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INHIBITION OF THE MUTAGENICITY AND METABOLISM OF 6-METHYL-BENZO[*a*]PYRENE AND 6-HYDROXYMETHYL-BENZO[*a*]PYRENE

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Abstract—Previously reported inhibitors of benzo[*a*]pyrene (BaP) mutagenicity in *Salmonella typhimurium* strain TA98 were tested for their effectiveness against the mutagenicity of 6-methyl-benzo[*a*]pyrene (6-CH_3-BaP), 6-hydroxymethyl-benzo[*a*]pyrene (6-CH_2OH-BaP) and 6-acetoxymethyl-benzo[*a*]pyrene (6-CH_3COOCH_2BaP). Dose–response curves obtained for phenothiazine (PTH), 2-chlorophenothiazine (2Cl-PTH), phenylisothiocyanate (PHN), phenethylisothiocyanate (PNE), *trans*-retinol (TR) and disulfiram (TETD) showed a variety of degrees of inhibition of mutagenicity. Additionally, glutathione (GSH) was found to inhibit the mutagenicity of 6-CH_3COOCH_2-BaP, and the mutagenicity of 6-CH_2OH-BaP was enhanced by the addition of supplemental ATP, Na_2SO_4 and EDTA. Only 2Cl-PTH was equally as good an inhibitor of 6-CH_3-BaP and BaP, reducing revertant colonies to less than 50% of control at 10 × BaP concentration. To probe the mechanism of inhibition, the effect of 2Cl-PTH on the binding of BaP and the 6-substituted benzo[*a*]pyrenes to cytochrome P-450 was investigated by difference spectroscopy. Also, the effect of 2Cl-PTH on the subsequent metabolism of 6-CH_3-BaP and 6-CH_2OH-BaP was investigated by rapid scan difference spectroscopy and high-performance liquid chromatographic separation of products. The results are consistent with a major mechanism of inhibition for 2Cl-PTH involving a competition for the cytochrome P-450 binding site.

6-Methyl-benzo[*a*]pyrene (6-CH_3-BaP†) is approximately ten times more mutagenic than benzo[*a*]pyrene (BaP) in *Salmonella typhimurium* strain TA98 [1] and twice as mutagenic in strain TA100 [2, 3]. The carcinogenicity of 6-CH_3-BaP has been reported to be similar to BaP [4, 5] or somewhat less than BaP [6–8] depending upon the protocol used. *In vitro* studies of metabolic activation [9, 10] have shown that the major products of metabolism of 6-CH_3-BaP by rat liver microsomes are hydroxymethyl derivatives, dihydrodiols and phenols. Dihydrodiol formation, in particular, is an order of magnitude less than that from BaP [10]. It therefore seems unlikely that the mutagenicity and/or carcinogenicity of 6-CH_3-BaP can be accounted for by 4,5-oxide or 7,8-diol-9,10-epoxide formation.

An alternate pathway of metabolism which has been proposed for methylated hydrocarbons in general [11–13] and 6-CH_3-BaP in particular [8, 14, 15] involves initial hydroxylation by a mixed-function oxidase (cytochrome P-450), possibly involving a cation radical intermediate (Fig. 1) [16]. This step is followed by *in vivo* or *in vitro* esterification to form reactive phosphate or sulfate esters. These esters, as well as synthetic methyl esters, have been implicated as reactive intermediates because of their ease of formation of reactive carbonium ions and because of their observed carcinogenic and mutagenic activity [5, 8, 13, 15, 17].

Previously we have identified several compounds which exert a substantial antimutagenic effect on BaP in strain TA98 of *S. typhimurium* [1, 18, 19]. The goal of this study was to test some inhibitors of BaP mutagenesis on 6-CH_3-BaP and derivatives, in a way that might demonstrate the involvement of activation at the 6-position, as related to the antimutagenic properties of the inhibitors. Therefore, effects of phenothiazine (PTH), 2-chlorophenothiazine (2Cl-PTH), phenylisothiocyanate (PHN), phenethylisothiocyanate (PNE), *trans*-retinol (TR), and disulfiram (TETD) on the mutagenicity of 6-CH_3-BaP, 6-CH_2OH-BaP and 6-CH_3COOCH_2-BaP have been investigated. Furthermore, since glutathione (GSH) and glutathione transferases have been implicated in the detoxification of some BaP metabolites [20, 21] and since GSH has been found to conjugate 7-hydroxymethyl-12-methylbenz[*a*]anthracene through an ester derivative [22], the antimutagenic role that GSH may play in the detoxification of 6-CH_3-BaP derivatives was also studied.

Additionally, the nature of the binding of BaP and the 6-substituted benzo[*a*]pyrenes to cytochrome P-450 and the effects of the phenothiazine inhibitors on this binding, as well as the subsequent metabolism, have been investigated. In this way, specific information was sought on possible mechanisms of inhibition, including direct inhibition of an ultimate

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† Abbreviations: BaP, benzo[*a*]pyrene; 6-CH_3-BaP, 6-methyl-benzo[*a*]pyrene; 6-CH_2OH-BaP, 6-hydroxymethyl-benzo[*a*]pyrene; 6-CH_3COOCH_2-BaP, 6-acetoxymethyl-benzo[*a*]pyrene; PTH, phenothiazine; 2Cl-PTH, 2-chlorophenothiazine; PHN, phenylisothiocyanate; PNE, phenethylisothiocyanate; TR, *trans*-retinol; TETD, disulfiram; GSH, glutathione; β-NF, β-naphthoflavone; DMSO, dimethyl sulfoxide; and TCPO, trichloropropene oxide.

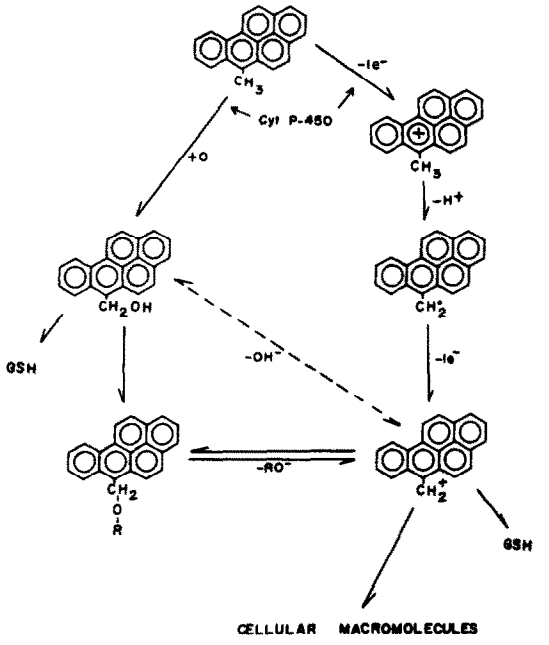

Fig. 1. Metabolic pathways that have been proposed for 6-CH_3-BaP.

mutagen, or competition between the inhibitor and mutagen/carcinogen for activating enzymes.

