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Genetics

Modification of DNA by the benzo[*a*]pyrene metabolite diol-epoxide *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

(ultimate carcinogen/binding and modification of DNA/stepwise digestion with endonuclease S1 and DNase/DNA base analysis)

TSUYOSHI KAKEFUDA AND HIRO-AKI YAMAMOTO

Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **The structural modification of double-stranded circular DNA of simian virus 40 and plasmid ColE1 by *in vitro* binding of *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene was studied. Stepwise hydrolysis with endonuclease S1 and DNase followed by DNA base analysis by thin-layer chromatography provided evidence that binding to adenine caused the local denaturation of DNA, whereas the more than 10-fold greater binding to guanine did not create such local denaturation. Of the two synthetic double-stranded polymers, poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC), bound to the diol-epoxide, only the former showed a marked hydrolysis after endonuclease S1 treatment, whereas binding occurred 24-fold more on the latter.**

Recent studies indicate that *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (diol-epoxide I) is a major metabolic derivative of the benzo[*a*]pyrene metabolite *trans*-7,8-diol-benzo[*a*]pyrene and that it binds covalently to DNA *in vitro* and *in vivo* (1–9). The level of binding of this compound to DNA *in vitro* as well as to mouse skin cellular DNA was found to be far higher than that of any other known metabolite. Very high mutagenic activity was observed in prokaryotic and eukaryotic test systems in culture (4, 10). Topical application of either diol-epoxide I or its precursor *trans*-7,8 diol-benzo[*a*]pyrene produced skin tumors in mice,* which indicates that diol-epoxide I may be an ultimate form of mutacarcinogen derived from benzo[*a*]pyrene.

Reaction between the 2-amino group of guanine and the 10 position of the diol-epoxide I is generally accepted as the major form of covalent attachment to DNA (5, 8). However, there is as yet no evidence showing that this major type of binding is in fact the binding most relevast to carcinogenesis and mutagenesis.

In the present study we describe the stepwise hydrolysis of diol-epoxide I-treated DNAs of plasmid ColE1, simian virus 40 (SV40), and calf thymus with S1 followed by treatment with deoxyribonuclease (DNase). This procedure allowed us to analyze the specific binding to different DNA bases that caused different forms of modification in the DNA double helix.

MATERIALS AND METHODS

Materials. ^{14}C-Labeled (53.9 mCi/mmol) and unlabeled diol-epoxide I were obtained through Ronald Harvey of the University of Chicago under National Cancer Institute Contract NOI-CP33387. Tritium-labeled and unlabeled ColE1 and SV40 DNA were prepared as described in previous papers (11, 12). Part of the ^{3}H-labeled SV40 DNA was purchased from Bethesda Research Laboratories. Calf thymus DNA and endonuclease S1 (*Aspergillus orysae*) were obtained from Miles Biochemicals. Unlabeled deoxyribonucleotides, alkaline phosphatase, and venom phosphodiesterase I were obtained from Sigma. DNase I (bovine pancreas) was from Worthington Biochemicals.

Reaction of Diol-Epoxide I with DNA. One hundred microliters of reaction mixture contained 5 μg of [^{14}C]diol-epoxide I in 36 μl of methanol, sodium cacodylate buffer, and 50 μg of ColE1 [^{3}H]DNA (1600 cpm/μg). For large-scale experiments, every ingredient was increased by a factor of 20–50. Unlabeled calf thymus DNA was also used for DNA base analysis in large-scale experiments. Incubation was at 37° for 5 hr in the dark, 10 ml of cold ethanol was added to the reaction mixture, and the precipitated DNA was washed twice with benzene, ethanol, and ether until no ^{14}C counts were released in the washing solution.

