Ramstein, J., & Leng, M. (1975) *Biophys. Chem. 3*, 234–240.
Ramstein, J., Hélène, C., & Leng, M. (1971) *Eur. J. Biochem. 21*, 125–136.
Ramstein, J., Dourlent, M., & Leng, M. (1972) *Biochem. Biophys. Res. Commun. 47*, 874–882.
Ramstein, J., Houssier, C., & Leng, M. (1973) *Biochim. Biophys. Acta 335*, 54–68.
Rigler, R., & Ehrenberg, M. (1973) *Q. Rev. Biophys. 6*, 139–199.
Rigler, R., Rabl, C. R., & Jovin, T. M. (1974) *Rev. Sci. Instrum. 45*, 580–588.
Seeman, N. C., Day, R. O., & Rich, A. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 1537.
Sobell, H. M., Tsai, C., Jain, S. C., & Gilbert, S. G. (1977) *J. Mol. Biol. 114*, 333–365.
Stone, A. L., & Bradley, D. F. (1961) *J. Am. Chem. Soc. 83*, 3627–3634.
Thomes, J. C., Weill, G., & Daune, M. (1969) *Biopolymers 8*, 647–659.
Tsai, C., Jain, S. C., & Sobell, H. M. (1977) *J. Mol. Biol. 114*, 301–315.
Weill, G. (1965) *Biopolymers 3*, 567–572.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465–497.

Mechanism of Benzo[*a*]pyrene Diol Epoxide Induced Deoxyribonucleic Acid Strand Scission†

Howard B. Gamper,* James C. Bartholomew, and Melvin Calvin

ABSTRACT: Approximately 1% of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BaP-diol epoxide) DNA alkylation sites rearrange with strand scission at neutral pH. Phosphotriester hydrolysis and depurination/depyrimidination strand scission were critically examined as possible mechanisms for this phenomenon. The catalysis of nicking by alkali and the inhibition of nicking by counterions were consistent with either mechanism. The kinetics of nicking, however, were characteristic of a multistep reaction such as depurination/depyrimidination strand scission and the detection of apurinic sites in BaP-diol epoxide alkylated DNA strongly supported this mechanism. The number of such sites, especially at lower reaction levels, was probably sufficient to account for strand scission. No direct evidence was obtained for nicking occurring through phosphotriester hydrolysis. Studies with model substrates, including dibutyl phosphate, DNA homopolymers, and TMV RNA, indicated that if BaP-diol epoxide forms phosphotriesters in DNA or RNA, they do not hydrolyze with strand scission. Besides apurinic/apyrimidinic sites, a second alkali-sensitive rearrangement product was present in BaP-diol epoxide modified DNA. These latter sites accumulated with time and after 24 h accounted for as much as 4% of the initial alkylation events. Although relatively stable at neutrality, they spontaneously nicked the DNA backbone at high pH. It is possible that these sites represent a rearrangement of the major N^2 guanine adduct.

The ubiquitous environmental pollutant benzo[*a*]pyrene is the most widely studied of the carcinogenic polycyclic aromatic hydrocarbons. In mammalian cells it is metabolically activated to either the syn or anti isomers of benzo[*a*]pyrene-7,8-diol 9,10-epoxide (Borgen et al., 1973; Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Ivanovic et al., 1976; Weinstein et al., 1976). Studies have shown this highly reactive electrophile to be cytotoxic (Landolph et al., 1978), mutagenic (Malaveille et al., 1975; Huberman et al., 1976; Wislocki et al., 1976a,b; Wood et al., 1977), transforming in tissue culture (Mager et al., 1977), and tumorigenic in vivo (Levin et al., 1977). It accounts for the majority of DNA and RNA modification (i.e., alkylation or adduct formation) by the hydrocarbon (Koreeda et al., 1978; Vahakangas et al., 1979). The primary adduct in both macromolecules has been shown to consist of a linkage between the C-10 position of the epoxide and the N^2 amino group of guanine (Koreeda et al., 1976; Osborne et al., 1976; Weinstein et al., 1976; Jeffrey et al., 1977; Meehan et al., 1977; Koreeda et al., 1978). The exocyclic amino groups of adenine and probably cytidine are also modified to lesser extents (Jennette et al., 1977; Jeffrey et al., 1977, 1979; Meehan et al., 1977; Straub et al., 1977; Ivanovic et al., 1978).

In a preliminary communication we reported that the synthetic anti isomer (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BaP-diol epoxide)[1] nicked superhelical Col E1 DNA at a low frequency relative to alkylation and proposed a mechanism for nicking based upon the hydrolysis of phosphotriesters rendered unstable by the presence of a β-hydroxyl group on the attached hydrocarbon (Gamper et al., 1977). However, in an independent study Shooter et al. (1977) postulated the same nicking as due to depurination strand scission. Indirect evidence exists for the reaction of BaP-diol epoxide with both DNA phosphate (Koreeda et al., 1976) and the N-7 position of guanine (Osborne et al., 1978; King et al., 1979), a modification known to labilize the glycosidic bond. In this paper we critically assess the relevance of the two proposed mechanisms utilizing as

† From the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received February 27, 1980.* This research was supported by the Division of Biomedical and Environmental Research, U.S. Department of Energy, under Contract No. W-7405-ENG-48.

[1] Abbreviations used: BaP-diol epoxide, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BaP-tetraol, 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid.

0006-2960/80/0419-3948$01.00/0

substrates superhelical SV40 DNA, DNA homopolymers, TMV RNA, and dibutyl phosphate.

Materials and Methods

Benzo[a]pyrene Derivatives. Crystalline BaP-diol epoxide was synthesized by K. Straub of this laboratory essentially using the method of McCaustland & Engel (1975). [^{3}H]-BaP-diol epoxide, 1.5 mg/mL in 19:1 tetrahydrofuran–triethylamine, was also synthesized by K. Straub according to published procedures (McCaustland et al., 1976) and had a specific activity of 1.23 Ci/mmol. The BaP-diol epoxide stocks were stored at −70 °C and dilutions were made with dimethyl sulfoxide. Hydrolysis of BaP-diol epoxide to 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BaP-tetraol) was effected by storing it overnight at 37 °C in dimethyl sulfoxide containing 10% 0.12 M HCl.

SV40 DNA. Confluent TC-7 African green monkey kidney cells were infected with wild-type SV40 small plaque strain Rh911 at 10–20 plaque forming units/cell. The DNA was labeled by adding 200 μCi of [^{14}C]thymidine or [^{3}H]thymidine (New England Nuclear) to 10^7 cells 18 h after infection. Superhelical SV40 (form I) DNA was isolated at the peak of viral replication 42 h postinfection by a modification of the Hirt extraction essentially as described by Hallick et al. (1978). DNA concentrations were determined assuming 50 μg/mL per 1.0 optical density unit at 257 nm (Peacocke & Walker, 1962). The DNA stocks were kept up to 1 year during which time there was a gradual increase in the amount of contaminating nicked circular (form II) DNA.

