MOLECULAR PHARMACOLOGY, 14, 306-322

Tentative Identification of Benzo[a]pyrene Metabolite-Nucleoside Complexes Produced *in Vitro* by Mouse Liver Microsomes

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(Received July 27, 1977)
(Accepted November 25, 1977)

SUMMARY

PELKONEN, OLAVI, BOOBIS, ALAN R., YAGI, HARUHIKO, JERINA, DONALD M. & NEBERT, DANIEL W. (1978) Tentative identification of benzo[a]pyrene metabolite-nucleoside complexes produced *in vitro* by mouse liver microsomes. *Mol. Pharmacol.*, 14, 306-322.

When [^{3}H]benzo[a]pyrene is incubated *in vitro* together with deproteinized salmon sperm DNA, NADPH, and mouse liver microsomes, the covalent binding of benzo[a]pyrene metabolites to DNA occurs. The metabolite-nucleoside complexes can be resolved into at least nine distinct peaks by elution of a Sephadex LH-20 column with a water-methanol gradient. These peaks are arbitrarily designated A (most polar) through I (least polar). With the use of synthetic and biologically produced metabolites, seven of nine peaks are tentatively assigned to one or more metabolites of benzo[a]pyrene. Peaks A and C are unidentified. Peaks B, D, F, and I include products of benzo[a]pyrene quinones that are further metabolized. Peak E reflects almost exclusively both the *cis*- and *trans*-7,8-diol 9,10-epoxides of benzo[a]pyrene. Peak G represents predominantly the K-region metabolite (the 4,5-oxide), interacting with one or more nucleosides. Peak H comprises reactive intermediates resulting from the further metabolism of benzo[a]pyrene phenols. The 7,8-oxide and the 9,10-oxide contribute to peaks E, F, G, and H. Benzo[a]pyrene thus may be metabolized to four different "types" of reactive intermediates capable of binding to DNA: (a) primary arene oxides, (b) diol epoxides, (c) phenols oxygenated further, and (d) quinones oxygenated further (or quinone-derived free radicals). These last three types of microsomally activated intermediates are therefore the result of two- or three-step enzymatic processes in which cytochrome P-450-mediated monooxygenations occur at least twice.

Portions of this work were presented at the Symposium on Active Intermediates: Formation, Toxicity, and Inactivation, Turku, Finland, July 1975 (1).

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INTRODUCTION

Carcinogenic polycyclic hydrocarbons, such as the ubiquitous environmental contaminant BP,[3] probably are metabolized

[3] The abbreviations used are: BP, benzo[a]pyrene; BP 7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; BP 4,5-dihydrodiol, *trans*-4,5-

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as a requirement for the initiation of tumorigenesis. Although RNA or protein might be the critical intracellular target at which chemical carcinogenesis is initiated, considerable interest has centered on DNA and on chemicals that bind covalently to DNA as possibly important early events in the initiation of tumors (2). Certain arene oxides of BP, which presumably act as alkylating agents, are mutagenic in several systems (3–8), induce malignant transformation in rodent cell cultures (9), and are highly carcinogenic in the mouse (10, 11).

To understand further the aromatic hydrocarbon–nucleic acid reaction, Baird and Brookes developed a method (12) for the enzymatic degradation of nucleic acid containing bound carcinogens and the fractionation of the resulting mixture by Sephadex LH-20 column chromatography. This method has shown great promise, in that distinct peaks eluted from the column can be demonstrated to change in elution profile, depending on the carcinogen incubated with microsomes and cofactors, on whether rat liver microsomes or cells in culture are used (12–18), and on which microsomal inhibitor is added *in vitro* (19). The nature of carcinogenic metabolites (from, e.g., BP, 7,12-dimethylbenz[a]anthracene, and 7-methylbenz[a]anthracene) bound to DNA nucleosides has been studied not only by column chromatography (12–19) but also recently by high-pressure liquid chromatography (20–22).

The *Ah* locus (see refs. 1 and 23 for reviews) is a regulatory gene controlling the cytochrome P-450-mediated metabolism of BP and other chemical carcinogens to reactive intermediates. Allelic differences at this locus in the mouse are associated with an increased risk of chemical carcinogenesis, mutagenesis, toxicity, and teratogenicity caused by a wide variety of chemicals (23). It therefore would be interesting if an association could be found between genetic differences in biological activity (e.g., BP tumorigenesis) and the binding of a specific BP metabolite(s) to DNA *in vitro*. Are BP metabolite–nucleoside peaks that are detectable by the column chromatographic method of Baird and Brookes (12) similar between mouse and rat liver microsomes? The present report answers this question; with the use of known intermediates and products of BP metabolism, we are able to identify tentatively the major (and in some cases, minor) contributions to seven of the nine chromatographic peaks observed.

MATERIALS AND METHODS

Source of reagents. NADPH was purchased from Calbiochem; glucose 6-phosphate dehydrogenase (bakers' yeast, type VII), BP, DNase I (from beef pancreas), alkaline phosphatase (*Escherichia coli*, type III), the sodium salt of salmon sperm DNA (type III), and D-glucose 6-phosphate, from Sigma Chemical Company; MC, from Eastman; sodium dodecyl sulfate (specially pure), from BDH Chemicals; corn oil, Mazola brand, from Best Foods; venom phosphodiesterase (type II), from Worthington Biochemical Corporation; generally tritiated [^{3}H]BP (20 Ci/mmole), from Amersham/Searle; 4-(*p*-nitrobenzyl)pyridine, from Aldrich Chemical Company; Sephadex LH-20, from Pharmacia Fine Chemicals; and Aquasol, from New England Nuclear. Greater than 99.9% purity of [^{3}H]BP was found by thin-layer chromatography.

Treatment of animals. All animals used were sexually immature (4–6-week-old) female C57BL/6N, DBA/2N, or C3H/HeN mice. Animals were obtained from the Veterinary Resources Branch, National Institutes of Health, about 1 week before treatment. The mice were housed in plastic cages, up to six mice per cage, on

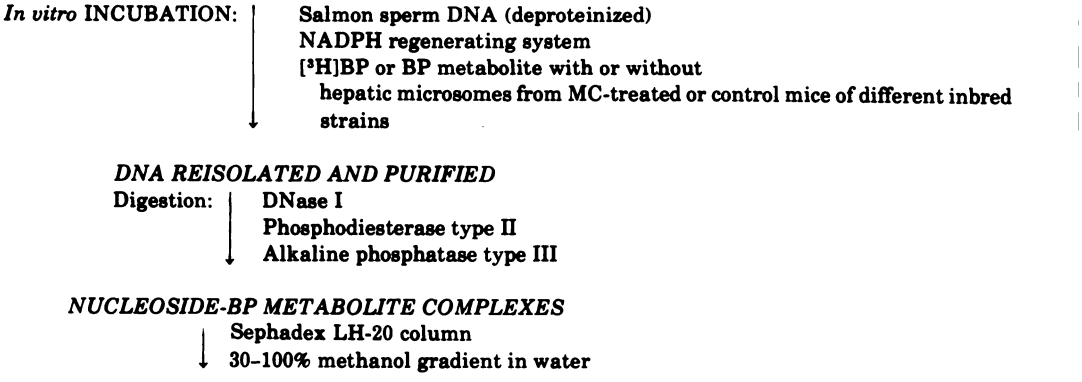
dihydroxy-4,5-dihydrobenzo[a]pyrene; BP 9,10-dihydrodiol, *trans*-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene; BP *cis*-7,8-diol 9,10-epoxide, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BP *trans*-7,8-diol 9,10-epoxide, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 3-HO-BP, 3-hydroxybenzo[a]pyrene; 6-HO-BP, 6-hydroxybenzo[a]pyrene; 9-HO-BP, 9-hydroxybenzo[a]pyrene; 1-HO-BP, 1-hydroxybenzo[a]pyrene; 7-HO-BP, 7-hydroxybenzo[a]pyrene; MC, 3-methylcholanthrene; TLC, thin-layer chromatography; B6, the responsive C57BL/6N inbred mouse strain; D2, the nonresponsive DBA/2N inbred mouse strain.

hardwood chip bedding in a controlled-environment animal room with controlled temperature and lighting (14 hr of light and 10 hr of darkness). The mice were permitted free access to food (Purina NIH open formula rat and mouse ration 5018) and tap water. MC treatment consisted of a single intraperitoneal dose (80 mg/kg of body weight) dissolved in corn oil 48 hr prior to killing.

