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EFFECTS OF MICROSOMAL ENZYME INDUCERS *IN VIVO* AND INHIBITORS *IN VITRO* ON THE COVALENT BINDING OF BENZO[a]PYRENE METABOLITES TO DNA CATALYZED BY LIVER MICROSOMES FROM GENETICALLY RESPONSIVE AND NONRESPONSIVE MICE*

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Abstract—The *in vitro* DNA binding of benzo[a]pyrene metabolites generated by mouse liver microsomes can be resolved into at least nine distinct peaks by elution of a Sephadex LH20 column with a water–methanol gradient. These peaks, representing metabolite–nucleoside complexes, are named A (most polar) through I (least polar). 3-Methylcholanthrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, phenobarbital, Aroclor 1254, pregnenolone-16α-carbonitrile, or ethanol was administered *in vivo* to genetically "responsive" C57BL/6N or "nonresponsive" DBA/2N mice, in an attempt to understand and identify increases or decreases in reactive BP intermediates that bind to DNA. Rises or falls in these peaks are also noted when liver microsomes from control or 3-methylcholanthrene-treated C57BL/6N or DBA/2N mice were incubated *in vitro* with [^{3}H]benzo[a]pyrene and microsomal enzyme inhibitors such as α-naphthoflavone, metyrapone or cyclohexene oxide. All of our interpretations concerning the binding of metabolites to DNA are consistent with non-K-region oxygenation of benzo[a]pyrene being mediated predominantly by cytochrome(s) P_1-450 and K-region oxygenation of benzo[a]pyrene being catalysed predominantly by form(s) of P-450 other than P_1-450. All of the biological perturbations are consistent with the following assignments. The major reactive intermediate of benzo[a]pyrene contributing to each peak is suggested to be: peaks A and C, an unknown dihydrodiol oxide; peaks B, D, F and I, quinones oxygenated further (or quinone-derived free radicals); peak E, both *cis*- and *trans*-7,8-diol-9,10-epoxides; peak F′, the 7,8-oxide; peak G, the 4,5-oxide; and peak H, an unknown phenol oxide. The DNA nucleosides are not identified in this study. Of the ten peaks listed here, it is of interest that the major metabolite(s) contributing to eight of the peaks (all except peaks F′ and G) involve(s) more than a single mono-oxygenation by forms of cytochrome P-450. All peaks, with the exception of peak G, appear to be predominantly associated with benzo[a]pyrene metabolism mediated by P_1-450 and, therefore, controlled by the *Ah* locus. The use of these microsomal enzyme inducers or inhibitors—combined with the underlying genetic predisposition of the individual, tissue, or cell culture system under study—demonstrates that the balance between P-450 and epoxide hydrase, and the ratio of each form of P-450 to the other forms of P-450, can influence markedly the quantity and quality of reactive intermediates of benzo[a]pyrene that bind to DNA.

Differences in the quantity or quality of reactive intermediates formed by the cytochrome P-450-mediated mono-oxygenases may be important in explaining genetic differences in individual risk for chemical carcinogenesis, mutagenesis, drug toxicity, and teratogenesis (as reviewed further in Refs. 1 and 2). When the chemical carcinogen BP† is incubated *in vitro* together with deproteinized DNA, NADPH, and mouse liver microsomes, covalent binding of BP metabolites to DNA occurs [3, 4]. The metabolite–nucleoside complexes can be resolved into at least nine peaks by the elution of a Sephadex

* Portions of this work were presented at the Symposium on Active Intermediates: Formation, Toxicity and Inactivation, Turku, Finland, July 1975 [D. W. Nebert, A. R. Boobis, H. Yagi, D. M. Jerina and R. E. Kouri, in *Biological Reactive Intermediates* (Eds. D. J. Jollow, J. J. Kocsis, R. Snyder and H. Vainio), pp. 125–45. Plenum Press, New York (1977)], and at the American Society for Pharmacology and Experimental Therapeutics Fall Meeting, New Orleans, LA, Aug. 1976 [A. R. Boobis and D. W. Nebert, *Pharmacologist* **18**, 210 (1976)].

† Abbreviations used in the text are as follows: BP, benzo[a]pyrene: MC, 3-methylcholanthrene; TCDD, 2,3,7,-8-tetrachlorodibenzo-*p*-dioxin: BP 7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; 4,5-dihydrodiol, *trans*-4,5-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; 6-HO-BP, 6-hydroxybenzo[a]pyrene: 9-HO-BP, 9-hydroxybenzo[a]pyrene: [^{3}H]BP, the generally tritiated form of BP; B6, the responsive C57BL/6N inbred mouse strain: D2, the nonresponsive DBA/2N inbred mouse strain: and GSH, reduced glutathione.

LH20 column with increasing methanol concentrations; these peaks are arbitrarily designated A (most polar) through I (least polar) [5]. With the use of synthetic and biologically produced metabolites [5], we have tentatively assigned (see Table 1) the major BP metabolite(s) generated by mouse liver microsomes that bind to DNA nucleosides and thereby contribute predominantly to each of ten peaks.

The rate at which one or another of these BP metabolites reacts with DNA is dependent upon a delicate balance between generation of the reactive intermediate and its detoxification. The half-life of the reactive intermediate must also be important. The balance of cytochromes* P-450 and P_1-450, epoxide hydrase, UDP glucuronosyltransferase, GSH-epoxide *S*-transferase, and phenol transulfonase obviously may vary, depending on different tissues, strains and species. Age, genetic expression, nutrition, hormone concentrations, diurnal rhythm, pH, saturating *vs* nonsaturating conditions of each enzyme for its substrate, the K_m and V_{max} for each enzyme, subcellular compartmentalization of each enzyme, the efficiency of DNA repair, and the immunological competence of the animal—all may be important factors contributing to the susceptibility of an individual to cancer or drug toxicity. Induction or suppression of one or more of these enzymes by chemicals also may affect this balance.

The induction of aryl hydrocarbon (BP) hydroxylase and its associated cytochrome P_1-450 by polycyclic aromatic compounds in the mouse is regulated by the *Ah* locus [1, 2]. Certain inbred strains such as B6 are genetically "responsive" and others such as D2 are genetically "nonresponsive," with respect to induction of P_1-450 and associated mono-oxygenase activities by polycyclic aromatic compounds. Different forms of P-450 produce different metabolite profiles from certain substrates (cf. Refs. 1, 2 and 8 for further discussion). For example, 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 forms of P-450 induced by MC (i.e. cytochrome P_1-450 associated with the *Ah* locus) oxygenate BP to a greater extent in the non-K-region, compared with K-region oxygenation [9–12].

In this report, we examine the increases or decreases in the nine BP metabolite–nucleoside peaks caused by the use of various microsomal enzyme inducers *in vivo* and inhibitors *in vitro*, with respect to genetically responsive or nonresponsive mice. The tentative identification of these BP metabolite nucleoside complexes, which was recently reported [5] with the use of known BP synthetic and biologically produced metabolites, is, therefore, complemented by these additional biological experiments.

