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MUTAGENESIS OF CERTAIN BENZO[a]PYRENE PHENOLS *IN VITRO* FOLLOWING FURTHER METABOLISM BY MOUSE LIVER *

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Abstract—Low concentrations of 1-hydroxy- and 3-hydroxybenzo[a]pyrene in the presence of NADPH and liver S-9 fraction from 3-methylcholanthrene-treated C57BL/6N mice are as much as 3-fold more mutagenic than benzo[a]pyrene in the bacteria *Salmonella typhimurium* LT_2 tester strain TA98. The level of mutagenicity rises with increasing phenol or S-9 protein concentration. In this system, 9-hydroxybenzo[a]pyrene is slightly mutagenic, while 2-hydroxy-, 7-hydroxy- and 12-hydroxybenzo[a]pyrene are not mutagenic at low concentrations. The S-9 fraction from 3-methylcholanthrene-treated DBA/2N mice or phenobarbital-treated C57BL/6N mice does not support significant levels of mutagenesis. The high level of mutagenicity by 1-hydroxy- or 3-hydroxybenzo[a]pyrene is inhibited by α-naphthoflavone but is not inhibited by metyrapone, 1,2-epoxy-3,3,3-trichloropropane or glutathione. The substrate for UDP-glucuronosyltransferase, UDP-glucuronic acid, prevents more than half of the mutagenicity caused by the further metabolism of 1-hydroxy- and 3-hydroxybenzo[a]pyrene. The combination of UDP-glucuronic acid and UDP-*N*-acetylglucosamine provides an even higher level of protection. The addition of the substrate for sulfotransferase(s), 3′-phosphoadenosine 5′-phosphate sulfate, also prevents about half of the mutagenesis caused by 1-hydroxy- or 3-hydroxybenzo[a]pyrene.

Polynuclear aromatic hydrocarbons, typically represented by benzo[a]pyrene (BP),† are widespread environmental contaminants and are of great concern to biologists because of evidence linking certain of these compounds to carcinogenesis in man [1]. BP *per se* is not active, but requires metabolic activation by cellular enzymes to reactive intermediates which can then bind covalently to critical cellular components. Cytochrome P-450-mediated mono-oxygenase activity is primarily responsible for catalyzing the formation of different epoxides, phenols and quinones of BP. The biological effects of BP arene oxides and diol epoxides have been studied extensively and are considered the active species involved in covalent binding to cellular nucleophiles [2, 3]. It is known that epoxides [4], as well as phenols [5–10], may be conjugated by related enzymes and rendered inert, or certain epoxides can be further metabolized to diols by the epoxide hydratase [5, 11]. Diols at certain positions in the molecule can then undergo metabolism via a second oxygenation producing diol epoxides which avidly bind to cellular DNA [12].

The metabolic fate of certain phenols of BP has been studied *in vitro* to a limited extent [13–16]. A major phenol, 3-OH-BP, has been shown to be further metabolized by MC-induced microsomes [14, 15]. With lung microsomes 3-OH-BP has been shown to inhibit competitively BP metabolism [15]. In addition, the major phenol, 9-OH-BP, is thought to be further metabolized in *in vitro* systems to 9-OH-BP-4,5-oxide [17]. It is generally considered, however, that the phenols are at most, weakly mutagenic [18, 19] and not carcinogenic [20, 21], and that these derivatives are largely converted to inert glucuronides by UDP-glucuronosyltransferase [5, 6, 8] or to sulfates by sulfotransferases [9, 10]. Although 2-OH-BP is not a mutagen [18] and has not been shown to form biochemically, it has been shown to be a potent carcinogen on mouse skin [22]. It was of interest, therefore, to examine further the consequences of metabolic activation of certain phenols of BP and to understand how conjugation reactions may alter these effects. Phenols which are produced enzymatically were of particular interest, especially the 3-OH-BP.