Preparation of microsomes. The preparation of liver microsomes was carried out as previously described (24), except that the livers were homogenized with a Polytron tissue homogenizer (Brinkmann) for 30 sec at setting 5. The livers from six animals were pooled for each determination. The microsomal pellets were resuspended in 30% glycerol–0.25 M potassium phosphate buffer, pH 7.25, and the protein content was determined by the method of Lowry *et al.* (25), with crystalline bovine serum albumin as a standard.

Microsome-catalyzed binding of [^{3}H]BP to DNA. The method used (Scheme 1) was essentially that of Baird and Brookes (12). The deproteinized salmon sperm DNA (20 mg) was dissolved in 5 ml of water. The cofactor pool ($MgCl_2$, 25 μmoles; EDTA, 1 μmole; NADPH, 7.0 μmoles; glucose 6-phosphate, 100 μmoles; glucose 6-phosphate dehydrogenase, 3.3 units), the buffer (potassium phosphate, pH 7.5; final concentration, 0.1 M), microsomes (4 mg of protein), and sufficient distilled water were added to make a final volume of 10 ml. The reaction was started by adding 60 nmoles of [^{3}H]BP (1.19 mCi) in 200 μl of acetone. The reaction mixture was incubated in a shaking water bath at 37° for 30 min in subdued light. The reaction was stopped by placing the flasks on ice. The microsomal fraction was centrifuged again at 105,000 × *g* for 60 min.

Isolation and purification of DNA. After centrifugation the supernatant fraction was extracted three times with 10 ml of diethyl ether each time (1 min of shaking per extraction, the phases separated by centrifugation at 1500 rpm for 15 min). After the extractions 0.4 g of NaCl was added to each sample (approximately 10 ml), and the DNA was precipitated by the addition of 20 ml of 95% ethanol. The DNA was transferred to 20 ml of 95% ethanol and allowed to stand at −20° overnight. The next day the DNA was dissolved in 10 ml of water. After dissolution, 0.46 ml of 5% sodium dodecyl sulfate solution in 45% ethanol was added and the mixture was stirred for 1 hr. Then 0.32 g of NaCl was added, and the samples were centrifuged at room temperature at 15,000 × *g* for 60 min. After centrifugation the DNA was precipitated by the addition of 10.6 ml of 95% ethanol, stirred for 2–3 hr in 20 ml of 95% ethanol, rinsed in acetone, and dried in air overnight. The next day the DNA was dissolved in 10 ml of water, 0.08 g of NaCl was added, and the sample was centrifuged at 15,000 × *g* for 60 min at room temperature. After centrifugation



SCHEME 1

0.44 g of NaCl was dissolved, and the DNA was precipitated by the addition of 10 ml of 95% ethanol, washed in 20 ml of 95% ethanol with stirring for 2–3 hr, rinsed in acetone, and dried overnight in air. Only metabolites covalently bound to DNA nucleosides (12) remain after this extensive isolation and purification of DNA. With [7,10-^{14}C]benzo[a]pyrene in our earliest experiments, we found no evidence of significant tritium exchange, even after these rather extensive enzymatic digestions of the DNA.

Hydrolysis of DNA. The purified DNA was dissolved in 10 ml of 0.01 M Tris buffer, pH 7.0, containing 0.01 M $MgCl_2$. After dissolution, 260 Kunitz units of DNase I were added and the sample was incubated at 37° in a shaking water bath for 6 hr. Then 1 ml of 0.1 M Tris buffer, pH 9.0, and 10 μl of venom phosphodiesterase were added, and the samples were incubated for 44–50 hr. Following this, 2.5 units of alkaline phosphatase were added and the samples were incubated for 48 hr. The hydrolyzed samples were stored at −20° until chromatography.

Column chromatography. The total DNA sample was thawed, lyophilized, dissolved in 4.4 ml of 30% methanol, filtered, and applied to a Pharmacia 90 × 1.5 cm column packed to a height of 80 cm with Sephadex LH-20 suspended in methanol and washed with 90% methanol (400–500 ml) and 30% methanol (400–500 ml). The elution of material from the column with 30% methanol in water–100% methanol involved a gradient of two eluting solvents: the initial solvent was prepared by adding 150 ml of methanol to 350 ml of water, and the second solvent was 800 ml of absolute methanol. The ultraviolet marker 4-(*p*-nitrobenzyl)pyridine (0.1 ml of a 500 μg/ml methanol solution) was added to the material placed on the column, and the positions of the BP-nucleoside peaks were standardized with this marker. Fractions (5.1 ml) were collected on an LKB Ultrorac fraction collector, type 7000, with an LKB type 8300 A Uvicord II monitoring the absorption of the effluent at 254 nm.

Measurement of radioactivity. One-milliliter portions from alternate fractions were added to 10 ml of Aquasol and counted on a Packard Tri-Carb model 3375 liquid scintillation spectrometer. The efficiency of tritium counting was found to be about 30%; 0.1-ml portions were taken from dissolved lyophilizates, mixed with 1 ml of "NCS" tissue solubilizer (Amersham/Searle) and 10 ml of Aquasol, and counted. After the lyophilizates were filtered, the filter paper was also added to the 10 ml of Aquasol before counting.

Preparation of Metabolites of BP

TLC-[^{3}H]BP-phenols and TLC-[^{3}H]BP-quinones. Ten millicuries of [^{3}H]BP were incubated in the same mixture as that used for binding studies, except that the DNA was omitted. After a 40-min incubation of the 10-ml reaction mixture, 10 ml of acetone were added and the mixture was extracted with three successive 10-ml portions of ethyl acetate. The pooled organic extracts were dried with Na_2SO_4, filtered, and evaporated under a stream of nitrogen. The residue was chromatographed on a 0.25-mm silica gel thin-layer chromatographic plate with benzene, followed by benzene–ethanol (19:1, v/v). The bands containing the various metabolites were separately extracted into methanol and purified by further chromatography. The recovered metabolites—which originally were identified by comparing their R_F values with co-chromatographed standards—were further identified by their ultraviolet spectra in methanol (26). Yields were about 10 nmoles for BP phenols and about 20 nmoles for BP quinones when 500 nmoles of BP had been used as the starting material.

Other metabolites. [^{3}H]BP 7,8-dihydrodiol (27), [^{3}H]BP 7,8-oxide (28), [^{3}H]BP 9,10-oxide (28), BP 4,5-oxide (29), and both *cis*- and *trans*-7,8-diol 9,10-epoxides (30) were prepared as described in the references cited.