MATERIALS AND METHODS

Materials. Sodium phenobarbital was purchased from Merck & Co. (Rahway, NJ); Aroclor 1254 from Analabs, Inc. (North Haven, CT); absolute ethanol from Publicker Industries Inc. (Philadelphia, PA); α-naphthoflavone (i.e. 7,8-benzoflavone) from Aldrich Chemical Co. (Milwaukee, WI); Tween 80 from Sigma Chemical Co. (St. Louis, MO); and cyclohexene oxide from Aldrich Chemical Co. The following were generous gifts: pregnenolone-16α-carbonitrile from Dr. Roger L. Bergstrom, G. D. Searle & Co. (Chicago, IL); metyrapone (2-methyl-1,2,3,3-pyridyl-1-propanone) from Dr. J. J. Chart, Ciba Pharmaceutical Co. (Summit, NJ); and TCDD from Dr. Alan P. Poland (University of Rochester Medical and Dental School, NY). The TCDD was originally from lot 851:144-II of Dow Chemical Co. (Midland, MI) and had been shown by gas–liquid chromatography–mass spectrometry to be 98.6 per cent pure, with 1.0% trichloro- and 0.4% pentachlorodibenzo-*p*-dioxin contaminants. The remainder of the chemicals and animals used in this study were obtained from sources previously cited [5].

Treatment of animals. MC in corn oil was administered at a dose of 80 mg/kg body weight 48 hr before killing. TCDD in *p*-dioxane was given at a dose of 100 μg or 1 μg/kg 72 hr before killing. Phenobarbital in 0.85% NaCl was administered at a dose of 80 mg/kg/day for 4 days; the dose for the first day was divided into equal amounts given 12 hr apart; the mice were killed 24 hr after the fourth dose. Aroclor 1254 was administered at a dose of 500 mg/kg 72 hr before killing. Pregnenolone-16α-carbonitrile was first dissolved in a minimal amount of Tween 80, and then water was added to make a suspension of 2 mg pregnenolone-16α-carbonitrile/ml. Pregnenolone-16α-carbonitrile was given at a dose of 50 mg/kg every 24 hr for three doses; the mice were killed 24 hr after the third dose. All of the above compounds were given intraperitoneally. Ethanol (30 per cent) was added to a solution of 30 g sucrose/100 ml of drinking water; the sucrose was necessary in order to disguise the taste of ethanol so that the mice would accept it. The ethanol in the drinking water was given continuously for 3 weeks before killing. Control animals in each experiment received the vehicle alone, by the same route and in the same amount and at the same times as had been given to the experimental animals: corn oil, *p*-dioxane (0.40 ml/kg), 0.85% NaCl, or Tween 80 in water intraperitoneally; or 30% sucrose as drinking water.

Other procedures. The preparation of mouse liver microsomes, incubation of the microsomes with NADPH, deproteinized salmon sperm DNA, and [^{3}H]BP, isolation of the DNA, and the purification of BP metabolite–nucleosides eluted by Sephadex

* The nomenclature for the various forms of cytochrome P-450 is currently inadequate, in view of four or more forms distinguishable by electrophoretic or immunochemical techniques. A better understanding of chemical and catalytic properties 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 mono-oxygenase activities. P_1-450 is defined in this report 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 [6] and rat [7] liver indicate that P_1-450 is distinctly different from the polycyclic aromatic-inducible form(s) of hemoprotein causing spectral shift to about 448 nm when reduced and combined with CO (i.e. cytochrome P-448). P-450 in a more specific sense in this report denotes all forms of P-450 other than P_1-450.

LH20 column chromatography were the same as previously described [3, 5]. The combined livers of five to seven mice/group were always studied. The microsomal enzyme inhibitors α-naphthoflavone, metyrapone or cyclohexene oxide were added in minimal amounts of methanol to the reaction mixture at 37° and shaken for 1 min, prior to the addition of [^{3}H]BP (6 μM final concn) and to the usual 37° incubation for 30 min [5].

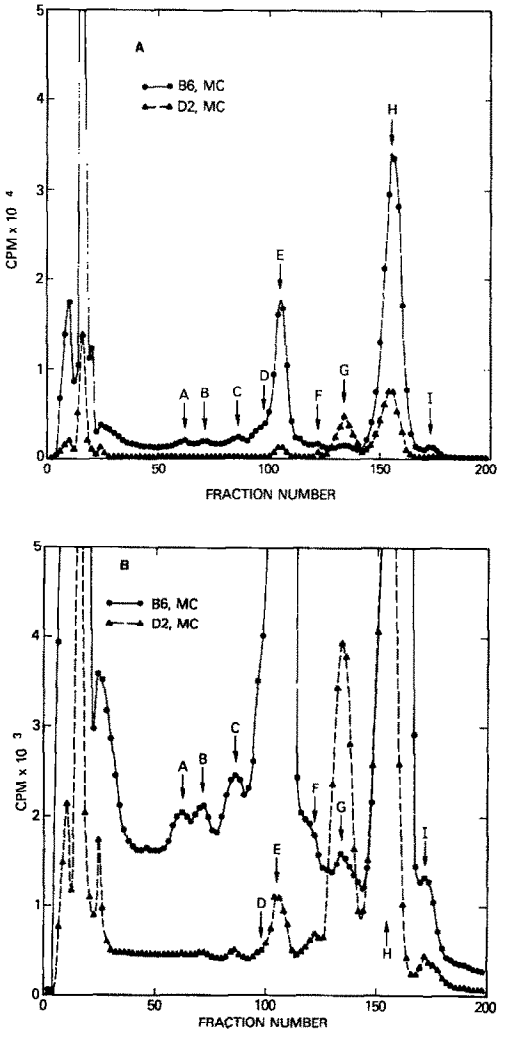

Fig. 1. Sephadex LH20 column chromatography of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from MC-treated B6 or D2 mice. In this figure and in subsequent figures, the treatment of the animals, the preparation of the hepatic microsomes and the separation of the metabolite–nucleosides after the incubation have been described previously [3, 5]. Deproteinized salmon sperm DNA (20 mg) was incubated with 4 mg of microsomal protein, 25 μmoles $MgCl_2$, 1 μmole EDTA, 7 μmoles NADPH, 100 μmoles glucose 6-phosphate, 1.4 units glucose 6-phosphate dehydrogenase, 1 m-mole potassium phosphate buffer, pH 7.5, and 60 nmoles [^{3}H]BP (1.19 mCi, sp. act. 20 Ci/m-mole) added in 200 μl acetone. The 10-ml reaction mixture was incubated at 37° for 30 min. The DNA was re-isolated, purified, digested with enzymes, then chromatographed on an 80-cm Sephadex LH20 column eluted with a 30–100% methanol gradient in water at a flow rate of approximately 1 ml/min. Two hundred fractions of 5.1 ml each were collected. Radioactivity (in cpm) was determined for 1-ml portions of alternate fractions. The ordinate in panel B is a 10-fold expansion of the ordinate (from the same experiment) in panel A.