BaP-diol Epoxide Alkylation. SV40 form I DNA (0.1–2.5 μg in 50–250 μL) was modified with BaP-diol epoxide or BaP-tetraol in 20 mM Tris-HCl (pH 8.0)–0.5 mM EDTA–5–10% dimethyl sulfoxide at the indicated molar reaction ratios (BaP-diol epoxide/DNA mononucleotide). Reaction mixtures were incubated in the dark at 37 °C for 1–48 h. Aliquots were analyzed for strand scission either directly after reaction or following ethyl acetate extraction by gel electrophoresis, electron microscopy, or ultracentrifugation. Alkylation was quantified by radioactivity counting using SV40 [^{14}C]DNA and [^{3}H]BaP-diol epoxide. The modified DNA was diluted to 1.0 mL with 20 mM Tris-HCl (pH 8.0)–0.5 mM EDTA–0.5 M NaCl and extracted 3 times with 1.0 mL of ethyl acetate. After the addition of 30 μg of carrier calf thymus DNA, the DNA was precipitated from the aqueous phase by addition of 2.0 mL of NaCl-saturated ethanol and by storage overnight at −20 °C. The DNA was pelleted, washed, and resuspended in 1.0 mL of NaCl-saturated 70% ethanol. It was then collected on Millipore 0.45-μm HAWP filters, combusted, and counted. Binding data were calculated by assuming SV40 contained 5200 base pairs (Reddy et al., 1978) and had a M_r of 3.6×10^6 (Tooze, 1973).

TMV RNA (2.6 μg in 50 μL) was reacted for 6 h at 37 °C with BaP-diol epoxide in 20 mM Tris–HCl (pH 8.0)–0.5 mM EDTA–10% dimethyl sulfoxide. The RNA was electrophoresed following two extractions with 50 μL of ethyl acetate. The DNA homopolymers (Miles; 25–75 μg in 100–200 μL) were reacted for 24 h at 37 °C with BaP-diol epoxide or BaP-tetraol in 20 mM Tris-HCl (pH 8.0)–10–20% dimethyl sulfoxide. Prior to electrophoresis the reaction mixtures were extracted twice with ethyl acetate. Poly(dA)·poly(dT), which was similarly modified, was prepared from equal amounts of the two homopolymers mixed together at 60 °C and slowly cooled to room temperature.

Preparative reaction of 59.9 mg of dibutyl [^{32}P]phosphate (Amersham) with 39.1 mg of BaP-diol epoxide (molar reaction ratio = 0.46) was carried out in 40 mL of acetone–H_2O (1:1; pH 7.5) for 24 h at 37 °C. The reaction products were loaded onto a Sephadex LH-20 column and eluted with a 0–50% ethanol log gradient in 0.03 M bicarbonate (pH 8.3). Fractions were analyzed for ^{32}P label and for absorption at 254 nm.

Small-scale reaction of 275 μg of dibutyl [^{32}P]phosphate with 2 mg of BaP-diol epoxide or BaP-tetraol (molar reaction ratio = 5.0) was carried out in 20 mM Tris-HCl (pH 7.4)–dimethyl sulfoxide (1:1) for 24 h at 37 °C. Before and after being heated at 85 °C for 15 min, reaction aliquots were diluted with cold carrier monobutyl phosphate and dibutyl phosphate and spotted on Eastman Kodak No. 6065 TLC plates. The plates were developed for 4 h in 2-propanol–NH_4OH–H_2O (7:2:1), dried, and sprayed with a phosphate visualizing reagent (Zweig & Sherma, 1972). The phosphate spots were excised and counted. A dibutyl [^{32}P]phosphate control was similarly incubated, chromatographed, and counted.

Gel Electrophoresis. Vertical slab gel electrophoresis was conducted in an apparatus constructed according to the design of Studier (1973). Gel dimensions were 13.3 × 13.0 × 0.3–0.5 cm. SV40 DNA was electrophoresed at 50 V for 12 h on 1.4% agarose gels prepared and run in 40 mM Tris–HCl (pH 7.9)–5 mM sodium acetate–1 mM EDTA. Disc gel electrophoresis of SV40 DNA was performed in a Hoefer Model EF301 apparatus. Samples were electrophoresed on 0.5 × 11.5 cm 1.4% agarose gels for 400 min at 75 V.

Gels containing SV40 DNA were stained for at least 1 h at 4 °C in electrophoresis buffer containing 0.5 μg/mL ethidium bromide. The fluorescent DNA bands were illuminated with short-wavelength UV (Ultra Violet Products, Inc., San Gabriel, CA) and photographed on Polaroid type 665 film through a yellow filter. For determination of radioactivity, DNA bands were excised under UV illumination, dried overnight at 37 °C on adsorbent pads, combusted in a Packard automatic combustion apparatus, and counted.

Several substrates were analyzed on 2% polyacrylamide–0.5% agarose gels including TMV RNA (4 h at 150 V), poly(dA) (1.5 h at 150 V), poly(dT) (3 h at 150 V), and poly(dC) (2.5 h at 150 V). These gels were prepared and run in 89 mM Tris–89 mM boric acid (pH 8.3)–2.8 mM EDTA (Peacock & Dingman, 1968). Denaturing polyacrylamide gels were prepared and run in formamide as described by Staynov et al. (1972). Poly(dA)·poly(dT) and poly(dG) were electrophoresed for 5 h at 150 V in denaturing 4% and 8% polyacrylamide gels, respectively.

TMV RNA gels were stained for 15 min in electrophoresis buffer containing 30 μg/mL acridine orange and then destained in the same buffer (McMaster & Carmichael, 1977). The fluorescent bands were illuminated with long-wavelength UV and photographed on Polaroid type 108 color film through a yellow filter. The DNA homopolymer gels were stained 1 h in a 1:1 solution of 0.4 M sodium acetate–0.4 M acetic acid (pH 4.7) containing 0.2% toluidine blue O and destained in 10% acetic acid. The gels were illuminated from below with white light and photographed on Polaroid type 665 film through a yellow filter. The negatives were traced with a Schoeffel Model SD3000 spectrodensitometer.

Electron Microscopy. Viral DNA was spread by using a modification of the Kleinschmidt procedure (Davis et al., 1971). The DNA monolayer was adsorbed onto Pelco No. 3HGC300 or No. 3HGC400 copper grids covered with parlodion film (Mallinckrodt), stained 15 s in 90% ethanol containing 0.05 mM uranyl acetate (Mallinckrodt) and 0.05 M HCl, rinsed 10 s in 90% ethanol, and air-dried. Grids were

rotary shadowed with 80% Pt–20% Pd (Ted Pella Co., Tustin, CA; No. 24 wire), and microscopy was carried out on a Zeiss EM-952 electron microscope.

Alkaline Sucrose Gradient Centrifugation. ^{14}C-Labeled SV40 DNA (in 50–100 μL) was layered on 5.0 mL of 5–20% sucrose gradients prepared in 0.7 M NaCl–0.3 M NaOH–2.5 mM EDTA and centrifuged for 90 min at 50000 rpm and 20 °C in a Beckman SW 50.1 rotor. The gradients were fractionated from the bottom by taking 7-drop aliquots into 1.0 mL of 0.12 M HCl. The fractions were counted for 2 min each in 4.0 mL of Aquasol-2 (New England Nuclear). Two peaks corresponding to form I DNA and forms II plus III DNA were present in the radioactivity profiles. The percent form I DNA was determined from the peak areas.

Nitrocellulose Filter Retention Assay. BaP-diol epoxide modified SV40 form I [^{14}C]DNA (0.4–0.8 μg in 100 μL) was diluted with 5.0 mL of 50 mM Tris-HCl (pH 8.2)–1 M NaCl and filtered through a Schleicher & Schuell type B-6 0.45-μm pore size filter at a flow rate of 2.5 mL/min. The filter was washed with 5.0 mL of 0.03 M sodium citrate–0.3 M NaCl, dried, and counted in Permafluor I (Packard). DNA containing approximately one adduct/10^2 base pairs was stably retained on the filter. Total DNA in a sample was determined as basically described by Kuhnlein et al. (1976).

