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Mechanism of phage φX174 DNA inactivation by benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide

(polycyclic aromatic hydrocarbons/DNA binding by benzo[*a*]pyrene-diolepoxide/infectious nucleic acid/DNA synthesis/chemical carcinogenesis)

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ABSTRACT **A previous report from this laboratory has shown that certain derivatives of polycyclic aromatic hydrocarbons bind to φX174 DNA and render it noninfectious. The present work describes the relationship between the extent of φX174 DNA binding by (±)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide and the effect on infectivity. The results suggest that one molecule of bound diolepoxide is sufficient to inhibit the replication of a single molecule of φX174 DNA. DNA synthesis studies, *in vitro*, indicate that when φX DNA bound by benzo[*a*]pyrene groups serves as template the rate of DNA polymerization is reduced and less product is formed. In addition, the propagation of synthetic DNA strands is blocked so that incomplete complementary chains are assembled. The relationship of these findings to the mutagenic and carcinogenic process associated with the action of benzo[*a*]pyrene-diolepoxide is discussed.**

Studies on the chemical and biological properties of (±)-*trans*-7, 8-dihydroxy-*anti*-9, 10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BP-diolepoxide) suggest that it may be intimately involved in benzo[*a*]pyrene (BP)-induced carcinogenesis. BP-diolepoxide is derived from the metabolic conversion of BP-7,8-diol by microsomal enzymes (1–3), it reacts covalently with nucleic acids (4, 5), and DNA conjugated with *anti*-BP-diolepoxide has been isolated from cultured cells exposed to BP (2, 6). In addition, several studies have shown that BP-diolepoxide is unusually active as a mutagenic agent for mammalian (1, 7) and bacterial cells (8, 9), and a more potent carcinogen than the parent hydrocarbon in newborn mice (10).

We have described a bacteriophage assay system for evaluation of the biological activity of polycyclic aromatic hydrocarbons (PAH) and related compounds (11–13). In this system, active PAH compounds inhibit viral replication. A simple procedure has been devised which allows us to determine whether a chemical agent prevents the biological expression of infectious viral nucleic acid by interacting with it directly, or only after intracellular activation (13). Among 31 PAH compounds examined, *anti*-benzo[*a*]pyrene-diolepoxide was the most active inhibitor of φX174 replication in *Escherichia coli* spheroplasts, and it was shown to react directly with φX174 DNA.

The present report describes the relationship between the extent of BP-diolepoxide binding to φX174 DNA and its effect on infectivity. Our results suggest that one molecule of bound diolepoxide is sufficient to block the replication of a single molecule of φX174 DNA. In support of this, studies *in vitro*, in which φX174 DNA conjugated with BP-diolepoxide was used as template for DNA replication, indicate that such complexes prevent the synthesis of intact replicative forms.

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METHODS

Preparation of φX174 Phage and Infectious DNA. Bacteriophage φX174am3 (a gift from L. B. Dumas, Northwestern University, Chicago, IL) was prepared by the procedure of Sinsheimer (14), except that the infected cell lysates obtained by lysozyme treatment were subjected to two successive centrifugations (15 min at 17,000 × *g* and 2 hr at 100,00 × *g*) for sedimentation of phage. The resuspended phages were then purified as reported (14). DNA was isolated from purified viruses by several extractions with phenol containing 1% sodium dodecyl sulfate, followed by dialysis against 10 mM Tris (pH 7.5)/1 mM EDTA. The concentration of φX DNA was determined on the basis that, in a 1-cm light path, 1 A_{260} unit is equivalent to 36 μg/ml or 1.3×10^{13} molecules per ml (14).

Radioactive φX174am3 DNA was prepared by the same procedure described above, except that infection was carried out in the presence of [^{3}H]thymidine (6 Ci/mmol) at a concentration of 20 nmol/ml. Isolation of DNA from the radioactive virus by phenol extraction gave a specific activity of 3×10^4 cpm/μg of DNA.

Infectivity Assay of φX174am3 DNA. The infectivity of φX174am3 DNA was assayed by absorption with spheroplasts prepared from *E. coli* K12W1485 and plating with its sensitive host, *E. coli* HF4714, for plaque development, as reported (11, 13).

