[CANCER RESEARCH 47, 3613–3617, July 15, 1987]

High Stereoselectivity in Mouse Skin Metabolic Activation of Methylchrysenes to Tumorigenic Dihydrodiols[1]

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ABSTRACT

The stereoselectivity of mouse skin metabolic activation to dihydrodiols of the strong carcinogen 5-methylchrysene (5-MeC) and the weak carcinogen 6-methylchrysene (6-MeC) was investigated. Synthetic 1,2-dihydro-1,2-dihydroxy-5-methylchrysene (5-MeC-1,2-diol), 5-MeC-7,8-diol, and 6-MeC-1,2-diol were resolved into their *R,R*- and *S,S*-enantiomers by chiral stationary phase high performance liquid chromatography. The absolute configurations of the enantiomers were assigned by their circular dichroism spectra. Using these enantiomers as standards, the metabolism of 5-MeC and 6-MeC *in vitro* in rat and mouse liver and *in vivo* in mouse epidermis was investigated. Only the *R,R*-enantiomers of each dihydrodiol predominated (>90%). The dihydrodiol enantiomers were tested for tumor initiating activity on mouse skin. In each case, the *R,R*-dihydrodiol enantiomer was significantly more tumorigenic than the *S,S*-enantiomer. The most tumorigenic compound was 5-MeC-1*R*,2*R*-diol; it was significantly more active than either 5-MeC-7*R*,8*R*-diol or 6-MeC-1*R*,2*R*-diol. The results of this study demonstrate that there is a high degree of stereoselectivity in the metabolic activation of 5-MeC and 6-MeC to proximate tumorigenic dihydrodiols in mouse skin. The bay region methyl group has no effect on the stereoselectivity of activation to 1,2-dihydrodiol metabolites in the chrysene system.

INTRODUCTION

5-MeC[3] (Fig. 1) is a strong carcinogen when tested on mouse skin but 6-MeC is a weak carcinogen (1). Investigations of the metabolic activation of 5-MeC have demonstrated that 5-MeC-1,2-diol and 5-MeC-1,2-diol-3,4-epoxide are major proximate and ultimate carcinogens (2). Comparative studies of the metabolic activation of 5-MeC and 6-MeC have shown that 6-MeC-1,2-diol is a major metabolite of 6-MeC in mouse skin, but that it is significantly less tumorigenic than is 5-MeC-1,2-diol (11). In addition, comparative tumorigenicity studies have established that 5-MeC-1,2-diol is more active than is 5-MeC-7,8-diol (3, 4). Based on these results, our hypothesis is that the high tumorigenicity of 5-MeC compared to 6-MeC is due to formation of 5-MeC-1,2-diol, which in turn can be metabolized to a diol epoxide having a methyl group and an epoxide ring in the same bay region; such a bay region diol epoxide could be produced from 5-MeC but not from 6-MeC. We believe that a bay region diol epoxide having a methyl group and an epoxide ring in the same bay region has exceptional tumorigenic properties; this is in agreement with available bioassay data (3).

However, another possible explanation for the differing tumorigenic activities of 5-MeC and 6-MeC is differing stereoselectivity in their metabolism to dihydrodiols and diol epoxides. Previous studies have shown that the stereoselectivity of dihydrodiol formation can be influenced by subtle differences in structure. For example, Yang and Fu demonstrated that the bay region 12-methyl group of 7,12-dimethylbenz(*a*)anthracene caused a change in the absolute configuration of the 5,6-dihydrodiol metabolite, compared to benz(*a*)anthracene and 7-methylbenz(*a*)anthracene (5). 5-MeC, like 7,12-dimethylbenz(*a*)anthracene, has a bay region methyl group. X-Ray crystallographic studies on 5-MeC have demonstrated that the presence of the methyl group in the bay region causes a widening of the bay region and a slight deformation from planarity (6, 7). These effects would not be expected in 6-MeC. Thus it seemed plausible that the stereoselectivity of either dihydrodiol formation or their conversion to diol epoxides might be affected by the presence of the bay region methyl group. In this study, we have investigated the metabolic formation and tumorigenicity in mouse skin of the enantiomers of 5-MeC-1,2-diol, 5-MeC-7,8-diol, and 6-MeC-1,2-diol.