The kinetics of strand scission at apurinic sites was monitored by using the filter retention assay. Apurinic DNA was prepared by incubating SV40 form I [^{14}C]DNA (8.4 μg in 50 μL) with 150 μL of 50 mM sodium citrate–20 mM Tris-HCl (pH 3.5)–0.5 mM EDTA at 50 °C for 12.5 min (Kirtikar et al., 1976). The solution was diluted 10 times with 20 mM Tris-HCl (pH 8.0)–0.5 mM EDTA and kept at 37 °C. To monitor strand scission, aliquots (100 μL) were taken as a function of time, pretreated 7 min at 30 °C with 200 μL of 0.3 M K_2HPO_4·KOH (pH 12.3), neutralized with 100 μL of 1 M KH_2PO_4·HCl (pH 4.0), and filtered as described above. Hydrolysis of apurinic sites during this pretreatment was negligible (Ross & Moses, 1978). The total number of apurinic sites was determined by pretreating an aliquot with 200 μL of 0.3 M K_2HPO_4·KOH (pH 12.3) for 120 min at 30 °C prior to neutralization and filtration. Under these conditions apurinic sites were quantitatively cleaved (Kuhnlein et al., 1976).

Apurinic Endonuclease Reaction. SV40 DNA (0.57 μg in 50 μL) was incubated 10 min at 37 °C with 45 μL of assay solution (44 mM Tris-HCl, pH 7.5–22 mM $MgCl_2$–0.011% Triton X-100) and 5 μL of human apurinic/apyrimidinic endonuclease stock (16000 units/mg of protein; 10 units/mL). The reaction was terminated by cooling to 4 °C and adding 10 μL of 0.2 M EDTA. The enzyme used here had been purified to near homogeneity from HeLa cells (Kane, 1979). One unit of activity is defined as the amount of enzyme necessary to produce 1 pmol of nicks/min at 37 °C with 5 nmol of PM2 DNA nucleotide containing two apurinic sites per molecule.

Results

Frequency of Alkylation and Strand Scission. Superhelical SV40 DNA was allowed to react with a concentration series of BaP-diol epoxide. After 24 h the reaction mixtures were analyzed for strand scission by agarose gel electrophoresis. The resultant electrophoretogram (Figure 1) demonstrates that at low molar reaction ratios BaP-diol epoxide nicked the DNA while at high ratios it broke the DNA generating linear form III as well as smaller fragments. The absence of form III DNA at lower molar reaction ratios implies that the fragmentation seen at high ratios resulted from two adjacent nicks on opposite strands as opposed to double-strand breaks. Furthermore, the heterodisperse tail of low molecular weight fragments extending from the form III band in the last two tracks indicates that, at least at higher reaction ratios, nicking occurred randomly. The tetraol hydrolysis product of BaP-diol epoxide, which does not covalently bind to DNA, had no effect on form I SV40 DNA (data not shown).

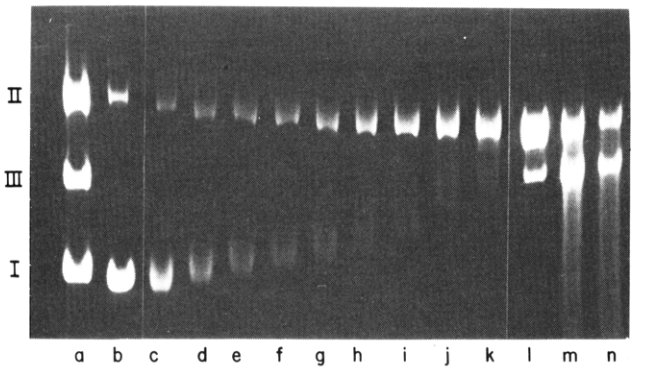

FIGURE 1: Agarose slab gel of superhelical SV40 DNA reacted with BaP-diol epoxide. Form I SV40 DNA was incubated with BaP-diol epoxide at hydrocarbon to DNA mononucleotide ratios of (b) 0, (c) 0.097, (d) 0.130, (e) 0.162, (f) 0.194, (g) 0.227, (h) 0.259, (i) 0.292, (j) 0.324, (k) 0.388, (l) 0.647, (m) 1.30, and (n) 3.24. After 24 h the reaction mixtures were extracted with ethyl acetate and electrophoresed. The direction of electrophoresis was downward. A partial *Eco*RI digest of SV40 DNA was run in track a. Forms I, II, and III denote superhelical, nicked circular, and linear SV40 DNA, respectively.

With increasing modification, the remaining form I DNA band broadened and exhibited a decreased mobility (Figure 1). This is attributed to local unwinding of the DNA helix brought about by covalently bound adducts. In the same reaction series the form II DNA band exhibited an increased mobility. This reflects a reduction in the intrinsic viscosity of the DNA together with an overloading of the form II band. These phenomena are discussed in a separate communication (Gamper et al., 1980).

Analysis of representative reaction mixtures by electron microscopy substantiated the gel data. Electron micrographs of superhelical SV40 DNA reacted with BaP-diol epoxide at molar reaction ratios of (A) 0, (B) 0.65, and (C) 3.2 show superhelical, nicked circular, and fragmented linear DNA forms, respectively (Figure 2).

The significance of strand scission was ascertained by quantifying the frequency of adducts and nicks per genome as a function of the molar reaction ratio. Covalent binding was determined after removal of BaP-tetraol from the DNA by ethyl acetate extraction and ethanol precipitation. The average number of nicks per DNA molecule (μ) was calculated from the equation $U = e^{-\mu}$ (Kuhnlein et al., 1976) following electrophoretic determination of the fraction of form I DNA (U) remaining in the reaction mixture after 24-h incubation. From Figure 3a it can be calculated that only 3% of the epoxide alkylated the DNA and that strand scission occurred to an extent less than 1% of the alkylation events. The proportion of nicking to covalent binding increased with the molar reaction ratio (Figure 3b), indicating a decreased binding efficiency for BaP-diol epoxide.

Over 97% of the labeled hydrocarbon which coprecipitates with DNA in ethanol has been identified as comprising guanine and adenine adducts modified at the N^2 and N^6 positions, respectively (Straub et al., 1977). For SV40 DNA modified by BaP-diol epoxide at a molar reaction ratio of 0.04 in Tris buffer, pH 8.0, 86% of the binding was to guanine and 14% was to adenine (Gamper et al., 1980). These purine adducts

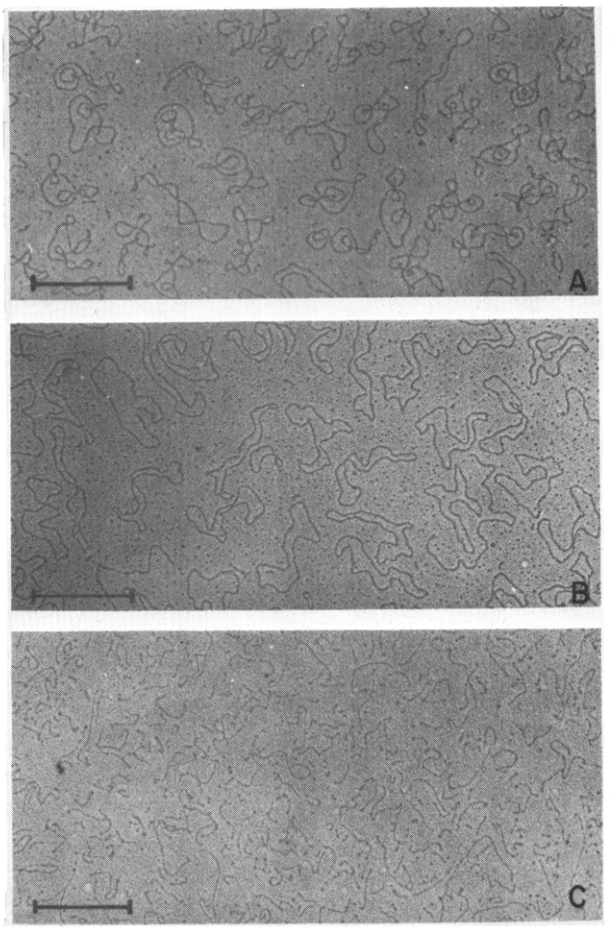

FIGURE 2: Electron micrographs of SV40 DNA reacted with BaP-diol epoxide at hydrocarbon to DNA mononucleotide ratios of (A) 0, (B) 0.647, and (C) 3.24. After 24 h of reaction the DNA samples were extracted with ethyl acetate and analyzed by electron microscopy. The bar equals 0.5 μm.

