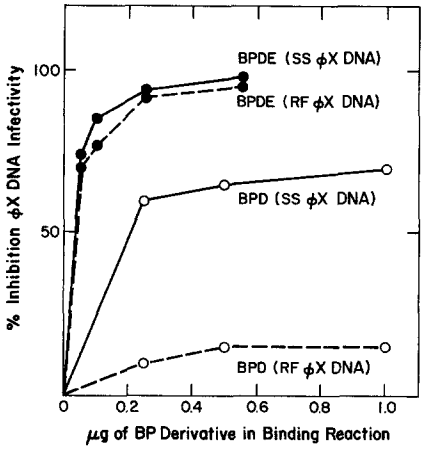
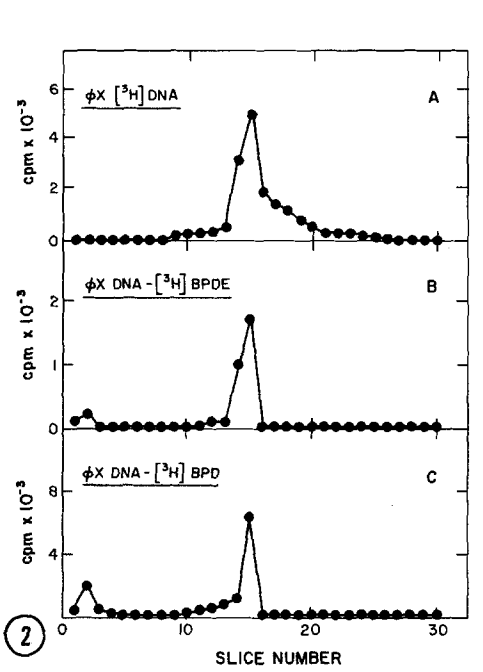


Figure 1. Effect of BPD and BPDE on the infectivity of ϕX174 DNA. The binding mixture (0.1. ml) contained either single-stranded (SS) or duplex (RF) ϕX174 DNA. BPD or BPDE was present in the amounts indicated. After incubation, DNA was isolated from the mixture and assayed for infectivity by transfection of E. coli spheroplasts as described in "Methods".



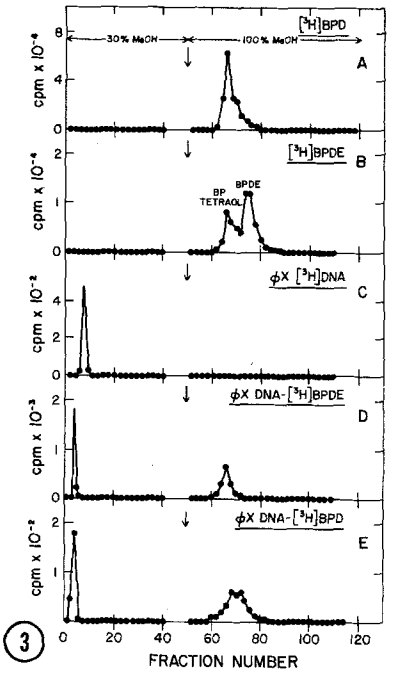


Figure 2. Agarose gel electrophoresis of BP-modified φX DNA. Single-stranded φX174 DNA (10 μg in 0.1 ml of binding mixture) was treated with 0.5 μg of either [^{3}H] BPD or [^{3}H] BPDE and reisolated as described in "Methods". Reacted and untreated DNA samples were applied to a 1% agarose slab gel made up in a buffered solution (pH 8) containing 0.04 M Tris base/0.2 M sodium acetate/0.15% glacial acetic acid/1 mM EDTA and run for 18 hours at a constant current of 40 mA. Each run was sliced into 0.5-cm sections; the sections were placed in vials containing 1 ml of water, briefly autoclaved, cooled to 60°, and 15 ml of Triton X-100 toluene scintillation fluid was added. Radioactivity was determined in a Nuclear Chicago Mark III scintillation counter. (A) Unreacted φX [^{3}H] DNA; (B) φX DNA bound with [^{3}H] BPDE; and (C) φX DNA bound with [^{3}H] BPD.

Figure 3. Sephadex LH20 chromatography of BP-modified φX DNA. Single-stranded φX174 DNA (100 μg in 1 ml of binding mixture) was treated with 5 μg of either [^{3}H] BPDE or [^{3}H] BPD, reisolated, and subjected to chromatography on Sephadex LH20, as described in "Methods". The Sephadex columns were eluted first with 30% methanol containing 5 mM NH_4HCO_3 (pH 8.4), followed by 100% methanol, as indicated above. A sample of 0.2 ml from each fraction was counted for its radioactive content. (A) Unreacted [^{3}H] BPD; (B) unreacted [^{3}H] BPDE and [^{3}H] BP-tetraol; (C) unreacted φX [^{3}H] DNA; (D) φX DNA bound with [^{3}H] BPDE; and (E) φX DNA bound with [^{3}H] BPD.

obtained suggest that BPD binds to single-stranded DNA, and that this complex remains largely intact under the conditions employed for electrophoresis and chromatography.