Preparation of Radioactive Replicative Intermediates RF1 and RF2. ^{3}H-Labeled φX174am3 phage, prepared as described above, was used to infect *E. coli* C in the presence of chloramphenicol (15 μg/ml). After 10 min of infection, DNA was extracted from the infected cells by the procedure of Brutlag *et al.* (15). The cell lysate was adjusted to contain 1 M NaCl and was extracted once with phenol; the nucleic acid was precipitated from the aqueous phase with ethanol. After resuspension in Tris/EDTA and sedimentation in a neutral sucrose gradient, the pellet showed two radioactive peaks, 21 S and 16 S, corresponding to the φX174 DNA replicative intermediates RF1 and RF2, respectively.

Reaction of φX DNA with BP-Diolepoxide. The reaction mixture (0.1 ml) contained 10 mM Tris (pH 7.5), 1 mM EDTA, 8 μg of φX DNA, and *anti*-BP-diolepoxide as indicated in *Results*. After incubation for 10 min at 25°, the DNA was precipitated with ethanol and washed with acetone, as described elsewhere (13). The precipitate was dried and dissolved in

Abbreviations: PAH, polycyclic aromatic hydrocarbons; BP, benzo[*a*]pyrene; BP-diolepoxide, (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydro-BP; MR, molar ratio.

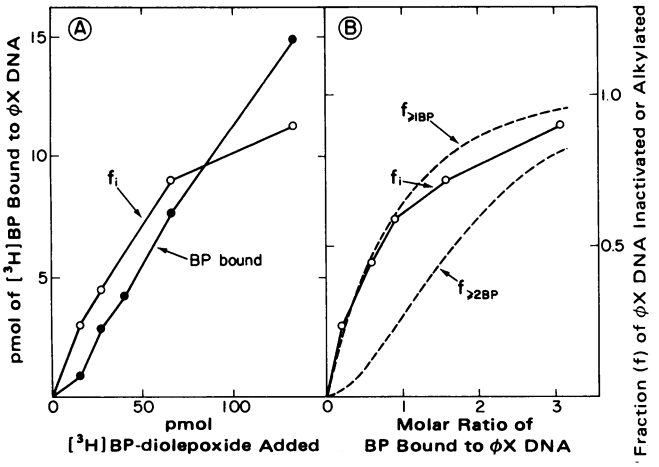

FIG. 1. Relationship between the molar quantity of φX DNA alkylated by BP-diolepoxide and the amount of infectivity lost. (*A*) Varying amounts of [³H]BP-diolepoxide, as indicated above, were added to different reaction vessels, each containing 4.8 pmol of φX DNA. After incubation, the [³H]BP–DNA complex was isolated and its DNA, radioactivity, and infectivity content was determined (see *Methods*). The term "f_i" represents the fraction of infectious φX DNA that lost infectivity as a consequence of the diolepoxide treatment. (*B*) The fraction of φX DNA inactivated (f_i) is plotted as a function of the molar ratio (MR) of the average number of BP molecules bound per molecule of φX DNA, calculated from the radioactivity and DNA content of the isolated [³H]BP–DNA complex. This curve is an average of four separate experiments. The terms $f_{\geq 1BP}$ and $f_{\geq 2BP}$ were calculated from the expressions $1 - P_0$ and $1 - P_0 - P_1$, respectively, where $P_0 = e^{-MR}$ and $P_1 = MR \times e^{-MR}$.

Tris/EDTA, and aliquots were taken for determination of the amount of A_{260} material present and its infectious content. When [³H]diolepoxide was used in the above reaction, radioactivity measurements of the dissolved precipitate were used for determinations of the extent of DNA alkylation. For DNA synthesis studies, the resuspended alkylated DNA was dialyzed overnight against 2 liters of Tris/EDTA prior to its use as a template for DNA polymerase reactions.