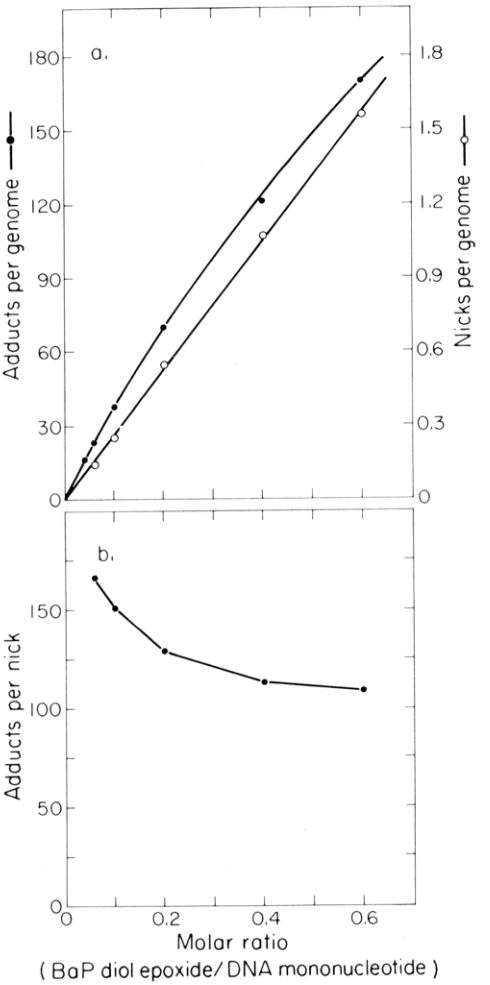

FIGURE 3: Comparison of SV40 DNA alkylation and strand scission by BaP-diol epoxide. SV40 form I [^{14}C]DNA (1.87 μg in 150 μL) was reacted with a concentration series of [^{3}H]BaP-diol epoxide. After 2-h incubation 100-μL aliquots were removed for determination of alkylation by radioactivity counting after ethyl acetate extraction and ethanol precipitation. After an additional 22-h incubation the remaining reaction mixtures were electrophoresed for estimation of strand scission.

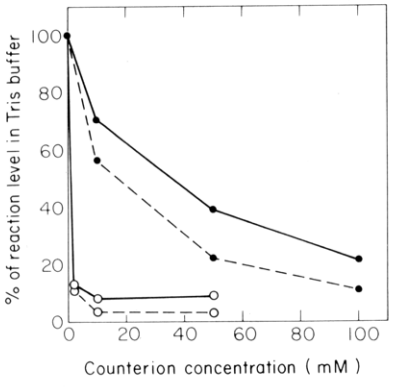

FIGURE 4: Inhibition of BaP-diol epoxide alkylation and strand scission of SV40 DNA by NaCl and $MgCl_2$. SV40 form I [^{14}C]DNA (1.5 μg in 145 μL) was reacted with [^{3}H]BaP-diol epoxide at a molar reaction ratio of 0.60 in the presence of the indicated concentrations of NaCl or $MgCl_2$. After 3 h 100-μL aliquots were removed to estimate covalent binding. The remaining reaction mixtures were incubated an additional 21 h and then electrophoresed to determine strand scission. The DNA control contained 197 adducts/genome and 2.45 nicks/genome. Covalent binding in the presence of (●—●) NaCl or (○—○) $MgCl_2$. Strand scission in the presence of (●---●) NaCl or (○---○) $MgCl_2$.

should not give rise to strand scission under physiological conditions. This implies that DNA nicking is due to one or more minor alkylation sites representing less than 1% of the ethanol precipitable adducts or to labile adducts which are lost in the ethanol supernatant. BaP-diol epoxide covalently bound to DNA phosphate or to the N-7 position of guanine would eventually be released into solution as free BaP-tetraol (Gamper et al., 1977) or as a hydrocarbon–guanine adduct (King et al., 1979).

Both covalent binding and nicking were inhibited by Na^+ and Mg^{2+} counterions (Figure 4). These ions bind electrostatically to DNA phosphate, the latter ion 50-fold more effectively (Dove & Davidson, 1962). The mirroring of this difference in the binding and nicking curves indicates that the interaction of these ions with DNA is responsible for the inhibition. Bound counterions inhibit alkylation of DNA phosphate and nearby sites such as N-7 guanine by electrostatic masking (Kriek & Emmelot, 1963; Rajalakshmi et al., 1978). Modification of these sites has been implicated in BaP-diol epoxide induced DNA strand scission. The counterions also reduce the probability of intercalation by shielding the DNA phosphates and winding the helix. Intercalation of BaP-diol epoxide is believed to precede its reaction with the exocyclic amino group of guanine (Gamper et al., 1980).

Kinetics of Covalent Binding and Nicking. In the standard pH 8.0 reaction buffer (20 mM Tris-HCl, 0.5 mM EDTA, and 5% dimethyl sulfoxide) at 37 °C, BaP-diol epoxide had a half-life of 21 min. The kinetics of hydrolysis to BaP-tetraol was determined indirectly by taking aliquots of an aqueous BaP-diol epoxide solution for reaction with superhelical DNA and plotting (on a semilogarithmic scale) the nicked circular

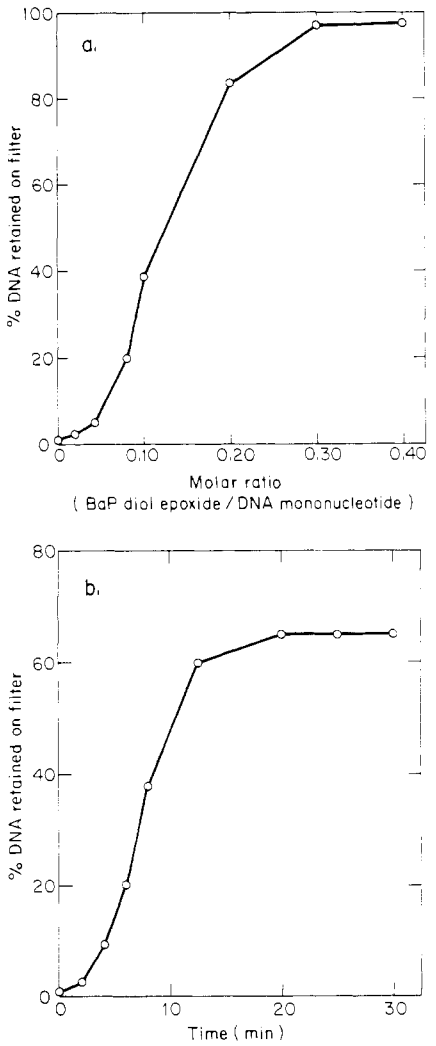

FIGURE 5: (a) Adsorption of BaP-diol epoxide modified SV40 DNA to nitrocellulose filters. SV40 form I [^{14}C]DNA was reacted with BaP-diol epoxide for 1 h and then filtered through nitrocellulose. (b) BaP-diol epoxide alkylation of SV40 DNA monitored by nitrocellulose filtration. SV40 form I [^{14}C]DNA (4.2 μg/mL) was reacted with BaP-diol epoxide at a molar reaction ratio of 0.15. Aliquots (100 μL) were taken as a function of time into 10 μL of 2.5 M 2-mercaptoethanol and filtered through nitrocellulose.