Binding of [^{3}H]BP metabolites to DNA. BP 4,5-oxide, [^{3}H]BP 7,8-oxide, [^{3}H]BP 9,10-oxide, and both *cis*- and *trans*-7,8-diol 9,10-epoxides were allowed to react separately with DNA in the absence of liver microsomes and the NADPH-regenerating

system. The incubation mixture consisted of 20 mg of DNA in 20 ml of 0.1 mM potassium phosphate buffer, pH 7.5, and the BP derivative added in either acetone or tetrahydrofuran-NH_4OH (1000:1, v/v). Acetone (7 ml) was added in order to prevent precipitation of the compound. After a 24-hr incubation, the residual acetone was removed by vacuum and the incubation mixture (approximately 10 ml) was extracted three times with 20 ml of water-saturated ethyl acetate each time; 0.32 g of NaCl was added after the extraction, and the DNA was precipitated by ethanol as described above. The further procedure was identical with that for [^{3}H]BP.

RESULTS

Requirement for mouse liver microsomes in formation of BP metabolite–nucleoside complexes. The typical profile of radioactivity eluted from the Sephadex LH-20 column depends upon the presence of mouse liver microsomes in the incubation mixture (Fig. 1). Between fractions 40 and 200, nine reproducible peaks were observed. The positions of these nine peaks (designated in Fig. 1 as A through I) were normalized using the ultraviolet marker 4-(*p*-nitrobenzyl)pyridine, which eluted maximally at fraction 67: peak A was centered at fraction 62; B, at 71; C, at 86; D, at 98 (usually a shoulder on the much larger peak E); E, at 105; F, at 122; G, at 134; H, at 155; and I, at 171. Peaks E and H [which correspond to peaks A and D named by Brookes and co-workers in studies with rat liver microsomes or cell culture (7)] were particularly large (Fig. 1) with microsomes from the genetically responsive MC-treated B6 mouse. The reproducibility of these maxima was excellent, and we are confident in distinguishing separate peaks (provided they are of similar height) as little as four fractions apart. Incubation of DNA and BP without microsomes or incubation of BP or metabolically produced BP metabolites with boiled microsomes produced no peaks between fractions 40 and 200, although there was some radioactivity in fractions 6–25 (Fig. 1). These data indicate that peaks A through I indeed represent microsomally catalyzed BP metabolite–nucleoside complexes rather than BP metabolites physically trapped in or adhered to nucleosides.

Nature of radioactivity eluting early. In every incubation with BP or its derivatives there were several (often quite large) peaks eluting between fractions 6 and 25. This material comprised 23–30% of the total radioactivity eluted from the column in all our experiments with microsomes present. To understand further the nature of this radioactivity, we pooled these fractions, concentrated them by evaporation, and subjected them to the same enzymatic hydrolysis a second time (Fig. 2). A small amount of this radioactivity (13–17% in three similar experiments) now eluted into the same nine peaks as had been seen for the DNA hydrolyzed the first time. Other experiments, in which pooled early fractions were subjected to hydrolysis with 0.3 M KOH and alkaline phosphatase, failed to liberate these nine peaks further. Incubations with BP with or without microsomes (Fig. 1) and incubations with previously generated BP metabolites in the presence of boiled microsomes (data not shown) revealed that a large amount (more than 90%) of the early peaks (fractions 6–25) was dependent on microsomal metabolism. Thus part of the material coming off the column early appears to represent BP metabolites bound to oligonucleotides incompletely hydrolyzed by the first procedure. Possibly BP metabolites already bound to DNA are oxygenated a second time and become attached to a second base, thereby forming metabolite-dinucleoside complexes that are eluted from the column in early fractions. The radioactivity eluting early may also represent, in part, BP metabolites physically trapped in or adherent to oligonucleotides or simply metabolites eluted in this region. BP metabolites also may bind to phosphate groups and elute in these early fractions. These early peaks might also represent, in small amounts, contaminating BP metabolite–protein complexes left behind after the sodium dodecyl sulfate–salt procedure.

In the remainder of this report, most of

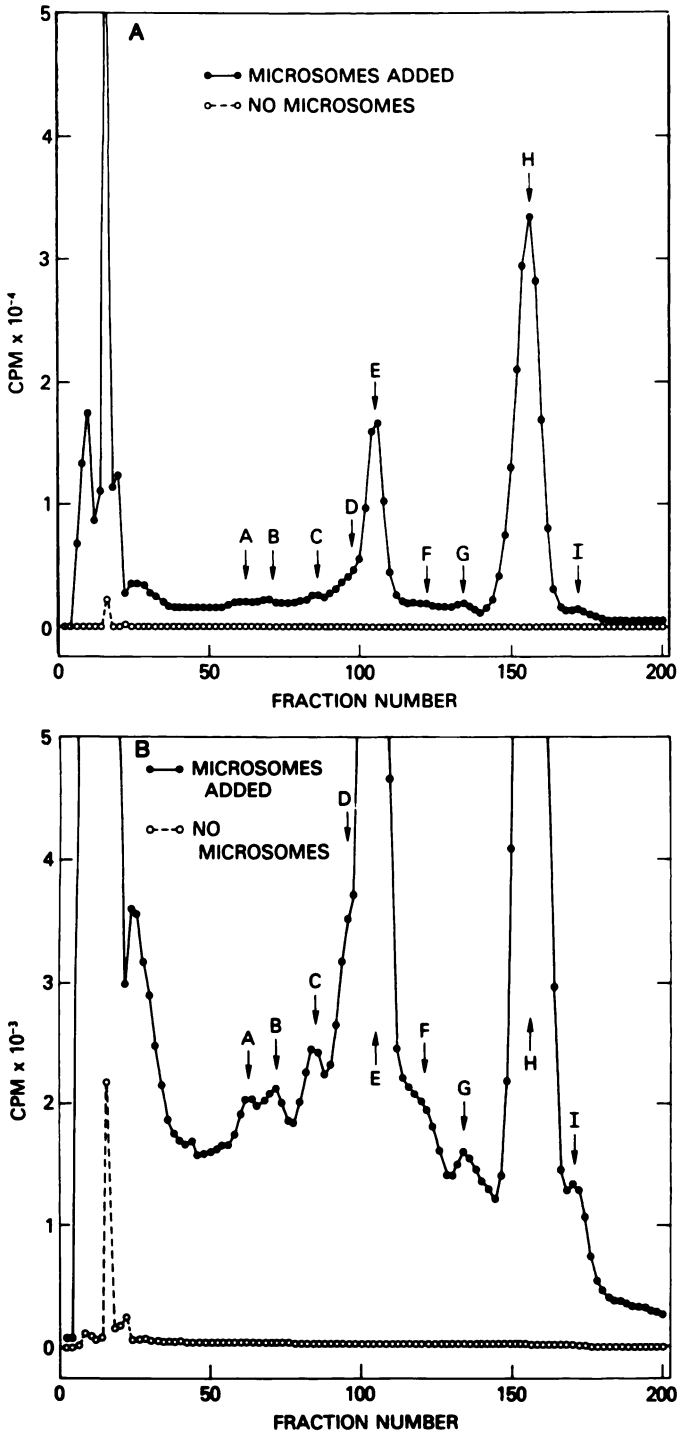


FIG. 1. *Sephadex LH-20 column chromatogram of enzyme digest of DNA with [³H]BP metabolites bound during incubation in vitro with or without hepatic microsomes from MC-treated B6 mice*

The hepatic microsomal fraction was prepared from livers combined from six mice, as described under MATERIALS AND METHODS. Fractions of 5.1 ml were collected. Radioactivity (in counts per minute) was determined in 10 ml of Aquasol for 1-ml portions of alternate fractions. The ordinate in Fig. 1B is a 10-fold expansion of the ordinate (from the same experiment) in Fig. 1A.