DNA Synthesis *In Vitro*. A partially purified extract from *E. coli* H560, prepared by the method of Wickner *et al.* (16), was used for the study of DNA synthesis *in vitro*, and was generously supplied by C. L. Peebles and N. R. Cozzarelli, The University of Chicago. The reaction mixture contained, in 0.1 ml, 20 mM Tris (pH 7.5), 4 mM dithiothreitol, 3 mM $MgCl_2$, 5 mM ATP, 0.04 mM each of dATP, dGTP, dCTP, and [³H]dTTP (1 Ci/mmol), 0.34 μg of φX174am3 DNA (untreated or alkylated with BP-diolepoxide), and 16 μl of the above *E. coli* enzyme preparation [40 mg/ml as determined by the method of Lowry *et al.* (17)]. After incubation at 25° for a given time period, 10% trichloroacetic acid was added and the precipitate was collected on Millipore filters (RA45). Filters were dissolved in a Triton X-100/toluene scintillant mixture and radioactivity was measured in a Nuclear Chicago Mark III scintillation counter. For product analysis by gradient centrifugation, 25 μl of 0.4 M EDTA was added to the reaction mixture, followed by 10 μl of a Pronase solution (10 mg/ml) that had been treated to inactivate DNase. After 25 min at 37°, a second portion of Pronase (5 μl) was added, and digestion was continued for another 25 min. The mixture was adjusted to contain 50 mM KOAc (pH 5.4), and a 10-fold volume of ethanol was added. After storage at −20° overnight, the precipitate was sedimented by centrifugation and resuspended in a minimum volume of Tris/EDTA for sedimentation analysis.

Sucrose Gradient Sedimentation Analysis. Neutral sucrose gradients (5–20%) contained 1 M NaCl, 1 mM EDTA, and 10 mM Tris (pH 7.5). Alkali gradients were the same, except that, in addition, they contained 0.2 M NaOH. The DNA synthetic product described above (0.10–0.15 ml) was layered on a 12-ml sucrose gradient and centrifuged at 39,000 rpm at 4° in a Beckman SW41 rotor for the time indicated in *Results*. Prior to alkali gradient analysis, the dissolved synthetic product was adjusted to contain 0.2 M NaOH.

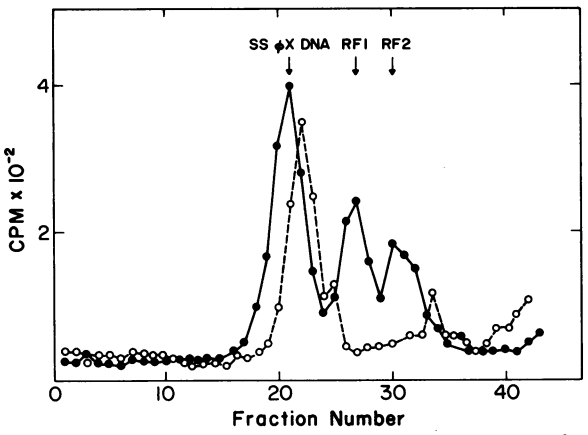

FIG. 2. Sucrose gradient analysis of φX174 DNA complexed with [³H]BP-diolepoxide. φX DNA (8 μg) was treated with [³H]BP-diolepoxide, and the [³H]BP-bound DNA was isolated (see *Methods*). An average of eight [³H]BP molecules bound per molecule of viral DNA was estimated. The [³H]BP–φX DNA (○) was subjected to neutral sucrose gradient analysis at 39,000 rpm for 5 hr, as described in *Methods*. A second gradient contained the radioactive markers (●), φX DNA, RF1, and RF2, prepared as described in *Methods*. The fractions collected at the end of the run were analyzed by direct measurement of radioactivity.

Chemicals and Radioisotopes. [³H]Thymidine (6 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, CA., and [³H]thymidine triphosphate (18.6 Ci/mmol) from New England Nuclear, Boston, MA. [³H]*Anti*-BP-diolepoxide (specific activity, 246 cpm/pmol) was synthesized under National Cancer Institute Contract CP-33387 by Midwest Research Institute, Kansas City, MO. This material had a chemical purity of more than 90% (Shen Yang, National Cancer Institute, Bethesda, MD, personal communication), which agreed with its biological activity as assayed in this laboratory (13). *Anti*-BP-diolepoxide was dissolved in dimethylformamide and stored at 4° in the dark. Under these conditions, no change in the dose response of this compound with respect to its capacity to inactivate φX74 DNA was detected over a 6-month period.