RESULTS AND DISCUSSION

Effect of MC treatment in vivo *on BP metabolites which bind to DNA,* in vitro. Genetically responsive B6 and nonresponsive D2 mice were treated with MC (Fig. 1). The BP metabolite–nucleoside profile from control B6 and D2 mice was similar to that from MC-treated D2 mice (data not illustrated). MC-treated responsive (Ah^b/Ah^d) and nonresponsive (Ah^d/Ah^d) offspring from the (B6D2)F_1 × D2 backcross were also examined (Fig. 2). Peaks A through F and H and I were increased in the responsive B6

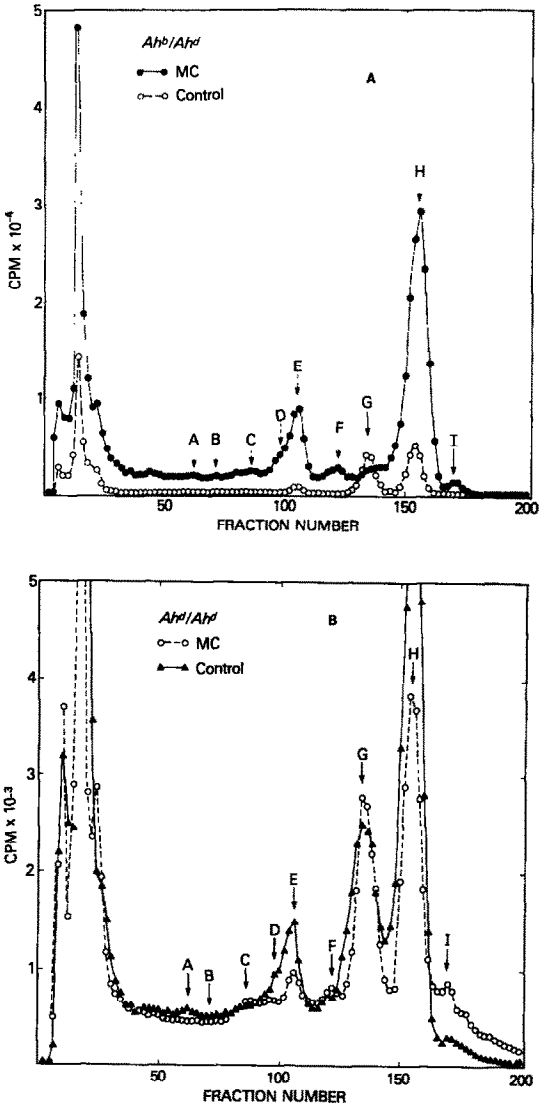

Fig. 2. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from MC-treated or control Ah^b/Ah^d (A) and Ah^d/Ah^d (B) mice. The genotypes of these (B6D2)D2 progeny were determined by zoxazolamine paralysis times [1] 2 weeks beforehand.

inbred parent and heterozygote, and peak G was larger in the nonresponsive D2 inbred parent and nonresponsive homozygote. We conclude, therefore, that the presence of the Ah^b allele which is necessary for the induction of P-450 by polycyclic hydrocarbons to occur [2]—causes increases in eight of nine peaks representing BP metabolite–nucleoside complexes: the only peak not increased is peak G (representing the interaction of BP 4,5-oxide with DNA [5]. Peak G is, in fact, actually decreased in the responsive B6 inbred parent and heterozygote.

Peaks E and H in Fig. 1A represent approximately 60 and 100 pmoles, respectively, of BP metabolites bound to nucleosides; the amount of DNA in these peaks could not be determined in this experiment because the DNA was not labeled. About 1.2 and 2.2 pmoles of BP metabolites were bound covalently/mg of DNA with B6 and D2 microsomes, respectively, and these are 0.30 and 0.055 per cent, respectively, of the total [^{3}H]BP that has been added to the incubation mixture.

In every incubation with BP there are several (often quite large) peaks eluting between fractions 4 and 40. In all of our experiments, this material comprised 15–30 per cent of the total radioactivity eluted from the column. Part of the material coming off the column early appears to represent BP metabolites bound to oligonucleotides incompletely hydrolyzed by the first procedure [5]. BP metabolites already bound to DNA are also possibly oxygenated a second time and bound to a second site on DNA, thereby forming metabolite–dinucleoside complexes which elute from the column in early fractions. The radioactivity eluting early may also represent, in part, BP metabolites bound to phosphate groups or simply metabolites physically trapped in oligonucleotides [5].

Effect of TCDD on BP metabolites which bind to DNA. The *Ah* locus has as a regulatory gene product the cytosolic receptor responsible for induction of P_1-450 and associated mono-oxygenase activities by polycyclic aromatic compounds [13]. TCDD has a much greater affinity for this receptor than MC [13] and, at a 100 μg/kg dose, induces P_1-450 and aryl hydrocarbon hydroxylase activity in both mice that are responsive and mice that are nonresponsive to MC [14]. At a 1 μg/kg dose, however, TCDD induces P_1-450 and the hydroxylase activity in mice that are responsive to MC but not in mice that are nonresponsive to MC [14].

As expected, the high dose of TCDD (Fig. 3) increased peaks A through F and H and I in the D2 mouse to levels as high as (and in some instances higher than) those in the B6 mouse. The large peak G found in MC-treated D2 mice (Fig. 1) is decreased in D2 mice by the large dose of TCDD to approximately the same level as that in B6 mice (Fig. 3). With 1 μg TCDD/kg (data not illustrated), the profile of the nine peaks in B6 and D2 mice was similar to that seen in Fig. 1 with MC-treated B6 and D2 mice respectively.

Effect of phenobarbital on BP metabolites which bind to DNA. There are no genetic differences in aryl hydrocarbon hydroxylase induction and, therefore, in BP metabolism between phenobarbital-treated B6 and D2 mice [15]. It, thus, was not surprising to find (Fig. 4) very similar profiles of BP metabolites