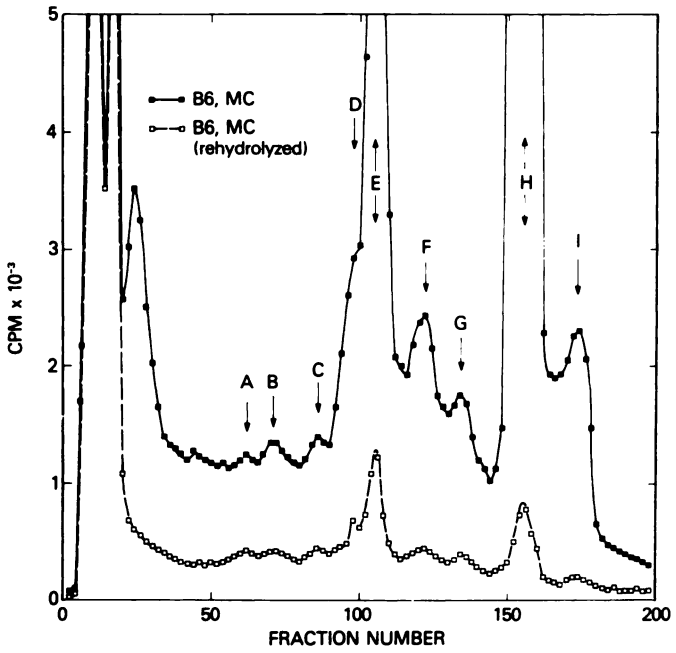


FIG. 2. *Sephadex LH-20 column chromatograms of enzyme digests of DNA with [³H]BP metabolites bound during incubation in vitro with hepatic microsomes from MC-treated B6 mice and an enzyme digest of pooled fractions 5–35 from the same experiment (B6, MC, rehydrolyzed)*

The experimental protocol was identical with that in Fig. 1 and is described under MATERIALS AND METHODS.

the nine peaks were tentatively identified by studying the interaction of reactive BP oxides or diol epoxides with DNA in the absence of microsomes and by studying the further microsomal metabolism of BP diols, phenols, or quinones to reactive intermediates that bind to DNA.

Binding of BP oxides to DNA in absence of microsomes and NADPH. Since BP 4,5-oxide was available only with low levels of radioactivity, we examined binding by this compound spectrophotometrically (Fig. 3A). Incubation of the 4,5-oxide with DNA yielded an adduct absorbing maximally at 272.5 nm. This optical maximum was used to determine that peak G alone contains the 4,5-oxide bound to a nucleoside(s) (Fig. 3B).

The reaction of BP 7,8-oxide with DNA in the absence of microsomal metabolism (Fig. 4A) gave rise to two peaks with maxima at fractions 105 and 118. The former peak corresponds to peak E. The latter peak consistently eluted earlier than peak F, and we refer to this adduct as peak F′. We believe that peak F′ is clearly distinct from peak F. We have found (19) that the presence of the epoxide hydrase inhibitor cyclohexene oxide *in vitro* appears to produce peak F′. The reaction of BP 9,10-oxide with DNA in the absence of microsomal metabolism (Fig. 4B) gave rise to three peaks: one corresponding to peak F; a second, consistently eluted two or three fractions earlier than peak G; and a third, corresponding to peak H. It should be noted that the amount of 7,8-oxide or 9,10-oxide binding to nucleosides is quite small, compared with that of the 4,5-oxide.

Binding of BP diol epoxides to DNA in absence of microsomes and NADPH. Diol epoxide adducts eluted from the Sephadex LH-20 column were monitored by ultraviolet absorption. The nucleoside adduct(s) obtained by allowing BP *cis*-7,8-diol 9,10-epoxide to react with deproteinized DNA *in vitro* (Fig. 5A) or with polyguanylic acid (data not illustrated) gave optical peaks at about 268, 278.5, 315, 329, and

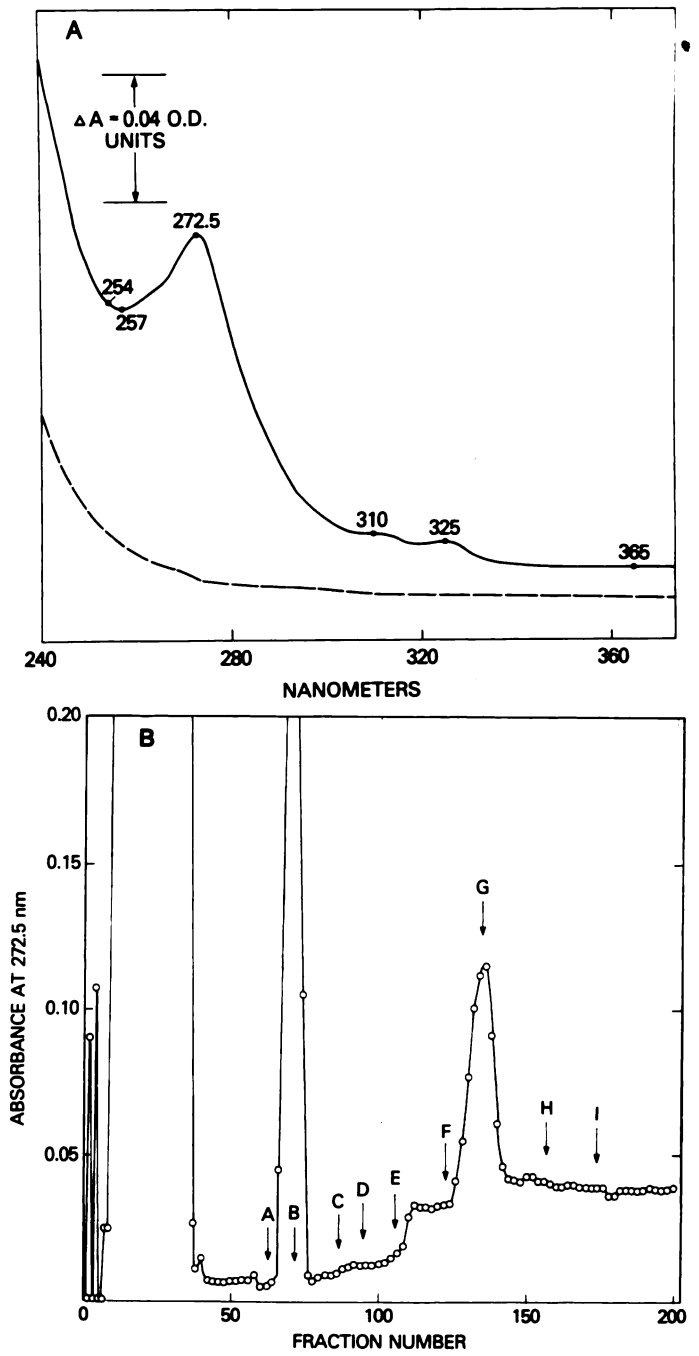


FIG. 3. *Spectrophotometric and chromatographic examination of the BP 4,5-oxide bound to DNA nucleoside(s).*

A. Absorption spectrum (——) after chromatography, of the digest of DNA which had been allowed to react *in vivo* with BP 4,5-oxide in the absence of the NADPH-regenerating system and hepatic microsomes. Twenty milligrams of DNA were incubated in 20 ml of 0.1 mM potassium phosphate buffer, pH 7.5, at 37° in the dark with 3.8 μmoles of BP 4,5-oxide for 24 hr. The incubation mixture was then extracted three times with 20 ml of water-saturated ethyl acetate each time. The DNA was reisolated, purified, hydrolyzed enzymatically, and chromatographed as described under MATERIALS AND METHODS. The control spectrum (- - -) represents the ultraviolet blank from a fraction eluted from a freshly packed column. Both spectra were obtained from fraction 134 (see Fig. 3B), with a blank of 30% methanol in water in the reference cuvette.

