DNA and RNA Adducts Formed in Hamster Embryo Cell Cultures Exposed to Benzo[a]pyrene†

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ABSTRACT: The time course of covalent binding of [^{14}C]-benzo[a]pyrene (BP) to RNA and DNA in hamster embryo cell cultures and types of nucleoside adducts have been investigated. Binding to RNA proceeded rapidly and followed a linear time course for at least 48 h. In contrast, the extent of binding to DNA reached a plateau after approximately 18 h of incubation with [^{14}C]BP. Fluorescence spectroscopy of the modified nucleic acids and high-pressure liquid chromatography of the nucleoside adducts provided evidence that benzo[a]pyrene-7,8-dihydrodiol 9,10-oxide (BPDE) is the major metabolite of BP responsible for covalent modification of hamster embryo cellular nucleic acids. Both BPDE I and II isomers are synthesized and participate in modification of the host cell's RNA and DNA. Although guanine is the major BPDE target during in vivo modification of RNA and DNA, there is a low but detectable modification of adenine and cytosine bases. The relative contributions of the various isomers of BPDE to in vivo nucleic acid modification vary between DNA and RNA. The types of RNA adducts do not depend on the length of time of exposure to BP. In contrast, the relative amounts of the multiple types of DNA adducts changed with time, suggesting that they underwent differential rates of excision. A greater heterogeneity of the DNA adducts in primary hamster embryo cell cultures was observed than in previous studies with human and bovine bronchial explants exposed to BP. Possible differences in DNA–BPDE adducts among species and cell types are discussed.

Although great advances have been made in our understanding of the metabolism of several chemical carcinogens and their interactions with cellular constituents, at the present time the critical biologic event is not known with certainty for any chemical carcinogen. Current evidence favors DNA as the critical cellular target, although other targets such as RNA and protein, and epigenetic effects, have not been excluded (Weinstein, 1976). With respect to covalent binding of carcinogens to DNA, there is increasing recognition of the fact that not only is the extent of DNA modification important but so too is the qualitative nature of the modification and the rates with which different types of DNA modifications are recognized and removed by DNA repair mechanisms (Shinohara and Cerutti, 1977; Westra et al., 1976; Goth-Goldstein, 1977; Yamasaki et al., 1977).

For the above reasons, the present study on the time course of covalent binding of benzo[a]pyrene[1] (BP) to cellular RNA and DNA and a detailed analysis, by fluorescence spectroscopy and high-pressure liquid chromatography (HPLC), of the types of nucleoside adducts was undertaken. Benzo[a]pyrene is a potent and ubiquitous environmental carcinogen that requires metabolic activation to exert its cytotoxicity, mutagenicity, and carcinogenicity (Gelboin et al., 1969; Huberman and Sachs, 1974; Kinoshita and Gelboin, 1972). Recent studies indicate that the most reactive metabolite of benzo[a]pyrene is BP-7,8-dihydrodiol 9,10-oxide (BPDE) (Borgen et al., 1973; Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Ivanovic et al., 1976; Weinstein et al., 1976; Huberman et al., 1976). BPDE has four possible isomeric forms (Figure 1); in isomer I the 7-hydroxyl and 9,10-oxide groups are on the opposite sides of the plane of the ring system and in isomer II they are on the same side. Each stereoisomer has two enantiomers, derived from either (7*R*)- or (7*S*)-BP-7,8-dihydrodiol, designated (7*R*)- or (7*S*)-BPDE, respectively. In human and bovine bronchial explants, BP becomes bound to DNA essentially as a single component (Jeffrey et al., 1977). Evidence has been presented that this occurs mainly via a specific isomer of BPDE, the 7*R* enantiomer of isomer I, resulting in trans addition of the 10 position of the hydrocarbon to the 2-amino group of guanine (Jeffrey et al., 1977). Binding to human and bovine bronchial RNA has also been observed, although there was greater heterogeneity of the nucleoside–BPDE adducts (Jeffrey et al., 1977).

At the present time, it is not known whether the exposure of normal human cell cultures to BP results in reproducible transformation. On the other hand, hamster embryo cultures (HEC) are readily transformed by BP and related polycyclic aromatic hydrocarbon carcinogens (Berwald et al., 1965; DiPaolo and Donavan, 1967). Moreover, recent studies indicate that BPDE is even more potent than BP in the transformation of HEC (Mager et al., 1977) and mouse fibroblast cultures (Sisskin, E., and Weinstein, I. B., unpublished studies). For these reasons, the present study has employed the HEC culture system, and our findings on the types of nucleoside adducts formed in hamster cells exposed to [^{14}C]BP are compared to those previously reported with human and bovine bronchial

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[1] Abbreviations used: BP, benzo[a]pyrene; HPLC, high-pressure liquid chromatography; BPDE, BP-7,8-dihydrodiol 9,10-oxide; BPDE isomer I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]-pyrene; BPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; (7*R*)-BPDE I, the 7*R*,8*S*,9*R*,10*R* enantiomer of BPDE I [or (+) BPDE I, derived from (−)-BP-7,8-dihydrodiol]; (7*S*)-BPDE I, 7*S*,8*R*,9*S*,10*S* enantiomer of BPDE I [or (−)-BPDE I, derived from (+)-BP-7,8-dihydrodiol]; (7*S*)-BPDE II, 7*S*,8*R*,9*R*,10*R* enantiomer of BPDE II [or (+)-BPDE II derived from (+)-BP-7,8-dihydrodiol]; HEC, hamster embryo cells; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