DNA produced as a function of hydrolysis time. The value obtained is comparable to the half-life of 38 min reported by Drinkwater et al. (1978) for BaP-diol epoxide in pH 7.4 buffer (10 mM Tris-HCl, 1 mM EDTA, and 20% dimethyl sulfoxide).

The sulfhydryl reagent 2-mercaptoethanol reacts instantaneously with BaP-diol epoxide and is quite effective in stopping its reaction with DNA. The covalent binding of BaP-diol epoxide to SV40 DNA has been followed by quenching reaction mixture aliquots with this reagent and analyzing the modified DNA by nitrocellulose filtration. Sufficiently modified form I SV40 DNA binds to nitrocellulose filters. The binding is probably mediated by externally bound hydrocarbon adducts or by localized single-stranded regions induced by alkylation (Gamper et al., 1980). At a molar reaction ratio of 0.15, ~60% of the DNA was retained by nitrocellulose (Figure 5a). When the reaction time course was monitored at this molar ratio (Figure 5b), it was apparent that covalent binding of the hydrocarbon to DNA was complete within 20 min. The initial lag in filter binding is not reflective of reaction kinetics and presumably arises from the requirement for multiple alkylation sites on the DNA if stable filter binding

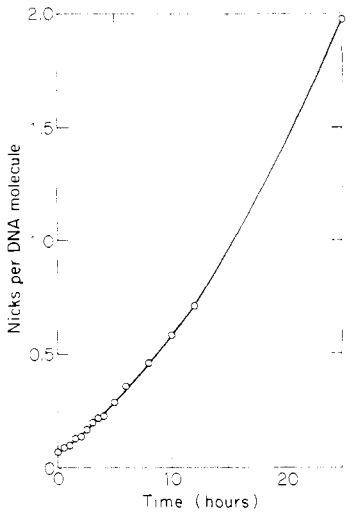

FIGURE 6: Kinetics of BaP-diol epoxide induced DNA strand scission. SV40 form I [^{14}C]DNA (8.4 μg/mL) was reacted with BaP-diol epoxide at a molar reaction ratio of 0.60. For quantitation of strand scission, aliquots of 30 μL were taken as a function of time into 10 μL of 2.5 M 2-mercaptoethanol and electrophoresed on 1.4% agarose disc gels. Brief exposure of the alkylated DNA to 2-mercaptoethanol did not introduce nicks.

is to occur. The rapidity of DNA modification, which has also been reported by others (Shooter et al., 1977; Kootstra et al., 1979), suggests that hydrolysis of BaP diol epoxide is in some way catalyzed by DNA.

The kinetics of strand scission was determined by disc gel electrophoresis of alkylated SV40 DNA at 30-min intervals. Figure 6 shows the time course of nicking for SV40 DNA modified at a molar reaction ratio of 0.60. In contrast to the rapid covalent modification of DNA, BaP-diol epoxide induced strand scission proceeded slowly with time throughout the 25-h experiment. This is consistent with nicking occurring through rearrangement of one or more BaP-diol epoxide adducts. Furthermore, the increase in slope of the curve in Figure 6 with time is indicative of a multistep rearrangement which terminates in nicking such as depurination/depyrimidination strand scission.

The stability of apurinic sites in SV40 DNA was investigated by the method of Kuhnlein et al. (1976) as described under Materials and Methods. At 37 °C conversion of these sites to nicks was unaffected by the presence of BaP-tetraol and occurred with a half-life of 16 h in the standard pH 8.0 reaction buffer (20 mM Tris-HCl, 0.5 mM EDTA, and 5% dimethyl sulfoxide). This value is significantly less than the 190-h half-life obtained by Lindahl & Andersson (1972) for apurinic sites in Hepes buffer and is attributed to the previously reported catalytic effect of Tris on β-elimination (Tamm et al., 1953).

Effect of Alkali on Strand Scission. The extent of strand scission in BaP-diol epoxide modified SV40 DNA was determined by gel electrophoresis or alkaline sucrose density gradient centrifugation of several reaction series (Figure 7). Electrophoretic analysis of alkylated SV40 DNA held at 37 °C for 24 or 48 h indicated that the rate of strand scission was dependent upon the level of alkylation. At molar reaction ratios below 0.20, DNA nicking appeared to terminate within 24 h. At higher molar reaction ratios, nicking continued past this time, perhaps reflecting the slow rearrangement of an adduct which was only present in highly modified DNA.

Alkaline sucrose density gradient analysis of representative SV40 DNA samples incubated 1 h with varying amounts of BaP-diol epoxide resulted in a level of strand scission identical

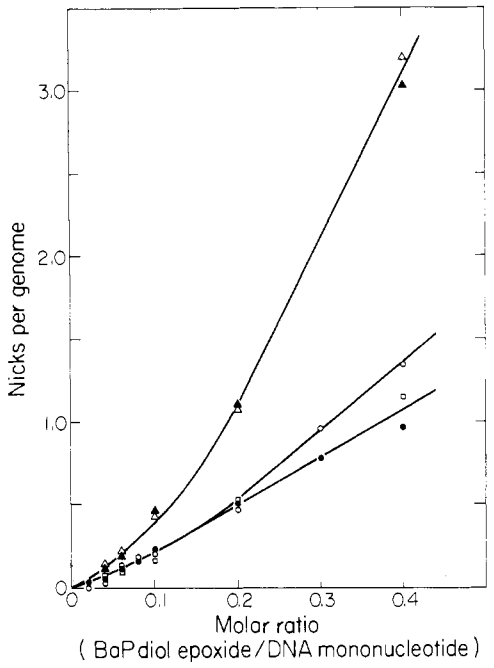

FIGURE 7: Strand scission of BaP-diol epoxide modified SV40 DNA determined by agarose gel electrophoresis and alkaline sucrose gradient centrifugation. SV40 form I [^{14}C]DNA was reacted with BaP-diol epoxide at the indicated molar reaction ratios. Strand scission was quantified by agarose gel electrophoresis or alkaline sucrose gradient centrifugation. (●) Electrophoretic analysis after 24-h incubation; (○) electrophoretic analysis after 48-h incubation; (□) alkaline gradient analysis after 1-h incubation; (▲) alkaline gradient analysis after 24-h incubation; (△) alkaline gradient analysis after 24-h incubation, followed by 4-h incubation at room temperature with an equal volume of 2 M glycine–NaOH (pH 13.1).

with that observed after 24-h incubation at neutrality (Figure 7). Thus, BaP-diol epoxide-induced nicking is catalyzed by brief exposure to high pH. Alkaline catalysis has been demonstrated for both deoxyribophosphotriester hydrolysis and β-elimination at apurinic sites (Shooter & Merrifield, 1976). BaP-tetraol-treated DNA controls retained their superhelicity in the alkaline sucrose gradients.