RESULTS

Fig. 1*A* illustrates the relationship between the binding of ³H-labeled *anti*-BP-diolepoxide to φX174 DNA and its effect upon the infectivity of this viral DNA. With increasing additions of [³H]diolepoxide to viral DNA, BP binding to φX DNA increases linearly, whereas the infectivity of φX DNA is inactivated exponentially. Fig. 1*B* shows the fraction (f_i) of infectious viral DNA inactivated and the Poisson distributions for the fraction of viral DNA bound by one or more ($f_{\geq 1BP}$) and two or more ($f_{\geq 2BP}$) molecules of BP, as a function of the molar ratio of BP bound to φX DNA. A comparison of the experimental and theoretical curves indicates that the inactivation of φX DNA (f_i) follows the $f_{\geq 1BP}$ curve rather closely, especially at the lower molar ratios.

When the complex [³H]BP–φX DNA (molar ratio = 8) is subjected to centrifugation in a neutral sucrose gradient, the BP-bound radioactivity sediments at a somewhat slower rate (25 S) than the untreated viral DNA (Fig. 2). If the alkylation

Table 1. Reaction requirements for the ϕX174 DNA-directed synthesis of DNA

Omission or addition	[^{3}H]dTMP incorporated into DNA, cpm
Complete	4530
Omit ϕX174 DNA	248
Omit dCTP, dATP, and dGTP	244
Omit ATP	243
Add EDTA	180
Add DNase	232

The reaction system (25 μl) was as described in *Methods* except for the omissions or additions indicated above. Where indicated, EDTA was present at a concentration of 13 mM and 70 ng of DNase was added to the reaction mixture. Incubation was for 20 min at 25°.

of single-stranded ϕX DNA by BP-diolepoxide were random and gave rise to DNA breakage, the S value of the BP-ϕX DNA complex would have been significantly lower and less uniform for high molar ratios of bound BP groups. The difference in S values between the untreated and BP-treated viral DNA is possibly due to conformational changes in the alkylated DNA and the hydrophobic nature of BP, and should be a function of the average number of BP groups attached to each DNA molecule.

In order to determine the effect of BP groups on the replicative process, we examined DNA synthesis *in vitro*, using alkylated ϕX DNA as template. In agreement with the findings of other groups (16), Table 1 shows that a partially purified extract of *E. coli* H560 catalyzes the incorporation of [^{3}H]thymidine triphosphate into a trichloroacetic acid-insoluble fraction that is dependent upon the presence of ϕX DNA, ATP, deoxynucleoside triphosphates, and magnesium ions in the reaction mixture, and is sensitive to the action of DNase. When analyzed by centrifugation in a neutral sucrose gradient, the product labeled *in vitro* sediments at a rate similar to that of the ϕX DNA replicative intermediate RF2 (Fig. 3).

Fig. 4 shows the kinetics of the polymerization reaction when unmodified and BP-modified ϕX DNA serve as templates. It

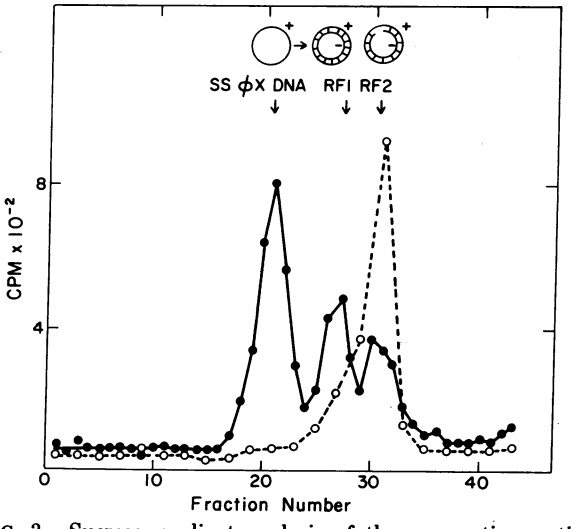

FIG. 3. Sucrose gradient analysis of the enzymatic reaction product directed by ϕX DNA. *In vitro* synthesis directed by ϕX DNA was carried out for 20 min as described in *Methods*. After Pronase treatment and ethanol precipitation, the resuspended product was subjected to sedimentation in neutral sucrose as described for Fig. 2. After centrifugation, the fractions collected were analyzed for radioactivity by trichloroacetic acid precipitation. ●, ^{3}H-Labeled markers; ○, synthetic product.