MATERIALS AND METHODS

Apparatus. HPLC was carried out with a system composed of a Rheodyne Model 7125 Injection Loop (Rheodyne Inc., Cotati, CA), two Model 510 pumps (Millipore, Waters Division, Milford, MA), an Automated Gradient Controller (Waters), a Model 116 UV Detector (Gilson, Middletown, WI), and a Flo-one/β Radioactive Flow Detector (Radiomatic Instruments, Tampa, FL). The following columns and programs were used: system 1, a 4.0- × 250-mm Lichrosorb RP-18 10μ column (EM Reagents, Cincinnati, OH) programmed with 50% CH_3OH in H_2O for 30 min, then a linear gradient from 50–80% CH_3OH in H_2O in 40 min, then a linear gradient from 80–100% CH_3OH in H_2O in 10 min, at 3 ml/min; system 2, the same column as in system 1, programmed with a linear gradient from 30–50% CH_3OH in H_2O in 20 min, then a linear gradient from 50–100% CH_3OH in H_2O in 1 h, at 3 ml/min; system 3, a 10.0- × 250-mm Pirkle Type I-A 5 column [γ-aminopropyl silanized silica to which (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine is ionically bonded], Regis Chemical Co., Morton Grove, IL, eluted with 25% [2:1, C_2H_5OH:CH_3CN], 75% hexane at 3 ml/min; system 4, a 4.6- × 250-mm Pirkle Type I-A 5 column eluted as in system 3 but at 2 ml/min.

UV spectra were run on a Cary Model 118 Spectrometer (Varian Instruments, Palo Alto, CA). CD spectra were done in tetrahydrofuran solution on a J-500C Spectropolarimeter (Jasco Inc, Easton, MD) courtesy of Professor Robert Bitteman, Chemistry Department, Queens College, City University of New York.

Chemicals. Racemic 5-MeC-1,2-diol, 5-MeC-7,8-diol, and 6-MeC-1,2-diol were synthesized (8–10). The *R,R*- and *S,S*-enantiomers of each dihydrodiol were separated and isolated using HPLC system 3. Their purities were established by reinjecting aliquots on HPLC system 4, and by their UV spectra. *R,R*- and *S,S*-enantiomers of chrysene-1,2-diol were kindly provided by Dr. Donald M. Jerina, NIH, Bethesda, MD.

[^{3}H]5-MeC and [^{3}H]6-MeC were prepared as described previously (11, 12). 12-*O*-Tetradecanoylphorbol-13-acetate was obtained from Consolidated Midland Corp., Brewster, NY.

Metabolism Experiments *in Vitro*. Supernatants (9,000 × *g*) were prepared from livers of mice which had been pretreated with either 3-MC in corn oil, or corn oil, and from F344 rats pretreated with Aroclor

Received 12/15/86; revised 4/1/87; accepted 4/2/87.

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[1] This study was supported by Grants CA-44377 from the National Cancer Institute and BC-132 from the American Cancer Society. This is paper 103 of the series "A Study of Chemical Carcinogenesis."

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[3] The abbreviations used are: 5-MeC, 5-methylchrysene; 6-MeC, 6-methylchrysene 5-MeC-1*R*,2*R*-diol, 1,2-dihydro-1*R*,2*R*-dihydroxy-5-methylchrysene; 5-MeC-1*S*,2*S*-diol, 1,2-dihydro-1*S*,2*S*-dihydroxy-5-methylchrysene, etc.; HPLC, high performance liquid chromatography; CD, circular dichroism; 3-MC, 3-methylcholanthrene; PAH, polynuclear aromatic hydrocarbons.