MATERIALS AND METHODS

Materials. NADPH and glutathione were purchased from CalBiochem (San Diego, CA); ANF and TCPE were from Aldrich (Milwaukee, WI); BP, bovine liver β-glucuronidase, UDPGA, UDP-*N*-acetylglucosamine, adenosine 3′:5′-diphosphate, and *p*-nitrophenyl sulfate were obtained from Sigma (St. Louis, MO); MC was from Eastman (Rochester, NY); and sodium phen-

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† Abbreviations used are: BP, benzo[a]pyrene; MC, 3-methylcholanthrene; TCPE, 1,2-epoxy-3,3,3-trichloropropane; UDPGA, UDP-glucuronic acid; MTP, metyrapone; ANF, α-naphthoflavone; GSH, glutathione; PAP, adenosine 3′:5′-diphosphate; PNP, *p*-nitrophenylsulfate; 1-OH-BP, 1-hydroxybenzo[a]pyrene; 3-OH-BP, 3-hydroxybenzo[a]-pyrene; 9-OH-BP, 9-hydroxybenzo[a]pyrene; 12-OH-BP, 12-hydroxybenzo[a]pyrene; BP-7,8-diol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; transferase, UDP-glucuronosyltransferase; hydrocarbons, polycyclic aromatic hydrocarbons; B6, C57BL/6N; and D2, DBA/2N.

obarbital was from Merck (Rahway, NJ). All metabolites of BP were obtained from the National Cancer Institute Chemical Repository, National Institutes of Health (Bethesda, MD) through Dr. D. G. Longfellow, Program Manager. MTP was a gift from Dr. J. J. Chart, Ciba (Summit, NJ) to Dr. D. W. Nebert, National Institutes of Health; the bacterial tester strain TA98, a mutant of *Salmonella typhimurium* LT_2, was obtained from Dr. B. Ames, University of California (Berkeley, CA); 4- to 6-week-old male C57BL/6N and DBA/2N mice were obtained from Veterinary Resources Branch, NIH; Swinnex filter units were obtained from Millipore (Bedford, MA).

Treatment of mice. The conditions of the animal room and the general care of the animals have been described elsewhere [23]. Certain mice were treated with a single intraperitoneal dose (200 mg/kg) of MC 5 days before being killed; control mice received corn oil alone. Some C57BL/6N mice were treated for at least 5 days with sodium phenobarbital dissolved (0.05%, w/v) in the drinking water.

Preparation of S-9 fraction. After exsanguination, each mouse liver was excised and homogenized as described previously [24]. All further manipulations were done at 0–4°. The homogenate was spun at 16,000 *g* for 15 min; the resulting supernatant fluid was designated S-9 fraction. S-9 fractions and other labile reagents used in the mutagenesis assay were sterilized by filtering through a Swinnex filter unit with 0.45 μm pore size. Protein concentrations were determined by the method of Lowry *et al.* [25] with crystalline bovine serum albumin as the standard. S-9 Fractions, used in any mutagenesis assay in which the substrates for sulfation or glucuronidation were included, were previously passed through a Sephadex G-25 column equilibrated with 0.25 M phosphate buffer, 0.15 M KCl, pH 7.2, in order to remove endogenous substrates.

Conjugation reactions. Glucuronidation of a phenol in the mutagenesis assay was generally assessed indirectly by determining protection against mutagenesis in the presence of both the S-9 fraction and 2 mM UDPGA. It was shown in a separate experiment, however, that [^{3}H]3-OH-BP glucuronide can form under the conditions of the mutagenesis assay. [^{3}H]3-OH-BP and UDPGA were added to the reaction components of the mutagenesis assay in 1.25 ml, but in the absence of bacteria. The conjugation reaction, initiated with [^{3}H]3-OH-BP, incubated for 15 min at 37°. Following the addition of 2 ml of 0.14 M sodium acetate buffer, pH 4.6, one-half (two tubes) of each system was incubated either for 60 min at 37° with approximately 1000 units of β-glucuronidase dissolved in 0.05 ml of sodium acetate buffer, pH 4.6, or in buffer alone. After the incubation period, 1 ml of cold acetone was added to each tube, and all tubes were spun at 1000 *g* for 10 min. A 0.1-ml aliquot of supernatant fluid from each tube was spotted on thin-layer silica gel plates and developed in the solvent system, developed by Nemoto and Gelboin [6]. The [^{3}H]3-OH-BP-glucuronide was fluorescent and migrated in our system with an R_f of 0.72. UDP-*N*-acetylglucosamine at 4 mM was added as an activator of UDP-glucuronosyltransferase [26]. Trace amounts of Triton X-100 were toxic to the TA98 tester strain and, therefore, it could not be used as an activator in the mutagenesis assay.

Likewise, sulfation of a phenol was assessed indirectly by determining protection against mutagenesis in the presence of the S-9 fraction, 5 mM PNP, and 20 μM PAP which together generate 3′-phosphoadenosine 5′-phosphate sulfate, the sulfation substrate [27]. It was not necessary to preincubate at 37° to generate 3′-phosphoadenosine 5′-phosphate sulfate before mixing with the other components of the mutagenesis assay.

