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FORMATION OF BENZO[a]PYRENE METABOLITE–NUCLEOSIDE ADDUCTS IN ISOLATED PERFUSED RAT AND MOUSE LIVER AND IN MOUSE LUNG SLICES

G. F. KAHL,† E. KLAUS,†* C. LEGRAVEREND,‡ D. W. NEBERT,‡ and O. PELKONEN§

†Department of Pharmacology, University of Mainz, Germany, ‡Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20014, U.S.A., and §Department of Pharmacology, University of Oulu, Finland

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Abstract—The formation of benzo[a]pyrene metabolite–nucleoside adducts in perfused rat and mouse liver and in mouse lung slices was studied by Sephadex LH20 chromatography. In liver from β-naphthoflavone-pretreated rats, four different deoxyribonucleoside complexes were observed; these are tentatively attributed to DNA modification by the 7,8-diol-9,10-epoxide(s), secondary metabolites of benzo[a]pyrene quinones, the 4,5-oxide, and secondary metabolites of benzo[a]pyrene phenols. The diol-epoxide–deoxyribonucleoside adduct was also detected in perfused liver and in lung slices from 3-methylcholanthrene-treated genetically responsive C57BL/6N mice, whereas no adducts were detectable in such samples from 3-methylcholanthrene-treated genetically nonresponsive DBA/2N mice. In perfused liver of phenobarbital-pretreated rats, the 4,5-oxide-deoxyribonucleoside adduct was present. These results suggest that some of the benzo[a]pyrene metabolite–nucleoside complexes generated by microsomes and deproteinized DNA *in vitro* also occur in the intact rodent liver and lung tissues.

Furthermore, complexes with the diol-epoxide(s) were observed with RNA from perfused liver of β-naphthoflavone-treated, but not from untreated or phenobarbital-treated rats. Complexes between ribonucleoside(s) and the diol-epoxide(s) were also found in perfused liver or lung slices from genetically responsive but not from genetically nonresponsive mice.

The carcinogenic potential of polycyclic aromatic hydrocarbons is probably related to their capacity to modify cellular macromolecules by covalent binding following metabolic activation [1–3]. Analysis of deoxyribonucleosides from DNA which had been incubated *in vitro* with benzo[a]pyrene (BP) in the presence of microsomal preparations has revealed that several adducts with reactive BP metabolites can be formed [4–7]. It has been reported that the most potent mutagens derived from BP are the 7,8-diol-9,10-epoxides [8–10]. Deoxyribonucleoside complexes with these compounds have been shown to be the only detectable adducts which occur in a number of intact cell models such as mouse embryo cells [11], human bronchial explants [12] and mouse skin [13] after exposure to BP.

The liver has the highest capacity of all tissues to metabolize BP, although the liver itself is usually considered not to be susceptible to BP-induced cancer. It is conceivable, however, that significant numbers of reactive BP metabolites which bind covalently in the target organs of BP-induced cancer are actually produced *in vivo* by the liver. Formation of DNA-modifying BP metabolites by hepatic microsomes has extensively been studied with liver microsomes [4–7]. These studies, however, involved artificial conditions of microsomes and deproteinized salmon sperm DNA incubated *in vitro*.

It therefore would be of considerable interest to investigate the capacity of the liver *in vivo* to metabolize BP. The deoxyribonucleoside adduct pattern may be different from that produced *in vitro*, because potent conjugation pathways are operative; these conjugation reactions are missing in a microsomal preparation. Moreover, DNA in the nuclei of cells from intact liver differs from the *in vitro* deproteinized DNA preparations.

This communication describes the formation of DNA- and RNA-containing adducts in perfused livers from rats and from mice which are genetically responsive to inducers of cytochrome P_1–450 [14]. Further, the formation of the BP diol-epoxide adduct in lung slices from these mice is also demonstrated.