1254, as previously described (11). Protein concentrations were 34 mg/ml (3-MC mouse), 39 mg/ml (uninduced mouse), and 38 mg/ml (Aroclor rat). Incubations of 5-MeC or 6-MeC with 9000 × *g* supernatant and cofactors were carried out as follows. A mixture of 5-MeC or 6-MeC (2.42 mg, 0.01 mmol) in 0.4 ml of dimethyl sulfoxide, 0.32 ml of 0.4 M $MgCl_2$, 0.32 ml of 1.65 M KCl, 0.08 ml of 1.0 M D-glucose-6-phosphate, and 0.64 ml of 0.1 M NADPH was brought to a total volume of 8.0 ml with potassium phosphate buffer (pH 7.4), and 8 ml of 9000 × *g* supernatant was added. The resulting mixture was incubated with shaking at 37°C for 20 min. The reactions were terminated by addition of 8 ml of acetone. The mixtures were extracted four times with 15 ml of ethyl acetate. The ethyl acetate extracts were analyzed by HPLC with system 1 for 5-MeC metabolites and system 2 for 6-MeC metabolites. The appropriate dihydrodiols were collected and reanalyzed by HPLC using system 4, for determination of enantiomeric composition.

Metabolism Experiments *in Vivo*. Twenty-five female CD-1 mice (age, 50–70 days) were shaved and, 24 h later, 15 mice were each treated with 0.15 ml of an acetone solution of [^{3}H]5-MeC (0.07 μmol/mouse, 0.7 Ci/mmol) and 10 mice were each treated with 0.15 ml of an acetone solution of [^{3}H]6-MeC (0.07 μmol/mouse, 1.7 Ci/mmol). Two h later the mice were killed and epidermal metabolites were isolated as described previously (13). The ethyl acetate extracts were analyzed by HPLC as in the *in vitro* experiments.

Bioassay for Tumor-initiating Activity. Each group consisted of 20 female CD-1 mice obtained at the age of 28 to 35 days from Charles River Breeding Laboratories, Inc., Kingston, NY. Animals were housed under standard conditions as previously described (3). At the age of 50 to 55 days, each mouse received a single initiating dose of the appropriate compound in 0.1 ml of acetone. Ten days later, promotion began by application of 2.5 μg of 12-*O*-tetradecanoylphorbol-13-acetate in 0.1 ml of acetone, three times weekly for 20 weeks. Mice were shaved when necessary and tumors were counted weekly. Statistical significance was evaluated using the Student's *t* test.

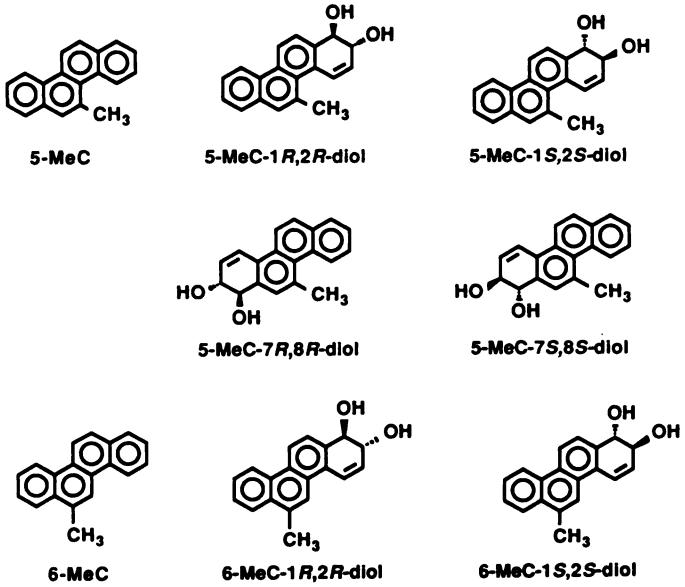

Fig. 1. Structures of 5-MeC, 6-MeC, and the enantiomeric dihydrodiols investigated in this study.

RESULTS

The enantiomers of 5-MeC-1,2-diol, 5-MeC-7,8-diol, and 6-MeC-1,2-diol were separated by chiral stationary phase HPLC under conditions similar to those described by Yang and coworkers for chrysene-1,2-diol and oxygenated metabolites of other PAH (14, 15). Fig. 2, *A–C* illustrate the separation of the enantiomers of 5-MeC-1,2-diol, 5-MeC-7,8-diol, and 6-MeC-1,2-diol and Table 1 summarizes the retention volumes and resolution values for the dihydrodiol enantiomers. The absolute configurations of the enantiomers of chrysene-1,2-diol have been previously determined by the exciton chirality method (16). The *R,R*-enantiomer purified in that study was injected under our HPLC conditions. It was more strongly retained than the *S,S*-enantiomer, confirming the assignments made by Weems et al. (14) and Weston et al. (17), based on retention volumes. The CD spectra of the enantiomers of 5-MeC-1,2-diol and 6-MeC-1,2-diol are shown in Fig. 3, *A* and *B*; similar spectra were obtained for the enantiomers of 5-MeC-7,8-diol. The CD spectrum of chrysene-1*R*,2*R*-diol was the same as those reported previously (14, 17), and was similar to the CD spectrum of each of the later eluting methylchrysene dihydrodiol enantiomers (*dashed lines* in Fig. 3, *A* and *B*). Thus, the later eluting enantiomers were assigned the *R,R*-configuration in each case.