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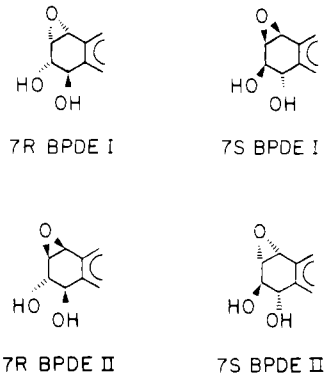

FIGURE 1: Structures of enantiomers of BPDE isomer I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, and BPDE isomer II, (±)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. For further descriptions of these compounds, see text.

cells. Possible qualitative changes in nucleoside adducts during the time course of [^{14}C]BP binding and removal have also been analyzed in the HEC cultures.

Experimental Procedure

Chemicals. [^{14}C]BP (specific activity 51 mCi/mmol) was purchased from Amersham/Searle Corp. and BPDE isomers I and II were kindly supplied by Dr. R. G. Harvey. Calf thymus DNA, micrococcal nuclease, bovine spleen phosphodiesterase, venom phosphodiesterase, and *Escherichia coli* alkaline phosphatase were purchased from Worthington Biochemicals. DNase I was obtained from Sigma Chemical Co. and proteinase K from Merck Co., Rahway, N.J.

Cell Culture. Primary cultures of LVG strain Syrian hamster embryo cells were prepared and cultured as described (Ivanovic et al., 1976). Confluent cultures were exposed to 1 μg/mL [^{14}C]BP in the absence of light for the indicated periods of time. In the "posttreatment incubation" studies, cells were exposed to [^{14}C]BP for 21 h, after which the radioactive medium was removed, the cell monolayer was rinsed twice with warmed Dulbecco's medium, and the cells were then incubated in BP-free medium for an additional 24 h.

Nucleic Acid Extraction and Purification Procedure. DNA was extracted from the pelleted nuclei as previously described (Ivanovic et al., 1976). RNA was isolated from the cytosol fraction by extracting six to seven times with phenol-chloroform (1:1) containing 0.1% 8-hydroxyquinoline, after adjusting the cytosol to 15 mM NaCl, 1.5 mM citrate buffer (pH 6.9), and 1% $NaDodSO_4$. Further purification was similar to the procedure for DNA. An alternative RNA extraction procedure was also used, which involved the following steps. After several phenol extractions and ethanol precipitation, the RNA was dissolved in an aqueous solution of 10 mM Tris, 5 mM EDTA, and 0.4 M NaCl; proteinase K (100 μg/mL) was added; and the mixture was incubated for 18 h at 37 °C. Following extraction with chloroform-isoamyl alcohol and ethanol precipitation, the RNA was treated with DNase I in 10 mM Tris (pH 7.9), 10 mM NaCl, and 3 mM $MgCl_2$ for 18 h at 37 °C. After repeating the phenol-chloroform extractions, the aqueous phase was extracted three times with 1–2 volumes of ethyl ether. Both methods of RNA extraction and purification gave HPLC profiles similar to those presented in Figure 4.

In Vitro Modified BPDE-Nucleic Acids. The in vitro reaction of DNA or RNA with BPDE in an aqueous ethanol mixture was similar to that described by Pulkrabek et al. (1977). Under these conditions, the major DNA adduct obtained with BPDE I corresponds to dGI-2 in Figure 5A.

Guanosine, cytidine, adenosine, deoxyguanosine, deoxyadenosine, and deoxycytidine adducts, prepared by in vitro reactions with BPDE I and II, were generous gifts from Drs. K. Grzeskowiak and K. W. Jennette. Preparation and HPLC elution profiles of these compounds have been previously described (Jennette et al., 1977; Jeffrey et al., 1977).

Enzymatic Hydrolysis of HEC BP-DNA. To assure maximum digestion of the modified DNA, a previously described method (Yamasaki et al., 1977) was used, with slight modifications: 50 A_{260} units of DNA in 2.5 mL of 100 mM NaCl, 10 mM Tris-HCl (pH 8.1), and 5 mM $MgCl_2$ was heat denatured at 100 °C for 3 min and rapidly cooled. Then, 60 units of *Neurospora crassa* endonuclease was added and the mixture incubated for 18 h at 37 °C. Subsequent steps involved incubation for an additional 18 h with 1000 units of DNase I and 500 units of *Staphylococcal* nuclease, followed by 1-h incubations with alkaline phosphatase (10 units), spleen phosphodiesterase (0.25 unit), crystalline snake venom phosphodiesterase (0.25 unit), repeated twice, and again alkaline phosphatase (10 units), each enzyme being added separately. Under these conditions, 80–85% of the radioactivity associated with the in vivo modified DNA was released as free deoxynucleoside adducts.