MATERIALS AND METHODS

Chemicals. TR, glucose-6-phosphate, histidine, biotin, GSH, adenine triphosphate (ATP), isocitrate dehydrogenase, sodium isocitrate, nicotinamide adenine dinucleotide phosphate (NADP) and NADPH were obtained from the Sigma Chemical Co., St. Louis, MO; sodium (di) ethylenediamine tetraacetate (EDTA) was obtained from Fisher Scientific, Pittsburgh, PA; PHN and PNE were purchased from the Eastman Kodak Co., Rochester, NY; β-naphthoflavone, PTH, 2Cl-PTH, dimethyl sulfoxide (DMSO), TETD and BaP were obtained from the Aldrich Chemical Co., Milwaukee, WI. All chemicals were used in their commercially available form. 6-CH_3-BaP and 6-CH_2-OH-BaP were prepared by literature methods [23] and 6-CH_3COOCH_2-BaP was prepared from 6-CH_2OH-BaP with a slight modification of a previously described procedure [24].

Mutagenicity assay. The bacterial strain, *S. typhimurium* TA98, was supplied by Dr. Bruce Ames, University of California, Berkeley, CA. The assay was performed as described by Ames *et al.* [25], with the following modification: histidine and biotin were added to the top agar before autoclaving. The mutagens were dissolved in 15 μl of DMSO and their concentrations were 2.8, 1.3, and 0.6 nmoles/plate for 6-CH_3-BaP, 6-CH_2OH-BaP and 6-CH_3COOCH_2-BaP respectively. The BP control was 6.0 nmoles/plate. These concentrations were chosen to be on the linear region of a dose versus mutagenicity plot and to provide a reasonable number of revertants per plate (200–500). The six inhibitors, for which dose–response experiments were performed, were added to the test system in 50 μl of DMSO to give final concentrations ranging from 0.6 to 150 nmoles/plate corresponding to 0.2 to 45 μM based on a final volume of 3.3 ml. GSH was added in 50 μl of water so that the final concentration in the top agar was 1 mM. Each plate also received 0.100 ml of a 12 to 15-hr culture and 0.605 ml of a freshly prepared S-9 mix, containing 0.040 ml of liver S-9 [26] obtained from β-naphthoflavone-induced rats and an NADPH-generating system. The dose–response tests with 6-CH_3COOCH_2-BaP, however, received no S-9 mix. In the additional experiments with BaP, 6-CH_3-BaP and 6-CH_2OH-BaP involving GSH, S-9 mix was used and with 6-CH_3COOCH_2-BaP, S-9 mix was used where indicated. The number of His^+ revertants was scored on a Lab-line digimatic colony counter after a 48-hr incubation in the dark at 37°. All dose–response experiments were performed at least three times, with 3–5 plates per antioxidant concentration. The results in Figs. 2 and 3 are expressed as the average of the percent of the mutagen to control, after spontaneous revertants have been substracted.

Artifactual inhibition and toxicity were monitored by routine inspection of background bacterial lawn and spontaneous reversion frequencies. In addition, toxicity towards 1 mM GSH was monitored by a survival study which consisted of plating 50 μl of revertant bacterial culture on minimal agar in the presence and absence of the inhibitor.

Binding and metabolism. Binding of BaP and derivatives to cytochrome P-450 obtained from β-naphthoflavone-induced rat livers was measured by difference spectroscopy [27–31]. Microsomes were prepared from the livers of male, white Sprague–Dawley rats weighing between 150 and 200 *g* according to standard procedures [32]. Microsomes were diluted in 0.1 M phosphate buffer, pH 7.4, 20% glycerol to a protein concentration of 1 mg/ml. Aliquots of BaP, BaP derivatives, and inhibitors were added in acetone from 10-μl Hamilton microsyringes; the volume of acetone did not exceed 20 μl. Protein concentrations were measured as in Ref. 33, using a kit from Bio-Rad Laboratories, Richmond, CA. Difference spectra were recorded on a Hewlett–Packard 8451A diode array spectrophotometer. Values of ΔA (500–418 nm) were used to quantitate the binding [29].

Metabolism was monitored by the BaP hydroxylase assay [34], by direct spectrophotometric observation of BaP phenol formation [35], and by high-performance liquid chromatographic (HPLC) separation of metabolites. A typical reaction mixture contained 5 mM sodium isocitrate, 5 mM $MgCl_2$, 0.15 M KCl, 80 μM BaP or derivative, 0.8 units isocitrate dehydrogenase/ml, and 0.5 mg microsomes/ml in 0.05 M Tris–chloride buffer, pH 7.4, and was incubated at 37°. The fluorescence assay was initiated by addition of NADPH (0.1 mM), and the specific enzymatic activity was obtained from times between 0 and 10 min. Direct spectrophotometric formation of phenols was performed on the HP 8451A diode array spectrophotometer at 37°. The microsomal suspension containing all components except NADPH was divided between sample and reference cuvettes.

Table 1. Effect of 1 mM GSH on the mutagenicity of BaP, 6-CH_3-BaP, 6-CH_2OH-BaP and 6-CH_3COOCH_2-BaP

Compound	System	His$^+$ revertants/plate* Control	1 mM GSH
6-CH_3COOCH_2-BaP (0.6 nmole/plate)	−S9	526 ± 58	295 ± 56
6-CH_3COOCH_2-BaP (0.6 nmole/plate)	+S9	182 ± 12	24 ± 18
6-CH_2OH-BaP (1.3 nmoles/plate)	−Suppl.† + S9	223 ± 46	173 ± 34
6-CH_2OH-BaP (1.3 nmoles/plate)	+Suppl.† + S9	409 ± 89	285 ± 88
6-CH_3-BaP (2.8 nmoles/plate)	+S9	483 ± 84	522 ± 30
BaP (6 nmoles/plate)	+S9	226 ± 38	248 ± 44

* Spontaneous revertants in the range of 30–50 per plate have been subtracted.
† Suppl. = PAPS-generating system = ATP (5 μmoles/plate), Na_2SO_4 (5 μmoles/plate), EDTA (0.1 μmole/plate).