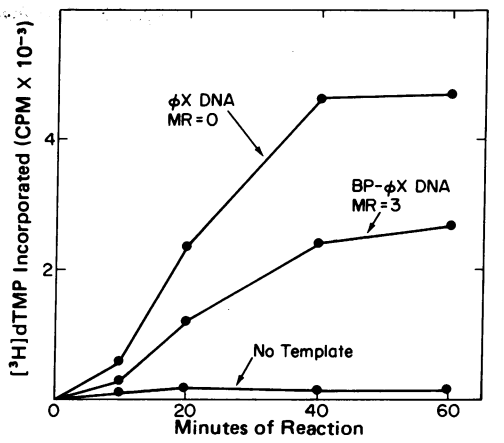

FIG. 4. Kinetics of the BP-ϕX DNA-directed synthesis of DNA. The reaction system (25 μl) was as described in *Methods;* both unmodified and BP-modified ϕX DNA were used as templates in separate reaction mixtures. At the time periods indicated above, samples were withdrawn, and the amount of [^{3}H]dTMP incorporated into DNA was determined by acid precipitation. The BP-ϕX DNA used in this experiment was prepared as described in *Methods* and had an average molar ratio (MR) (BP/ϕX DNA) of 3, as estimated from Fig. 1*B*, based on the fraction of infectivity inactivated, f_i.

is apparent that the incorporation of [^{3}H]dTMP into an acid-insoluble product for the alkylated ϕX DNA template occurs at a significantly reduced rate, and that the amount of label incorporated is less than with unmodified template. At molar ratios of BP/ϕX DNA higher than 3, the rate of formation of labeled product is reduced further, but not completely eliminated (data not shown). Fig. 4 also shows that, in this reaction system, there is an induction period of 5–10 min preceding the linear incorporation of substrate into a DNA product, for both types of templates.

The products of the reaction directed by the various templates described for Fig. 4 were analyzed by sucrose gradient centrifugation. As shown in Fig. 5, for each type of ϕX DNA template used, regardless of the degree of BP alkylation, the labeled product synthesized sediments like the replicative intermediate RF2 in neutral sucrose gradients. Although a similar amount of RF2 product was synthesized for the nonalkylated template and the alkylated template modified to a molar ratio of 0.2 (Fig. 5*A*), less RF2 product was formed with those templates containing higher amounts of bound BP.

When the RF2 products were examined by sedimentation under alkaline conditions, marked differences in the profiles were observed for the dissociated complementary strands polymerized on the two types of templates. Under alkaline conditions (Fig. 6), a large fraction of the DNA product made with BP-ϕX DNA serving as template sediments more slowly and less uniformly than the nonalkylated template product, suggesting the synthesis of complementary DNA strands that are incomplete and partially heterogeneous in size. When the time of synthesis is extended, the labeled products made from the alkylated templates, at molar ratios of both 0.2 and 2.1, appear to become more heterodispersed.

DISCUSSION

Our study on the relationship between the extent of BP-diolepoxide binding with ϕX174 DNA and its effect on infectivity indicates that the alkylation of a molecule of viral DNA by a single molecule of benzo[*a*]pyrene-diolepoxide is sufficient to render it noninfectious. The Poisson distribution formula can be used to generate a family of theoretical curves for single or