Alkaline Hydrolysis of HEC BP-RNA. The procedure was similar to that used by Jennette et al. (1977). RNA samples were hydrolyzed at 37 °C for 18 h with 0.3 N NaOH. The nucleotide mixtures were neutralized with acetic acid, incubated with alkaline phosphatase (1 unit/A_{260}) for 24 h at 37 °C, and chromatographed on Sephadex LH-20 columns (see below).

HPLC and Sephadex LH-20 Column Chromatography. Separation of BP-modified nucleosides from unmodified nucleosides, following hydrolysis of the modified DNA or RNA, was achieved by Sephadex LH-20 column chromatography as described previously (Jennette et al., 1977). The isolated modified nucleosides were then analyzed by HPLC as described by Jeffrey et al. (1976a). Samples were eluted with a concave (Du Pont No. 2) gradient from 25 to 80% methanol in water over an 80-min period.

Fluorescence. Low-temperature fluorescence spectra of HEC RNA and DNA or in vitro reaction products were obtained with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer as previously described (Ivanovic et al., 1976).

Results

Time Course for the in Vivo Binding of [^{14}C]BP to HEC DNA and RNA. Figure 2 compares the time course of in vivo covalent binding of [^{14}C]BP to the RNA and DNA of confluent cultures of HEC. The binding to RNA proceeded rapidly and followed an approximately linear time course for at least 48 h. This indicates that BP and/or its reactive metabolites were available for nucleic acid binding during this time period. The time course for binding to DNA was quite different. After approximately 18 h of incubation with [^{14}C]BP, DNA binding reached a plateau. Previous studies with the BHK cell line indicate that hamster cells are capable of excising BP-deoxyguanosine lesions (Shinohara and Cerutti, 1977). Therefore, the most likely explanation for the plateau seen with DNA binding, but not RNA binding, is that the DNA plateau is due to an equilibrium between de novo binding and the excision repair process. To confirm this hypothesis, we performed the following study. After exposure of confluent HEC cultures to [^{14}C]BP for 21 h, the cells were washed twice with warmed media and incubated in complete growth medium lacking BP for 24 h, and cellular DNA and RNA were ex-

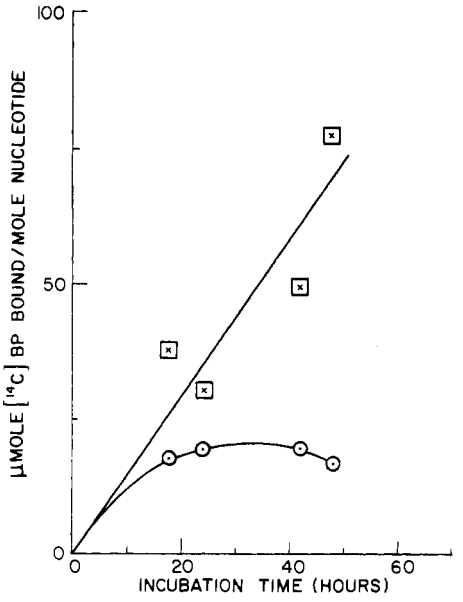

FIGURE 2: Time course of binding of [^{14}C]BP to DNA and RNA in confluent HEC cultures: HEC DNA (⊙-⊙), HEC RNA (⊡-⊡). The results represent mean values obtained from five independent studies.

TABLE I: Effect of Posttreatment Incubation on the Extent of [^{14}C]BP Binding to DNA and RNA in Confluent HEC Cultures.

Incubation conditions	μmol of [^{14}C]BP bound/mol of nucleotides	
	DNA	RNA
[^{14}C]BP for 21 h	13	23
[^{14}C]BP for 45 h	12	42
[^{14}C]BP for 21 h; then no [^{14}C]BP for 24 h	7.9	29

tracted. The extents of [^{14}C]BP binding, together with appropriate controls, are presented in Table I. It is apparent that removal of [^{14}C]BP from the medium between the 21 and 45 h interval led to an approximate 40% decrease in the amount of [^{14}C]BP bound to DNA, when compared to the plateau value obtained when [^{14}C]BP was left in the medium during this period. With RNA, however, removal of [^{14}C]BP from the medium did not result in a decrease in specific activity during the subsequent 24 h, presumably because of the lack of an RNA repair mechanism.