Incubation of Diol-Epoxide-Bound DNA with S1 Enzyme. The washed, diol-epoxide I-treated DNA (1 mg) was dissolved in 10 ml of sodium acetate buffer (pH 4.5) containing 0.5 M NaCl, 1 mM $ZnSO_4$, and 5% glycerol (S1 buffer). Five micrograms of S1 enzyme protein (69×10^3 units/mg of protein) was added and the reaction mixture was incubated for 30 min at 37°. The enzyme activity and specificity to single-stranded polynucleotide chains were tested according to Vogt (13). Incubation was stopped by the addition of 10 ml of cold ethanol, and the mixture was then centrifuged at 15,000 rpm (27,000 *g*) for 30 min. The supernatant was air dried, dissolved in 2 ml of 10 mM sodium acetate buffer (pH 9.0) containing 0.1 unit of venom phosphodiesterase, and incubated for 24 hr. Then 2.5 units of alkaline phosphatase were added and incubation was continued for 24 hr. The sample was stored at −20°.

Preparation of DNase Hydrolysate. DNA precipitated by ethanol after S1 incubation (described above) is termed "residual DNA" in this paper. The residual DNA and DNA reacted with diol-epoxide I but not incubated with S1 enzyme were hydrolyzed extensively with DNase, phosphodiesterase, and alkaline phosphatase as described (14, 15).

Preparation of Deoxyribonucleoside-Diol-Epoxide I Complexes. Deoxyguanosine and deoxyadenosine (0.5 μmol) were incubated with equal moles of unlabeled or [^{14}C]diol-epoxide I in 2 ml of sodium cacodylate buffer (pH 8) with 20% methanol for 5 hr at 37°. The chemical reactivity of diol-epoxide I to deoxycytosine and thymidine was markedly less than with the other nucleosides. Portions of the compound that did not bind to deoxyribonucleosides and that were converted to 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (7, 9) after incubation were extracted twice with equal volumes

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Abbreviations: diol-epoxide I, *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; SV40, simian virus 40.

* Yang, S. K., Geloin, H. V. & Kakefuda, T. (1977) *Abst. Proc. Am. Assoc. Cancer Res.*

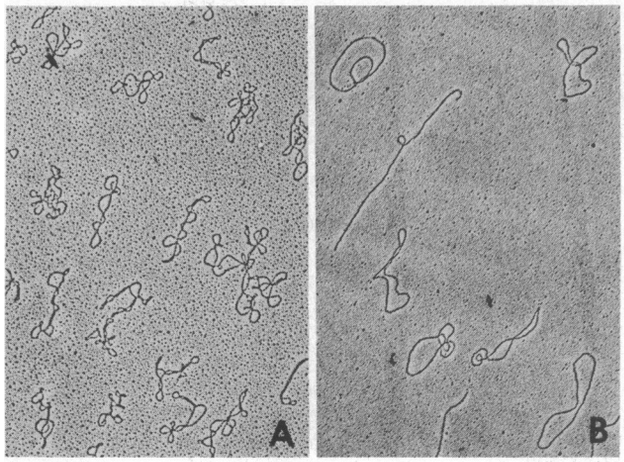

FIG. 1. (*A*) Intact ColE1 DNA with tightly twisted configuration. (*B*) Diol-epoxide I-bound DNA. DNA (0.32 μg) was incubated for 30 min at 37° with 5.36×10^{-12} mol of diol-epoxide I. A few μg of DNA were spread with 50% formamide and 0.04% cytochrome *c* on 17% formamide solution, picked up on a grid with supporting film, stained with uranyl acetate, and shadowed for electron microscopy. Diol-epoxide I-treated DNA shows a marked decrease in superhelical turns and is "partially relaxed" (text).

of ethylacetate. These were then chromatographed on a Sephadex LH-20 column as described below.

Incubation of Synthetic Polydeoxyribonucleotides with Diol-Epoxide I. Two milliliters of reaction mixture containing 100 μg each of double-stranded poly(dA-dT)·poly(dA-dT) or poly(dG-dC)·poly(dG-dC), 10 μg of [^{14}C]diol-epoxide I (specific activity 53.9 mCi/mmol), and 0.3 ml of methanol in 5 mM Tris·HCl (pH 8) were incubated for 5 hr at 37°. The polymers precipitated in ethanol were washed extensively with benzene and ether and dissolved in 2 ml of S1 buffer. Subsequent digestions with S1, phosphodiesterase, and alkaline phosphatase were similar to the digestion of DNA.