The stereoselectivity of dihydrodiol formation from 5-MeC and 6-MeC was examined *in vitro* using liver 9000 × *g* supernatants from F344 rats pretreated with Aroclor 1254 and from

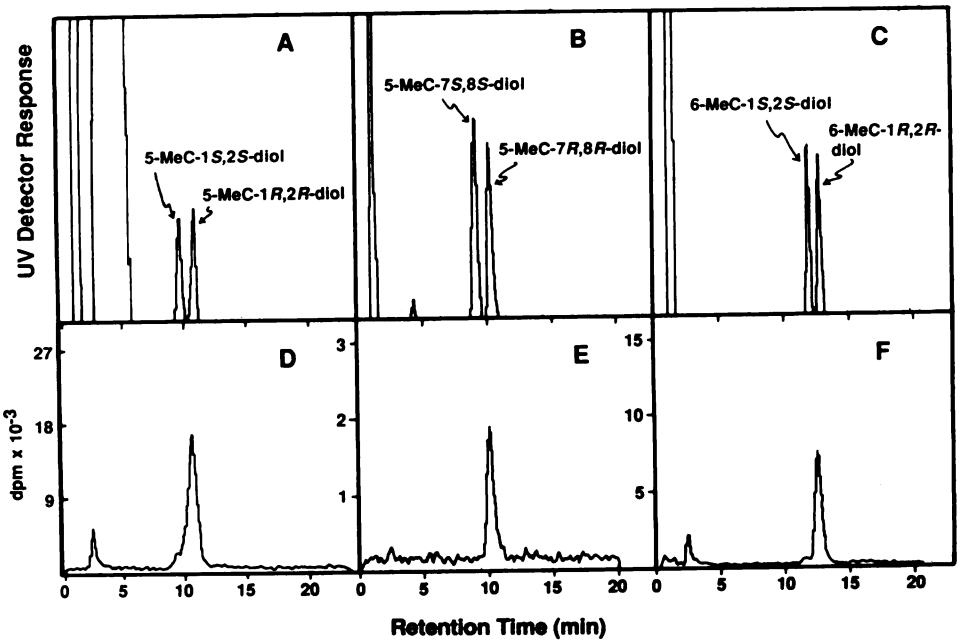

Fig. 2. Chromatograms of dihydrodiols isolated from the epidermis of mice treated with [^{3}H]5-MeC or [^{3}H]6-MeC, then mixed with synthetic racemic dihydrodiols, and analyzed by chiral stationary phase HPLC using system 4 (see "Materials and Methods"). *A*, UV detection of added synthetic racemic 5-MeC-1,2-diol; *D*, dpm corresponding to metabolically formed [^{3}H]5-MeC-1,2-diol (4.0 pmol/mg dry epidermis); *B*, UV detection of synthetic racemic 5-MeC-7,8-diol; *E*, dpm corresponding to metabolically formed [^{3}H]5-MeC-7,8-diol (2.6 pmol/mg dry epidermis); *C*, UV detection of synthetic racemic 6-MeC-1,2-diol; *F*, dpm corresponding to metabolically formed [^{3}H]6-MeC-1,2-diol (8.0 pmol/mg dry epidermis). In each case the *R,R*-enantiomer predominated in mouse epidermis.

Table 1 *Retention volumes and resolution of methylchrysene dihydrodiol enantiomers*

Resolved by chiral stationary phase HPLC, system 3 (see "Materials and Methods").