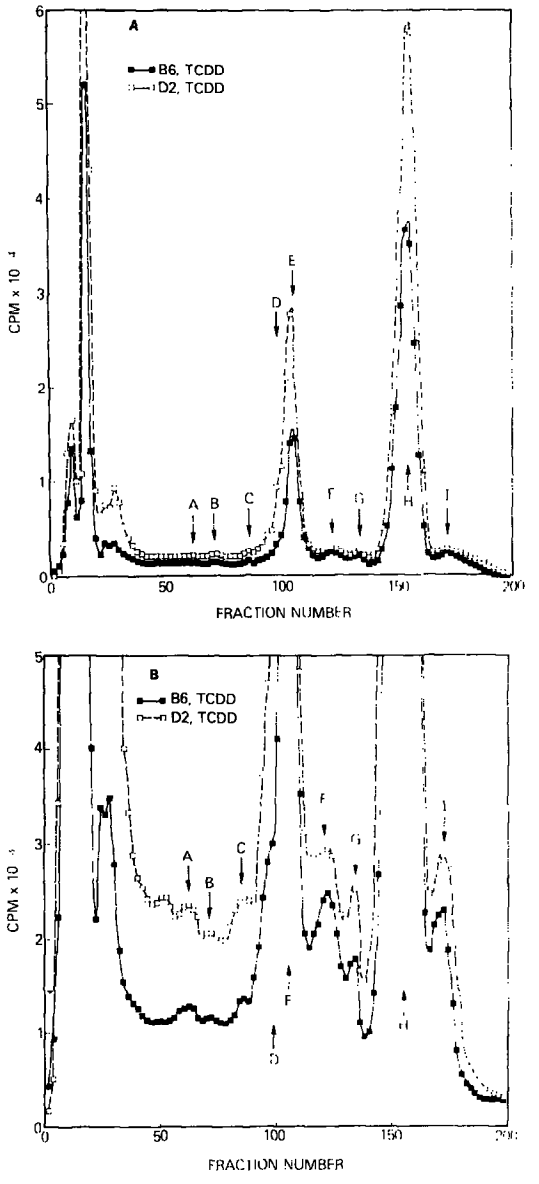

Fig. 3. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from TCDD-treated B6 or D2 mice. A dose of 100 μg TCDD/kg body weight was given intraperitoneally 72 hr before killing. The ordinate in panel B is a 10-fold expansion of the ordinate (from the same experiment) in panel A.

binding to DNA nucleosides when hepatic microsomes from phenobarbital-treated B6 and D2 mice were used. In comparison with control D2 mice, phenobarbital increased all peaks 2- to 6-fold. These increases are consistent with the greater amount of microsomal P-450 (per mg of microsomal protein) induced by phenobarbital. These increases are also in agreement with the observed apparent induction of some P_1-450 by phenobarbital observed previously in these mice [15, 16].

Effect of polychlorinated biphenyls on BP metabolites which bind to DNA. Aroclor 1254 is composed of

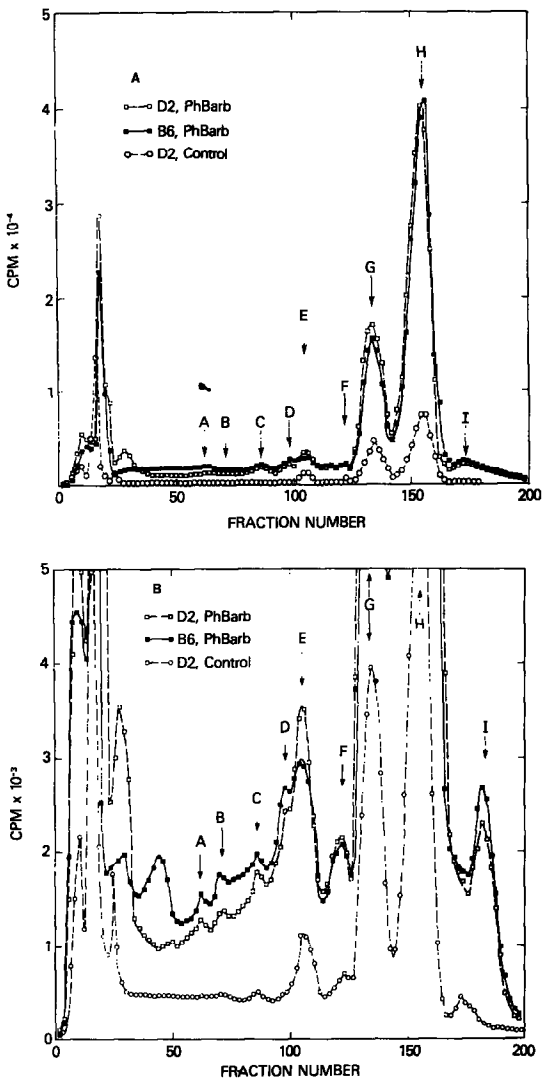

Fig. 4. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from phenobarbital-treated (PhBarb) B6 or D2 mice. The ordinate in panel B is a 10-fold expansion of the ordinate (from the same experiment) in panel A.

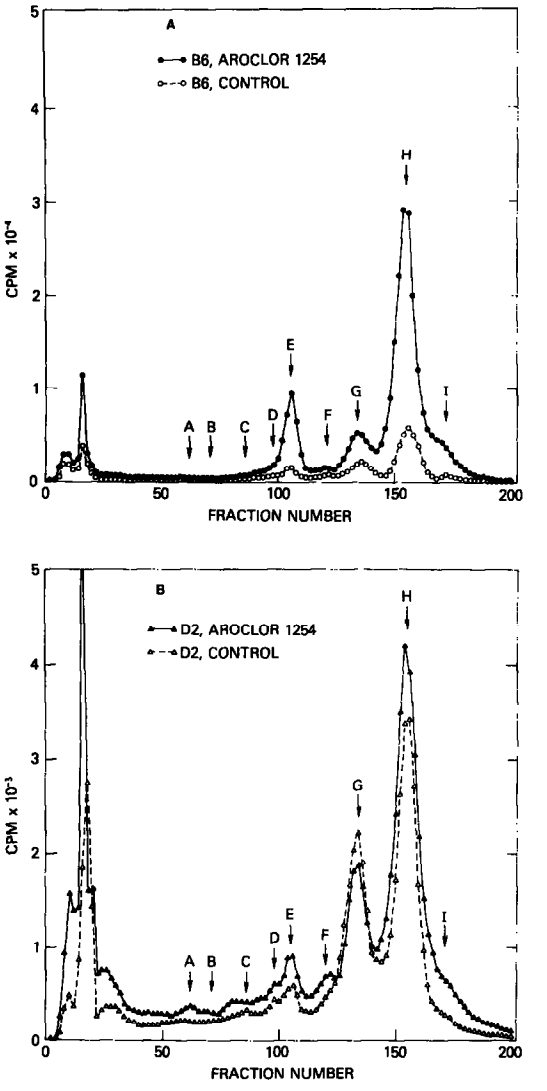

Fig. 5. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from control or Aroclor 1254-treated B6 (A) or D2 (B) mice.

approximately (by weight) 48% penta-, 23% hexa-, 21% tetra-, 6% heptachlorobiphenyls, and 2% lesser chlorinated biphenyls [17]. Commercial mixtures of polychlorinated biphenyls have been reported [18–20] also to contain trace amounts of chlorinated naphthalenes and chlorinated dibenzofurans. Certain of the chlorinated dibenzofurans are potent inducers of P_1-450 and aryl hydrocarbon hydroxylase activity [13]. It was claimed [21] that certain of the chlorobiphenyl congeners exert both an MC-like and a phenobarbital-like inducing effect. It now appears clear [22, 23], however, that any given halogenated congener produces only an MC-like (if planar) or a phenobarbital-like (if nonplanar) response but not both responses (or the congener may be devoid of either inducing activity). Aroclor 1254 is known [24] to exert both MC-like and phenobarbital-like effects in responsive mice but only phenobarbital-like effects in nonresponsive mice.