After taking a reference spectrum NADPH was added to the sample cuvette. Spectra were automatically taken between 350 and 650 nm at 1-min intervals for 10 min. The absorbance of cytochrome b_5 was compensated for by addition of 100 μM NADH to both cuvettes. The rates of phenol formation could be determined by monitoring the change in absorbance with time at 428–432 nm relative to 454 mm [35].

HPLC separation of metabolites was carried out on ethyl acetate/acetone (2:1) extracts of a 60-min microsomal metabolism. The solvent was evaporated and the residue was dissolved in 500 μl of methanol. The separation was carried out on an ISCO liquid chromatograph using reverse phase gradient elution on a 5 μM Ultrasphere ODS C_{18} column (4.6 mm × 25 cm) with 254 nm u.v. detection. The composition of the initial and final solvents was 65% CH_3OH:35% H_2O and 100% CH_3OH (Burdick & Jackson). A linear gradient was used for 35 min followed by 5 min at 100% CH_3OH at a flow rate of 1 ml/min.

RESULTS

Mutagenicity studies. The mutagenicity of 6-CH_3-BaP and 6-CH_2-OH-BaP to strain TA98 was found to be approximately 200 and 180 revertants/nmole, respectively, in the presence of rat liver S-9 mix. No mutagenicity was detected in the absence of S-9. 6-CH_3COOCH_2-BaP, on the other hand, was active as a direct mutagen, with an activity of 760 revertants/nmole. In the presence of S-9 mix, the mutagenicity of 6-CH_3COOCH_2-BaP was reduced to approximately one-third of its value in the absence of S-9 (Table 1).

Dose–response curves of the six strong inhibitors of BaP mutagenicity were constructed from data obtained from mutagenicity experiments with 6-CH_3-BaP and 6-CH_2OH-BaP in the presence of S-9 and 6-CH_3COOCH_2-BaP in the absence of S-9. The inhibitors were studied in the range from 1 to 50 times the concentration of the hydrocarbon. Figures 2 and 3 show the dose–response curves for 6-CH_3-BaP and 6-CH_2OH-BaP. PTH (Figs. 2a and 3a) at low doses led to an increase in the mutagenicity of 6-CH_3-BaP and 6-CH_2OH-BaP; however, at higher doses a slight, although statistically insignificant, inhibition was observed. On the other hand, 2Cl-PTH inhibited 6-CH_3-BaP strongly (Fig. 2b), but did not inhibit 6-CH_2OH-BaP (Fig. 3b), in fact showing again an increased mutagenicity at low doses. PHN did not inhibit 6-CH_3-BaP (Fig. 2c); however, it moderately inhibited 6-CH_2OH-BaP (Fig. 3c). PNE and TR were both moderate inhibitors of both 6-CH_3-BaP and 6-CH_2OH-BaP (Figs. 2d, 3d and 2e, 3e), while TETD (Figs 2f, 3f) showed little inhibition towards either 6-CH_3-BaP or 6-CH_2OH-BaP. None of the inhibitors tested inhibited the direct acting mutagen 6-CH_3COOCH_2-BaP in the absence of S-9 mix (data not shown).

The inhibition of the mutagenicity of 6-CH_3COOCH_2-BaP by 1 mM GSH in the presence or absence of S-9 was pronounced (Table 1), the mutagenicity being reduced to essentially zero in the presence of S-9 and GSH. However, the mutagenicity of 6-CH_2OH-BaP was reduced only slightly by the presence of GSH and that of 6-CH_3-BaP was not affected significantly (Table 1). Additionally, it was observed that the mutagenicity of 6-CH_2OH-BaP was increased markedly in the presence of additional ATP, sulfate and EDTA, as previously observed by others [13].

Binding and metabolism. From the above results the most striking observation was the strong inhibitory effect of 2Cl-PTH on 6-CH_3-BaP in contrast to its lack of effect on 6-CH_2OH-BaP. To investigate the inhibitory mechanism of this compound in more detail, the effect of 2Cl-PTH on the binding of BaP, 6-CH_3-BaP, 6-CH_2OH-BaP and 6-CH_3COOCH_2-BaP to cytochrome P-450 and on the subsequent metabolism was investigated. BaP, the three derivatives and 2Cl-PTH all gave Type I difference spectra upon binding to microsomal P-450 from β-NF-induced rats. Spectral dissociation constants, K_S, and A_{max} values were obtained from plots of $1/\Delta A$ (500–