Mutagenesis test with S-9 fraction. With mouse liver S-9 preparations, the assay was carried out as described previously [28] but with slight modifications as to the order of adding reaction components. In a test tube, sodium phosphate (pH 7.4), KCl, $MgCl_2$ and 0.48 mM NADPH were included as a pool, followed by any modifier compound at 0°; the TA98 tester strain (10^8/plate), S-9 fraction, and mutagen were then added at room temperature. The final volume was always 2.5 ml, and concentrations were as described [28], except where indicated. Top agar at 45° was added immediately, mixed and poured on an agar plate. Colonies (histidine revertants) were counted after a 2-day incubation at 37°. Benzo[a]pyrene and its metabolites and the modifiers, MTP, TCPE and ANF, were dissolved in dimethylsulfoxide; maximal amounts per plate were 30 μl dimethylsulfoxide. The background mutagenic rate for 2.5 μg phenol/plate was approximately 100 colonies for 1-OH- or 3-OH-BP and 35 colonies for BP. The data are always corrected for the background rate of reversion.

RESULTS

Potent mutagenicity resulting from activation of certain BP phenols. Initially, several phenols of BP were examined for effects on histidine reversion with the bacterial tester strain TA98 in the *Salmonella* mutage-

Table 1. Comparative activation of phenols of benzo[a]pyrene to mutagens by mouse liver S-9 fraction and protection by UDPGA*

	BP	1-OH-BP	2-OH-BP	3-OH-BP	7-OH-BP	9-OH-BP	12-OH-BP	BP-7,8-diol
"Mutagen" alone	540	1775	0	677	0	121	0	796
+ UDPGA (2.0 mM) + UDP-*N*-AcgluNH$_3$ (4.0 mM)	46	53	0	51	0	89	0	710

*C57BL/6N mice were treated with MC, and liver S-9 fractions were prepared as described under Materials and Methods. Test agar plates contained 10^8 TA98 bacteria, 2.5 μg BP or BP metabolite, and 2.0 mg protein/plate, except for the BP-7,8-diol which was present at 1.25 μg/plate in the presence of 0.25 mg protein. Some plates contained UDP-glucuronic acid (UDPGA) and UDP-*N*-acetylglucosamine (UDP-*N*-AcgluNH$_3$). These data were corrected for background revertants. Values are of histidine revertant colonies. This represents a typical experiment repeated three times.

Table 2. Evidence for 3-OH-benzo[a]pyrene glucuronide*

	^{3}H (dis./min/100 μl)	
	No β-glucuronidase treatment	After β-glucuronidase treatment
[^{3}H]3-OH-BP alone	620	176
[^{3}H]3-OH-BP + UDPGA	2686	168
[^{3}H]3-OH-BP + UDPGA + UDP-*N*-AcgluNH$_3$	6635	752
[^{3}H]3-OH-BP + UDP-*N*-AcgluNH$_3$	296	75

*[^{3}H]3-OH-BP (2.5 μg/tube) was added to the reaction components of the mutagenesis assay, as described under Materials and Methods. Either 2.0 mM UDP-glucuronic acid (UDPGA) and/or 4.0 mM UDP-*N*-acetylglucosamine (UDP-*N*-AcgluNH$_3$) was included in the incubation with 0.75 mg S-9 protein from MC-treated C57BL/6N mice. Disintegrations per min (dis./min) represent a tritiated compound(s) which migrates with an R_f of 0.72 on thin-layer silica gel plates. The β-glucuronidase-sensitive compound was extracted from developed [6] plates and counted in aquasol.

nesis assay [28] and an activating system consisting of NADPH and the S-9 fraction of the cell. Liver S-9 fraction from MC-treated C57BL/6N mice was determined to be a more efficient source of mono-oxygenase activity than microsomes. Other tester strains were not examined since TA98 is one of the most sensitive strains available. 1-OH-BP was found to be highly mutagenic, i.e. at least 3-fold more active than BP (Table 1). 3-OH-BP was found to be a potent mutagen as well, causing a slightly higher level of revertants than BP. 9-OH-BP was less mutagenic than BP, while 2-OH-, 7-OH- and 12-OH-BP were not mutagenic at 2.5 μg/plate with MC-induced S-9 protein. Differences in mutagenicity are not related to toxicity of phenols at these low concentrations since revertants appear and increase in number with increasing concentrations of 7-OH- (data not shown) and 12-OH-BP (see Fig. 3). The maximum number of revertants is approximately 175–250 at 30 μg/plate. 2-OH-BP is also mutagenic at the appropriate concentration with control S-9 protein.* The level of mutagenicity in this assay by BP-7,8-diol can be affected by toxicity of the highly reactive and mutagenic epoxide [29] derived from the diol. Toxicity can be altered by either increasing the enzyme concentration [18] or the 7,8-diol concentration (data not shown). BP-7,8-diol at the nontoxic level of 1.25 μg/plate is shown to be very mutagenic (Table 1).