Chromatography on Sephadex LH-20 Column. The S1 hydrolysates of DNA and synthetic polydeoxyribonucleotides reacted with diol-epoxide I, DNase hydrolysates of residual DNA and total DNA that was not preincubated with S1, and the deoxyribonucleotide-diol-epoxide I adducts described above were chromatographed on a Sephadex LH-20 column (1.5 × 90 cm) with 500 ml each of 30% and 100% methanol gradient as described (14, 15). ^{14}C radioactivity was determined in 1-ml fractions with 10 ml of Hydromix.

Thin-Layer Chromatography. Pooled peak samples and diol-epoxide I alone from Sephadex LH-20 chromatography (570 ml elution volume) were analyzed for the identification of deoxyribonucleotides bound to the hydrocarbons with a Silica Gel GF Uniplate (250 μm thick, Analtech, Inc.) and acetone/28% NH_4OH (100:3) as solvent.

Electron Microscopy of DNA Molecules. ColE1 DNA and SV40 DNA treated with diol-epoxide I and the samples that had been incubated with S1 enzyme were examined by electron microscopy by the methods described previously (11).

RESULTS

Binding of Diol-Epoxide I with ColE1 DNA. Under the experimental conditions used, a portion of reaction mixture containing 0.32 μg of ColE1 [^{3}H]DNA (8.1×10^{-14} mol) bound to 5.36×10^{-12} mol of [^{14}C]diol-epoxide I. Since ColE1 DNA consists of 6000 base pairs, it was calculated that 67 diol-epoxide I molecules were bound to each DNA molecule. This was far greater than the number bound by any of the other benzo[*a*]-

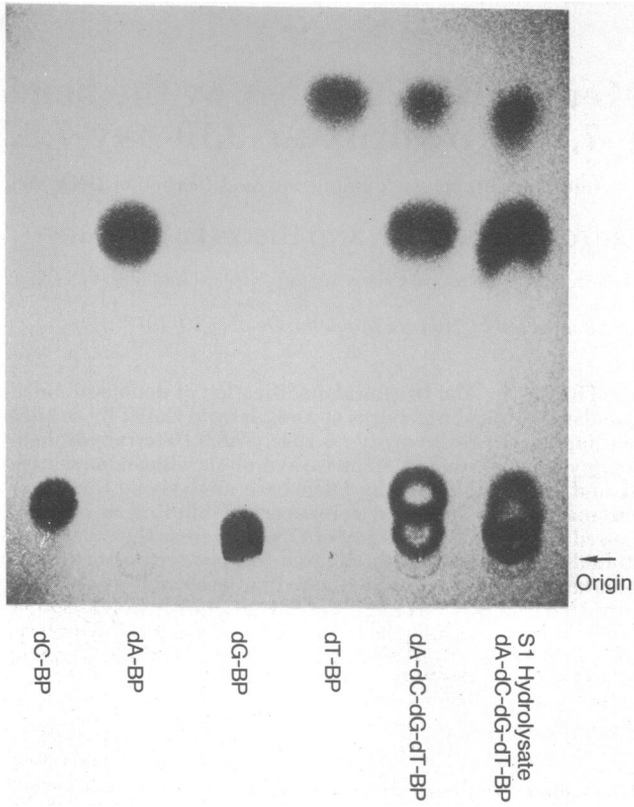

FIG. 2. The S1 hydrolysate of calf thymus DNA reacted with [^{14}C]diol-epoxide I and deoxyribonucleotides reacted with unlabeled diol-epoxide I were chromatographed on a Sephadex LH-20 column. Peak samples from the 570-ml elution volume were analyzed by thin-layer chromatography. Individual reference samples are as marked. A mixture of the four reference samples with and without addition of the DNA S1 hydrolysate are on the right. An experiment with ColE1 DNA provided the same result (not shown). Spots were identified by UV lamp and photographed. (BP, diol-epoxide I adduct).

pyrene metabolites we tested (K-region epoxide benzo[*a*]pyrene and *trans*-7,8-diol-benzo[*a*]pyrene with microsomes).