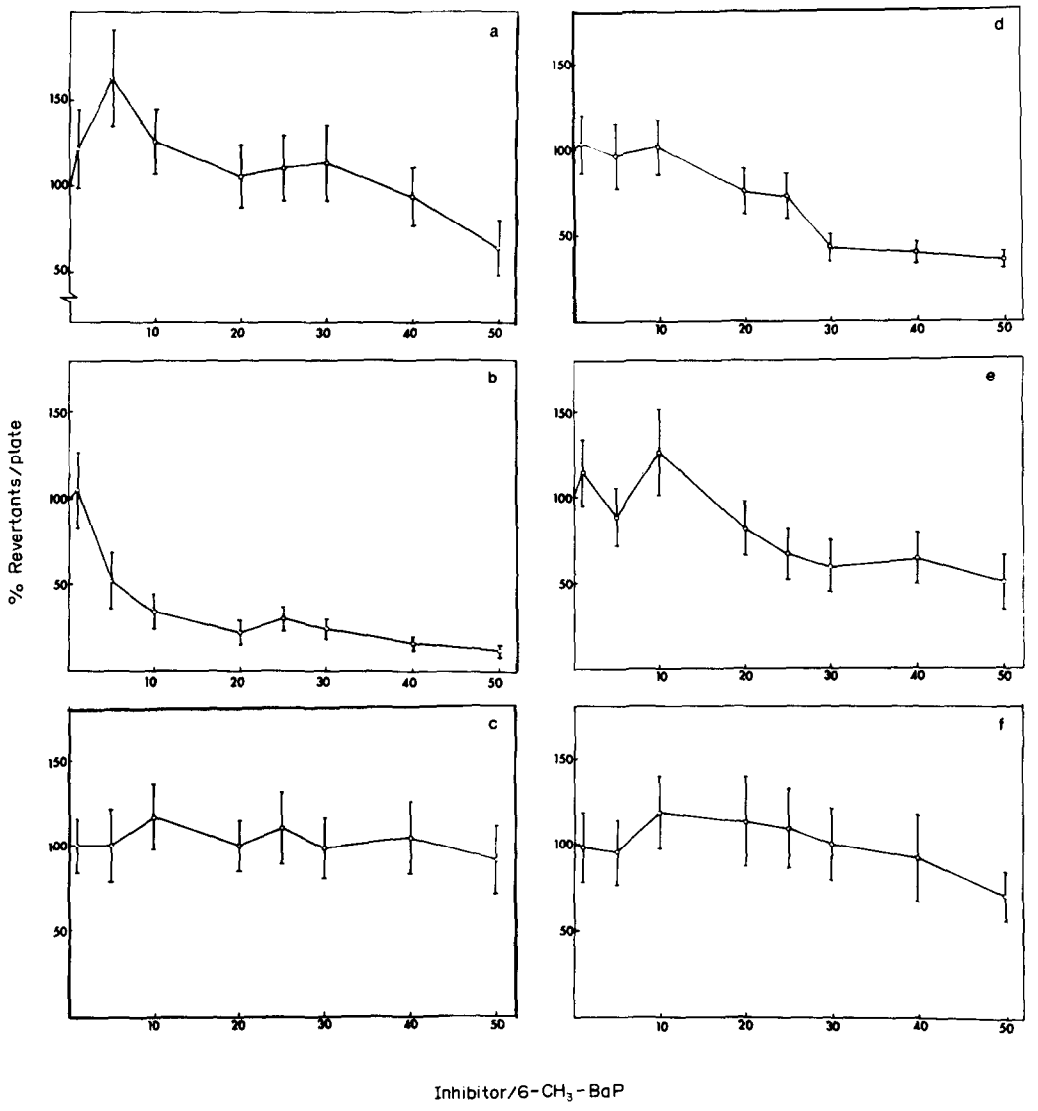

Fig. 2. Effects of six selected antioxidants on the mutagenicity of 6-CH_3-BaP (2.8 nmoles/plate) in strain TA98 of *S. typhimurium*: (a) PTH, (b) 2Cl-PTH, (c) PHN, (d) PNE, (e) TR, and (f) TETD. Each plate received 40 μl of hepatic S-9 from β-naphthoflavone-induced rats. Error bars represent ± the standard deviation calculated as a percentage of counts. See Methods for complete assay conditions. Numbers on the abscissa represent the ratio of the concentration of the inhibitor to that of 6-CH_3-BaP.

418 nm) versus 1/concn of BP or derivative using a weighted linear least squares analysis, and are listed in Table 2. As can be seen, the affinity for P-450 is in the order 6-CH_2OH-BaP > 6-CH_3-BaP > 6-CH_3COOCH_2-BaP > BaP. The effect of 2Cl-PTH on the dissociation constants is also indicated in Table 2. At a 2Cl-PTH concentration of 2 μM, an apparent 16-fold increase in K_S was observed for 6-CH_3-BaP. This change is much larger than that predicted by a simple competitive binding mechanism for inhibition [31] and suggests that 2Cl-PTH might exert a strong effect on the metabolism of 6-CH_3-BaP. On the other hand, inhibition of the binding of 6-CH_2OH-BaP and 6-CH_3COOCH_2-BaP more nearly obeys the expected kinetics for competitive binding which is characterized by an increase in K_S and a decrease in ΔA_{max}. The binding of BaP itself was found to be inhibited non-competitively by 2Cl-PTH as evidenced by a decrease in ΔA_{max} while K_S was essentially unchanged.

Rapid scan difference spectroscopy was used to monitor directly the formation of phenols from BaP, 6-CH_3-BaP and 6-CH_2OH-BaP assuming that the u.v. spectra of the phenols from these three derivatives are similar. From data such as Fig. 4, the initial rate of phenol formation was determined to be 1.82 nmoles/mg protein/min for 6-CH_2OH-BaP, 1.36 for BaP, and 1.06 for 6-CH_3-BaP. A molar absorptivity of 13,200 l/mole cm was assumed [35] for each phenolic product and applied to the dif-