A significant increase in the frequency of alkali-sensitive sites was observed for BaP-diol epoxide modified SV40 DNA incubated 24 h prior to centrifugation (Figure 7). At higher molar reaction ratios, these sites actually outnumbered DNA nicks. The sites were rapidly cleaved at high pH, and no additional sites were exposed by a 4-h pretreatment with pH 13.1 buffer. Although extremely labile in alkali, at neutral pH these sites were relatively stable. Thus, despite the appreciable number of such sites in alkylated DNA after 24-h incubation, only minor strand scission at high molar reaction ratios was observed in the subsequent 24-h period. The time-dependent accumulation of these sites indicated that most if not all were secondary rearrangement products. The stability of the DNA backbone at these sites in neutral buffer suggests that they are not responsible for the nicking of DNA observed at physiological pH. As such, they are unlikely to be phosphotriesters or apurinic/apyrimidinic sites. As will be shown, the DNA backbone at these sites appears to be resistant to apurinic endonuclease.

Susceptibility of BaP-diol Epoxide Modified DNA to Apurinic Endonuclease. The existence of apurinic/apyrimidinic sites in alkylated SV40 DNA was probed with HeLa apurinic/apyrimidinic endonuclease, an enzyme which nicks the helix adjacent to free sugars. Viral DNA, modified with a concentration series of BaP-diol epoxide, briefly was incubated with a saturating amount of the endonuclease 2 and 24 h after addition of the hydrocarbon and then analyzed for nicking by gel electrophoresis. For comparison, parallel DNA samples were assayed at the same time for spontaneous and alkali-catalyzed strand scission. The results are presented in Figure 8.

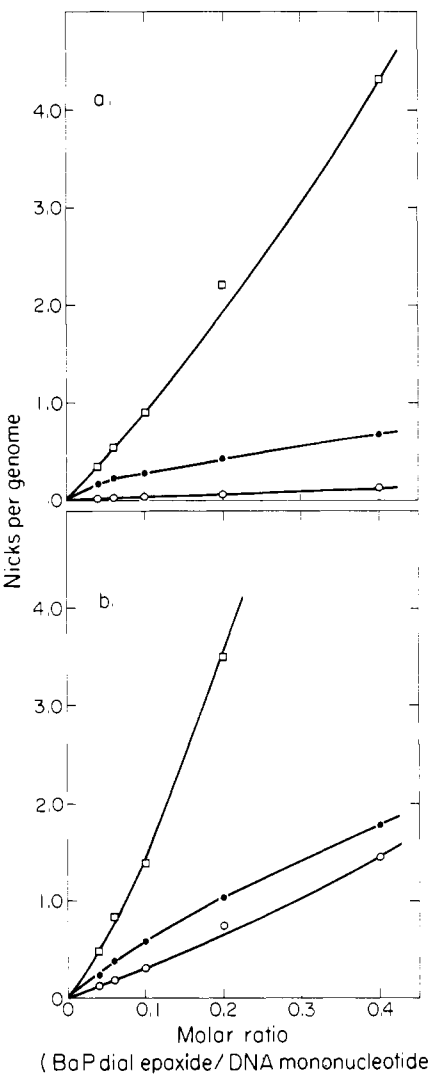

FIGURE 8: Comparison of strand scission elicited by apurinic endonuclease and alkali treatment of BaP-diol epoxide modified SV40 DNA. SV40 form I [^{14}C]DNA (12.5 μg/mL) was reacted with BaP-diol epoxide at the indicated molar reaction ratios. After (a) 2- and (b) 24-h incubation, three sets of 50-μL aliquots were withdrawn. One set was layered onto 5.0-mL alkaline sucrose gradients and centrifuged. The other two sets were electrophoresed after incubation for 10 min with either 0 or 0.05 EU of apurinic endonuclease. (●) Gel electrophoresis after apurinic endonuclease treatment; (○) gel electrophoresis after mock apurinic endonuclease treatment; (□) alkaline sucrose gradient centrifugation.

The plots demonstrate that BaP-diol epoxide modified DNA is a substrate for apurinic endonuclease. The apurinic/apyrimidinic sites appeared rapidly and were readily detectable after 2-h incubation. The level of these sites after 2-h incubation is consistent with spontaneous nicking proceeding through a depurination/depyrimidination strand scission mechanism, especially at lower molar reaction ratios.

At both reaction times, treatment of modified SV40 DNA with alkali generated more strand scission than did reaction with apurinic endonuclease. Therefore, apurinic/apyrimidinic sites represent only a fraction of the total alkali-sensitive sites present in modified DNA. The identity of the remaining sites, which account for up to 4% of the modification, is under investigation.

Table I: Percent Monobutyl Phosphate Present in Dibutyl Phosphate

sample	% ± standard deviation[a] after 24 h at 37 °C	after additional 15 min at 85 °C
control	0.56 ± 0.09	
BaP-tetraol treated	0.56 ± 0.04	0.51 ± 0.05
BaP-diol epoxide treated	0.53 ± 0.02	0.59 ± 0.01

[a] Each percent is an average from at least four determinations.

Reaction of BaP-diol Epoxide with Dibutyl Phosphate. The difficulty of isolating the putative hydrocarbon phosphate adduct intact from a DNA reaction mixture prompted us to investigate the binding of BaP-diol epoxide to the model phosphodiester dibutyl [^{32}P]phosphate. Reaction was allowed to proceed in 50% aqueous acetone, pH 7.5. After 24 h the products were analyzed by Sephadex LH-20 chromatography. Less than 0.1% of the label was retained by the column and it eluted as a shoulder on a BaP-tetraol peak. The low recovery and the contamination with BaP-tetraol precluded characterization of the hydrocarbon phosphate adduct as either a phosphotriester or a phosphodiester. Although a phosphodiester would be expected from rearrangement of the triester, it could also arise from reaction of the diol epoxide with contaminating monobutyl [^{32}P]phosphate. Given this ambiguity, the small amount of adduct found does not conclusively demonstrate triester formation.

In an attempt to indirectly detect phosphotriester formation and subsequent rearrangement to the diester, the monobutyl phosphate content of BaP-diol epoxide and BaP-tetraol reaction mixtures with dibutyl phosphate was determined by thin-layer chromatography either immediately after reaction or following heating at 85 °C for 15 min. If BaP-diol epoxide formed phosphotriesters, they would be expected to rearrange to phosphodiesters with release of *n*-butyl alcohol (Gamper et al., 1977). Upon exposure to brief heating or to the high pH of the chromatography solvent, the diesters should rapidly convert through an S_N1 mechanism to monobutyl phosphate and BaP-tetraol (Koreeda et al., 1976). Thus, an increase in monobutyl phosphate content would be indicative of phosphotriester formation with subsequent hydrolysis through a cyclic intermediate. No significant increase in monoester content was found even after heat treatment (Table I). A maximal increase of 0.12% would have been expected from the frequency of DNA strand scission, assuming it was mediated by phosphotriester hydrolysis.

Reaction of BaP-diol Epoxide with DNA Homopolymers. A second approach undertaken to detect phosphotriester-induced nicking involved reacting each of the four homopolydeoxyribonucleotides with BaP-diol epoxide and analyzing for degradation. If nicking occurred through a triester mechanism, then each of the polymers should have been degraded. The experiment with poly(dT) could be particularly revealing since the diol epoxide does not react with thymine (Meehan et al., 1977). After reaction and ethyl acetate extraction, the polymers were electrophoresed and the tracings in Figure 9 were obtained. At a molar reaction ratio of 4.0, poly(dA), poly(dG), and poly(dC) were all degraded to varying extents. Poly(dT), however, remained intact whether it was reacted as a random-coil single strand or as part of an ordered duplex with poly(dA). The selectivity of strand scission is consistent with nicking originating from a base modification as opposed to a phosphate or deoxyribose modification.