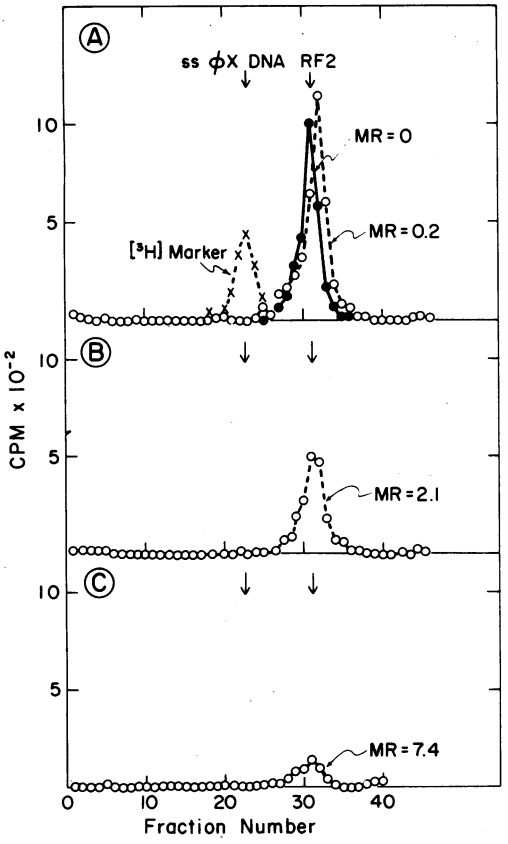

FIG. 5. Sedimentation analysis of the synthetic DNA products under neutral conditions. The reaction system (0.1 ml) was as described in *Methods*. Separate reaction mixtures contained unmodified and BP-modified φX DNA as templates at different molar ratios (MR) of BP/φX DNA. After 20 min of incubation at 25°, the individual reaction products were isolated as described under *Methods* and subjected to centrifugation in neutral sucrose gradients for 6 hr at 39,000 rpm. The gradient region corresponding to RF2 was collected and precipitated with alcohol, and the precipitate was resuspended in 0.3 ml of Tris/EDTA. A sample of 0.1 ml of this suspension was again subjected to centrifugation in neutral sucrose; the remaining material was used for alkali gradient analysis (Fig. 6). The radioactivity of fractions collected from the second neutral gradient was measured directly. Single-stranded φX [³H]DNA was used as marker in a separate gradient and is shown only in *A*. BP–φX DNAs, alkylated with different concentrations of BP-diolepoxide, were prepared as described in *Methods*. The plaque-forming capacity for three different BP–φX DNA preparations indicated f_i (fraction inactivated) values of 0.775, 0.215, and 0.009, which correspond to average molar ratios of 0.2, 2.1, and 7.4, respectively, as estimated from the f_i plot in Fig. 1*B*.

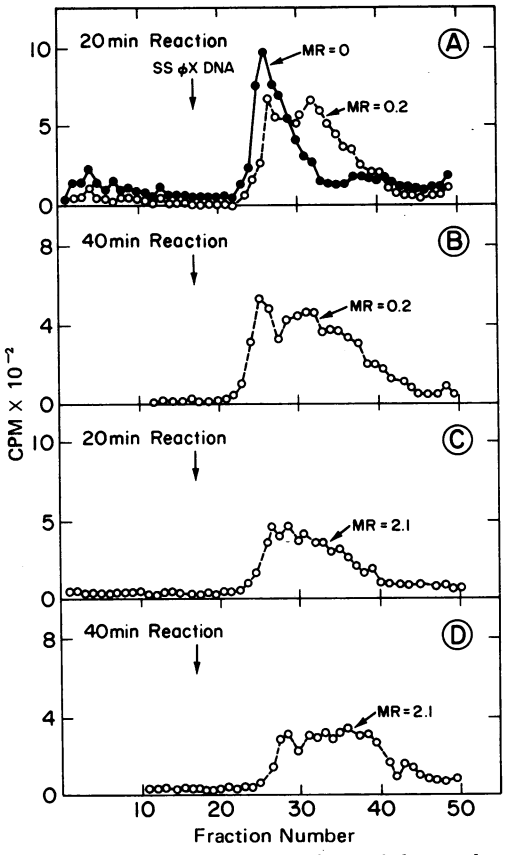

FIG. 6. Alkali sucrose-gradient analysis of the synthetic DNA products. The 20-min synthetic RF2 reaction products isolated by sedimentation in neutral sucrose (0.15 ml) in the experiment described in Fig. 5 were subjected to alkaline sucrose gradient centrifugation for 10 hr at 39,000 rpm. Similar RF2 reaction products, with the same type of BP–φX DNA templates, were prepared from 40-min reaction mixtures and subjected to the same type of centrifugation in alkaline gradients. The fractions collected at the end of the run were analyzed for radioactivity.