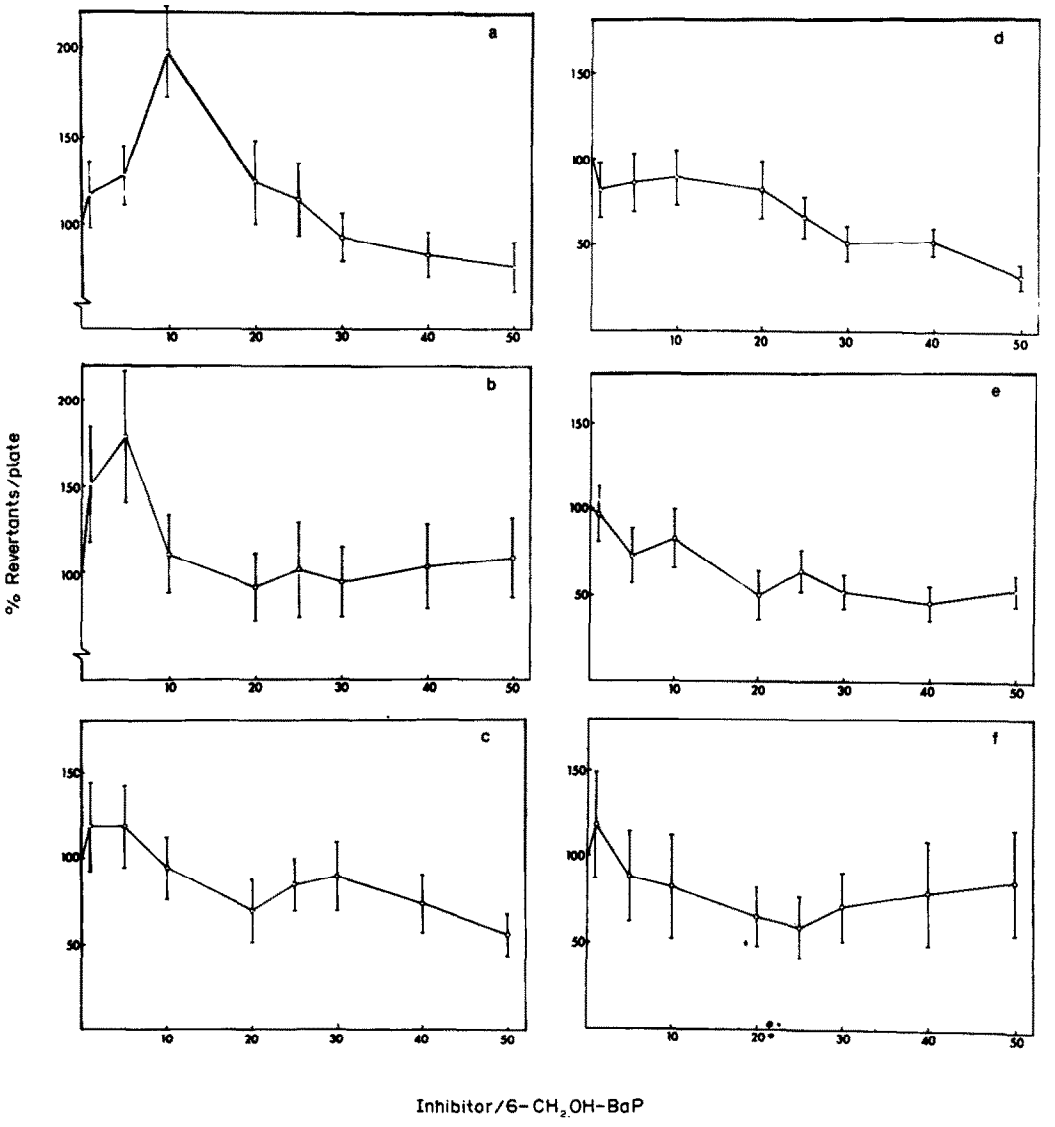

Fig. 3. Effects of six selected antioxidants on the mutagenicity of 6-CH_2OH-BaP (1.3 nmoles/plate) in strain TA98 of *S. typhimurium*: (a) PTH, (b) 2Cl-PTH, (c) PHN, (d) PNE, (e) TR, and (f) TETD. Each plate received 40 μl of hepatic S-9 from β-naphthoflavone-induced rats. Error bars represent ± standard deviation calculated as a percentage of counts. See Methods for complete assay conditions. Numbers on the abscissa represent the ratio of the concentration of the inhibitor to that of 6-CH_2OH-BaP.

Table 2. Summary of substrate and inhibitor binding to cytochrome P-450 as measured by difference spectroscopy

Compound	K_s (μM)	ΔA_{max}	K_s^* (app) (μM)	ΔA^*_{max} (app)
BaP	0.69 (0.13)†	0.041	0.62 (0.14)	0.019
6-CH_3-BaP	0.41 (0.06)	0.035	6.75 (0.25)	0.112
6-CH_2OH-BaP	0.26 (0.10)	0.029	0.53 (0.08)	0.017
6-CH_3COOCH_2-BaP	0.53 (0.16)	0.029	0.78 (0.18)	0.018
2Cl-PTH	3.09 (0.21)	0.029		

* Apparent values were measured in the presence of 2 μM 2Cl-PTH.

† Values in parentheses are relative standard deviations calculated from inverse absorbance values used to determine spectral dissociation constants.

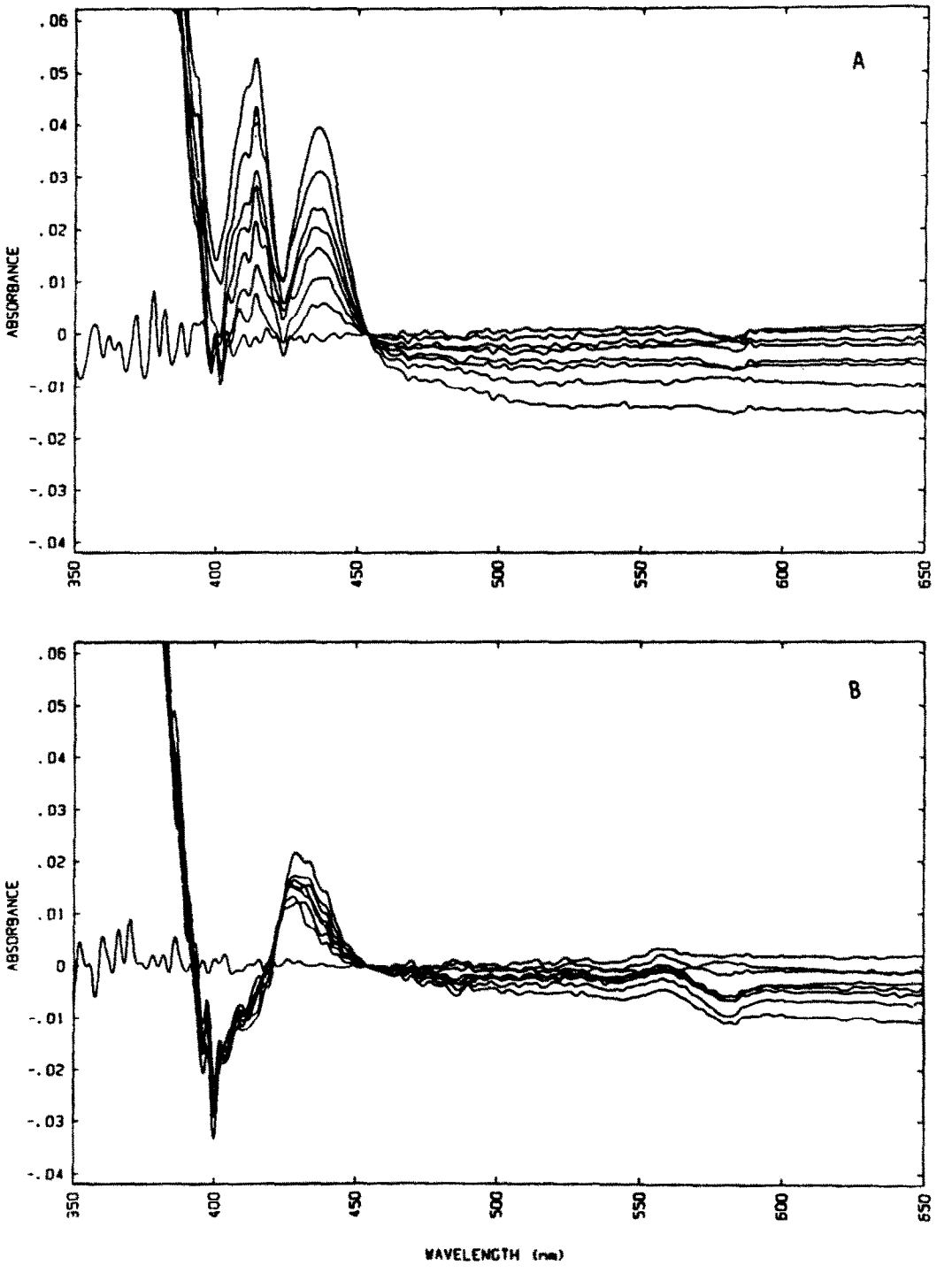

Fig. 4. Rapid scan spectrophotometric measurement of 80 μM 6-CH_3-BaP metabolism in the absence (A) and the presence (B) of equimolar 2Cl-PTH. Spectra were recorded at 1, 2, 3, 4, 5, 7 and 10 min after addition of NADPH. Control experiments with 2Cl-PTH alone showed no spectral changes in the 350–650 nm wavelength range.