B. Sephadex LH-20 column chromatogram of the digest of the same material from which the sample examined spectrophotometrically in Fig. 3A was obtained. The nine arrows, A through I, denote the nine positions where the nucleoside-BP metabolite complexes appear (as shown in Fig. 1). The large peak centered around fraction 67 represents the ultraviolet marker 4-(*p*-nitrobenzyl)pyridine.

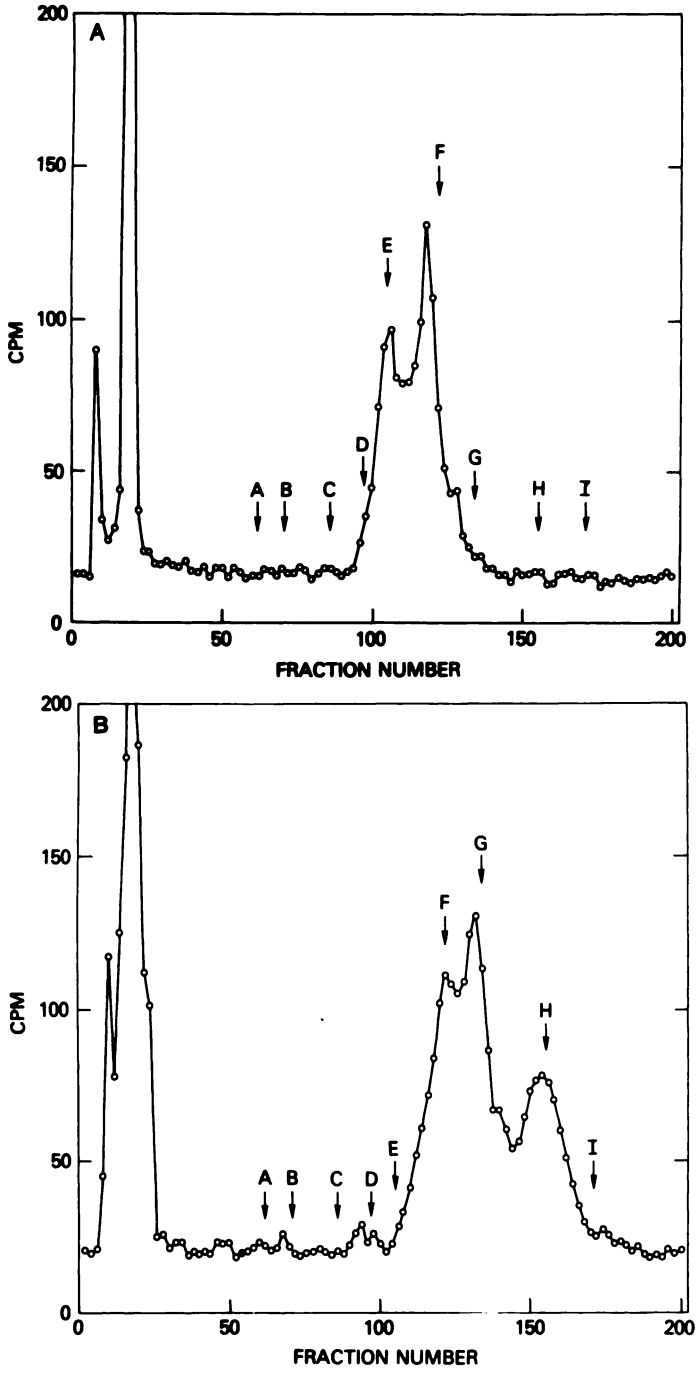


FIG. 4. *Sephadex LH-20 column chromatogram of enzyme digest of DNA that had reacted in vitro with [^{3}H]BP 7,8-oxide (A) or [^{3}H]BP 9,10-oxide (B) in the absence of the NADPH-regenerating system and hepatic microsomes*

The amount of BP 7,8-oxide added was 6 μmoles (about 65 μCi). The quantity of BP 9,10-oxide added was 1.9 μmoles (about 47 μCi). The experimental protocol was similar to that in Fig. 3, except that the amount of BP metabolite-nucleoside complexes in each fraction was determined by liquid scintillation counting, as described under MATERIALS AND METHODS.

344.5 nm. Both BP *cis*-7,8-diol 9,10-epoxide and BP *trans*-7,8-diol 9,10-epoxide (data not shown) behaved similarly.

Figure 5B compares radiometric analyses with spectrophotometric analyses of fractions eluted from the column. The broad absorption peak at 254 nm (between fractions 111 and 198) is the ultraviolet blank for the gradient and was also found with newly packed columns to which no nucleosides or BP metabolites had been added. It is noteworthy that whereas peak E (determined by radioactive samples) was maximal in fraction 105, the 254-nm absorption was maximal in fraction 107 and the $\Delta A_{344-365}$ was maximal in fraction 109 of Fig. 3B. The radioactive peak therefore does not coincide exactly with the absorption peak at 254 nm, and neither of these maxima corresponds exactly with the maximal $\Delta A_{344-365}$, which represents the nucleoside complex with BP *cis*-7,8-diol 9,10-epoxide. That the radioactive peak does not coincide exactly with the spectrophotometrically detected peak may reflect an "isotope effect."[4] These data suggest that the absorption at 254 nm is insufficient as a method for determining BP metabolite–nucleoside complexes and that the radioactive peak E generated by microsomes (Fig. 1) comprises more than a single complex between nucleoside and the *cis*- or *trans*-diol epoxide. Hence peak E may also contain complexes between nucleoside(s) and other BP metabolites (as will be shown below). The same results shown in Fig. 5B with BP *cis*-7,8-diol 9,10-epoxide were also found with BP *trans*-7,8-diol 9,10-epoxide (data not illustrated).

It has been suggested (17) that the "diol epoxide peak" (i.e., peak E) contains some BP not covalently bound to nucleosides. We did not find this to be the case, since the incubation of BP or microsomally produced BP products in excessive amounts with heat-denatured microsomes failed to produce any peaks between fractions 40 and 200.

[4] A tritiated 7,12-dimethylbenz[a]anthracene metabolite was recently shown (20) to be slightly delayed in its elution from a high-pressure liquid chromatographic column, compared with the same metabolite not containing tritium.