Since we were also interested in the relative specificity of UDP-glucuronosyltransferase(s) (EC 2.4.1.17) for the various phenols of BP, the effect of UDPGA and UDP-*N*-acetylglucosamine on mutagenesis was examined. UDP-*N*-acetylglucosamine is considered an endogenous activator of membrane-bound transferase activity [26]. Table 1 also shows that transferase activity in the S-9 fraction from MC-treated C57BL/6N mice apparently conjugates 1-OH-BP and 3-OH-BP almost completely, whereas 9-OH-BP is conjugated to a lesser extent as determined by levels of mutagenicity in the presence of UDPGA. Histidine reversion due to BP-7,8-diol under these conditions was not affected significantly by the presence of the transferase substrate. Mutagenicity by concentrations of 7,8-diol as low as 0.08 μg/plate was not affected significantly by the presence of UDPGA and UDP *N*-acetylglucosamine. UDPGA alone always allowed less protection than in the presence of UDP-*N*-acetylglucosamine, and UDP-*N*-acetylglucosamine alone did not alter significantly the level of mutagenesis.

Evidence for formation of a glucuronide of a benzo[a]pyrene phenol under conditions of the mutagenesis assay is shown in Table 2. With [^{3}H]3-OH-BP and UDPGA in the presence of S-9 protein from MC-treated C57BL/6N mice, a β-glucuronidase-sensitive product isolated by the method of Nemoto and Gelboin [6] is formed. The addition of UDP-*N*-acetylglucosamine to the system further increased the level of 3-OH-BP-glucuronide.

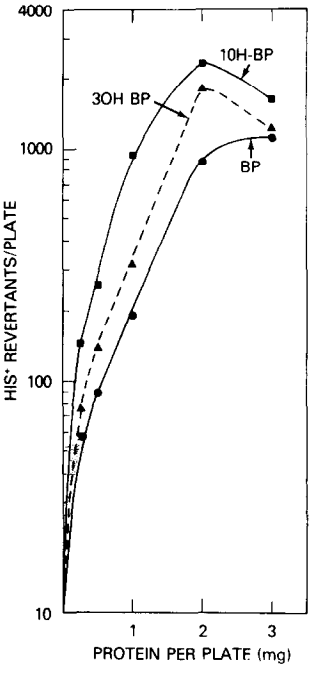

Fig. 1. Mutagenicity *in vitro* of 1-OH-BP, 3-OH-BP and BP as a function of S-9 protein concentration. The S-9 fraction was prepared as described under Materials and Methods from the livers of four MC-treated C57BL/6N mice. Each mutagen was present at 2.5 μg/plate; the background level of revertants was subtracted in each case.

*I. S. Owens and M. Warner, manuscript in preparation.

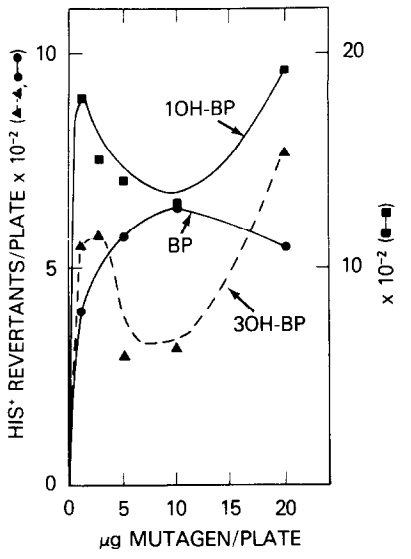

Fig. 2. Mutagenicity *in vitro* as a function of 1-OH-BP, 3-OH-BP and BP concentration. The S-9 fraction from livers of four MC-treated C57BL/6N mice was prepared and filtered as described in Materials and Methods, and 1.0 mg of S-9 protein was used in each assay.