Aroclor 1254 produced both MC- and phenobarbital-like responses in B6 mice (Fig. 5A): the eight peaks reflecting BP metabolism to reactive intermediates by induced P_1-450, were increased: and peak G reflecting BP metabolism to the 4,5-oxide predominantly by an induced form of P-450 other than P_1-450 was also enhanced. The small increases in all peaks in Aroclor 1254-treated D2 mice (Fig. 5B) appear to represent the phenobarbital-like inducing effect without the MC-like inducing effect.

Effect of pregnenolone-16α-carbonitrile on BP metabolites which bind to DNA. Pregnenolone-16α-carbonitrile is a distinctly different inducer of microsomal enzyme activities [25] and increases a form of P-450 [16] electrophoretically distinct from those

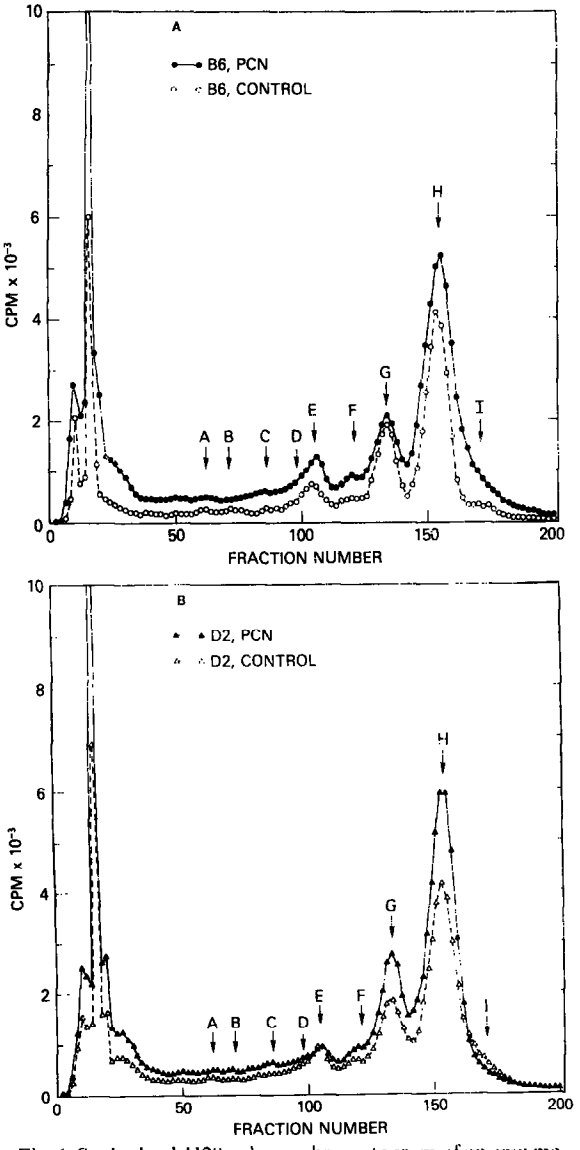

Fig. 6. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from control or pregnenolone-16α-carbonitrile-treated (PCN) B6 (A) or D2 (B) mice.

forms induced by polycyclic aromatic compounds or phenobarbital. There are no detectable genetic differences between B6 and D2 mice treated with pregnenolone-16α-carbonitrile [16, 26]. No important differences in the BP metabolite–nucleoside profile (Fig. 6) between pregnenolone-16α-carbonitrile-treated B6 and D2 mice were seen. Peak E was slightly increased in B6, compared with D2 mice treated with pregnenolone-16α-carbonitrile. Peak G was somewhat increased in D2, compared with B6 mice treated with pregnenolone-16α-carbonitrile. Because a small rise in aryl hydrocarbon hydroxylase occurs [26] in pregnenolone-16α-carbonitrile-treated mice, only small increases in the amount of BP metabolites bound to DNA (compared with control levels) were expected, and this is what was found.

Effect of ethanol in vivo on BP metabolites which bind to DNA. Ethanol is oxidized principally to acetaldehyde by alcohol dehydrogenase and to a lesser extent by the microsomal ethanol-oxidizing system believed to be mediated by some form of P-450 [27, 28]. Chronic ethanol administration *in vivo* induces certain mono-oxygenase activities to varying degrees and not others [29]. Tetrahydrofuran *in vitro* inhibits preferentially a form of P-450 induced by chronic ethanol administration [30]. Chronic ethanol treatment increases a form of P-450 electrophoretically distinct from those forms induced by polycyclic aromatic compounds, phenobarbital, or pregnenolone-16α-carbonitrile: there is no association of the induction process by ethanol with the *Ah* locus (J. S. Felton and D. W. Nebert, manuscript in preparation).

Small increases were found in all nine peaks in ethanol-treated B6 mice (Fig. 7A), compared with

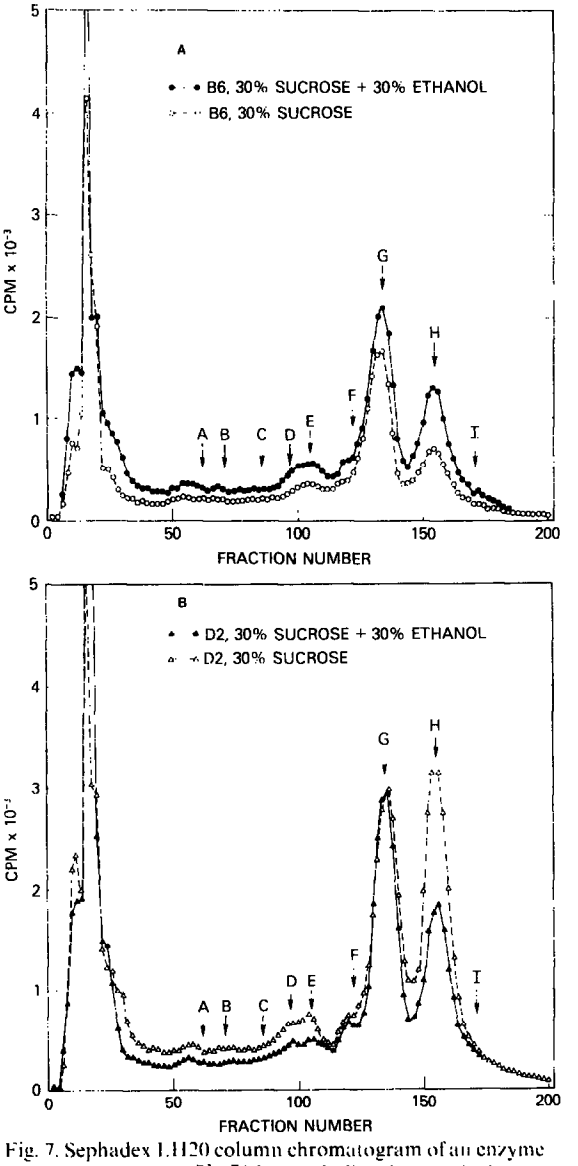

Fig. 7. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation with hepatic microsomes from B6 (A) or D2 (B) mice kept on a control diet supplemented with either 30% sucrose plus 30% ethanol in water or 30% sucrose alone in water.