multiple interactions between two types of molecules, with the implicit assumption that the interactions are random. The molar ratios estimated for the amount of BP bound to DNA represent average values, since we have assumed that the distribution of the alkylated groups among DNA molecules is random. The rather close fit of the experimental curve (f_i) to the theoretical curve describing the fraction of φX DNA alkylated by one or more BP molecules (Fig. 1*B*) strongly supports the conclusion of a single-hit inactivation for φX DNA molecules and justifies the assumption that alkylation is largely random. In addition, the close coincidence of the experimental and $f_{\geq 1BP}$ curves suggests that repair of BP–φX DNA is minimal or does not occur; hence alkylation is essentially irreversible in our *E. coli* assay system. The small difference between the f_i and $f_{\geq 1BP}$ curves for the higher molar ratios may not be significant experimentally; however, we cannot exclude the occurrence of some nonrandom alkylation of DNA, or perhaps replication of some alkylated DNA molecules that might take place with or without DNA repair, possibly after some recombinant event (18).

Sedimentation analysis of BP-modified φX DNA (Fig. 2) indicates homogeneity in size and a sedimentation rate close to that of unmodified viral DNA. It is unlikely, therefore, that inactivation of viral DNA by BP alkylation results from single-strand cleavage or intermolecular DNA crosslinking, as reported for other types of alkylating agents (19). Some insight into the mechanism of inactivation of viral DNA is provided by our studies on DNA synthesis *in vitro*, in which alkylated templates were used.

The rate of DNA synthesis is lowered when BP–φX DNA serves as template; the total amount of DNA polymerized is also reduced. Centrifugation analysis under neutral conditions indicates that the DNA products made on BP–φX DNA templates behave like RF2 intermediates, but under denaturation conditions the synthetic complementary strands sediment largely as fragmented chains. With increasing time of synthesis, the degree of fragmentation seems to increase. These results suggest that benzo[*a*]pyrene groups on DNA templates block the propagation of new chains to full-sized molecules and imply that polymerization through the alkylated template site does not occur.

If DNA polymerization is blocked by BP groups and the synthetic complementary chains are incomplete, why do the

BP-ϕX DNA products sediment like RF2 intermediates? It is possible that, when chain elongation is blocked, the polymerizing enzyme initiates synthesis at another site on the same template. The question of single or multiple initiation sites for the replication of ϕX DNA is still unresolved (20). The reduced rate of incorporation found with BP-ϕX DNA might result from the cumulative induction periods required for initiation of new chains, but multiple initiations would not readily explain the marked decrease in the level of DNA synthesis observed with alkylated templates. Isolated RF2 products, synthesized under the direction of ϕX [^{3}H]DNA and BP-ϕX [^{3}H]DNA (molar ratio = 4) are rendered acid-soluble by the action of S1 nuclease to the extent of 9 and 32%, respectively (data not shown). Since S1 nuclease digests single-stranded DNA, the RF2 products made with alkylated templates appear to be only partially duplex in nature. The conversion of single-stranded ϕX DNA to a partial DNA duplex may be sufficient to cause these products to sediment like RF2 intermediates under neutral conditions.

Certain PAH compounds are known mutagens and carcinogens. If PAH binding to DNA is directly responsible for these biological effects (21, 22), it is difficult to understand their mechanism of action in view of our present results which suggest that DNA copying is blocked on PAH-modified templates. *In vivo*, PAH-alkylated duplex DNA could undergo excision and repair (23); this process itself might promote some misrepair. It is also possible that error-prone DNA repair activity might be responsible for PAH mutagenesis similar to that observed for UV mutagenesis (24). On the other hand, mechanisms other than PAH binding to DNA (e.g., modification of other types of cellular macromolecules) might contribute and be important for an understanding of PAH action in biological systems.

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