Electron Microscopic Appearance of Diol-Epoxide I-Bound ColE1 DNA. ColE1 DNA covalently bound to about 70 hydrocarbon molecules showed a marked decrease of superhelical turns, which resulted in the molecules as a whole becoming more relaxed (Fig. 1). We call this type of change "partial relaxation." Occasional linear molecules and completely relaxed open circles of unit length were also observed under the experimental conditions we used. Single- and double-stranded nicks on DNA occurred more frequently when the ratio of diol-epoxide I to DNA mononucleotides was increased above 0.12 (data not shown). Taking into consideration the conformational change induced in superhelical DNA by intercalation of ethidium bromide (16), the changes observed in diol-epoxide I-bound ColE1 DNA could be explained by the fact that accumulated numbers of local unwinding (denaturation) sites were created by insertion of hydrocarbon molecules between the DNA double helix. Similar changes were observed by Weinstein and Grunberger (19) with *N*-acetoxyaminofluorene bound to DNA.

Effect of S1 Enzyme on Diol-Epoxide I-Bound DNA. Since local unwinding sites of DNA were assumed to be created by insertion of hydrocarbon molecules, a diol-epoxide I-treated ColE1 DNA sample was incubated with S1 (see *Materials and Methods*). About 7% of the total ^{14}C radioactivity was released

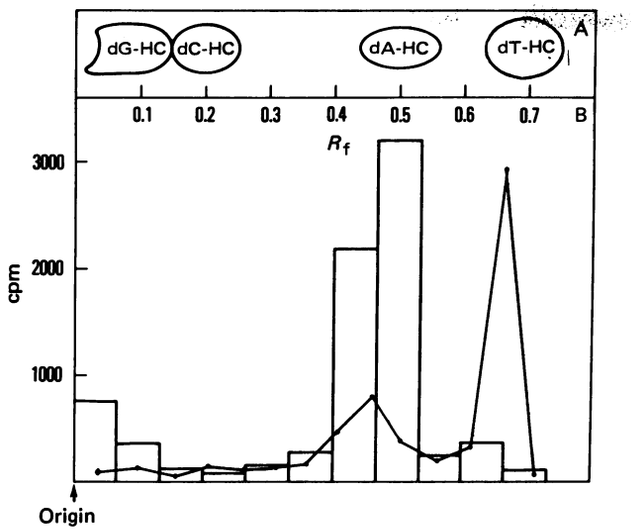

FIG. 3. (*A*) Spot positions of each reference sample (HC, diol-epoxide I adduct). (*B*) The ^{14}C radioactivity of the right corner sample of Fig. 2 is shown as open columns. When [^{14}C]diol-epoxide I was used for preparation of reference samples, the radioactivity was determined at the same corresponding spot positions observed under UV illumination. The ^{14}C radioactivity of the S1 hydrolysate coincided with deoxyadenosine–diol-epoxide I adduct. The solid line represents [^{14}C]diol-epoxide I alone subjected to thin-layer chromatography under the same conditions as the S1 hydrolysate of the DNA sample. A major peak at the running front was characteristic of diol-epoxide I. No radioactivity of S1 hydrolysate was found at the running front position.