Low-Temperature Fluorescence Spectra of HEC DNA and RNA Following in Vivo Exposure to BP. The above studies on [^{14}C]BP labeling of nucleic acids do not, of course, provide information on the type(s) of nucleoside adducts in DNA and RNA at various time points. We utilized, therefore, low-temperature fluorescence spectroscopy, a technique previously developed in our laboratory (Ivanovic et al., 1976), to obtain information regarding the structure of the DNA and RNA-bound BP chromophores at different time points. Figure 3 indicates that the fluorescence emission spectra of DNA and RNA samples obtained from HEC following an 18-h incubation with the parent hydrocarbon closely resembled those obtained when DNA or RNA reacted with BPDE in vitro. The pyrene-like nature of the spectrum of the in vivo samples provides evidence that the major bound chromophore arose from the BPDE metabolite, a result consistent with previous studies (Daudel et al., 1975; Ivanovic et al., 1976). The fluorescence spectra of the 24- and 42-h DNA and RNA samples (Figure 3) and of 48-h and posttreatment incubation samples

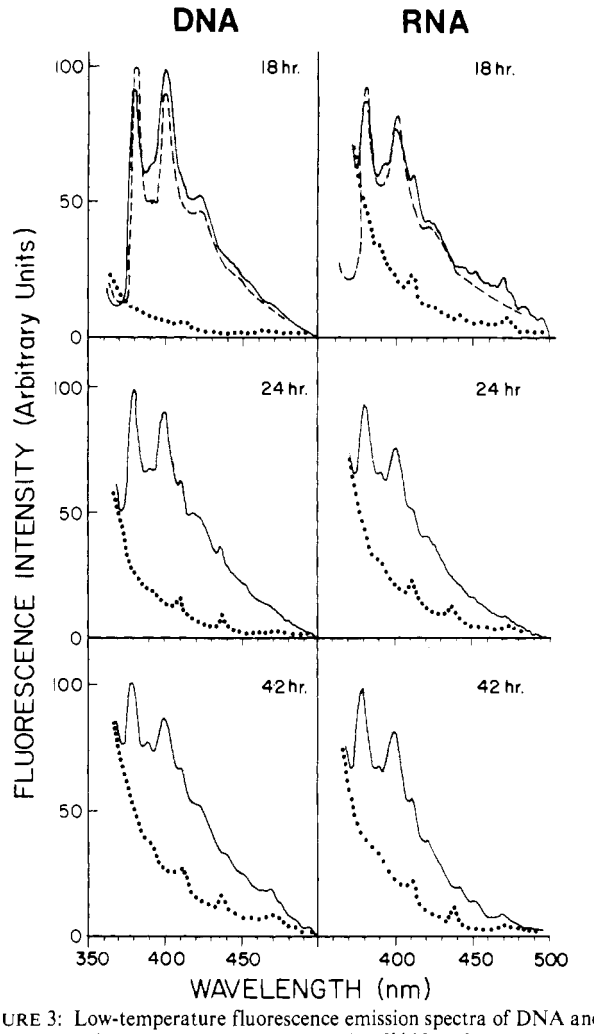

FIGURE 3: Low-temperature fluorescence emission spectra of DNA and RNA obtained from HEC cultures exposed to [^{14}C]BP for 18, 24, or 42 h, with comparisons to in vitro modified DNA: in vivo modified nucleic acid samples (—), control NA (···), DNA or RNA modified in vitro with BPDE (- - -).

(not shown here) were qualitatively siimilar to the 18-h samples.

As mentioned in the introduction and as described in Figure 1, two stereoisomers of BPDE have been synthesized, designated I and II, and each stereoisomer has two enantiomers, designated 7*R* and 7*S*. We have found that the fluorescence spectra of DNA which reacted in vitro with isomer I were identical to that obtained with DNA which reacted with isomer II. The fluorescence spectra also do not distinguish between different nucleoside adducts formed with BPDE, i.e., the guanosine, adenosine, or cytidine adducts, since the emission is predominantly from the pyrene moiety of the adduct. To obtain further information on these aspects, it was necessary to analyze the samples by HPLC.

Analysis of Ribonucleoside-BP Adducts by HPLC. RNA samples obtained from confluent HEC cultures exposed to [^{14}C]BP for either 18 or 42 h were hydrolyzed with KOH, and the released nucleosides were analyzed as follows. The BP-modified nucleosides were separated from unmodified nucleosides on a Sephadex LH-20 column. The former, which were retained by the LH-20 column and then eluted with 80% methanol, were analyzed by HPLC, utilizing as markers various BPDE–nucleoside adducts synthesized in vitro. Structural assignments of the covalent guanosine adducts derived from the in vitro reaction of poly(G) with either BPDE I or II have