MATERIALS AND METHODS

Generally labelled [^{3}H]BP (40 Ci/m-mole) was obtained from Amersham Buchler (Braunschweig, Germany). Unlabelled BP obtained from Aldrich Europe was added to give a specific activity of 1.7 mCi/μmole in the liver perfusion experiments. BP was purified as described previously [15]. β-Naphthoflavone (BNF) was purchased from Aldrich Europe. RNase (Type II-A) was purchased from Sigma Chemical Co. (St. Louis, MO). The sources of all other reagents have been previously described [7].

Male Sprague–Dawley rats weighing 250 g received 1 × 80 mg BNF/kg dissolved in peanut oil 48 hr prior to killing or 3 × 80 mg phenobarbital (PhBarb)/kg dissolved in 0.90% NaCl at 24 hr intervals, the last injection given 24 hr prior to killing. Control rats received oil or 0.90% NaCl. 3-Methylcholanthrene (MC) in corn oil was administered at a dose of 80 mg/

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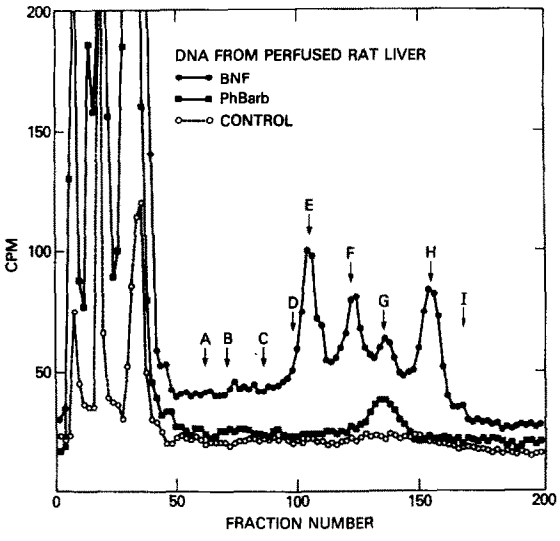

Fig. 1. Elution profile from Sephadex LH20 chromatography of hydrolyzed DNA from rat liver perfused with [^{3}H]BP. The perfusion was performed with an erythrocyte-containing medium for 1 hr in the presence of 100μM BP. Livers from single oil-treated (O), phenobarbital-treated (■), or BNF-treated (●) animals were examined. In this and in subsequent figures, the letters and arrows indicate the point of elution of the major peaks observed when BP is reacted with exogenous DNA in the presence of mouse liver microsomes [7]. These data represent single animals, although similar profiles were found in repeated experiments when other single animals had been similarly treated.

kg to C57BL/6N mice and DBA/2N mice 48 hr before killing.

Liver perfusions were performed for 1 hr with an erythrocyte-containing medium by the technique of Miller *et al.* [16] as modified by Hems *et al.* [17]. For incubation of lung slices, the lungs of 10–30 mice were pooled. Incubation was performed at 37° for 2 hr under an atmosphere of O_2/CO_2 (95:5). The isolation procedure for DNA and RNA was essentially that described by Kuroki and Heidelberger [18] as a modification of the procedure of Diamond *et al.* [19]. The nucleic acids were extensively washed with ethanol and ether. Hydrolysis of DNA and Sephadex LH20 chromatography of deoxyribonucleosides were performed according to Baird and Brookes [20], as described in detail in a preceding paper [7]. RNA was treated like DNA, except that RNase was used for the first digestion step.

RESULTS AND DISCUSSION

DNA in perfused rat or mouse liver or mouse lung slices. The deoxyribonucleoside complexes of BP metabolically activated by liver microsomes from polycyclic hydrocarbon-pretreated rats or mice can be resolved into at least nine peaks by Sephadex LH20 chromatography [6, 7]. These peaks are arbitrarily designated A (most polar) to I (least polar) and have been tentatively identified by the use of known BP metabolites [7] as described in Table 1. We have now investigated in liver perfusion experiments the formation of nucleoside adducts with metabolically activated BP by the intact liver. The Sephadex LH20 elution profile of the deoxyribonucleosides is given in Fig. 1 (rat liver) and Fig. 2 (mouse liver). No specific peak which can be attributed to any of the known adducts was detected in the livers of untreated rats or MC-treated D2 mice. A large amount of unidentified polar material is eluted with the first 50 fractions in all experiments; this may partially consist of incompletely digested oligonucleosides [7] but also probably represents binding of reactive BP metabolites to constituents of the DNA other than those eluting with fractions 50–200, e.g. binding to the phosphate groups.