In order to optimize the conditions for mutagenesis with the phenols, the histidine reversion was determined with respect to S-9 protein and phenol concentrations. In Fig. 1 the level of revertants with BP, 1-OH-BP and 3-OH-BP is shown to increase rapidly with increasing S-9 protein up to 2.0 mg/plate. Maximum activity was about 2000 colonies for the phenols and approximately 1000 colonies for BP. Revertants due to 1-OH-BP and 3-OH-BP fluctuated with various S-9 preparations at a particular protein concentration, but the phenols always gave the same relative levels of revertants.

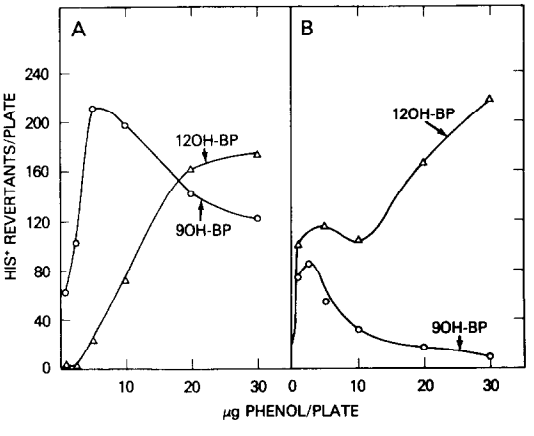

Fig. 3. Mutagenicity *in vitro* as a function of 9-OH-BP or 12-OH-BP concentration. Panel A represents the level of revertants by 9-OH-BP and 12-OH-BP using S-9 protein prepared from the liver of MC-treated C57BL/6N mice, as described in Materials and Methods. Panel B represents the reversion by 9-OH-BP and 12-OH-BP using phenobarbital-treated C57BL/6N mice. Two mg of S-9 protein was used in each assay.

In Figs. 2 and 3, the reversion as a function of phenol concentration is shown with liver S-9 fraction. 1-OH- and 3-OH-BP at 1 μg/plate caused a high level of mutagenicity with a decline in activity between 5 and 10 μg/plate, and finally a second rise with 20 μg/plate using S-9 fraction from MC-treated C57BL/6N mice. There was no obvious explanation for the decline in activity, such as the existence of a toxic "lawn effect" [30]. These effects were also seen at 0.5 mg S-9 protein/plate (data not shown). It is unlikely that toxicity is a factor since mutagenesis increased again at higher phenol concentrations. In contrast, reversion due to BP rose with increasing concentrations up to 10 μg/plate and then declined slightly. The highest level of histidine reversion (Fig. 3, panel A) by either 9-OH-BP or 12-OH-BP with S-9 fraction from MC-treated mice was about 200 colonies, i.e. the reversion was much below the level of approximately 600 and 1800 colonies caused by 1.0 μg/plate of 3-OH-BP and 1-OH-BP respectively.

Since plates without S-9 fraction showed little mutagenesis, it was evident that enzymatic activation was necessary for significant levels of reversion. It was of interest, therefore, to determine if a cytochrome P-450-mediated mono-oxygenase system, inducible in mice [23, 31] by phenobarbital, was more efficient at metabolizing the 9-OH- and 12-OH-BP, compared with a cytochrome P_1-450 system, inducible in mice [23, 31] by MC (Fig. 3, panel A). Figure 3, panel B, shows revertants as a function of 9-OH-BP or 12-OH-BP in the presence of the S-9 fraction from phenobarbital-treated mice. Reversion due to 9-OH-BP was significantly less in the presence of the S-9 fraction from phenobarbital-treated mice compared with the S-9 fraction from MC-treated mice. However, there was a higher number of revertants at low levels of 12-OH-BP (compared to Fig. 3, panel A) and a secondary rise at higher concentrations. 9-OH-BP and 12-OH-BP at 30 μg/plate did occasionally cause toxic effects in the absence of enzyme.