Binding of BP 7,8-dihydrodiol to DNA requires microsomal metabolism. The radioactive 7,8-dihydrodiol was allowed to react with NADPH and hepatic microsomes from various control and MC-treated inbred mouse strains (Fig. 5C). Only peak E was found, regardless of whether MC-treated or control mice were used or which strain of mouse was used. No significant peaks were found when BP 7,8-dihydrodiol was incubated with DNA in the absence of microsomes. Peak E was larger in MC-treated genetically responsive B6 or C3H/HeN than in control D2 or C3H/HeN mice. The further metabolism of BP 7,8-dihydrodiol to the 7,8-diol 9,10-epoxides [which then react with DNA nucleoside(s) to form peak E] therefore may occur more readily via P_1-450 than P-450.[5] This hypothesis is supported by a recent study (27) comparing the further metabolism of the 7,8-dihydrodiol to the 7,8-diol 9,10-epoxides in liver microsomes from control and MC- and phenobarbital-treated rats: such metabolism proceeded almost 4 times more readily in MC-treated rats than in phenobarbital-treated rats.

Binding of BP phenols and quinones to DNA requires microsomal metabolism. When a phenolic fraction of BP prepared by thin-layer chromatography was incubated with NADPH, DNA, and microsomes from MC-treated B6 mice (Fig. 6A),

[5] The nomenclature for various forms of cytochrome P-450 is currently inadequate, in view of four or more forms distinguishable by electrophoretic (31) or immunochemical (32) techniques. Better understanding of chemical and catalytic properties (33–36) should permit, in time, a more suitable nomenclature to be devised. In this report "P-450" in the general sense denotes all forms of CO-binding hemoproteins associated with membrane-bound, NADPH-dependent monooxygenase activities. P_1-450 is defined as that form(s) of cytochrome increased during polycyclic aromatic inducer treatment and concomitantly associated with induced aryl hydrocarbon hydroxylase activity. Recent data in rabbit (37), rat, and mouse (T. M. Guenthner and D. W. Nebert, manuscript in preparation) liver indicate that P_1-450 is distinctly different from the polycyclic aromatic-inducible form(s) of hemoprotein causing the spectral shift to about 448 nm when reduced and combined with CO (i.e., cytochrome P-448).

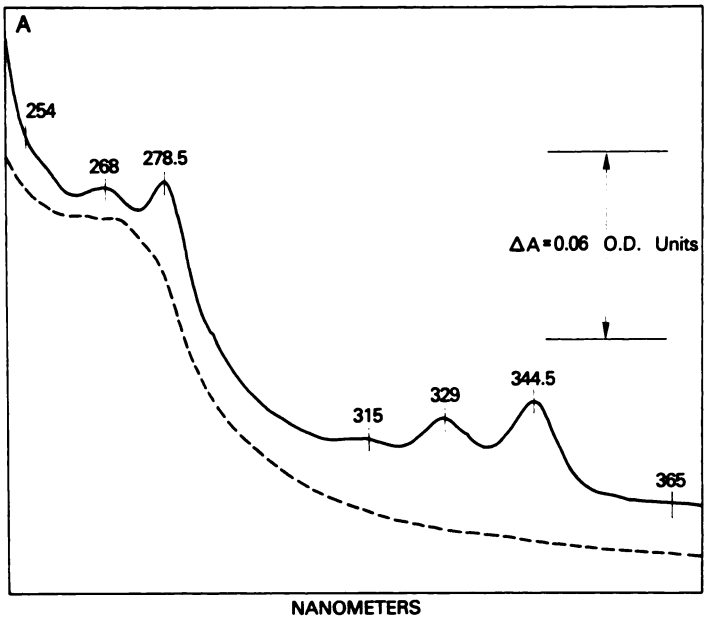


FIG. 5. *Spectrophotometric and chromatographic examination of the BP cis-7,8-diol 9,10-epoxide bound to DNA nucleoside(s).*

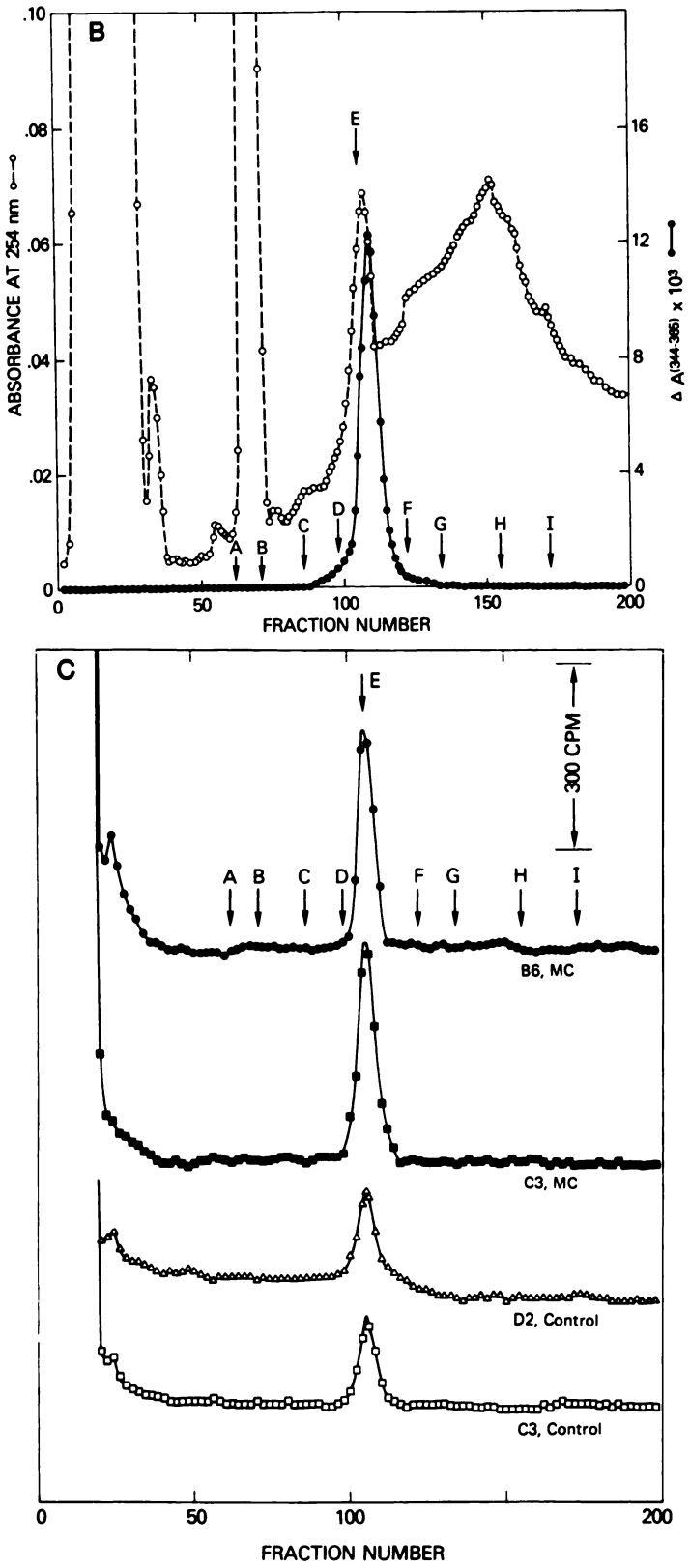
A. Absorption spectrum (——) after chromatography, of the digest of DNA which had been allowed to react *in vitro* with BP *cis*-7,8-diol 9,10-epoxide in the absence of the NADPH-regenerating system and hepatic microsomes. Twenty milligrams of DNA were incubated in 20 ml of 0.1 M potassium phosphate buffer, pH 7.5, at 37° in the dark. Six additions of 1.65 mg of the diol epoxide in 0.5 ml of tetrahydrofuran were made over a 6-hr period; this amounts to a total of approximately 33 μmoles of the diol epoxide. Sufficient acetone was added during the course of the 6 hr to prevent precipitation. The DNA was incubated further for 18 hr at 37° and then extracted three times with 20 ml of water-saturated ethyl acetate each time. The DNA was subsequently precipitated, purified, hydrolyzed enzymatically, and chromatographed as described under MATERIALS AND METHODS. The control spectrum (- - -) represents the ultraviolet blank from a fraction eluted from a freshly packed column. The experimental spectrum (——) was obtained from fraction 109 (see Fig. 5B), with a blank of 30% methanol in water in the reference cuvette. Very similar results were found with the *trans*-7,8-diol 9,10-epoxide.