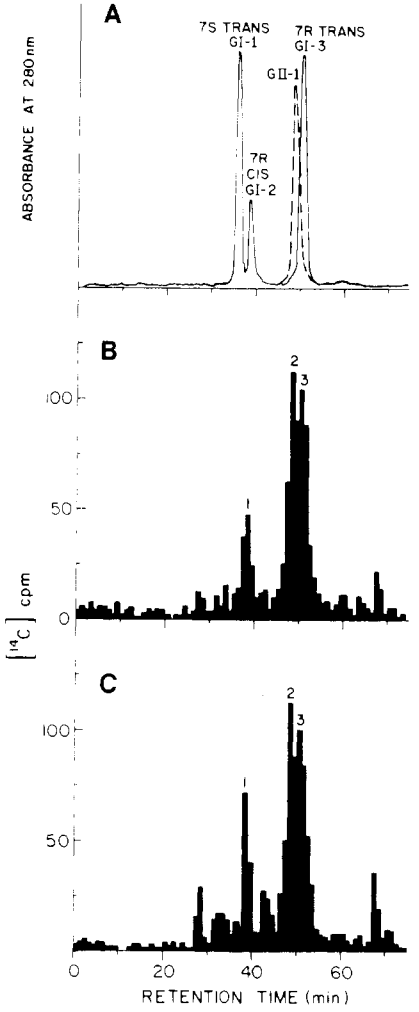

FIGURE 4: HPLC profiles of RNA adducts formed in confluent HEC cultures incubated with [^{14}C]BP. (A) In vitro markers: Elution positions of guanosine adducts formed by in vitro reactions with BPDE I ("GI-1,2,3") with designation of enantiomeric form (7*S* or 7*R*) and type of addition to 9,10-oxide (trans or cis) and of a guanosine adduct formed with BPDE II ("GII-1"). For a further description of these markers, see the text. (B) RNA isolated from HEC after 18-h exposure of the culture to [^{14}C]BP. (C) RNA isolated from HEC after 42-h exposure of the culture to [^{14}C]BP.

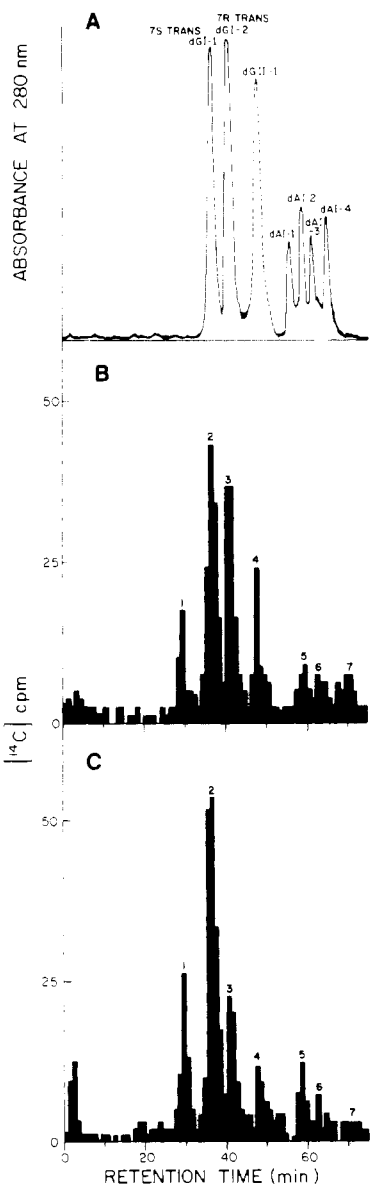

FIGURE 5: HPLC profiles of DNA adducts formed in confluent HEC cultures incubated with [^{14}C]BP. (A) In vitro markers: Elution position of deoxyguanosine and deoxyadenosine adducts formed by in vitro reactions with BPDE I ("dGI-1,2; dAI-1,2,3,4") with designation of enantiomeric form (7*S* or 7*R*) and type of addition to 9,10-oxide (trans or cis) and of a deoxyguanosine adduct formed with BPDE II ("dGII-1"). For a further description of these adducts, see the text. (B) DNA isolated from HEC after 18-h exposure of the culture to [^{14}C]BP. (C) DNA isolated from a culture of HEC exposed to [^{14}C]BP for 21 h and then incubated for an additional 24 h in the absence of BP ("posttreatment incubation").

also been described elsewhere (Weinstein et al., 1976; Moore et al., 1977; Jeffrey et al., 1977). The HPLC elution characteristics of BPDE adducts formed with adenosine and cytidine have also been described (Jennette et al., 1977; Jeffrey et al., 1977), although their precise structures are not known.

HPLC analysis of the HEC [^{14}C]BP-RNA adducts revealed the presence of three major products, designated HEC RNA-1-3, and possibly a few minor products (Figure 4B). The elution positions of guanosine adducts synthesized in vitro with BPDE isomer I (GI-1, GI-2, GI-3) and of a guanosine adducts with isomer II (GII-1) are indicated in Figure 4A. A comparison of these elution positions indicated the following. HEC RNA peak 3 (Figure 4B) coincides with the in vitro product GI-3 (Figure 4A), whose structure and stereochemistry have been previously elucidated (Jeffrey et al., 1975a,b; Moore et al., 1977). This compound results from trans addition of the 2-amino group of guanine to the 10 position of the 7*R* enantiomer of BPDE I (Jeffrey et al., 1976a,b). The same compound has been found as one of the major BP adducts in human and bovine bronchial RNA and mouse skin RNA (Jeffrey et al., 1977; Moore et al., 1977). HEC RNA peak 1 (Figure 4B) is cochromatographic with GI-2 (Figure 4A); it is analogous to GI-3 but is formed by cis addition. This nucleoside adduct was also previously identified in human, bovine, and mouse skin RNA (Jeffrey et al., 1977; Moore et al., 1977). HEC RNA peak 2 (Figure 4B) corresponds in its elution position to the marker GII-1 (Figure 4A). The latter results from trans addition of the 2-amino group of guanine to the 10 position of the 7*S* enantiomer of BPDE II. The presence of this adduct and of peak 3 in the HEC RNA digest provides indirect evidence that both BPDE isomers I and II are synthesized by HEC. It is of interest to note that the guanosine adduct derived from the 7*S* enantiomer of BPDE I, represented in Figure 4A by marker peak GI-1, was not detected in significant amounts in our HEC RNA nor has it been detected thus far in any of the mammalian systems analyzed. The absence of this product is