	Retention volume (ml)	RV[a]
5-MeC-1*S*,2*S*-diol	75	2.2
5-MeC-1*R*,2*R*-diol	82.5	
5-MeC-7*S*,8*S*-diol	73.5	1.8
5-MeC-7*R*,8*R*-diol	81	
6-MeC-1*S*,2*S*-diol	90	1.6
6-MeC-1*R*,2*R*-diol	96	
Chrysene-1*S*,2*S*-diol[b]	75	1.0
Chrysene-1*R*,2*R*-diol	78	

[a] RV, resolution value = $2(V_2 - V_1)/(W_2 + W_1)$, where V is the retention volume and W is the peak width at base.

[b] Absolute configuration determined by the excitation chirality method (16). Samples were kindly provided by Dr. Donald M. Jerina.

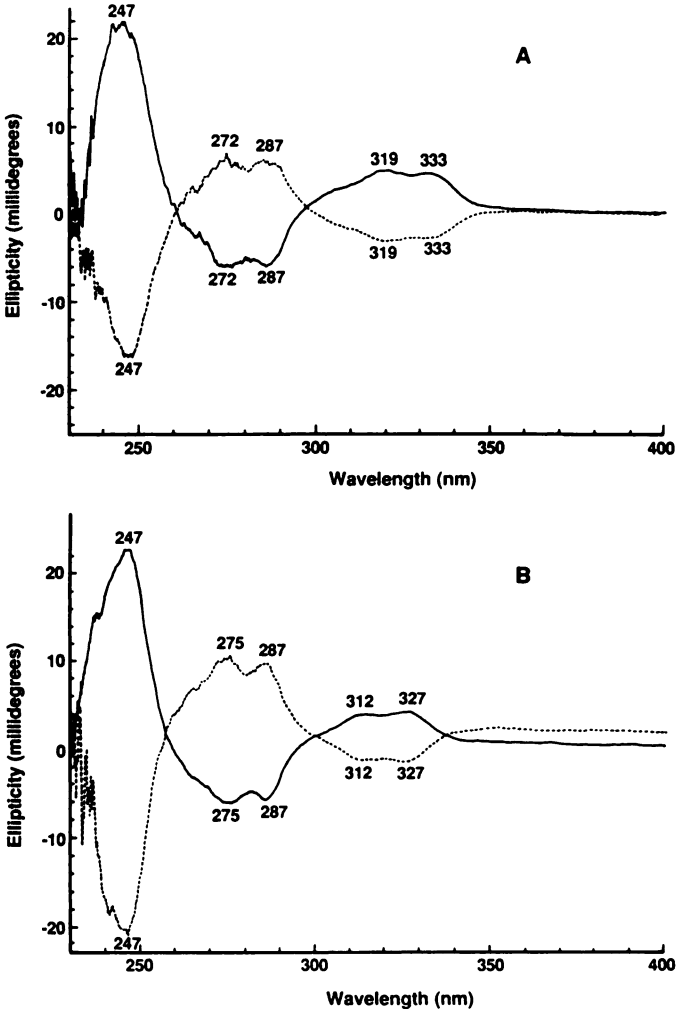

Fig. 3. CD spectra of early eluting (——) and late eluting (– – –) enantiomers of (*A*) 5-MeC-1,2-diol and (*B*) 6-MeC-1,2-diol.

livers of control or 3-MC pretreated mice. Incubations were carried out for 20 min. In previous studies, we have shown that dihydrodiol formation is linear for at least 30 min, that 70–95% of MeC substrate is not metabolized, and that secondary metabolites of the dihydrodiols are minimal under these conditions (11, 18, 19). The dihydrodiols produced in these incubations were collected and analyzed by chiral stationary phase HPLC. The results of these analyses are illustrated in Fig. 4, *A* and *B* for 5-MeC-1,2-diol in 3-MC pretreated and uninduced mouse liver. Only a single peak was observed; coinjection with synthetic 5-MeC-1,2-diol established that the peak was 5-MeC-