With control rat liver (Fig. 1), no distinct peaks beyond fraction 50 were found. With phenobarbital pretreatment, however, only peak G—representing BP 4,5-oxide bound to nucleoside(s)—was detected. These data probably reflect the enhanced induction by phenobarbital of a form of P-450 other than P_1-450 and the preferential formation of the K-region oxide by this form of P-450 (discussed in [14]). In livers from BNF-treated rats, at least four different deoxyribonucleoside adducts were formed in comparable amounts of (peaks E, F, G, and H in Fig. 1). The 7,8-diol-9,10-epoxides of BP [5, 7, 22, 23] are the major contributing compo-

Table 1. Postulated identification of DNA-bound BP metabolites produced *in vitro* by mouse liver microsomes*

Peak	Fraction number at which peaks occur	Precursor	Ultimate active form of BP metabolite which is the major contributor to the peak [7, 21]
A	62	Dihydrodiol(s)	Unknown dihydrodiol oxide(s)
B	71	Quinone(s)	Quinone-oxide(s) (or quinone-derived free radicals)
C	86	Dihydrodiol(s)	Unknown dihydrodiol oxide(s)
D	98	Quinone(s)	Quinone-oxide(s) (or quinone-derived free radicals)
E	105	7,8-Dihydrodiol	7,8-Diol-9,10-epoxides (both *cis* and *trans*)
F	122	Quinone(s)	Quinone-oxide(s) (or quinone-derived free radicals)
F′	115	BP	7,8-Oxide
G	134	BP	4,5-Oxide
H	155	Phenol(s)	Unknown phenol oxide(s)
I	171	Quinone(s)	Quinone-oxide(s) (or quinone-derived free radicals)

* No attempt has been made to identify the nucleoside(s) bound covalently with these various BP metabolites. In the case of peak E, the 7,8-diol-9,10-epoxides are believed to bind predominantly to the 2-amino group of guanine, though other nucleosides may also participate to a small extent (< 10 per cent discussed in detail in [7] and [14]).

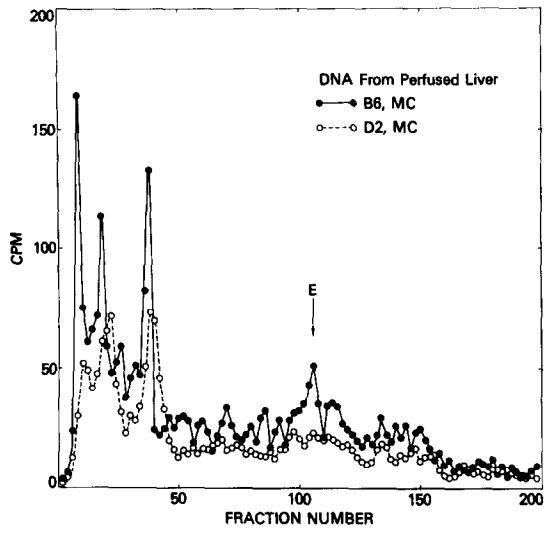

Fig. 2. Elution profile from Sephadex LH20 chromatography of hydrolyzed DNA from mouse liver perfused with [^{3}H]BP. The perfusion was performed with an erythrocyte-containing medium for 1 hr in the presence of 2.5 μM BP. Livers from single MC-treated B6 (●) or MC-treated D2 mice (O) were examined.

nents of peak E. These compounds are assumed to be the most important carcinogenic forms of BP [8–10, 24, 25]. This deoxyribonucleoside adduct has also been found under other *in vivo* conditions such as cell culture [11], tissue explants [12], BP-painted skin [13] and perfused rat lung [26, 27]. This adduct was also found in perfused liver (Fig. 2) and in lung slices (Fig. 3) from genetically responsive mice which had been pretreated with an inducer of cytochrome P_1-450, whereas no deoxyribonucleoside adducts could be detected in perfused liver or lung slices from genetically nonresponsive animals.