controls receiving sucrose–water, and no new peaks or no striking increases in any one peak were seen. Ethanol treatment decreased all peaks in D2 mice (Fig. 7B), except peak G, which remained the same as controls receiving sucrose–water; however, this is believed not to be a genetic difference in ethanol-induced forms of P-450. B6 is one of the inbred strains of mice which prefers ethanol if given a choice, whereas the D2 inbred strain has an almost total aversion to ethanol [31]. We believe this difference in sensitivity of the central nervous system to the effects of ethanol [32] between these two strains explains the dissimilarity in response of BP metabolism seen in Figs. 7A and 7B, because the D2 appeared somewhat ill compared with B6 mice during the same 3-week experiment.

Effect of α-naphthoflavone in vitro *on BP metabolites which bind to DNA.* α-Naphthoflavone *in vitro* inhibits mono-oxygenase activities associated with P_1-450 in MC-treated responsive mice but has no effect on, or enhances, mono-oxygenase activities associated with other forms of P-450 in control mice or in MC-treated nonresponsive mice [33]. α-Naphthoflavone has been shown to inhibit the binding of BP to polyguanosylic acid catalyzed by microsomes from MC-treated rat liver [34] and the binding of 3-HO-BP to DNA catalyzed by lung microsomes from MC-treated rats [35, 36]. With microsomes from MC-treated B6 mice (Fig. 8A), α-naphthoflavone caused striking decreases in all peaks except peak G. This finding suggests that BP metabolism to the 4,5-oxide is mediated by an α-naphthoflavone-insensitive form of P-450 rather than P_1-450.

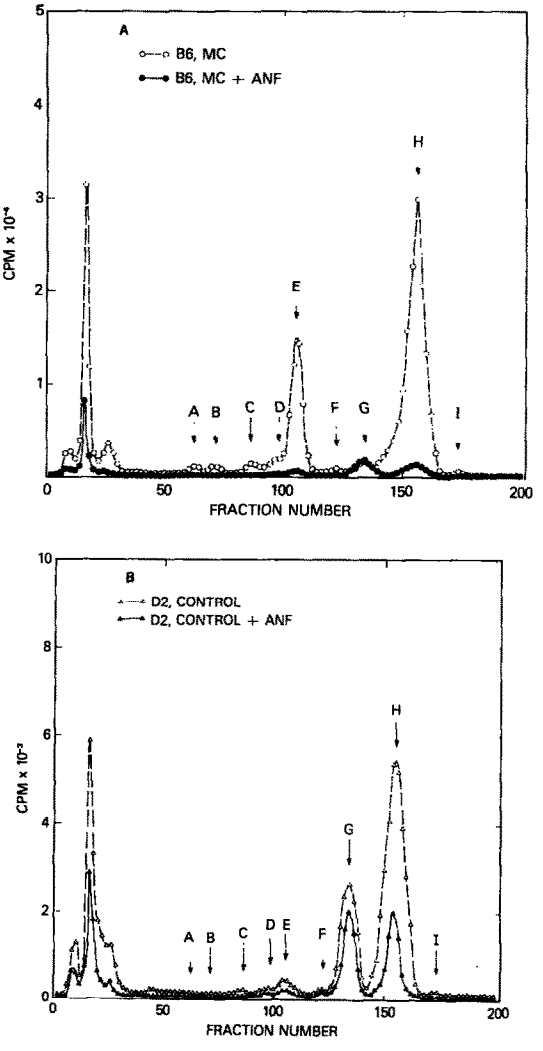

Fig. 8. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation in the presence or absence of 0.05 mM α-naphthoflavone (ANF) with hepatic microsomes from MC-treated B6 (A) or control D2 (B) mice.

With microsomes from control D2 mice (Fig. 8B), α-naphthoflavone caused no appreciable decrease in peak G but decreased the other eight peaks to at least one-half their size. Results similar to those in Fig. 8B were found with control B6 and MC-treated D2 mice (data not illustrated). The results with control D2 microsomes (Fig. 8B) suggest that the hepatic P-450-mediated BP metabolism in these mice can be partially inhibited by α-naphthoflavone or that there is a "background level" of P_1-450 in D2 mouse liver microsomes. The P_1-450 might even be present in nonhepatic cells in the liver (e.g. mesenchymal cells or macrophages).

Effect of metyrapone in vitro *on BP metabolites which bind to DNA.* Metyrapone *in vitro* inhibits mono-oxygenase activities associated with form(s) of P-450 other than P_1-450 but has little effect on activities associated with P_1-450 in MC-treated responsive mice. [33]. With microsomes from MC-treated B6 mice (Figs. 9A and 9B), metyrapone had no effect on the BP metabolite nucleoside profile except for more than a 2-fold increase in peak G and significant increases in peaks H and I. We believe this result indicates that, if the conversion of BP to BP 4,5-oxide is preferentially inhibited by this dose of metyrapone *in vitro*, other pathways of BP metabolism will become more prominent. Thus, increases in peaks G and H via further metabolism of BP phenols or the 9,10-oxide and increases in peak I via further metabolism of BP quinones (Table 1) are consistent with the preferential inhibition of a form(s) of P-450 other than P_1-450 by metyrapone.

The effect of metyrapone on microsomes from control D2 mice is shown in Figs. 9C and 9D. Similar results were found with microsomes from control B6 or MC-treated D2 mice. Metyrapone significantly decreased peaks D, E, F, G and H and had no significant effect on peaks A, B, C and I. If all peaks except G reflect BP metabolism predominantly by P_1-450 in MC-treated D2 mice, we would not have expected metyrapone to decrease peaks D, E, F and H: thus peaks D, E, F and H must represent P-450-catalyzed metabolites as well as P_1-450-caralyzed metabolites.