B. Sephadex LH-20 column chromatogram of the digest of the same material from which the sample examined spectrophotometrically in Fig. 5A was obtained. ●, $\Delta A_{344-365}$, which is the peak-to-trough difference of the absorbance spectrum from samples containing the nucleoside–diol epoxide complex. Very similar results were found with the *trans*-7,8-diol 9,10-epoxide. ○, absorbance at 254 nm. The large peak centered around fraction 67 represents the ultraviolet marker 4-(*p*-nitrobenzyl)pyridine.

C. Sephadex LH-20 column chromatogram of an enzyme digest of DNA with bound metabolites of [^{3}H]BP 7,8-dihydrodiol (52 μCi/μmole) during incubation *in vitro* with hepatic microsomes from MC-treated B6 or C3H/HeN (C3) or control D2 or C3 mice, respectively. The further procedure was identical with that in Fig. 1 and is described under MATERIALS AND METHODS.

a large peak H and a small peak E were seen. If microsomes from MC-treated D2 mice were used instead, peak H was less than half as large and peak E was insignificant. No significant peaks were found in fractions 40–200 if the BP phenols were incubated with DNA in the absence of microsomes.

When a quinone fraction of BP prepared by thin-layer chromatography was incubated with NADPH, DNA, and microsomes from MC-treated B6 mice (Fig. 6B), peaks corresponding quite well to D, F, H, and I occurred. Peak H in Fig. 6B may represent a contamination of this quinone sample (prepared by thin-layer chromatography) with BP phenols. With microsomes from MC-treated D2 mice, all four of these peaks (but especially D and H) were diminished and a peak centering at fraction 74 was found. This peak in the region of peak B found only in D2 mice is


B
ABSORBANCE AT 254 nm
Δ A(344-365) x 10³
FRACTION NUMBER
A
B
C
D
E
F
G
H
I
C
300 CPM
B6, MC
C3, MC
D2, Control
C3, Control
FRACTION NUMBER

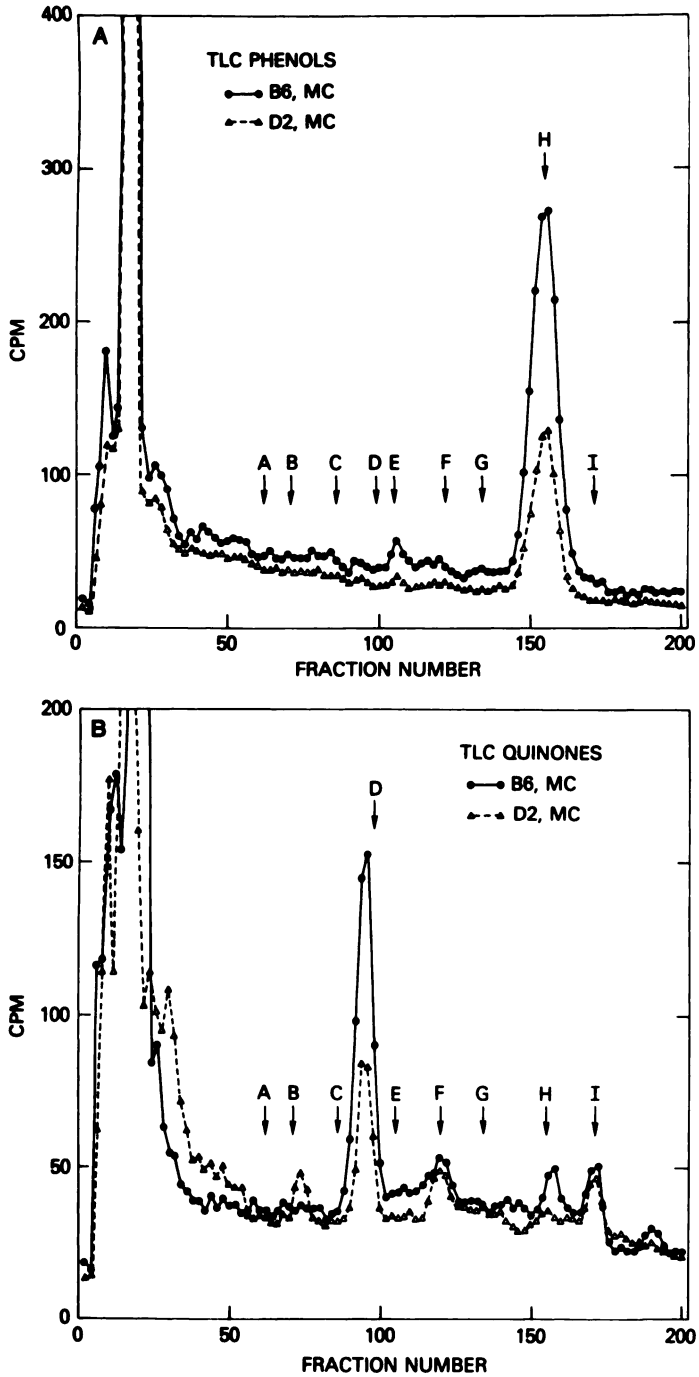


FIG. 6. *Sephadex LH-20 column chromatogram of enzyme digest of DNA with bound metabolites of TLC-BP phenols (A) and TLC-BP quinones (B) during incubation in vitro with hepatic microsomes from MC-treated B6 or D2 mice*

The amounts of BP derivatives added were as follows: TLC-BP phenols, 2 nmoles (23 μCi); TLC-BP quinones, 4.9 nmoles (49 μCi). The experimental protocol was identical with that in Fig. 1 and is described under MATERIALS AND METHODS.

puzzling. How the relatively polar peak B and the relatively nonpolar peak I can both be derived from the further metabolism of quinones is not understood. A second hydrolysis of fractions 1–25 from the material shown in Fig. 6A and B produced a small amount of radioactivity eluting with the same profile as that shown in Fig. 6A and B (data not illustrated). No significant peaks were found in fractions 40–200 if the BP quinones were incubated with DNA in the absence of microsomes.

Relative amounts of BP metabolites bound to DNA. We attempted to quantify (Table 1) the amounts of various BP metabolites that bind to DNA, in terms both of picomoles and of percentage of the compound added *in vitro*. BP 4,5-oxide and the 7,8-diol 9,10-epoxides of BP were readily detectable spectrophotometrically, whereas we were unsuccessful in detecting spectrophotometrically BP 7,8-oxide or BP 9,10-oxide complexes with nucleoside(s). We thus conclude that the K-region oxide and the diol epoxides of BP represent the greatest quantities of the metabolic intermediates that bind covalently to DNA nucleoside(s). Among the dihydrodiols, phenols, and quinones (requiring further microsomal metabolism before binding to DNA occurs), BP 7,8-dihydrodiol was bound in by far the largest amount, followed by the phenols; the quinones bound only to a very minor extent. This observed high amount of covalent binding of the 7,8-dihydrodiol (38) to DNA *in vitro* was of course the finding that first led to postulating the BP 7,8-diol 9,10-epoxide (16).