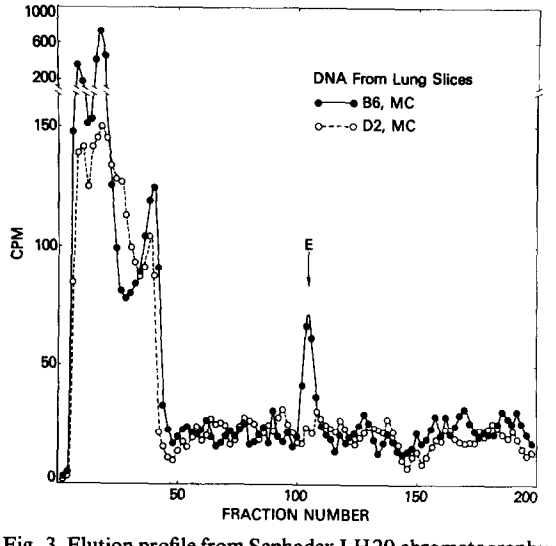

Fig. 3. Elution profile from Sephadex LH20 chromatography of hydrolyzed DNA from mouse lung slices incubated with [^{3}H]BP. In several experiments, lungs from 10 to 30 MC-treated B6 (●) or MC-treated D2 mice (O) were combined. The slices were incubated in the presence of 6.25 μM BP.

In perfused rat livers which had been exposed to higher BP concentrations (100 μM) than the perfused mouse livers (2.5 μM) and from which higher amounts of DNA could be obtained to be applied to the column, three peaks in addition to peak E were detected when the animals had been treated with an inducer of cytochrome P_1-450 (Fig. 1). Increases in the 4,5-oxide adduct represented by peak G were observed neither in previous *in vivo* studies nor *in vitro* under the conditions of cytochrome P_1-450 induction, though peak G does appear in the perfused liver of the BNF-treated rat. The major components contributing to peak H are further oxygenated metabolites of phenolic BP products (Table 1). This phenol oxide adduct is predominant *in vitro* when BP is metabolically activated by hepatic microsomes from polycyclic hydrocarbon-pretreated animals [5–7]. It has also been observed in perfused lungs from BNF-treated rats [26]. *In vivo*, however, secondary oxygenation of BP phenols does not occur so readily as *in vitro*, probably because of the action in intact liver of effective conjugating enzymes which rapidly remove the phenolic compounds. 9-Hydroxy-BP, the 4,5-oxide of which is assumed to be a principle component of peak H [7, 28], has been shown to be a good substrate for both glucuronidation [29] and sulfation [30, 31] *in vitro*. Removal of BP phenols appears to be relatively complete in control and phenobarbital-pretreated animals, but the large increase in the concentration of these compounds caused by BNF-induced cytochrome P_1-450 may exhaust the conjugation capacity of the organ.

The BP concentrations to which the human population is exposed are much lower than that used in the rat liver perfusion experiments and are also lower than the concentration used in the mouse liver perfusion experiments. Whereas the total amount of BP metabolites available for DNA modification will be lower in this situation, the proportion of secondarily oxygenated metabolites will be high as related to the amount of carcinogen available [32]. This may favor the formation of peak E, but possibly also may favor the formation of peaks F and H, all of which represent adducts with secondary metabolites of BP. The only metabolite undergoing a single monooxygenation by P-450 before binding covalently to DNA is the K-region epoxide adduct (peak G).

RNA in perfused rat or mouse liver or mouse lung slices. Figure 4 demonstrates the ribonucleoside adducts with BP metabolites formed in the perfused rat liver. Peaks E, G, and H are detectable in control liver. No specific peaks attributable to the known adducts can be separated clearly in the liver RNA digest from phenobarbital-pretreated rats. This result again suggests that phenobarbital-induced enhancement of the conjugation enzymes may be important. BNF treatment markedly increases total binding to rat liver RNA [15] and, accordingly, adducts are found. The main peak is the 7,8-diol-9,10-epoxide adduct. Peaks B and F are also detected; these peaks both have been attributed to nucleoside adducts with secondary metabolites from BP quinones (Table 1). In contrast to the results obtained with rat liver DNA, however, peaks G and H (representing the 4,5-oxide complex and the phenol oxide complex) cannot clearly be resolved. The