Effect of cyclohexene oxide in vitro *on BP metabolites which bind to DNA.* Cyclohexene oxide is one of many inhibitors of epoxide hydrase [37] action on BP oxides in the following order: 9,10-oxide > 7,8-oxide > 4,5-oxide [38]. Cyclohexene oxide has no effect on 3-HO-BP or BP quinone formation [38]. With microsomes from MC-treated B6 mice (Figs. 10A and 10B), cyclohexene oxide caused a large increase in peak H. This result suggests that conversion of non-K-region oxides to the correspond-

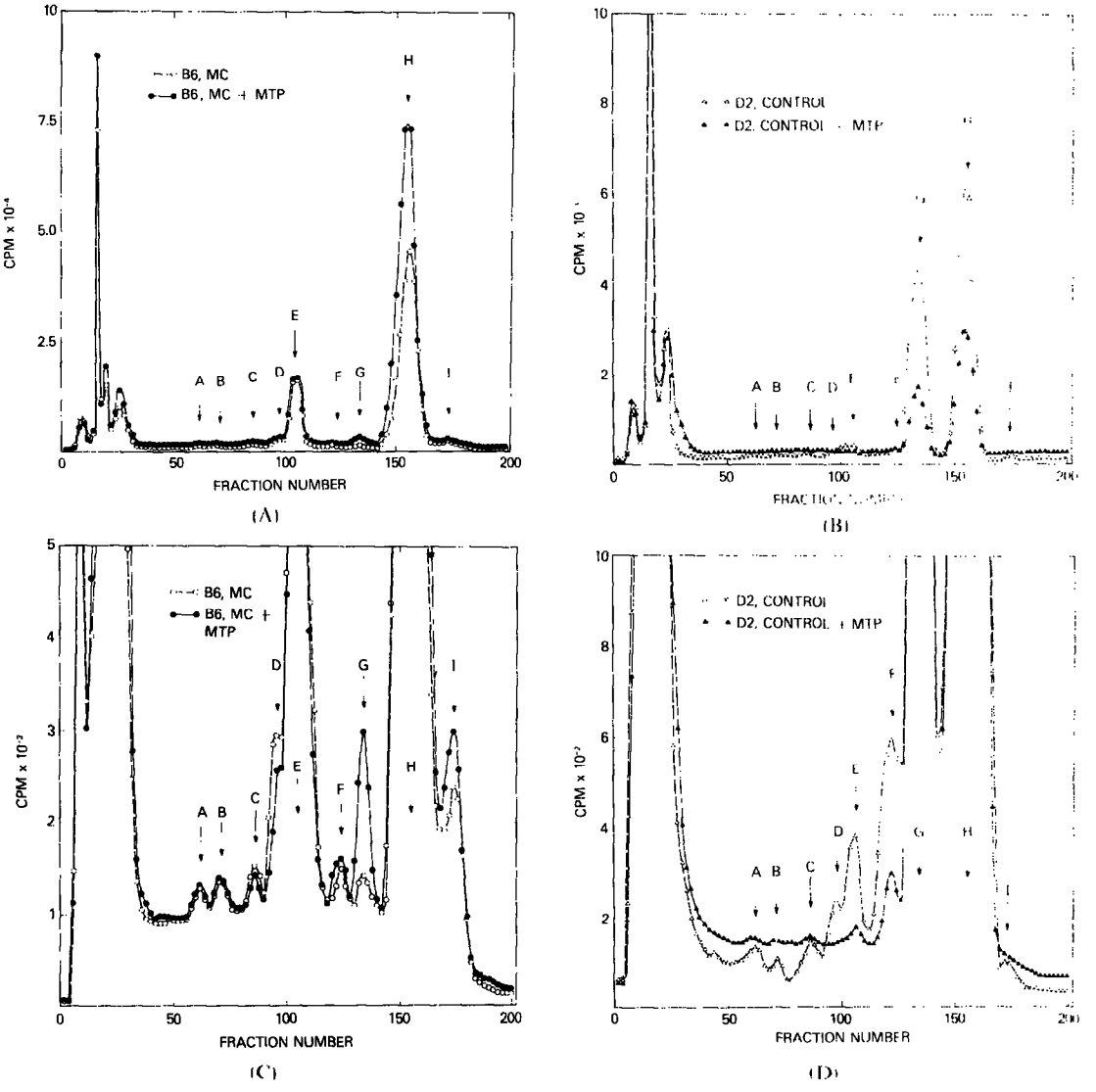

Fig. 9. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation in the presence or absence of 0.10 mM metyrapone (MTP) with hepatic microsomes from MC-treated B6 (A) or control D2 (C) mice. The ordinates in panels B and D are 10-fold expansion of the ordinates (from the same experiment) in panels A and C respectively.

ing phenols is effectively inhibited by cyclohexene oxide, thereby causing increased formation of phenol-oxides which contribute to peak H (Table 1). Cyclohexene oxide caused a large decrease in peak E (Figs. 10A and 10B): this finding points out the importance of epoxide hydrase in the three-step formation of the BP-7,8-diol-9,10-epoxides (Table 1), which comprise peak E almost exclusively. Cyclohexene oxide caused about a 2-fold increase in peak G (Figs. 10A and 10B). This result may reflect the block in BP 4,5-oxide metabolism to the 4,5-diol. Similar effects by cyclohexene oxide on BP binding to DNA with liver microsomes from MC-treated rats have been reported [39].

Peak C was increased with microsomes from MC-treated B6 mice, and was blocked by cyclohexene oxide. This suggests that peak C represents a dihydrodiol that requires further metabolism to a reactive intermediate which binds to DNA.

Cyclohexene oxide caused the appearance of peak F′ (between peaks E and F in Fig. 10B), which had been found [5] when authentic BP 7,8-oxide was reacted with DNA in the absence of microsomes. Small increases in peaks D, F and I (Figs. 10A and 10B) suggest that cyclohexene oxide also enhances the pathway involving the further metabolism of quinones to intermediates which react with DNA. This observation may lend support to our hypothesis [5] that the quinone-oxide pathway is important in the formation of certain BP metabolite–nucleoside adducts.

With microsomes from control D2 mice (Figs. 10C and 10D), cyclohexene oxide also caused a large increase in peak H and doubled the size of peak G, just as had been seen in Figs. 10A and 10B. Cyclohexene oxide had no effect on peak F, because there