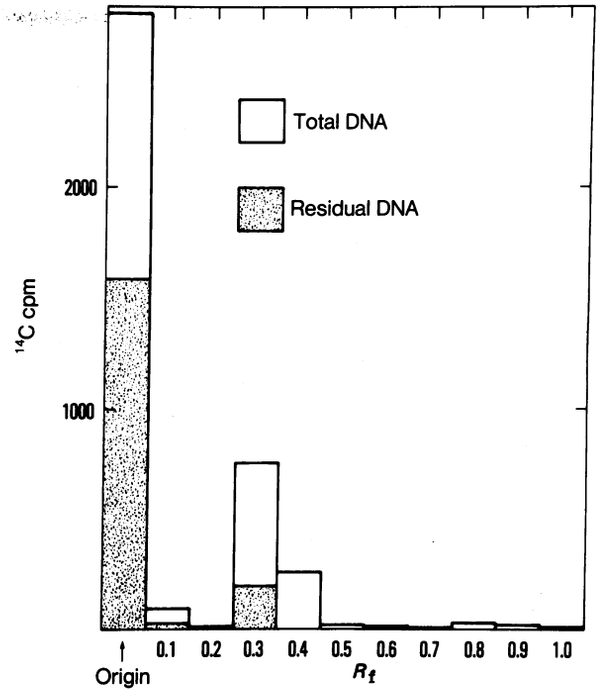

FIG. 4. Calf thymus DNA reacted with [^{14}C]diol-epoxide I was incubated with S1 and precipitated in ethanol. This residual DNA was further incubated with DNase, phosphodiesterase, and alkaline phosphatase. The sample peaking at an elution volume of 570 ml on Sephadex LH-20 column chromatography was analyzed by thin-layer chromatography (shaded column). Calf thymus DNA not preincubated with S1 was also subjected to DNase, phosphodiesterase, and alkaline phosphatase digestion and to Sephadex LH-20 and thin-layer chromatography (open column). Under this thin-layer chromatography condition the deoxyguanosine–diol-epoxide I adduct spotted near the origin and the deoxyadenosine adduct at R_f 0.3. Running direction is left to right.

into the supernatant after S1-treated DNA was precipitated by ethanol. Electron microscopic observation revealed random fragmentation of DNA with a mean length of 0.21 μm. Assuming the ColE1 DNA to be 2.12 μm long, we estimated that less than 10 nicks occurred on each DNA molecule on which 67 molecules of hydrocarbon were bound. It is conceivable, therefore, that only a small fraction of the bound molecules is responsible for the local unwinding of the DNA double helix, which causes sensitivity to the S1 enzyme. No such fragmentation occurred after S1 treatment of DNA that was not treated with diol-epoxide I.

Chromatography of S1 Hydrolysates and Reference Samples. S1 hydrolysates of calf thymus and ColE1 DNA and synthetic polymers reacted with diol-epoxide I were incubated with phosphodiesterase and alkaline phosphatase and subjected to Sephadex LH-20 column chromatography (see *Materials and Methods*). In the S1 hydrosate a major peak of [^{14}C]hydrocarbon bound to DNA bases appeared at the elution volume 570 ml and a minor peak at 620 ml. The reference samples of deoxyguanosine and deoxyadenosine bound to diol-epoxide I peaked at the same elution volume (570 ml) as did the S1 hydrolysate. It is already known that nucleosides are eluted in the early fractions. We observed early elution patterns in DNase hydrolysates similar to those reported by Sims *et al.* (1), but these will not be discussed in the present study. The S1 hydrolysate of [^{14}C]diol-epoxide I-treated synthetic deoxyribonucleotides also eluted at a volume of 570 ml.

Identification of Binding that Caused Local Unwinding of DNA. The S1 hydrolysate and its major peak at 570 ml elution volume were analyzed by thin-layer chromatography together with reference samples of deoxyguanosine and deoxyadenosine bound to diol-epoxide I, which were also collected from the same peak position. Fig. 2 shows a typical thin-layer chromatogram of a S1 hydrolysate isolated from a LH-20 column at an elution volume of 570 ml. Seventy-one percent of the radioactivity of this sample surprisingly migrated to the deoxyadenosine position and only 7% was found at the deoxyguanosine position (Fig. 3). The deoxycytosine adduct hydrolyzed by S1 varied from one experiment to another, ranging from 5 to 20% of the S1 hydrolysis product but only 1.5% of the total binding. ^{14}C-Labeled diol-epoxide I alone showed two peaks under the same conditions. The peak with the highest radioactivity migrated with the running front (Fig. 3). No such two-peak pattern was observed in the S1 hydrolysate, which indicates that neither free diol-epoxide I nor its hydrolysis product (6, 7, 9) was involved in the S1 hydrolysate. Similar experiments on base analysis with ColE1 DNA gave essentially the same result as did calf thymus DNA. The minor peak at 620 ml elution volume contained deoxycytosine and deoxyguanosine in varying proportions, as analyzed by thin-layer chromatography.

Binding Pattern of S1-Insensitive Portions of DNA. In order to examine bound molecules that did not cause local denaturation of the DNA double helix, we hydrolyzed the residual DNA with DNase, phosphodiesterase, and alkaline phosphatase and chromatographed the hydrolysate on Sephadex LH-20. A sample from the major peak at 570-ml elution volume was subjected to thin-layer chromatography. The results indicated that 89% of the hydrocarbon was bound to deoxyguanosine and only 11% to deoxyadenosine. The binding patterns of the residual DNA and of diol-epoxide I-treated total DNA without S1 treatment were identical and showed marked dominance by guanine (Fig. 4).