TABLE II: Effect of Duration of Exposure of HEC to BP on the HPLC Profile of Deoxynucleoside Adducts.[a]

HPLC peak	Structure of the coincident in vitro marker	HPLC t_R (min)	% of total[b] At 18 h	% of total[b] Posttreatment incubation (21/45 h)[c]
1	BPDE–deoxycytidine?	30	9	16
2	(7*S*)-BPDE I–9,10-*trans*-deoxyguanosine	37	35	50
3	(7*R*)-BPDE I–9,10-*trans*-deoxyguanosine	41	28	15
4	BPDE II–deoxyguanosine	48	13	8
5–7	BPDE–deoxyadenosine	54–66	15	6

[a] HEC cultures were exposed to radioactive BP for 18 or 21 h, followed by a 24-h incubation in the absence of BP [designated "posttreatment incubation (21/45 h)"]. DNA was then extracted, hydrolyzed, and analyzed by HPLC. For additional details, see Methods and Figures 2 and 5. [b] Results obtained from HPLC profiles (see Figure 5) are expressed as the percent contribution of a given peak to the total radioactivity eluted. [c] The remaining 5% is due to the presence of an early peak eluted at 2–4 min from the HPLC; see Figure 5C.

consistent with the observation that the 7*S* enantiomer of BPDE I has not been detected in a microsomal preparation (Yang et al., 1977), presumably reflecting negligible synthesis or extremely rapid degradation.

Figure 4C represents the HPLC profile of modified nucleosides obtained from the RNA of HEC exposed to [^{14}C]BP for 42 h. The profile is qualitatively similar to that obtained with the 18-h sample (Figure 4B), with the exception of increased prominence of the minor peaks in the 42-h sample. Thus far we have had insufficient amounts of these minor peaks for further analysis. Their retention times, however, suggest that they correspond to previously described BPDE–cytidine and BPDE–adenosine adducts (Jennette et al., 1977).

Analysis of Deoxynucleoside–BP Adducts by HPLC. Figure 5B presents the HPLC profile of modified deoxynucleosides obtained after enzymatic digestion of a DNA sample extracted from HEC exposed to [^{14}C]BP for 18 h. This profile accounts for about 80% of the radioactivity associated with the DNA. The remaining 20% apparently represents incompletely digested material, since it eluted in the void volume of the LH-20 column and has not been further characterized. Similar difficulties with digests of BP-modified DNA have been noted by others (Sims et al., 1974; Grover et al., 1976; Osborne et al., 1976; Jeffrey et al., 1977). The digest of HEC DNA revealed four major radioactive peaks, designated HEC-DNA-1-4, and a few minor products (Figure 5B and Table II).

The elution positions of markers synthesized in vitro with BPDE I or II are given in Figure 5A. Comparisons of the HEC products with these markers led to the following assignments. HEC DNA peak 3 (Figure 5B) coincides in its elution position with the marker dGI-2 (Figure 5A). The latter results from trans addition of the 2-amino agroup of guanine to the 10 position of the 7*R* enantiomer of BPDE I (Jeffrey et al., 1977). This adduct corresponds to the single major deoxynucleoside adduct described in the DNA of human and bovine segments. HEC DNA peak 2 (Figure 5B) is cochromatographic with the marker dGI-1, synthesized by reaction of dG with a racemic mixture of BPDE I. The circular dichroism spectrum of dGI-1 is nearly identical to that of dGI-2 but of opposite sign, suggesting that it is derived from the 7*S* enantiomer of BPDE I (Grzeskowiak, K., et al., unpublished studies). The presence of considerable amounts of this product in DNA is surprising, since the corresponding product was not observed in cytoplasmic RNA, nor was the intermediate detected in a microsomal system (see above). HEC DNA peak 4 (Figure 5B) corresponds in its elution position to the marker dGII-1 (Figure 5A), a product of the reaction of dG with a racemic mixture of BPDE II. HEC DNA peak 1, which elutes earlier than any of the marker dG adducts, is in the same region as dC adducts formed in vitro with BPDE I (Jennette et al., 1977). HEC DNA peaks 5–7 account for about 15% of the radioactivity eluted (Figure 5B and Table II). Their retention times are characteristic of the multiple dA adducts formed by in vitro reactions of dA with BPDE I (peaks dAI-1,2,3,4 in Figure 5A) and the multiple dA adducts formed in vitro with BPDE II (data not shown here). In view of the small amounts of these materials and their relatively poor resolution, precise assignments have not yet been possible.