The nature of the association of BPD with single-stranded DNA was examined further after enzymatic hydrolysis of the φX DNA complex to nucleoside monomers. Binding of φX DNA with BPD and BPDE was carried out either with [^{3}H] guanosine-

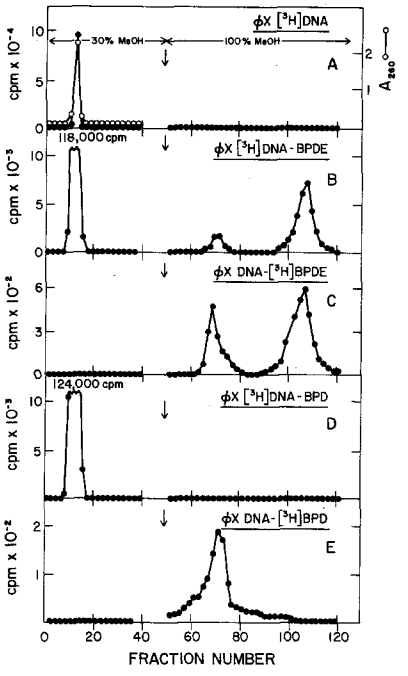


Figure 4. Sephadex LH20 chromatography of nuclease-digested BP-modified φX DNA. The binding mixture (0.4 ml) contained 50 μg of single-stranded φX 174 DNA and 25 μg of either BPDE or BPD. Following binding, the φX DNA was isolated as described in "Methods" and hydrolyzed to nucleosides in two steps. The initial digestion was carried out in a reaction mixture which contained, per ml, 70 mM $ZnSO_4$, 10 mM $MgCl_2$, 23 mM NaCl, 7 mM sodium acetate (pH 4.5), 10,000 units of S1 nuclease, 100 μg of DNase, 1, and 200 μg of DNA. After incubation for 2 hours at 37°, the digestion mixture was neutralized with 1 N NaOH, adjusted to contain 50 mM $MgCl_2$, 70 mM Tris · HCl (pH 8.1), and 30 μg/ml each of snake venom phosphodiesterase and alkaline phosphatase, and was further incubated for 1 hour at 37°. The hydrolysates were stored at -20° until used. Sephadex LH20 chromatography was carried out under the conditions described in Fig. 3. (A) Digested guanosine-labeled φX [^{3}H] DNA plus 4 A260 units of deoxyguanosine; (B) digested guanosine-labeled φX [^{3}H] DNA bound with BPDE; (C) digested φX DNA bound with [^{3}H] BPDE; (D) digested guanosine-labeled φX [^{3}H] DNA bound with BPD; and (E) digested φX DNA bound with [^{3}H] BPD.

labeled φX DNA or with ^{3}H-labeled BP derivatives. Following enzymatic digestion of the washed φX DNA complex, the digest was subjected to chromatography on LH20 Sephadex. Figure 4A shows that chromatography of hydrolyzed φX [^{3}H] DNA yields a single radioactive peak following elution with 30% methanol; this peak is coincident with deoxyguanosine added as marker. Elution with 100% methanol gives no further counts. Hydrolysis of labeled φX DNA pretreated with BPDE yields, in addition to [^{3}H] nucleosides, two peaks eluted with 100% methanol (Fig. 4B). The second and larger of these two peaks has been identified as the BPDE adduct of deoxyguanosine (data not shown), in agreement with other reports (3-5), whereas the smaller,

unidentified peak could represent either the BPDE adduct of deoxyadenosine or incomplete digestion products. Chromatography of hydrolyzed ϕX DNA pretreated with [^{3}H] BPDE yields no radioactivity in the 30% methanol eluate, but gives two labeled peaks with 100% methanol (Fig. 4C). The first peak is believed to be BP-tetraol, which was shown to be present in the unhydrolyzed ϕX DNA-BPDE complex (Fig. 3D); the second peak coincides with the position of the BPDE-deoxyguanosine adduct. Similar chromatographs of digested ϕX [^{3}H] DNA pretreated with unlabeled BPD failed to reveal any radioactive nucleoside migrating in a position other than that where labeled deoxyguanosine is found (Fig. 4D). Chromatography of hydrolyzed ϕX DNA bound with [^{3}H] BPD (Fig. 4E) showed the presence of a single peak eluting with 100% methanol in a position where free BPD was located (Fig. 3A).

DISCUSSION

We had reported earlier that the 7,8-dihydrodiol derivative of benzo[a]pyrene selectively binds to single-stranded DNA and inhibits the infectious capacity of ϕX174 DNA (1). In addition, we had observed that BPD binds extensively to poly(dG) and poly(dI), but only slightly to other DNA homopolymers as well as natural RNAs and ribohomopolymers. In confirmation of the above findings, the present study shows that reaction of ϕX174 DNA with BPD results in the formation of a DNA-BPD complex that is precipitable with ethanol, resists washing with different organic solvents and remains intact after dissolution in aqueous medium and reprecipitation with ethanol. The stable nature of this complex is further emphasized by its capacity to withstand dissociation during gel electrophoresis and chromatography on LH20 Sephadex. However, when this complex is digested with a mixture of nucleases in the presence of alkaline phosphatase, chromatography on LH20 Sephadex indicates the release of free BPD, and no BPD-nucleoside adducts are observed, suggesting that the association of BPD with ϕX DNA is noncovalent. Ibanez et al. have also reported on the noncovalent binding of the BP-tetraol, derived from anti-BPDE, to DNA (12). The possibility of triester formation between BPD and DNA has not been eliminated; however, if this were so, it would be difficult to explain the lack of BPD reaction with duplex DNA and with RNA.