Reaction of BaP-diol Epoxide with TMV RNA. Strand scission of alkylated RNA is considered diagnostic for phosphotriester hydrolysis (Shooter, 1975), and we previously reported that MS2 RNA was so degraded by BaP-diol epoxide (Gamper et al., 1977). However, a more careful study with TMV RNA failed to demonstrate nicking even after several hours incubation at high molar reaction ratios. The modified RNA, which from spectral analysis contained ~300 adducts per genome at a molar reaction ratio of 1.0 (Pulkrabek et al.,

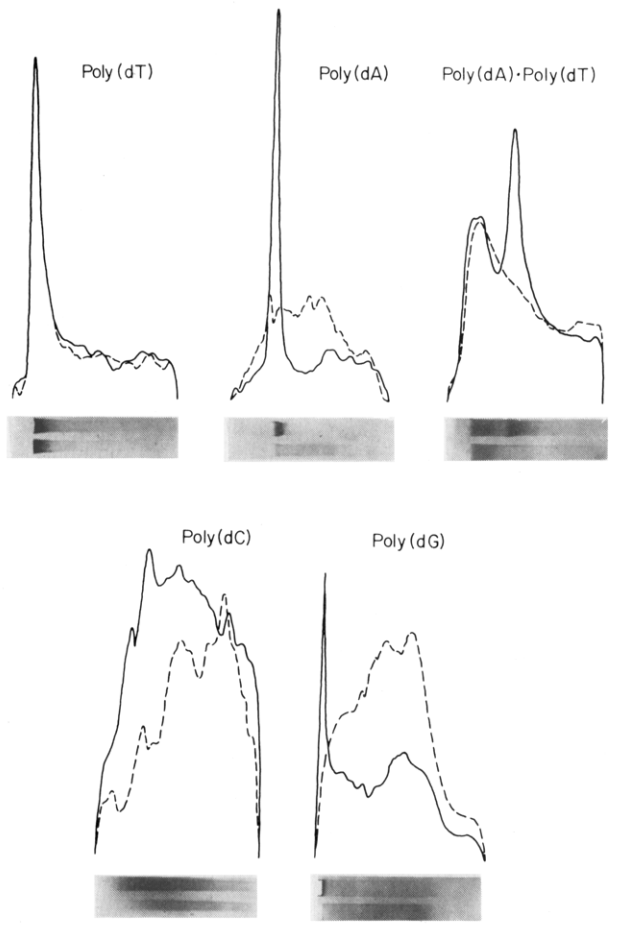

FIGURE 9: Electrophoretic pattern of DNA homopolymers after prior reaction with BaP-diol epoxide or BaP-tetraol. The DNA homopolymers were reacted with BaP-diol epoxide or BaP-tetraol at a molar reaction ratio of 4.0. After 24 h the reaction mixtures were extracted with ethyl acetate and electrophoresed. Poly(dA)·poly(dT) and poly(dG) were electrophoresed in formamide to minimize secondary structure. The upper track (—) contained homopolymer reacted with BaP-tetraol, and the lower track (---) contained the same homopolymer reacted with BaP-diol epoxide. Electrophoresis was from left to right.

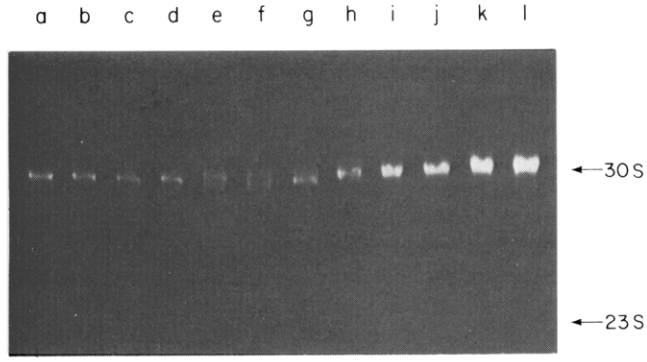

FIGURE 10: Polyacrylamide–agarose slab gel of TMV RNA reacted with BaP-diol epoxide. TMV RNA was reacted with BaP-diol epoxide at hydrocarbon to RNA mononucleotide ratios of (a) 0, (b) 0.05, (c) 0.10, (d) 0.20, (e) 0.30, (f) 0.40, (g) 0.60, (h) 0.80, (i) 1.0, (j) 1.5, (k) 2.0, and (l) 3.0. After 6 h the reaction mixtures were extracted with ethyl acetate and electrophoresed downward. The gel was strained with acridine orange.

1977), was analyzed electrophoretically in Tris–borate buffer (Figure 10) and in formamide (H. Gamper and B. Singer, unpublished results). No fragmentation was detectable by either technique. A similar conclusion regarding the integrity of BaP-diol epoxide modified RNA has been reached by Shooter et al. (1977) using bacteriophage R17 RNA.

Although unable to fragment TMV RNA, Figure 10 shows that BaP-diol epoxide induced a distinct conformational change in the RNA at molar reaction ratios of 0.20–0.40. The abrupt increase in mobility was linked to a change in fluorescence from green to red. Acridine orange intercalates into double-stranded nucleic acid and stacks onto single-stranded nucleic acid to give green or red fluorescence, respectively (McMaster & Carmichael, 1977). The green fluorescence of native TMV RNA indicates the presence of considerable secondary structure in the molecule despite the low ionic strength of the electrophoresis buffer, a phenomenon studied in detail by Boedtker (1960). The appearance of red fluorescence upon alkylation suggests that the abrupt mobility change is due to hydrocarbon-induced loss of secondary structure.

Discussion

BaP-diol epoxide could elicit DNA strand scission through four potential pathways: photochemical attack, free radical reaction, phosphotriester hydrolysis, or depurination/depyrimidination strand scission. The first two mechanisms have not been seriously considered since diol epoxide reacts as a highly stabilized benzylic carbonium ion (Whalen et al., 1979). Furthermore, the modification of SV40 DNA reported here was carried out under subdued lighting and neither covalent binding or nicking were affected by 0.5 M isopropyl alcohol, a free radical scavenger.

Attempts to indirectly demonstrate strand scission arising from phosphotriester hydrolysis were unsuccessful. Thus, the inability of BaP-diol epoxide to modify the model substrate dibutyl phosphate or to degrade poly(dT) and TMV RNA strongly argue against this mechanism. If phosphotriesters are formed in DNA and RNA, they rearrange to diesters with exclusive loss of the hydrocarbon. In DNA the loss is mediated by an S_N1 mechanism, while in RNA it could proceed through a cyclic triester intermediate as well. It is possible that they are extremely short-lived, in which case a mechanism is provided for the catalytic hydrolysis of BaP-diol epoxide in the presence of nucleic acid. If this is so, the hydrocarbon phosphate adducts detected by Koreeda et al. (1976) could represent terminal phosphodiesters.

The recognition of BaP-diol epoxide modified DNA by apurinic endonuclease conclusively demonstrates depurination/depyrimidination. Considering the rapid appearance of apurinic sites and their 16-h half-life (for rearrangement of the sugar with strand scission), it is highly likely that nicking proceeds through depurination/depyrimidination strand scission. Attempts to isolate and characterize the liberated hydrocarbon–base adducts have been unsuccessful. At low reaction levels the quantity of these adducts formed may be too low to permit detection. Utilizing highly modified DNA, Osborne et al. (1978) have obtained evidence for the formation of a labile N-7 guanine derivative. This adduct was spontaneously liberated from alkylated DNA with a half-life of 3 h (King et al., 1979).