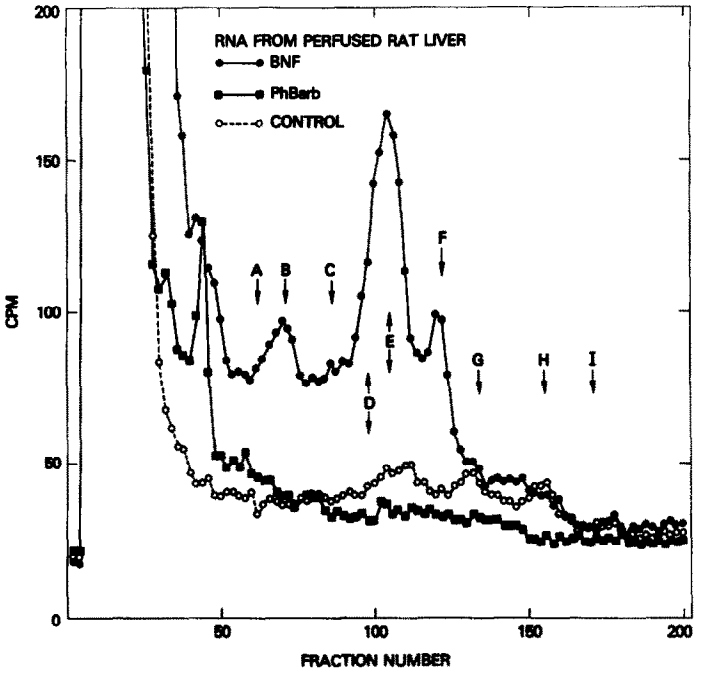

Fig. 4. Elution profile from Sephadex LH20 chromatography of hydrolyzed RNA from rat liver perfused with [^{3}H]BP. The perfusion was performed with an erythrocyte-containing medium for 1 hr in the presence of 100 μM BP. Livers from single oil-treated (O), phenobarbital-treated (■), or BNF-treated (●) animals were examined.

failure of RNA to bind these metabolites may be related to steric differences between the nucleic acids. Also, phenolic compounds formed by the action of the nuclear monooxygenase [33] may be less likely to be removed prior to secondary oxygenations than those formed in the endoplasmic reticulum.

The diol-epoxide adduct represented by peak E is also found in RNA from perfused liver (Fig. 5) and in lung slices (Fig. 6) from MC-treated genetically responsive mice. No specific peaks were resolved from the RNA digest of perfused liver and of lung slices from MC-treated genetically nonresponsive mice.

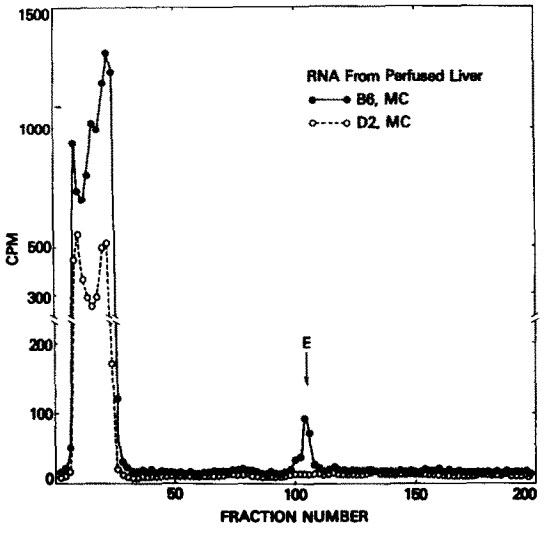

Fig. 5. Elution profile from Sephadex LH20 chromatography of hydrolyzed RNA from mouse liver perfused with [^{3}H]BP. The perfusion was performed with an erythrocyte-containing medium for 1 hr in the presence of 2.5 μM BP. Livers from single MC-treated B6 (●) or MC-treated D2 mice (O) were examined.