Figure 5C provides the profile of deoxynucleoside adducts found in HEC DNA exposed to [^{14}C]BP for 21 h and then incubated for an additional 24 h in the absence of BP ("posttreatment incubation"). It is apparent that the relative abundance of individual peaks shifted appreciably during the posttreatment incubation (compare Figure 5B,C). The data are summarized quantitatively in Table II. Although the differences are complex, perhaps the most striking one is the decrease in the relative abundance of DNA peak 3 and increase of peak 2 that occurred with posttreatment incubation. When expressed quantitatively, the ratio of picomoles of BP adducts in peak 2 to picomoles in peak 3 was 3 in the posttreatment incubation sample, whereas the corresponding value for the 18-h incubation sample was 1. As shown in Table I and discussed above, the posttreatment incubation period is associated with a decrease in the total specific activity of the DNA, apparently due to DNA excision repair. The associated changes in the HPLC BP-deoxynucleoside profiles suggest that the peak 3 adduct is excised at a more rapid rate than the peak 2 adduct and that other peaks may undergo differential rates of excision.

In the above experiments, we used [^{14}C]BP to avoid possible artifacts due to isotope exchange. Because of the low specific activity of [^{14}C]BP and the low extents of in vivo modification of cellular DNA, the absolute cpm present in the HPLC peaks are low. Nevertheless, the results obtained are reproducible. The data presented in Figure 5 and Table II were obtained with DNA samples pooled from two separate experiments. In an additional study (not shown here), HEC cultures were incubated with [^{14}C]BP for 24 h. The HPLC profile of modified deoxynucleosides was similar to that of an 18-h sample (Figure 5B), except that there was a relative decrease in peaks 3 and 4. This is consistent with the trend noted in comparing Figure 5B to 5C. Furthermore, in recent studies we have labeled HEC with high specific activity [^{3}H]BP. The HPLC peaks contain a much larger number of cpm, and the profiles are similar to those shown in Figure 5.

Discussion

The present results indicate that multiple ribonucleoside and deoxyribonucleoside adducts are formed in the RNA and DNA of HEC cultures exposed to [^{14}C]BP. All of the major in vivo adducts cochromatographed with ribonucleoside or deoxyribonucleoside adducts synthesized in vitro with BPDE isomer I or BPDE isomer II as the reactive species. These results, as well as the low-temperature fluorescence spectra of

the cellular RNA and DNA, provide evidence that BPDE is the major metabolite of BP responsible for covalent modification of hamster embryo cellular nucleic acids.

We must emphasize that at the present time our identification of the in vivo adducts is based largely on comparisons of their retention times on HPLC to those of known marker compounds. More precise identification is complicated by the extremely small amounts of materials available from in vivo sources. As described under Results, it would appear that in HEC both BPDE I and II are synthesized and participate in modification of the host cell's RNA and DNA. We have also presented indirect evidence that both the 7*R* and 7*S* enantiomers of BPDE I and II appear to be involved in this process. The relative contributions of the various isomers of BPDE to in vivo nucleic acid modification vary between DNA and RNA. HPLC analysis revealed profiles of BPDE–RNA adducts in HEC that were similar to those previously reported for human, bovine (Weinstein et al., 1976; Jeffrey et al., 1977), and mouse skin RNA (Moore et al., 1977). Moreover, the types of RNA adducts did not change appreciably between 18 and 45 h in culture. In contrast, the profiles of DNA adducts changed as a function of incubation time. In recent studies with human and bovine bronchial segments, almost all of the radioactivity associated with DNA was derived from (7*R*)-BPDE I (Jeffrey et al., 1977). However, in certain rodent cell cultures, both isomers I and II of BPDE appear to be involved in DNA binding (Shinohara and Cerutti, 1977; Baird and Diamond, 1977). The high resolution obtained by HPLC in the present studies provides further evidence for the latter conclusion. It appears, therefore, that there can exist major differences in types and relative abundance of DNA–BPDE adducts between species and cell types. The basis for such variations is not known. The most likely explanations relate to variations in specificities of microsomal monooxygenase systems in the synthesis of the various isomers of BPDE, perhaps in response to various physiologic and genetic determinants. Variations in rates of excision of the multiple adducts between species and cell types could also play an important role.

The present results indicate that, although guanine is the major BPDE target during in vivo modification of RNA and DNA in HEC, there is also modification of adenine and cytosine bases. Only small amounts of such adducts were tentatively identified and this aspect requires further studies. In vitro studies indicate that BPDE can react with all three bases (Jennette et al., 1977), presumably because they share an exocyclic amino group.

Our results suggest that not only is the profile of nucleoside modification by BP complex but so too is the process of excision from DNA of the multiple types of modified deoxynucleosides. It appears that in HEC cultures certain types of deoxynucleosides modified by BPDE are excised from the DNA at a more rapid rate than others. There is precedent for differential excision by cells of multiple DNA adducts formed with other carcinogens, including the N-7 and O-6 alkylated bases resulting from exposure to diethylnitrosamine (Goth-Goldstein, 1977) and the N^2 and C-8 adducts formed from *N*-2-acetylaminofluorene (Westra et al., 1976). In the latter case, this appears to be due to differences in nucleic acid conformation associated with the two different nucleic acid adducts (Yamasaki et al., 1977) and presumably similar phenomena underlie the differential excision of the multiple BPDE–DNA adducts.