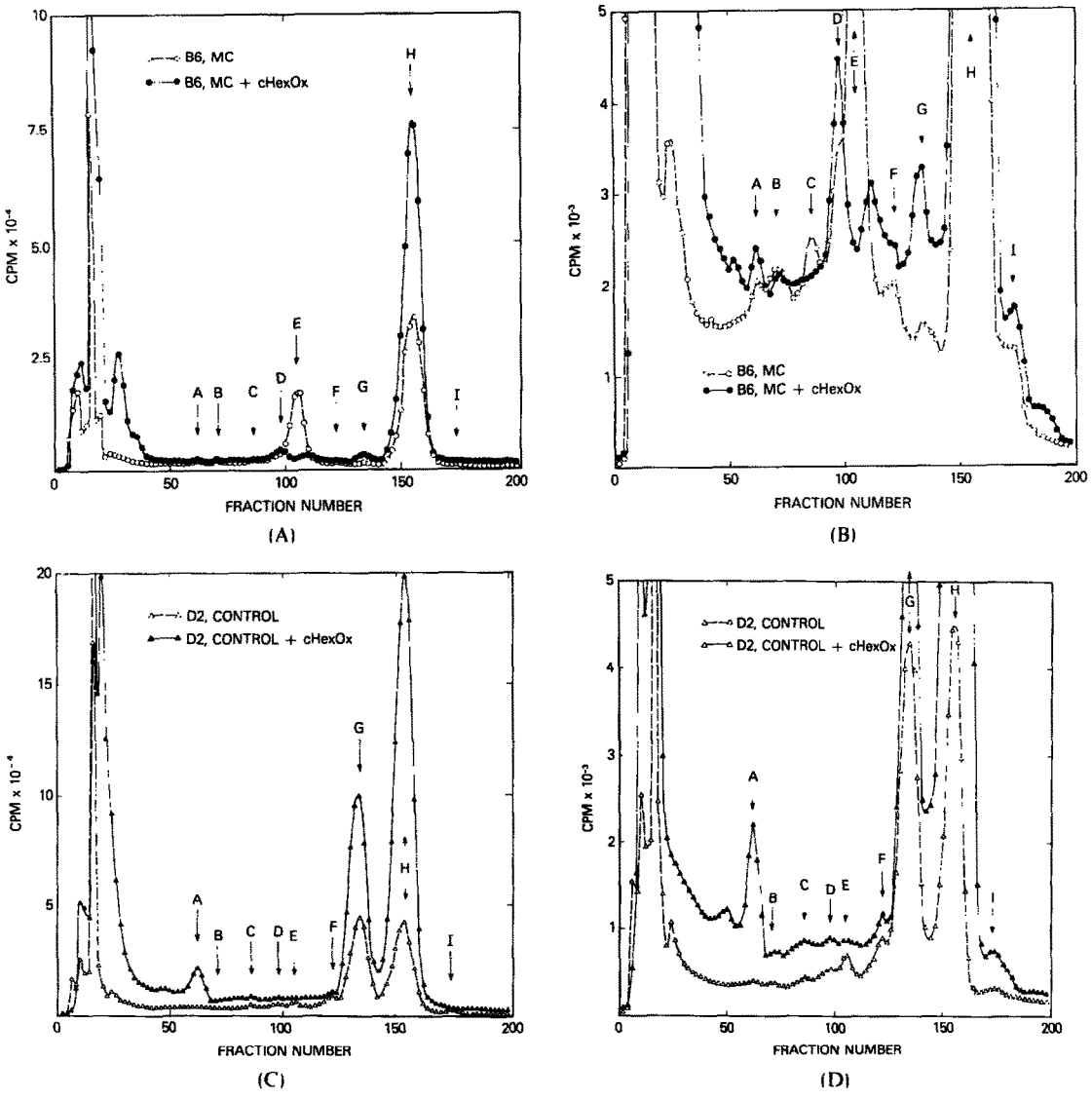

Fig. 10. Sephadex LH20 column chromatogram of an enzyme digest of DNA with [^{3}H]BP metabolites bound during an *in vitro* incubation in the presence or absence of 2.0 mM cyclohexene oxide (cHexOx) with hepatic microsomes from MC-treated B6 (A) or control D2 (C) mice. The orindates in panels B and D are 10-fold expansion of the ordinates (from the same experiment) in panels A and C respectively.

was no significant peak E in the control sample. No peak F' was found in Figs. 10C and 10D, probably because non-K-region oxygenation by control P-450 is relatively small. Small increases in peaks D, F and I also occurred, as had been seen in Figs. 10A and 10B.

Peak A was unusually large with microsomes from control D2 mice (Figs. 10C and 10D). We suggest that peak A is a dihydrodiol which undergoes a second mono-oxygenation and that this dihydrodiol oxide then interacts with DNA. Results similar to those in Figs. 10C and 10D were found with microsomes from control B6 or MC-treated D2 mice (data not shown).

A recent study [40] compared the effects of α-naphthoflavone or 1,1,1-trichloropropene 2,3-oxide as *in vitro* inhibitors of BP metabolite binding to liver nuclei DNA in MC- and phenobarbital-treated and control rats. Although these authors found only five peaks representing metabolite–nucleoside adducts and only identified tentatively two of the five peaks [40], all of the observed rises or falls in these peaks effected by *in vivo* inducers or *in vitro* inhibitors are in agreement with our findings in this present report.

Conclusions. Several noteworthy conclusions arise from our study. First, eight of the nine BP metabolite–nucleoside peaks are more closely associated with P_1-450 induction than with P-450 induction and, therefore, are under control of the *Ah* locus [1]. Peaks D, E, F and H contain, as well, metabolites catalyzed by forms of P-450 other than P_1-450. Second, microsomal enzyme inducers *in vivo* and inhibitors *in vitro* can have profound effects on which BP metabolites are formed and which react covalently with DNA. Knowledge of these effects and of the genetic constitution of the laboratory animals (or cells in culture) being studied may influence the interpretations of results when studying polycyclic

Table 1. Postulated identification of BP metabolites produced *in vitro* by mouse liver microsomes that bind to DNA

Peak	Fraction number at which peaks occur	Precursor	Ultimate active form of BP metabolite which is the major contributor to the peak
A	62	Dihydrodiol(s)	Unknown dihydrodiol oxide
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)

hydrocarbon carcinogenesis or mutagenesis. For example, the predominant form of P-450 existing in control and polycyclic hydrocarbon-treated cells in culture is P_1-450 [41], which would account for BP metabolism predominantly in the non-K-region by cell cultures [12]. Third, of the reactive intermediates which are believed to contribute predominantly to each of the ten peaks listed in Table 1, it is of interest that eight (all peaks except F′ and G) require more than a single oxygenation via cytochromes P-450. When the substrate is nonsaturating to the P-450 mono-oxygenase system, increased secondary metabolism of BP is known [42] to occur. When a carcinogen *in vivo* is administered topically or subcutaneously or when the human population is exposed to an environmental carcinogen, the substrate concentration in all likelihood will be nonsaturating, i.e. far below its K_m value. Hence, in terms of the amount of carcinogen available, there will be increased formation of these important reactive intermediates involving more than one mono-oxygenation by P-450, resulting in more covalent binding to DNA per unit of substrate available (compared with a substrate under saturating conditions). Fourth, other factors such as diet, concentration of microsomal protein *in vitro*, use of perfused tissues, or use of tissues in the intact animals also affect greatly the profile of BP metabolites bound to DNA, and these data will be the subject of further reports from this laboratory. Fifth, the *Ah* locus has been shown to be associated with genetic differences in BP tumorigenesis (reviewed in Refs. 2 and 8), toxicity (Ref. 24, reviewed in Ref. 1), and teratogenesis [43]. It will be of interest to identify the ultimate carcinogen, toxic chemical, or ultimate teratogen of BP, in each of these respective experimental instances, that most likely is formed predominantly by cytochrome P_1-450.

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