DISCUSSION

The data in this report with mouse liver microsomes confirm and extend the earlier work from several other laboratories that have examined the binding of metabolites to DNA nucleosides following metabolism of BP by rat liver microsomes *in vitro* or by cells in culture. Five major peaks shown previously (17) have been tentatively identified as one or more nucleosides complexed with BP 4,5-oxide (17), the 7,8-diol 9,10-epoxides (16, 39), and presumably 9-HO-BP 4,5-oxide (40). Recent evidence (41) has also suggested that BP quinones, BP 9,10-dihydrodiol, and BP 4,5-dihydrodiol are further metabolized to intermediates that react with nucleoside(s). These complexes, however, are present in low amounts.

It has become increasingly evident (see discussions in refs. 1, 23, and 36) that different forms of P-450 generate from one substrate different metabolite profiles. Hence forms of hepatic P-450 from control or phenobarbital-treated rats oxygenate BP to a greater extent in the K-region, compared with non-K-region oxygenation, and P_1-450 induced by MC oxygenates BP to a greater extent in the non-K-region, compared with K-region oxygenation (23, 42). The form of P-450 that predominates

TABLE 1

Relative amounts of binding of BP metabolites to DNA with or without MC-treated B6 microsomes and NADPH

The amounts of metabolites bound were estimated by determining the total radioactivity eluted from fractions 50–200, except in the case of nonradioactive 4,5-oxide and *cis*-7,8-diol 9,10-epoxide, which were estimated with the use of established ultraviolet extinction coefficients (29, 30).

BP Metabolite	Addition of microsomes to incubation mixture	Amount added to incubation mixture	Estimated binding of metabolite(s) to nucleoside(s)	
			Amount bound to DNA	Percentage of metabolite(s) added
		nmoles	*pmoles*	%
4,5-Oxide	No	3,800	29,000	0.76
7,8-Oxide	No	6,000	1,400	0.025
9,10-Oxide	No	3,800	525	0.03
cis-7,8-Diol 9,10-epoxide	No	33,000	179,000	0.54
7,8-Dihydrodiol	Yes	60	432	0.72
TLC-BP phenols	Yes	2	2.3	0.11
TLC-BP quinones	Yes	4.9	0.9	0.02

in control and especially polycyclic hydrocarbon-treated cells in culture is P_1-450 (43). It is therefore not surprising to find peak E (the BP 7,8-diol 9,10-epoxide–nucleoside complex) and peak H (BP phenols metabolized further) as the two major peaks when DNA has reacted with MC-induced P_1-450 from animal liver (Fig. 1) or skin or with polycyclic hydrocarbon-treated cells in culture (17, 39, 40), because these adducts comprise BP metabolites in which principally non-K-region oxygenation has occurred. Only a few BP metabolites undergoing a single oxygenation bind to DNA nucleosides in significant amounts. Compared with BP 4,5-oxide, the 7,8-oxide and the 9,10-oxide are both low in amount (Table 1), and phenols and quinones are unreactive unless further metabolized. As substrates, BP 4,5-oxide, 7,8-oxide, and 9,10-oxide are all converted to their respective dihydrodiols by epoxide hydrase at approximately equal rates (43). If epoxide hydrase becomes inhibited in control or phenobarbital-treated mice, therefore, we predict that BP 4,5-oxide binding to DNA (peak G) will increase. If epoxide hydrase becomes inhibited in genetically responsive MC-treated mice, however, we suggest that binding of BP 7,8-oxide (peaks E and F′) and BP 9,10-oxide (peaks F, G, and H) to DNA would be enhanced. We have experimental data (19) in support of these predictions.

The further metabolism of phenols to intermediates reacting with DNA (Fig. 6A) is consistent with other studies suggesting that 9-HO-BP (40) and 3-HO-BP (44) are metabolized to reactive intermediates that bind to DNA nucleosides. In an experiment with [^{14}C]BP and [^{3}H]BP together (45), the phenol-related peak had an isotope ratio similar to the 9-HO-BP product, whereas the 3-HO-BP product lost about 30% of its tritium; these data thus suggested that peak H is formed predominantly by the further metabolism of 9-HO-BP rather than 3-HO-BP. Supportive data have been obtained via experiments with UDP glucuronosyltransferase,[6] for which 9-HO-BP is a considerably better substrate than 3-HO-BP. The role of other physiologically produced phenols, 1-HO-BP and 7-HO-BP (46), in the further metabolism and subsequent binding to DNA is probably negligible.

Peak D was the main peak representing the further metabolism of quinones to reactive intermediates interacting with DNA. What these reactive intermediates are remains unknown, but at least two different types of reactions are conceivable: quinone oxides and quinone-derived free radicals (47). The finding that the further metabolism of the BP phenols or the BP quinones is greater in microsomes from MC-treated B6 mice than MC-treated D2 mice (Fig. 6) suggests that P_1-450 and therefore predominantly non-K-region oxygenation of BP may occur in this further metabolism. However, an oxygenated product of BP may appear entirely different from BP itself to the enzyme active sites of the various forms of P-450. It would be of interest to prepare sufficiently radioactive 1-HO-BP, 6-HO-BP, 7-HO-BP, 9-HO-BP, and the 1,6-, 3,6-, and 6,12-quinones, to allow each of these to react with deproteinized DNA and microsomes, and to see whether any specific peaks can be attributed to the further metabolism of any of these specific metabolites that have already undergone an initial monooxygenation.

Our studies thus lead to the tentative identification of seven of the nine peaks consistently found during the binding of BP metabolites to DNA *in vitro*. The degree of certainty with respect to the assignment of each peak to a given metabolite varies considerably. In several instances one peak probably comprises more than one metabolite bound to DNA. Moreover, we have not attempted to identify which nucleoside(s) is (are) involved with each of these peaks. It is clear that BP can be metabolized enzymatically via four different pathways to reactive intermediates that bind to DNA: (a) formation of arene oxides; (b) the "diol epoxide pathway," in which the first monooxygenation is followed by epoxide hydration and a second monooxygenation; (c) the "phenol

[6] I. S. Owens and O. Pelkonen, manuscript in preparation.

oxide pathway," in which two (or more?) monooxygenations occur (with or without epoxide hydrase action?); and (d) the "quinone oxide pathway," which may involve monooxygenation of a quinone or may involve quinone-derived free radicals. These four different pathways are represented, respectively, by (a) peaks G, E, F', and H; (b) peak E; (c) peak H and, to a small extent, peak E; and (d) peaks B, D, F, and I. Although the 7,8-diol 9,10-epoxides of BP have created much interest and attention (16, 18, 21, 22, 27, 30, 48–50) in the etiology of chemical mutagenesis (5–8) or tumorigenesis (10, 11), it cannot be unequivocally excluded that other pathways of BP metabolism may also be significant in certain tissues or under certain experimental conditions.

ACKNOWLEDGMENT

We appreciate the expert secretarial assistance of Ms. Ingrid E. Jordan.

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