Table 3. Effects of equimolar PTH and 2Cl-PTH on the rate of BaP, 6-CH_3-BaP and 6-CH_2OH-BaP phenol formation in rat liver microsomes as determined by repetitive scan difference spectroscopy

Time (min)	Rate of phenolic product formed (nmoles/mg protein/min)								
	BaP			6-CH_3-BaP			6-CH_2OH-BaP		
	Control	+PTH	+2Cl-PTH	Control	+PTH	+2Cl-PTH	Control	+PTH	+2Cl-PTH
1	1.36	0.61	0.06	1.06	1.37	0.45	1.82	1.36	1.44
	(0.22)	(0.50)	(0.00)	(0.29)	(0.30)	(0.33)	(0.25)	(0.22)	(0.001)
4	1.10	0.53	0.17	0.87	0.60	0.34	1.44	1.02	0.99
	(0.08)	(0.21)	(0.25)	(0.17)	(0.33)	(0.33)	(0.16)	(0.07)	(0.08)
10	0.76	0.36	0.09	0.59	0.41	0.15	1.02	0.76	0.70
	(0.02)	(0.17)	(0.10)	(0.04)	(0.25)	(0.09)	(0.08)	(0.08)	(0.07)

Values are averages of three to five trials; relative standard deviations are shown in parentheses. Conditions for assay are given in Methods.

ference in absorbance between 430 and 454 nm. 2Cl-PTH and PTH were observed to exert significant inhibitory effects on phenol formation of BaP, 6-CH_3-BaP and 6-CH_2OH-BaP at concentrations equimolar to BaP and/or its derivatives (Table 3). The substrate susceptibility to inhibition has BaP > 6-CH_3-BaP > 6-CH_2OH-BaP. At equimolar 2Cl-PTH concentrations phenol formation from BaP and 6-CH_3-BaP was inhibited by 90 and 75% during the 10-min incubation period, whereas phenol formation from 6-CH_2OH-BaP was only inhibited by 25% in the same period (Table 3). PTH decreased the rate of BaP phenol formation by about 50%, but exerted a much weaker effect on 6-CH_3-BaP and 6-CH_2OH-BaP (Table 3).

The metabolism of BaP in the presence and absence of 2Cl-PTH was also monitored by fluorescence spectroscopy to corroborate the results from spectral binding and rapid scan spectroscopy. Fluorescence of 3-OH-BaP was monitored at 520 nm in alkali solution with excitation at 466 nm. After 30 min of incubation aryl hydrocarbon hydroxylase metabolism of BaP was 300 pmoles/mg protein/min. In the presence of 2Cl-PTH, the activity dropped to 173 pmoles/mg protein/min.

The reverse phase HPLC profile of 6-CH_3-BaP metabolites from organic solvent extracts of a 1-hr incubation is shown in Fig. 5A and is very similar to the previously published data of Hammernik *et al.* [10]. By comparison to the previous data [10], peaks labeled 3 and 4 probably represent the 1 and 3 phenol derivatives of 6-CH_2OH-BaP. Figure 5B shows the chromatogram of 6-CH_3-BaP metabolites spiked with authentic 6-CH_2OH-BaP which elutes at 21.5 min. There is no corresponding peak in the metabolic profile of 6-CH_3-BaP (Fig. 5A). This suggests that virtually all of the metabolically formed 6-CH_2OH-BaP is converted to secondary phenol metabolites. This is consistent with the rapid scan difference spectroscopy which indicated that 6-CH_2OH-BaP is metabolized to phenols almost twice as fast as the parent 6-CH_3-BaP under our experimental conditions. Additionally, HPLC profiles of 6-CH_2OH-BaP also showed that the major products of the metabolism of this compound had the same retention time as peaks 3 and 4 from 6-CH_3-BaP. When 2Cl-PTH was added to the incubation mixture at the same concentration as 6-CH_3-BaP, a large decrease (90%) was clearly observed for the peak due to 1- and 3-OH-6-CH_3-BaP (peak 5); the other metabolite peaks were more difficult to unequivocally observe due to overlap with 2Cl-PTH metabolite peaks (Fig. 5C). However, the formation of the phenolic metabolites of 6-CH_2OH-BaP, as measured by HPLC, was only slightly decreased in total by the addition of equimolar amounts of 2Cl-PTH to the incubation mixture (data not shown). These results are also consistent with the rapid scan difference spectroscopy discussed above.

DISCUSSION

The mutagenicity of 6-CH_3-BaP, 6-CH_2OH-BaP and 6-CH_3COOCH_2-BaP towards strain TA98 of *S. typhimurium* was found to be similar to the data reported by Flesher *et al.* [13]. The data are essentially consistent with the hypothesis that 6-CH_3-BaP is converted to 6-CH_2OH-BaP which in turn may be converted into an activated ester which is a powerful mutagen and carcinogen (Fig. 1). However, the rapid metabolism of 6-CH_2OH-BaP to phenolic derivatives suggests perhaps that the ultimate mutagens may be activated esters of the phenolic derivatives of 6-CH_2OH-BaP. The increased mutagenicity of 6-CH_2OH-BaP in the presence of ATP, Na_2SO_4 and EDTA is also consistent with the formation of sulfate esters and parallels the increased binding of 6-CH_2OH-BaP to DNA under the same conditions [14]. However, the experiments with GSH seem somewhat contradictory. In the absence of S-9, 1 mM GSH reduced the mutagenicity of 6-CH_3COOCH_2-BaP by *ca.* 45%, indicating a substantial non-enzymatic conjugation by GSH [20]. In the presence of S-9 alone, the mutagenicity of 6-CH_3COOCH_2-BaP was reduced by 65% possibly due to contributions from conjugation reactions, reactions with S-9 proteins, or hydrolysis reactions followed by further cytochrome P-450 oxidations. Addition of GSH reduced the mutagenicity to almost zero, no doubt due to the occurrence of both enzymic and non-enzymic conjugation reactions. The minimal effect of GSH on the mutagenicity of 6-CH_2OH-BaP and