The detection of a second class of alkali-sensitive sites resistant to apurinic endonuclease was unexpected. A recent report (Mizusawa & Kakefuda, 1979) that heat–alkali treatment of BaP-diol epoxide alkylation sites leads to strand scission suggests that the major N^2 guanine adduct may slowly rearrange to an alkali-sensitive product under physiological conditions. Such a product could account for the alkali-catalyzed nicking we have observed, and this is currently being investigated.

Acknowledgments

We especially thank Agatha S.-C. Tung for suggesting alkaline sucrose gradient centrifugation as a technique to differentiate at high pH between BaP-diol epoxide induced nicking and helix destabilization of form I DNA and Caroline Kane for treating the modified viral DNA with her apurinic endonuclease. We also express appreciation to Ken Straub for providing BaP-diol epoxide and for assistance in the dibutyl phosphate experiments, to Hisao Yokota for help in preparing the SV40 DNA, to Bea Singer for donating TMV RNA, and to Steven G. Platt for critical reading of the manuscript.

References

Boedtker, H. (1960) *J. Mol. Biol. 2*, 171.

Borgen, A., Darvey, H., Castagnoli, N., Crocker, T. T., Rasmussen, R. E., & Wang, I. Y. (1973) *J. Med. Chem. 16*, 502.

Daudel, P., Duquesne, M., Vigny, P., Grover, P. L., & Sims, P. (1975) *FEBS Lett. 57*, 250.

Davis, R. W., Simon, M., & Davidson, N. (1971) *Methods Enzymol. 21*, 413.

Dove, W., & Davidson, N. (1962) *J. Mol. Biol. 5*, 467.

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

Gamper, H. B., Tung, A. S.-C., Straub, K., Bartholomew, J. C., & Calvin, M. (1977) *Science 197*, 671.

Gamper, H. B., Straub, K., Calvin, M., & Bartholomew, J. C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2000.

Hallick, L. M., Yokota, H. A., Bartholomew, J. C., & Hearst, J. E. (1978) *J. Virol. 27*, 127.

Huberman, E., Sachs, L., Yang, S. K., & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 607.

Ivanovic, V., Geacintov, N. E., & Weinstein, I. B. (1976) *Biochem. Biophys. Res. Commun. 70*, 1172.

Ivanovic, V., Geacintov, N. E., Yamasaki, H., & Weinstein, I. B. (1978) *Biochemistry 17*, 1597.

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

Jeffrey, A. M., Grzeskowiak, K., Weinstein, I. B., Nakanishi, K., Roller, P., & Harvey, R. G. (1979) *Science 206*, 1309.

Jennette, K. W., Jeffrey, A. M., Blobstein, S. H., Beland, F. A., Harvey, R. G., & Weinstein, I. B. (1977) *Biochemistry 16*, 932.

Kane, C. (1979) Ph.D. Thesis, University of California at Berkeley.

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

King, H. W. S., Osborne, M. R., & Brookes, P. (1979) *Chem.-Biol. Interact. 24*, 345.

Kirtikar, K. M., Cathcart, G. R, & Goldthwait, D. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4324.

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

Koreeda, M., Moore, P. D., Yagi, H., Yeh, H. J. C., & Jerina, D. M. (1976) *J. Am. Chem. Soc. 98*, 6720.

Koreeda, M., Moore, P. D., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., & Jerina, D. M. (1978) *Science 199*, 778.

Kriek, E., & Emmelot, P. (1963) *Biochemistry 2*, 733.

Kuhnlein, U., Penhoet, E. E., & Linn, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1169.
Landolph, J. R., Becker, J. F., Gamper, H., Bartholomew, J. C., & Calvin, M. (1978) *Chem.-Biol. Interact. 23*, 331.
Levin, W., Wood, A. W., Wislocki, P. G., Kapitulnik, J., Yagi, M., Jerina, D., & Conney, A. H. (1977) *Cancer Res. 37*, 3356.
Lindahl, T., & Andersson, A. (1972) *Biochemistry 11*, 3618.
Mager, R., Huberman, E., Yang, S. K., Gelboin, H. V., & Sachs, L. (1977) *Int. J. Cancer 19*, 814.
Malaveille, C., Bartsch, H., Grover, P. L., & Sims, P. (1975) *Biochem. Biophys. Res. Commun. 66*, 693.
McCaustland, D. J., & Engel, J. F. (1975) *Tetrahedron Lett. 30*, 2549.
McCaustland, D. J., Fischer, D. L., Duncan, W. P., Ogilvie, E. J., & Engel, J. F. (1976) *J. Labelled Compd. Radiopharm. 12*, 583.
McMaster, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835.
Meehan, T., Straub, K., & Calvin, M. (1977) *Nature (London) 269*, 725.
Mizusawa, H., & Kakefuda, T. (1979) *Nature (London) 279*, 75.
Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Int. J. Cancer 18*, 362.
Osborne, M. R., Harvey, R. G., & Brookes, P. (1978) *Chem.-Biol. Interact. 20*, 123.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668.
Peacocke, A. R., & Walker, I. O. (1962) *J. Mol. Biol. 5*, 550.
Pulkrabek, P., Leffler, S., Weinstein, I. B., & Grunberger, D. (1977) *Biochemistry 16*, 3127.
Rajalakshmi, S., Rao, P. M., & Sarma, D. S. R. (1978) *Biochem. Biophys. Res. Commun. 81*, 936.
Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, S., Pan, J., Ghosh, P. K., Celma, M. L., & Weissman, S. M. (1978) *Science 200*, 494.
Ross, S. L., & Moses, R. E. (1978) *Biochemistry 17*, 581.
Shooter, K. V. (1975) *Chem.-Biol. Interact. 11*, 575.
Shooter, K. V., & Merrifield, R. K. (1976) *Chem.-Biol. Interact. 13*, 223.
Shooter, K. V., Osborne, M. R., & Harvey, R. G. (1977) *Chem.-Biol. Interact. 19*, 215.
Sims, P., Grover, P. L., Swaisland, A., Pal, K., & Hewer, A. (1974) *Nature (London) 252*, 326.
Staynov, D. Z., Pinder, J. C., & Gratzer, W. B. (1972) *Nature (London), New Biol. 235*, 108.
Straub, K. M., Meehan, T., Burlingame, A. L., & Calvin, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5285.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237.
Tamm, C., Shapiro, H. S., Lipshitz, R., & Chargaff, E. (1953) *J. Biol. Chem. 203*, 673.
Tooze, J., Ed. (1973) *The Molecular Biology of Tumour Viruses*, pp 269–419, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Vahakangas, K., Nebert, D. W., & Pelkonen, O. (1979) *Chem.-Biol. Interact. 24*, 167.
Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Nakanishi, K. (1976) *Science 193*, 592.
Whalen, D. L., Ross, A. M., Montemarano, J. A., Thakker, D. R., Yagi, H., & Jerina, D. M. (1979) *J. Am. Chem. Soc. 101*, 5086.
Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Jerina, D. M., & Conney, A. H. (1976a) *Biochem. Biophys. Res. Commun. 68*, 1006.
Wislocki, P. G., Chang, R. L., Levin, W., Yagi, H., Jerina, D. M., & Conney, A. H. (1976b) *Proc. Am. Assoc. Cancer Res. 17*, 136.
Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Thakker, D. R., Jerina, D. M., & Conney, A. H. (1977) *Biochem. Biophys. Res. Commun. 77*, 1389.
Zweig, G., & Sherma, J., Eds. (1972) *CRC Handbook of Chromatography*, p 162, CRC Press, Cleveland, OH.