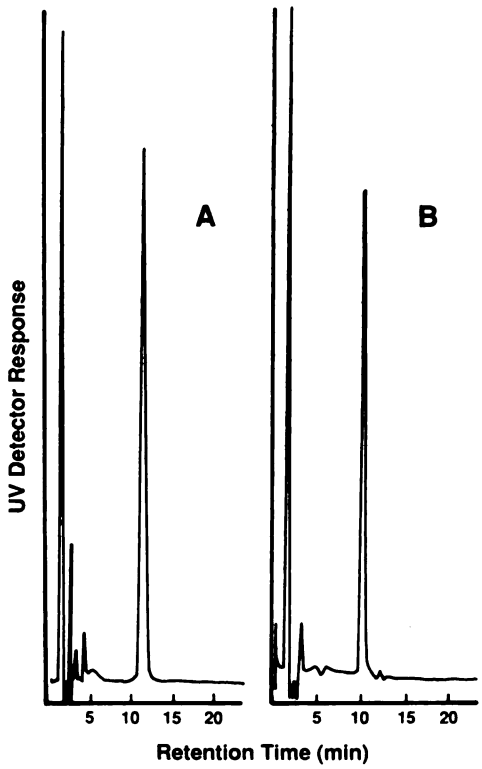

Fig. 4. Chromatograms obtained upon chiral stationary phase HPLC analysis of 5-diol-1,2-diol formed in incubations of 5-MeC with liver 9000 × *g* supernatant from (*A*) 3-MC pretreated mice and (*B*) control mice. The peak in each case was established as the *R*,*R*-enantiomer by coinjection with synthetic 5-MeC-1,2-diol (see Fig. 2*A*). Levels of 5-MeC-1*R*,2*R*-diol formed were 0.47 (*A*) and 0.44 (*B*) nmol/mg protein.

1*R*,2*R*-diol. Similar results were obtained in the analyses of 5-MeC-7,8-diol and 6-MeC-1,2-diol formed in the three *in vitro* systems. Only the *R*,*R*-diol was detected in each case.

One possible explanation for this observation was that both diol enantiomers were formed but the *S*,*S*-enantiomer was rapidly metabolized to other products. To test this hypothesis, either racemic 5-MeC-1,2-diol or 6-MeC-1,2-diol was incubated with 9000 × *g* supernatant from Aroclor pretreated rats, under conditions identical to those used for the parent MeC. Unmetabolized diol was collected and analyzed by chiral stationary phase HPLC. The results demonstrated that preferential metabolism of neither 5-MeC-1*S*,2*S*-diol nor 6-MeC-1*S*,2*S*-diol occurred under these conditions.

Dihydrodiol formation in mouse epidermis was examined 2 h after application of either [^{3}H]5-MeC or [^{3}H]6-MeC. Previous studies have shown that dihydrodiol concentrations are maximal at that time point (11, 13). The recoveries of radioactivity from epidermis were 60% ([^{3}H]5-MeC) and 47% ([^{3}H]6-MeC) in these experiments. The dihydrodiols were collected and analyzed by chiral stationary phase HPLC (Fig. 2, *D–F*). In each case the *R*,*R*-diol predominated. Levels of *S*,*S*-diols, if formed, did not exceed 7% of total dihydrodiols.

The results of the assays for tumor-initiating activity are summarized in Table 2. In each case, the *R*,*R*-dihydrodiol enantiomer was significantly more tumorigenic than the *S*,*S*-enantiomer. The most tumorigenic compound was 5-MeC-1*R*,2*R*-diol; it was significantly more active than either 5-MeC-7*R*,8*R*-diol or 6-MeC-1*R*,2*R*-diol.

DISCUSSION

The results of these studies clearly show that there is a high degree of stereoselectivity in the metabolism of 5-MeC and 6-

Table 2 *Tumor-initiating activity of methylchrysene dihydrodiol enantiomers in mouse skin*

Groups of 20 female CD-1 mice (age, 50–55 days) were shaved and treated with a single dose of each compound in 0.1 ml of acetone. Ten days later, each group was treated three times weekly with 2.5 μg of 12-*O*-tetradecanoylphorbol-13-acetate in 0.1 ml of acetone, for 20 weeks.