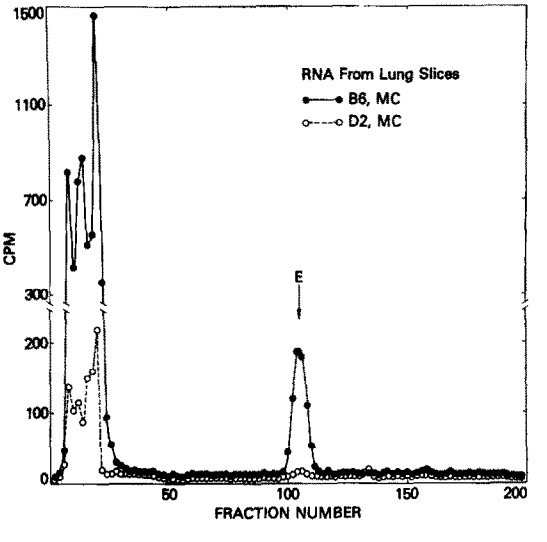

Fig. 6. Elution profile from Sephadex LH20 chromatography of hydrolyzed RNA from mouse lung slices incubated with [^{3}H]BP. In several experiments, lungs from 10 to 30 MC-treated B6 (●) or MC-treated D2 mice (O) were combined. The slices were incubated in the presence of 6.25 μM BP.

Possible relevance of DNA binding of BP metabolites to carcinogenesis. Studies with several inbred strains of mice [34, 35] have demonstrated that allelic differences at the *Ah* locus are associated with MC- and BP-initiated subcutaneous fibrosarcomas and MC-initiated pulmonary tumors. Recent data also indicate the importance of P_1-450-catalyzed BP activation to the ultimate carcinogen in mouse; however, the dose of intratracheal BP needed to initiate lung tumors is at least six times greater than the dose of intratracheal MC (R. E. Kouri, personal communication). The genetically responsive individual therefore has an increased risk for these types of chemically-induced cancers, most likely because tissues at the site of carcinogen exposure have higher induced aryl hydrocarbon hydroxylase activity and its associated cytochrome P_1-450, when compared with the same tissues from the genetically nonresponsive individual treated identically. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin has been shown [36] to be a cocarcinogen presumably because it induces P_1-450 in so-called genetically nonresponsive mice, thereby enhancing MC-initiated tumorigenesis. MC and BP are thus believed to be metabolized to ultimate carcinogenic forms in the mouse via quantitative increases and/or qualitiative changes in the forms of polycyclic hydrocarbon-induced P_1-450 (discussed in detail in [14]). All peaks except G (Table 1) are known to be associated with increased amounts of P_1-450 and, hence, the Ah^b allele [6].

It is well known that BP is a good carcinogen for lung tissue but almost never causes hepatomas. Since genetic differences in peak E can be shown for both perfused mouse liver and mouse lung slices, additional factors obviously must prevent tumorigenesis in the liver. From the similarity in DNA binding of BP metabolites (Figs. 2 and 3), these factors probably do not represent significant differences in the further metabolism of BP to glucuronide and sulfate conjugates, diols, quinones, etc. Cancer initiated by BP therefore may include P_1-450-mediated metabolism to reactive BP intermediates that attack a critical subcellular target, but there must exist additional important (tissue-specific) factors such as the rate of DNA repair, differences in immunosuppression, etc. It is interesting, however, that the ratio of peak E to the rest of the background radioactivity (fractions 50–200) is larger in lung (Fig. 3) than in liver (Fig. 2).

The data in this report suggest that the levels of induced P_1-450 in the genetically responsive animal—that appear to be causally related to BP tumorigenesis [14, 36]—are correlated with the larger amounts of DNA-bound diol-epoxides found in tissues of these cancer-prone individuals. It should be emphasized, however, that the amount of P_1-450-catalyzed metabolite of BP that is sufficient for binding to a particular "critical target" in the cell, thereby initiating cancer, may be extremely minute and represent less than 0.01 per cent of peak E or could be totally undetectable under these experimental conditions.

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