Because we observed a much higher level of revertants by 3-OH- or 1-OH-BP in the bacterial mutagenesis assay using strain TA98 than had been reported previously [18], it was of interest to characterize the enzymatic activation of these two phenols, comparing activation by cytochrome P-450 and cytochrome P_1-450-dependent mono-oxygenase systems in the presence of known modifiers of these enzymes. In Table 3, histidine reversion in the presence of 3-OH-BP is shown with control or induced S-9 fraction. With each S-9 fraction NADPH is shown to be a requirement. The complete assay system shows that the S-9 fraction from phenobarbital-treated C57BL/6N mice increased activation above control levels slightly, whereas the S-9 fraction from MC-treated C57BL/6N mice increased mutagenesis 6-fold. Since MC induces primarily ANF-sensitive cytochrome P_1-450-dependent mono-oxygenase activity [31] and phenobarbital induces primarily MTP-sensitive cytochrome P-450-dependent mono-oxygenase activity [31] in the C57BL/6N mice, the effect of ANF and MTP on these activities was examined. It can be seen that 50 μM ANF inhibited MC-induced activity and greatly enhanced phenobarbital-induced activity. In contrast, 100 μM MTP significantly inhibited phenobarbital-induced activity and slightly inhibited MC-induced activity. Generally,

Table 3. Modification of the mutagenesis resulting from activation of 3-OH-benzo[a]pyrene by mouse liver S-9 fraction*

	Concn (mM)	Pretreatment of mice *in vivo*			
		B6			D2
		Control	Phenobarbital	3-Methylcholanthrene	3-Methylcholanthrene
Complete		100	135	595	254
−NADPH		0	2	5	8
+ANF	0.05	95	533	227	280
+MTP	0.1	130	70	473	157
+TCPE	0.372	157	92	684	
	3.72			510	125
	7.44			473	
+UDPGA	2.0	50	23	280	153
+UDPGA +	2.0	0	−10	120	111
+UDP-*N*-AcgluNH$_3$	4.0				
+UDP-*N*-AcgluNH$_3$	4.0	109	142	495	208
+PAP +	0.02			138	142
+PNP	0.005				
+PAP	0.02			453	231
+PNP	0.005			425	217
+GSH	0.5	95	127	430	183

*S-9 fractions (1.5 mg/plate) were either prepared from C57BL/6N (B6) mice which were controls, phenobarbital-treated, or MC-treated; or fractions were prepared from DBA/2N (D2) mice which were MC-treated. 3-OH-BP was present at 2.5 μg/plate in each case. UDP-glucuronic acid (UDPGA), UDP-*N*-acetylglucosamine (UDP-*N*-AcgluNH$_3$), adenosine-3′:5′-diphosphate (PAP), *p*-nitrophenylsulfate (PNP), glutathione (GSH), α-naphthoflavone (ANF), metyrapone (MTP), 1,2-epoxy-3,3,3-trichloropropane (TCPE), and the 3-OH-BP were added as described under Materials and Methods. Each test plate contained 10^8 bacteria. Values are of histidine revertant colonies. This represents a typical experiment repeated three times.

these same effects of ANF and MTP on cytochrome P_1-450- and cytochrome P-450-dependent mono-oxygenase activities, respectively, were seen previously for the *in vitro* metabolism of BP [31] and for MC mutagenesis [32]. TCPE at one or three concentrations was shown not to alter significantly the level of revertants by 3-OH-BP activation with S-9 fraction from C57BL/6N mice, although the hydratase inhibitor decreased activity approximately 50 per cent with S-9 fraction from DBA/2N mice.

Also in Table 3 (columns 3 and 4), the relative activation of 3-OH-BP by S-9 fractions from either MC-treated C57BL/6N or DBA/2N mice can be compared. Cytochrome P_1-450-associated mono-oxygenase activity has been shown to be induced by MC treatment in the responsive C57BL/6N mice and, essentially, unaffected by MC treatment of the nonresponsive DBA/2N mice [23]. This difference in responsiveness has been shown to relate to genetic regulation by one or two alleles at the *Ah* locus in mice [23, 33]. Mutagenesis by 3-OH-BP is not induced in DBA/2N mice, whereas one sees a 6-fold induction in C57BL/6N mice. Activity with the S-9 fraction from control DBA/2N mice (data not shown) did not differ significantly from activity from MC-treated DBA/2N mice. ANF slightly enhanced the level of revertants due to the S-9 fraction from MC-treated DBA/2N mice, and MTP slightly inhibited the reversion.

Since glucuronidation is the primary detoxifying mechanism for phenolic substrates in mammalian cells, the effect on mutagenesis of adding the substrate, UDPGA, for UDP-glucuronosyltransferase activity was studied. In Table 3 the addition of UDPGA in the assay resulted in 40–50 per cent protection against mutagenicity in each case, except for the S-9 fraction from phenobarbital-treated mice which allowed 83 per cent protection. The combination of UDPGA and UDP-*N*-acetylglucosamine further enhanced the protection due to glucuronidation of 3-OH-BP or its metabolites. This nucleoside sugar alone slightly protected against mutagenesis with the S-9 fraction from either MC-treated strain. The results of this study suggest that transferase activity was slightly induced only after phenobarbital treatment.