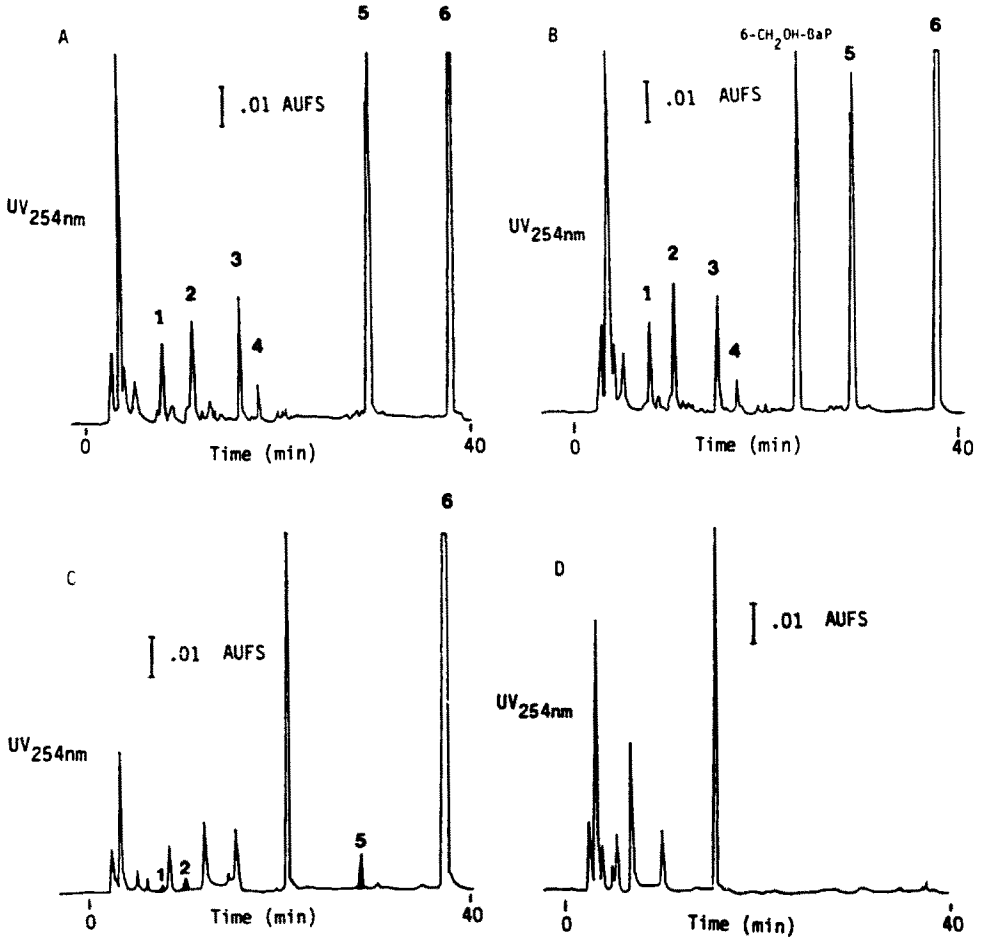

Fig. 5. HPLC metabolic profiles of (A) 6-CH_3-BaP, (B) 6-CH_3-BaP + 6-CH_2OH-BaP standard, (C) 6-CH_3-BaP + 2Cl-PTH, and (D) 2Cl-PTH. Numbered peaks in (A) probably correspond to the 6-CH_3-BaP metabolites: 1 = 4,5-diol; 2 = 7,8-diol; 3 = 1-OH-6-CH_2OH; 4 = 3-OH-6-CH_2-OH; 5 = 3-OH and 1-OH; and 6 = 6-CH_3-BaP. The metabolite peaks 1, 2, 5 and 6 are also indicated in C. Identification of peaks is based on retention times relative to chromatogram in Ref. 10. 6-CH_3-BaP and 2Cl-PTH concentrations were 40 μM. Microsomal protein concentration was 1.0 mg/ml.

the lack of effect on 6-CH_3-BaP and BaP indicate that the enzymic conjugation of GSH is not very effective in the S-9 systems and may further indicate that the concentration in the aqueous phase of the free electrophile from the hydroxymethyl ester is very low. These results contrast with the observations of Watabe *et al.* [22] who found that 4 mM GSH completely inhibits the mutagenicity of 7-hydroxymethyl-benz[*a*]anthracene in the presence of a hepatic supernatant fraction with a PAPS-generating system.

The results of the dose–response experiments may be compared to the strong inhibition of the mutagenicity of BaP caused by these same compounds [18]. The mutagenicity of BaP is, however, mainly due to the production of the mutagenic metabolites, BaP-4,5-oxide and BaP-7,8-diol-9,10-epoxides, by reaction of BaP with cytochrome P-450 enzymes [36]. In contrast, our working hypothesis for 6-CH_3-BaP, as outlined in Fig. 1, also involves a common initial reaction with cytochrome P-450. The fact that the inhibitors did not behave in the same way for both BaP and 6-CH_3-BaP can be taken as suggestive evidence that these two compounds are activated to ultimate mutagens by different pathways. Additional evidence for different pathways was also obtained from the effect of TCPO added to the mutagenicity assay. TCPO inhibited the formation of diols from both BaP and 6-CH_3-BaP by inhibition of epoxide hydrase. The observed [37] decrease in the mutagenicity of BaP (52%) on addition of 0.2 mM TCPO indicates a substantial contribution to the mutagenicity from BaP-7,8-diol-9,10-epoxides. The effect of added TCPO on the mutagenicity of 6-CH_3-BaP was minimal, indicating that the formation of epoxides is a much less important contributor to the mutagenicity of 6-CH_3-BaP.