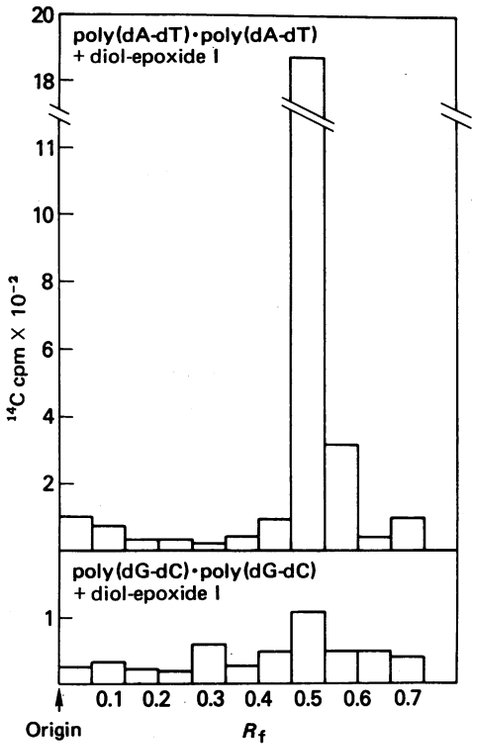

FIG. 5. Synthetic double-stranded deoxyribonucleotide polymers were reacted with diol-epoxide I. S1 hydrolysates that peaked at an elution volume of 570 ml from a Sephadex LH-20 column were subjected to thin-layer chromatography.

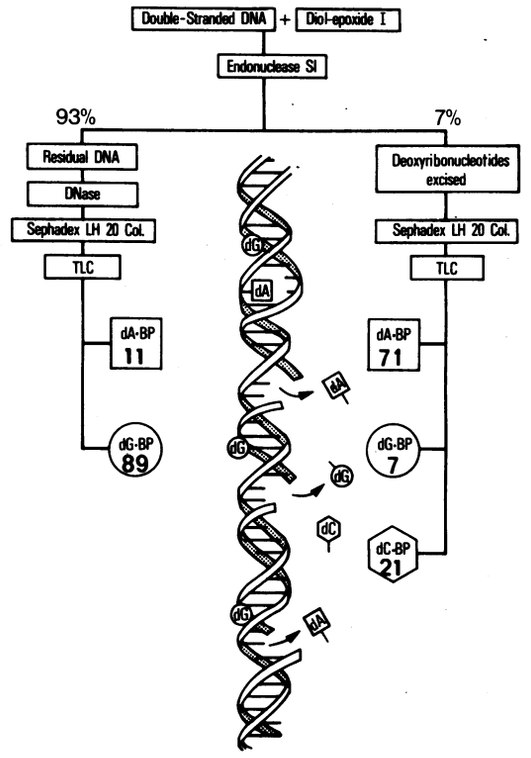

FIG. 6. Schematic illustration of the general binding pattern and modification of the DNA double helix caused by diol-epoxide I. Diol-epoxide I-bound DNA was hydrolyzed stepwise with S1 and DNase and chromatographed and analyzed by thin-layer chromatography. The numbers indicate the percent of binding with different DNA bases. The bindings that modified DNA so that it became sensitive to S1 are illustrated at the right. The binding to phosphate groups was not taken into consideration.

Binding and Structural Modification of Synthetic Deoxyribonucleotides by Diol-Epoxide I. In order to define the difference in possible structural modifications induced by the binding of diol-epoxide I to different deoxyribonucleotides, the double-stranded synthetic polymers poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) were reacted with [^{14}C]diol-epoxide I. The binding was 24 times more prevalent on the latter than the former. However, when the reacted polymers were incubated with S1 only poly(dA-dT)·poly(dA-dT) yielded a large amount of ethanol-unprecipitable ^{14}C radioactivity. The polymer solution became visibly less viscous after S1 treatment. The S1 hydrolysate of poly(dA-dT)·poly(dA-dT) obtained from the Sephadex LH-20 column at an elution volume of 570 ml showed the same thin-layer chromatogram pattern as the S1 hydrolysate of calf thymus and ColE1 DNA and as deoxyadenosine reacted with diol-epoxide I. On the other hand, the poly(dG-dC)·poly(dG-dC)-diol-epoxide I adduct yielded virtually no hydrolysate after incubation with S1 (Fig. 5).