	Dose (nmol)	% Tumor-bearing animals		Tumors/animal	
		10 weeks[a]	20 weeks[a]	10 weeks	20 weeks
5-MeC	33	32	84	0.9	4.8
	10	30	90	0.4	2.7
5-MeC-1*S*,2*S*-diol	33	20	85	0.2	3.0
	10	10	60	0.2	0.9[b]
5-MeC-1*R*,2*R*-diol	33	50	85	1.7	7.9[c]
	10	60	100	1.5	9.4[c,d]
5-MeC-7*S*,8*S*-diol	33	0	30	0	0.4[e]
5-MeC-7*R*,8*R*-diol	33	15	75	0.3	2.3[f,g]
6-MeC	33	0	5	0	0.1
6-MeC-1*S*,2*S*-diol	33	0	5	0	0.1
6-MeC-1*R*,2*R*-diol	33	10	45	0.1	0.7[h,i]
Acetone		0	0	0	0

[a] Weeks of treatment with 12-*O*-tetradecanoylphorbol-13-acetate.
[b] Less tumorigenic than 5-MeC, $P = 0.006$.
[c] More tumorigenic than 5-MeC-1*S*,2*S*-diol, $P < 0.001$.
[d] More tumorigenic than 5-MeC, $P < 0.001$.
[e] Less tumorigenic than 5-MeC, $P < 0.001$.
[f] More tumorigenic than 5-MeC-7*S*,8*S*-diol, $P < 0.001$.
[g] Less tumorigenic than 5-MeC, $P = 0.03$.
[h] More tumorigenic than 6-MeC-1*S*,2*S*-diol, $P = 0.008$.
[i] More tumorigenic than 6-MeC, $P = 0.008$.

MeC to the three dihydrodiols examined. In each case, the *R*,*R*-enantiomer was formed to the virtual exclusion of the *S*,*S*-enantiomer. These results demonstrate that the position of the methyl group has no apparent effect on the stereoselectivity of 5-MeC or 6-MeC metabolism to precursors to bay region diol epoxides, in mouse epidermis or using the *in vitro* systems employed in the present study. Previous investigations of the metabolism of chrysene to its 1,2-dihydrodiol have demonstrated stereoselective metabolism, depending on the system employed (14, 17, 20). A high degree of stereoselectivity was observed in the formation of the *R*,*R*-enantiomer, as in the present study, in experiments carried out with cultured mouse, rat, or human skin, or with liver microsomes from 3-MC-pretreated rats. However, liver microsomes from untreated or phenobarbital pretreated rats were less stereoselective.

The results of the tumor initiation studies demonstrate that the metabolically formed *R*,*R*-enantiomer is in each case more tumorigenic than the corresponding *S*,*S*-enantiomer. Taken together with the metabolic studies and previous results (2), these data establish the stereoselectivity of 5-MeC metabolic activation to dihydrodiols in mouse epidermis. The major pathway leading to tumor development is clearly through 5-MeC-1*R*,2*R*-diol, with a lesser contribution from 5-MeC-7*R*,8*R*-diol.

Comparison of the tumor-initiating activities of 5-MeC-1*R*,2*R*-diol, 6-MeC-1*R*,2*R*-diol, and 5-MeC-7*R*,8*R*-diol shows the important effect on tumorigenicity of a methyl group in the bay region where the epoxide ring will form. Although previous bioassays of methylchrysene dihydrodiols had also shown the same effect (3, 4), the *R*,*R*- and *S*,*S*-enantiomers of these three dihydrodiols had not been compared. The results suggest that the tumor-initiating activity of a specific enantiomer of 5-MeC-1,2-diol-3,4-epoxide will be greater than that of 6-MeC-1,2-diol-3,4-epoxide. Assays currently in progress indicate that this is the case.

The results of the present study are consistent with those of previous investigations of the stereoselective metabolism and activation of PAH. Several unsubstituted PAH including chrysene, benzo(*a*)pyrene, and benz(*a*)anthracene, are metabolized stereoselectively to their *R*,*R*-dihydrodiols which are in turn more tumorigenic in mouse skin than are the corresponding *S*,*S*-enantiomers (20–25). However, the tumorigenic activities of enantiomeric dihydrodiols of methylated PAH have not been previously examined. The results indicate that, at least in the chrysene system, the stereoselectivity of metabolic activation *via* the diol epoxide pathway for the parent hydrocarbon is predictive of the pathway for methylated derivatives. It will be interesting to determine if similar results are seen in other PAH ring systems.

ACKNOWLEDGMENTS

We thank Sharon Dolan for her excellent technical assistance and Gail Thiede for editing the manuscript.

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