Since none of the compounds studied exhibited any inhibition of the ultimate mutagen, 6-CH_3COOCH_2-BaP, it may be concluded that any effects on the mutagenicity of 6-CH_3-BaP or 6-CH_2OH-BaP involve modifications of their enzymatic processes or metabolic intermediates. Since the first step in the metabolism of BaP and 6-CH_3-

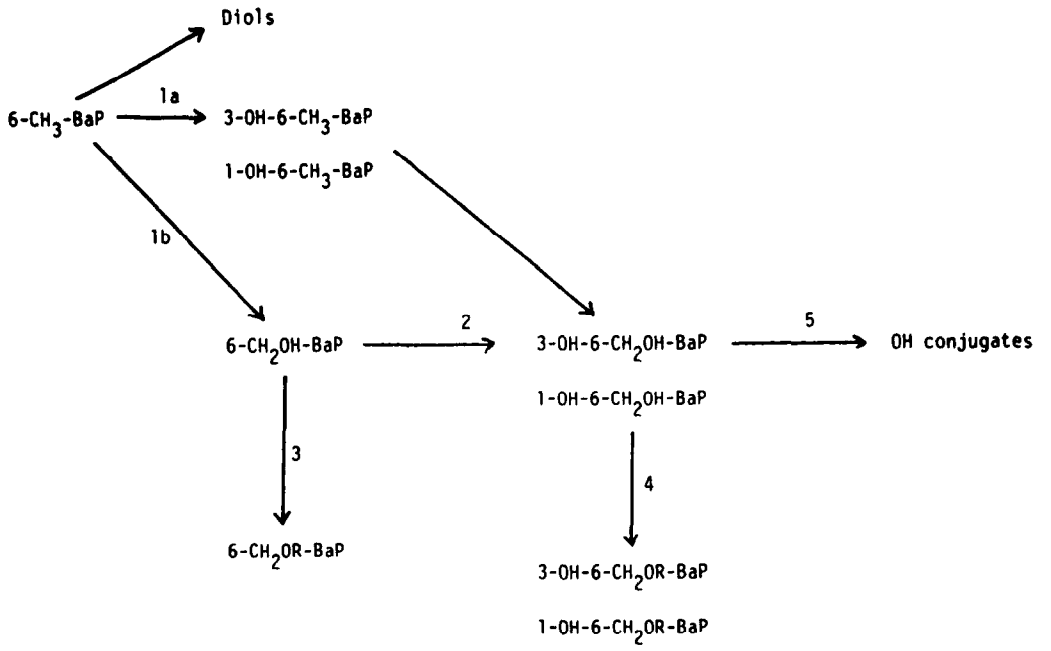

Fig. 6. Proposed reaction scheme for the *in vitro* metabolism of 6-CH_3-BaP and 6-CH_2OH-BaP. R can represent sulfate, phosphate or acetate groups.

BaP is assumed to be common, any inhibitor whose primary mechanism of inhibition involves this step should affect BaP and 6-CH_3-BaP equally. Only 2Cl-PTH fell into this category. The compounds that were good inhibitors of BaP mutagenicity but only moderate inhibitors of 6-CH_3-BaP, i.e. PTH, PNE and TR, must have other mechanisms of inhibition toward BaP metabolism in addition to modifying the cytochrome P-450 reaction. Those compounds that did not inhibit 6-CH_3-BaP, i.e. PHN and TETD, apparently had little effect on the cytochrome P-450 enzymes. Those compounds that inhibited the mutagenicity of 6-CH_2OH-BaP (PHN, PNE and TR) may prevent the formation of reactive esters by unknown mechanisms.

Overall, the most striking observation was that 2Cl-PTH inhibited 6-CH_3-BaP as well as it inhibited BaP but did not inhibit 6-CH_2OH-BaP. This suggests that the major mechanism of inhibition of 2Cl-PTH is its effect on the common step in the metabolism of BaP and 6-CH_3-BaP, namely the initial reaction with cytochrome P-450. This is consistent with the known metabolism of 2Cl-PTH by aryl hydrocarbon hydroxylase systems [38–40] and was further substantiated by the binding and metabolism studies which showed that 2Cl-PTH inhibited the binding of BaP and 6-CH_3-BaP to cytochrome P-450, although in a non-competitive manner for BaP and in a complex manner for 6-CH_3-BaP. The lack of inhibition exerted by PTH on the mutagenicity of 6-CH_3-BaP may be explained by the following observations. PTH is known to be a weaker inhibitor of BaP mutagenicity [19] than 2Cl-PTH. Table 3 shows that PTH was also a much weaker inhibitor of metabolic phenol formation than was 2Cl-PTH, for all three hydrocarbons investigated, and, in fact, inhibited BaP metabolism to a greater extent than 6-CH_3-BaP metabolism. 2Cl-PTH was, however, much less inhibitory of the binding of 6-CH_2OH-BaP to cytochrome P-450 and reduced the rate of phenol formation from this compound much less than that of BaP or 6-CH_3-BaP. These results also provide at least a partial explanation for the inhibition of the mutagenicity of BaP and 6-CH_3-BaP by 2Cl-PTH and its lack of effect on 6-CH_2OH-BaP. The major mechanism of inhibition for BaP and 6-CH_3-BaP seems to be an overall reduction in metabolism, due to competition for the active site on the cytochrome P-450 enzyme. The lack of an inhibitory effect upon 6-CH_2OH-BaP is less easily explained. However, a possible hypothesis may be given in terms of the pathways illustrated in Fig. 6. As noted above, the conversion of 6-CH_2OH-BaP to phenolic derivatives (step 2) was faster than the conversion of 6-CH_3-BaP to its phenolic derivatives (step 1a). If step 1b were similarly slower than step 2, it would explain the lack of observed production of 6-CH_2OH-BaP from 6-CH_3-BaP and the observation of the 3-OH and 1-OH derivatives of 6-CH_2OH-BaP. This would explain the similar mutagenicity of 6-CH_3-BaP and 6-CH_2OH-BaP. If the mutagenicity of 6-CH_2OH-BaP were produced by reactive sulfate or phosphate esters of 6-CH_2OH-BaP produced by reaction with sulfate or phosphate transferase enzymes (step 3) present in S-9 preparations, it is also possible that esters of the 1-OH and 3-OH-6-CH_2OH-BaP would contribute to the mutagenicity (step 4). Obviously, the extent of the mutagenicity of 6-CH_2OH-BaP would depend on the relative rates of reactions 2, 3 and 4, and on other conjugation reactions (step 5). The increased mutagenicity of 6-CH_2OH-BaP on addition of ATP, $MgCl_2$ and Na_2SO_4 would be

explained by an increase in reactions 3 and 4 at the expense of 2 and 5. The increased mutagenicity of 6-CH_2OH-BaP at low concentrations (Fig. 3b) of 2Cl-PTH might also be explained if reactions 2 or 5 were retarded so that more reactive ester could be produced in reactions 3 and 4. Obviously, these speculations require more quantitative determinations and careful kinetic studies of the relationship between cytochrome P-450 metabolism and sulfate ester conjugation at polycyclic aromatic hydrocarbon methyl side chains in order to fully understand all of the observations reported in this paper.

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