Further studies are required to determine which of the multiple types of nucleic acid modification produced by activated metabolites by BP are most closely correlated with the carcinogenic process and to what extent the role of differential excision from the DNA of such adducts affects target cell susceptibility to BP-induced carcinogenicity.

Acknowledgments

The authors thank Drs. K. Grzeskowiak and A. M. Jeffrey for providing the in vitro BPDE–nucleoside model compounds and helpful discussions of the HPLC analyses. We are grateful to Mr. Douglas Eisenberg for preparing the hamster embryo cell cultures.

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Mechanism of Selective Inhibition of 3′ to 5′ Exonuclease Activity of *Escherichia coli* DNA Polymerase I by Nucleoside 5′-Monophosphates†

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ABSTRACT: The 3′ to 5′ exonuclease activity of *Escherichia coli* DNA polymerase I can be selectively inhibited by nucleoside 5′-monophosphates, whereas the DNA polymerase activity is not inhibited. The results of kinetic studies show that nucleotides containing a free 3′-hydroxy group and a 5′-phosphoryl group are competitive inhibitors of the 3′ to 5′ exonuclease. Previous studies by Huberman and Kornberg [Huberman, J., and Kornberg, A. (1970), *J. Biol. Chem. 245,* 5326] have demonstrated a binding site for nucleoside 5′-monophosphates on DNA polymerase I. The K_{dissoc} values for nucleoside 5′-monophosphates determined in that study are comparable to the K_i values determined in the present study, suggesting that the specific binding site for nucleoside 5′-monophosphates represents the inhibitor site of the 3′ to 5′ exonuclease activity. We propose that (1) the binding site for nucleoside 5′-monophosphates on DNA polymerase I may represent the product site of the 3′ to 5′ exonuclease activity, (2) the primer terminus site for the 3′ to 5′ exonuclease activity is distinct from the primer terminus site for the polymerase activity, and (3) nucleoside 5′-monophosphates bind at the primer terminus site for the 3′ to 5′ exonuclease activity.

The ability of bacterial and phage DNA polymerases to copy DNA templates in vivo and in vitro with such high fidelity has been ascribed in part to the associated 3′ to 5′ exonuclease activity of these DNA polymerases (Brutlag and Kornberg, 1972; Muzcyzka et al., 1972). The 3′ to 5′ exonuclease activity has a proof-reading function as it excises a mismatched nucleotide incorporated at the primer terminus prior to further chain extension, thereby helping to maintain replication fidelity. We have recently demonstrated that the proofreading 3′ to 5′ exonuclease activity of both *E. coli* DNA polymerase I and mammalian DNA polymerase δ can be selectively inhibited by nucleoside 5′-monophosphates, whereas the DNA polymerase activity is not inhibited (Byrnes et al., 1977). The present study was undertaken to elucidate the mechanism of this selective inhibition.

Materials and Methods

Unlabeled nucleosides, nucleotides, and nucleotide analogues were obtained from either P-L Biochemicals Inc. or Sigma Chemical Co. The purity of nucleoside 5′-monophosphates was examined by PEI-cellulose thin-layer chromatography as described by Cashel et al. (1969) and no contamination by nucleoside diphosphates or triphosphates was detectable (<0.1%). However, because of the high concentrations of nucleoside 5′-monophosphates used in the experiments, they were further purified: dAMP was purified by chromatography on Dowex-1 (chloride) as described by Cohen (1966) and TMP by chromatography on Dowex-1 (formate) as described by Hulbert and Furlong (1967). PEI-cellulose thin-layer chromatography plates were obtained from Brinkman Instruments. [^{3}H]dTTP and γ-[^{32}P]ATP were purchased from Amersham Corp. Poly(dA-dT) was purchased from Grand Island Biological Co. and was dialyzed before use against 0.01 M Tris-HCl[1] (pH 7.4), 0.06 M KCl. Oligo[d(T)$_4$] was obtained from Collaborative Research, Inc. Polynucleotide kinase was purchased from Boehringer Mannheim Corp. *E. coli* B (¾ log) was purchased from Grain Processing Corp. Calf thymus was purchased from Pel-Freez Biologicals Inc. *Hin*II and *Hin*III restriction endonuclease fragments of ^{32}P-labeled SV-40 DNA were the generous gift of Dr. Walter Scott.

DNA polymerase I was prepared from *E. coli* B according to Jovin et al. (1969) through step VII. Terminal deoxynucleotidyltransferase was prepared from calf thymus according to Bollum et al. (1974) through step 5′.

[^{3}H]poly[d(T)$_{50}$] was synthesized with terminal deoxynucleotidyltransferase using oligo[d(T)$_4$] as primer and [^{3}H]-dTTP (25–200 cpm/pmol) according to the method of Bollum (1968). The chain length of [^{3}H]poly[d(T)$_{50}$] was determined by enzymatic end-group labeling using γ-[^{32}P]ATP and

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‡ Research Career Development Awardee (NIH-K04-HL-00031).
§ Investigator of Howard Hughes Medical Institute.

[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/78/0417-1603$01.00/0