Sulfotransferase(s) also plays a physiological role in detoxifying phenolic compounds via sulfation. Therefore, the effect of adding the substrate for sulfotransferase, 3′-phosphoadenosine 5′-phosphate sulfate, on mutagenesis was examined (Table 3). 3′-Phosphoadenosine 5′-phosphate sulfate was formed enzymatically from 3′-phosphoadenosine 5′-phosphate and PNP as described previously [27]. 3′-Phosphoadenosine 5′-phosphate sulfate in the presence of the S-9 fraction from either MC-treated C57BL/6N or DBA/2N mice protected against 45–75 per cent of the mutagenicity by 3-OH-BP. Either 3′-phosphoadenosine 5′-phosphate or PNP alone slightly reduced reversion. Generally, glucuronidation in the presence of UDPGA and UDP-*N*-acetylglucosamine was a more efficient protective reaction than sulfation.

Glutathione was tested as a protective agent against reversion caused by 3-OH-BP since highly reactive epoxides formed from hydrocarbons can be inactivated through conjugation with glutathione via glutathione-*S*-epoxide transferase(s) [4]. It can be seen that the number of revertants is decreased slightly by the addi-

Table 4. Modification of the mutagenesis resulting from activation of 1-OH-benzo[a]pyrene by mouse liver S-9 fraction *

	Concn (mM)	Pretreatment of mice *in vitro*			
		B6			D2
		Control	Phenobarbital	3-Methylcholanthrene	3-Methylcholanthrene
Complete		221	475	1478	273
−NADPH		54	8	10	5
+ANF	0.05	2775+ or 200	384	680	1370+ or 250
+MTP	0.1	2043+ or 210	588	1463	177
+TCPE	0.372	295	476	1408	
	3.72	441	320	1410	259
	7.44			1624	
+UDPGA	2.0	223	235	425	176
+UDPGA + UDP-*N*-AcgluNH$_3$	2.0 + 4.0	18	16	113	75
+UDP-*N*-AcgluNH$_3$	4.0	327	611	1563	271
+PAP + PNP	0.02 + 0.005			705	153
+PAP	0.02			1718	245
+PNP	0.005			1650	286
+GSH	0.5	236	459	1523	275

*S-9 fractions (1.5 mg/plate) were either prepared from C57BL/6N (B6) mice which were controls, phenobarbital-treated, or MC-treated; or fractions were prepared from DBA/2N (D2) mice which were MC-treated. 1-OH-BP was present at 2.5 μg/plate in each case. Modifier compounds are abbreviated as described in the legend of Table 2. Modifier compounds and 1-OH-BP were added as described under Materials and Methods. This represents a typical experiment repeated three times. Values are of histidine revertant colonies.
+ See Results for discussion of these data.

tion of glutathione. Because excess glutathione could possibly prevent oxidation of a hydrocarbon by depressing the re-dox potential of the microsomal electron transport chain and allow protection due to a low level of epoxide, the effect of higher concentrations of glutathione was not explored.

The effects of modifier compounds on the oxidation of the 3-OH-BP and 1-OH-BP are parallel, with few exceptions (Table 4). 1-OH-BP at 2.5 μg/plate is at least 2.5 times more mutagenic than 3-OH-BP in the presence of the S-9 fraction from MC-treated C57BL/6N mice. Mutagenic activity in the presence of the S-9 fraction from MC-treated C57BL/6N mice is sensitive to ANF and not to MTP. MTP slightly enhanced the number of revertants with the S-9 fraction from phenobarbital-treated mice instead of inhibiting as with 3-OH-BP. In the presence of 1-OH-BP, ANF slightly inhibited reversion by the S-9 fraction from phenobarbital-treated mice, whereas it enhanced reversion with 3-OH-BP. ANF, added to the control S-9 fraction in two of four experiments with 1-OH-BP, but not with 3-OH-BP, caused an unexpected 5- to 10-fold increase in mutagenesis. Likewise, ANF sometimes caused a large increase in reversion by 1-OH-BP with S-9 fraction from MC-treated DBA/2N mice. TCPE and glutathione were not effective in reducing 1-OH-BP mutagenicity.