DISCUSSION

The results of these experiments strongly suggest that there are three different types of binding of the ultimate mutacarcinogen diol-epoxide I to DNA *in vitro*. These are: (*i*) numerically minor binding that causes local unwinding (denaturation) of the DNA double helix, presumably due to the insertion of hydrocarbon molecules between DNA strands; (*ii*) binding that does not create a severe steric hindrance between hydrocarbon and DNA base pairs; and (*iii*) binding that induces single- or double-stranded nicks by cleaving the phosphodiester bonds of DNA.

Our results are consistent with previous findings that the majority of the diol-epoxide I binding to DNA occurs on guanine bases. However, when the diol-epoxide I-bound DNA was incubated with S1 endonuclease, only 7% of the S1 hydrolysate consisted of guanine–hydrocarbon adducts (Fig. 6). Seventy-one percent of the S1 hydrolysate consisted of an adenine–hydrocarbon adduct. This finding led us to believe that the binding of hydrocarbon to adenine caused local denaturation of DNA and subsequently resulted in the conformational change of the superhelical DNA that was observed on electron microscopy (Fig. 1). On gel electrophoresis, SV40 DNA reacted with diol-epoxide I migrated significantly more slowly than untreated DNA. The rate of retardation depended on the amount of diol-epoxide I bound to DNA, which indicated that the conformational change (increase in the overall dimension by partial relaxation) is the result of direct interaction between diol-epoxide I and the circular DNA (data not shown).

Experiments in which the double-stranded synthetic polymers, poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC), reacted with [^{14}C]diol-epoxide I showed that only the former induced S1-sensitive strand separation. Evidently, cleavage of dG·dC base pairing did not result from the binding.

Based upon these findings and studies from other laboratories (5, 6, 8), we assumed that the carbon 10 position of the hydrocarbon bound to the N2 position of adenine unstabilized hydrogen binding with thymine, whereas binding with guanine leaves two hydrogen bonds still available for stable base pairing. If this is the case, the functional impact on the genetic machinary of binding of benzo[*a*]pyrene metabolites to adenine is highly significant. A DNA sequence consisting of clusters of adenine bases was found in ColE1 DNA near the initiation site of replication (17). Musso *et al.* reported that promotors analyzed in bacteria have sequences highly rich in A·T base pairs that may facilitate local denaturation of the DNA by RNA

polymerase (18). AAA is the template for UUU which is a codon for phenylalanine-tRNA.

The discrepancy between the facts that a large number of hydrocarbon molecules bind to DNA particularly with guanine and that a relatively small number of base modifications (base substitution or deletion) may induce a mutation has been problematic for a long time. The present study suggests that local DNA modification induced by binding of diol-epoxide I is restricted to adenine-thymine pairs. Local denaturation sites induced by *N*-2-acetylaminofluorene bound to guanine were reported to be large enough to decrease the intrinsic viscosity of double-stranded DNA (19). Such an obvious viscosity change was not found in the binding with polycyclic aromatic hydrocarbons (20). These findings also support the concept that the DNA modifications induced by diol-epoxide I binding are relatively small numerically and geometrically.

Open circle and linear DNAs of unit length present in diol-epoxide I-treated DNA are no doubt produced by scission of phosphodiester bonds. Our recent experiments indicated that breakage of phosphodiester bonds was caused by binding of hydrocarbon to phosphate as well as DNA bases.

During the preparation of this manuscript Gamper *et al.* (21) reported that the diol-epoxides elicit a concentration-dependent nicking of superhelical ColE1 DNA in an *in vitro* reaction. The formation of unstable phosphotriesters was implicated. We observed that the presence of metal ions such as La^{3+} greatly enhanced the binding of diol-epoxide I to phosphate groups of DNA which were readily hydrolyzed in alkali (H. Yamamoto and T. Kakefuda, unpublished observations). The cocarcinogenic effect of such ions would be of considerable importance.

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