Although the binding of BPD to DNA appears not to be covalent and is different, therefore, from the covalent binding by BPDE, the BPD-DNA complex is sufficiently stable to inhibit the infectivity of ϕX174 DNA. This inhibition is much less efficient than that with BPDE, which probably reflects the difference in the type of DNA binding exhibited by the two different BP derivatives. The finding that BPD is incapable of binding effectively to duplex DNA and inactivating the RF form of ϕX DNA suggests that the BPD-binding sites in double-stranded DNA are masked.

PAH-dihydrodiols are known to be precursors for the formation of diolepoxide metabolites, considered to be ultimate carcinogens (13). The present study indicates that dihydrodiols can also bind directly to DNA, and that they may do so when single-stranded regions are available, i.e., during replication and repair. In this sense, PAH-diols may also be considered as ultimate carcinogens, although it is presently not known whether the bound diol undergos further oxidation in vivo. Nagao and coworkers (14) have suggested that the (-)-enantiomer of BP-trans-7,8-diol may be a direct mutagen in Salmonella typhimurium TA100, and Levin et al. (15) have reported marked differences in the tumor-initiating activity of (+)- and (-)-enantiomers of this same BP-diol on mouse skin. A study of the DNA-binding activity of these two enantiomers may offer important information regarding their biological activities.

ACKNOWLEDGEMENTS

This study was supported by funds from the United States Department of Energy under Contract Number DE-AC02-80EV10328 and by a grant from the American Cancer Society, BC-132D.

References

1. Hsu, W.-T., Sagher, D., Lin, E. J., Harvey, R. G., Fu, P. P., and Weiss, S. B. (1979) Biochem. Biophys. Res. Commun., 87, 416-423.
2. Reviews: (a) Harvey, R. G. and Fu, P. P. in "Polycylic Hydrocarbons and Cancer: Environment, Chemistry, and Metabolism" (1978) Vol. 1, Gelboin, H. V. and Ts'o, P.O.P. Eds., Academic Press, New York, Chap. 6, pp. 133-165; (b) Yang, S. K., Deutsch, J. and Gelboin, H. V., Ibid, Chap. 10, pp. 205-231; (c) Miller, E. C. (1978) Cancer Res., 38, 1477-1496.
3. Osborne, M. R., Thompson, M. H., Tarmy, E. M., Beland, F. A., Harvey, R. G., and Brookes, P. (1976) Chem. Biol. Interact., 14, 343-348.
4. Weinstein, I. B., Jeffrey, A. M., Autrup, H., Kasai, H., and Nakanishi, K. (1976) Science, 193, 592-595.

5. Koreeda, M., Moore, P. D., Yagi, H., Yeh, H.J.C., and Jerina, D. M. (1976) J. Am. Chem. Soc., 98, 6720-6722.
6. Straub, K. M., Meehan, T., Burlingame, A. L., and Calvin, M. (1977) Proc. Natl. Acad. Sci. USA, 74, 5285-5289.
7. Jennette, K. W., Jeffrey, A. M., Blobstein, S. H., Beland, F. A., Harvey, R. G. and Weinstein, I. B. (1977) Biochemistry 16, 932-938.
8. Hsu, W.-T., Lin, E.J.S., Harvey, R. G., and Weiss, S. B. (1977). Proc. Natl. Acad. Sci. USA, 74, 3335-3339.
9. Lovett, M. A., Gurney, D. G., and Helinski, D. R. (1974) Proc. Natl. Acad. Sci. USA, 71, 3854-3857.
10. Hsu, W.-T., Harvey, R. G., Lin, E.J.S., and Weiss, S. B. (1977) Proc. Natl. Acad. Sci. USA, 74, 1378-1382.
11. Baird, W. M. and Brookes, P. (1973) Cancer Res., 33, 2378-2385.
12. Ibanez, V., Geacintov, N. E., Gagliano, A. G., Brandimarte, S., and Harvey, R. G. (1980) J. Am. Chem. Soc., 102, 5661-5666.
13. Review: Harvey, R. G. (1981) Accts. Chem. Res., in press.
14. Nagao, M., Sugimura, T., Yang, S. K., and Gelboin, H. V. (1978) Mutation Res., 58, 361-365.
15. Levin, W., Wood, A. W., Chang, R. L., Slaga, T. J., Yagi, H., Jerina, D. M., and Conney, A. H. (1977) Cancer Res., 37, 2721-2725.