The presence of both UDPGA and UDP-*N*-acetylglucosamine for glucuronidation protected against reversion by 1-OH-BP activation to a greater extent than they protected against mutagenesis due to 3-OH-BP activation. Induction of glucuronidating activity for 1-OH-BP was higher by MC treatment of C57BL/6N mice than similar activity for 3-OH-BP. On the other hand, induction of glucuronidating activity for 3-OH-BP by phenobarbital was greater than the activity observed for 1-OH-BP. 3′-Phosphoadenosine 5′-phosphate sulfate again provided about 50 per cent protection against mutagenesis, and GSH did not protect against reversion by 1-OH-BP.

DISCUSSION

Activation of 1-OH- and 3-OH-BP to potent bacterial mutagens by the S-9 fraction isolated from MC-treated C57BL/6N mice and not by the S-9 fraction isolated from MC-treated DBA/2N mice suggests that cytochrome P_1-450-dependent mono-oxygenase activity can further metabolize these two phenols to highly reactive species in a manner similar to its metabolism of the native BP molecule. Since cytochrome P_1-450 metabolizes BP primarily in the non-K-region [34] to generate, among other intermediates, a highly reactive BP-7,8-diol-9,10-oxide, it is possible that cytochrome P_1-450 can likewise further metabolize 1-OH- and 3-OH-BP in the non-K-region to highly reactive intermediates. If the potent mutagenic species generated from activation of the 1-OH- or 3-OH-BP is a 7,8-diol-9,10-oxide, it would indicate that BP may ultimately cycle through a cytochrome P-450 system at least three

times. Furthermore, BP may initially serve as substrate for more than one type of cytochrome P-450 system, whereas its primary and secondary metabolites may be better subtrates for only one type of cytochrome P-450. Hence, the levels and types of cytochrome P-450 systems present may control the extent to which BP is further metabolized.

The weak, or absence of, mutagenicity of 7-OH-, 9-OH- and 12-OH-BP which already contain an hydroxyl group in or near the non-K-region further suggests that metabolism in the non-K-region by this mono-oxygenase system is responsible for the high level of mutagenicity seen with 1-OH- and 3-OH-BP.

On the other hand, the lack of mutagenicity by 2-OH-BP in the presence of cytochrome P_1-450 activity suggests that a small change in the position of the hydroxyl groups around the benzo[a]pyrene molecule may cause significant changes in its metabolism by a particular cytochrome P-450 system. Preliminary evidence suggests that this phenol is a better substrate for activation by the cytochrome P-450 system.

The much higher revertant rate of tester strain TA98 by 1-OH- and 3-OH-BP in this study with crude S-9 fraction compared to a similar previous study [18] with a reconstituted microsomal mono-oxygenase and epoxide hydratase system is probably due to the crude but more efficient microsomal system used in this study. Reconstitution may not have been complete [18]. Also, a mammalian cell-mediated mutagenesis assay [35] or a mammalian system without activation [36] did not show significant mutagenicity by 1-OH- or 3-OH-BP, and an earlier study with less sensitive tester strains failed to show significant mutagenicity due to BP phenols [37]. Thus, mutagenicity by these phenols is likely dependent upon the presence of both the appropriate tester strain and activating system.

If certain phenols are potential mutagenic derivatives of BP, the conjugating enzymes, UDP-glucuronosyltransferase [8] and sulfotransferase [9], are probably important in removing these promutagens from the cell. Furthermore, the extent to which one or both of these conjugating activities is present, and their substrate preferences, may affect the results of any *in vivo* assay involving phenols. It is appreciated that UDP-glucuronosyltransferase activity exists in most tissues, including skin [38]. Since mono-oxygenase and transferase activities are important in the metabolism of phenols and since both are functionally located in the drug metabolism pathway, the efficiency of coupling these enzymatic activities will necessarily control effects of the phenols. Induction by MC of mono-oxygenase and UDP-glucuronosyltransferase activities for 1-OH-BP seems to be quite high. Thus, highly coupled mono-oxygenase and transferase activities *in vivo* for 1-OH-BP might neutralize any potentially harmful effects of this phenol and may account for low levels of this compound *in vivo* [39]. In lung, 3-OH-BP production, for example, is increased in rats after exposure to cigarette smoke [40]. It remains to be seen whether conjugating activity for 1-OH-BP or 3-OH-BP is also increased by cigarette smoke. Thus, the level of cytochrome P_1-450 associated mono-oxygenase activity for further activation of potentially mutagenic BP phenols compared with the level of conjugating activities for detoxifying critical phenols may be important factors relating to the basis of target tissue for BP.

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