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Biochemistry

Metabolic pathways of 7,12-dimethylbenz[*a*]anthracene in hepatic microsomes

(carcinogen/adrenocorticolytic agent/high pressure liquid chromatography)

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ABSTRACT **High pressure liquid chromatography has enabled quantitative analysis of the *in vitro* metabolism of 7,12-dimethylbenz[*a*]anthracene, 7-methyl-12-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, and 7,12-dihydroxymethylbenz[*a*]anthracene by 3-methylcholanthrene-induced and control rat liver microsomes. The following previously unrecognized metabolites have been tentatively identified: 5,6-dihydro-5,6-dihydroxy-7-methyl-12-hydroxymethylbenz[*a*]anthracene, 3-hydroxy-7,12-dihydrodihydroxymethylbenz[*a*]anthracene, 4-hydroxy-7,12-dihydrodihydroxymethylbenz[*a*]anthracene, and 8,9-dihydro-8,9-dihydroxy-7,12-dihydroxymethylbenz[*a*]anthracene. The epoxide hydratase inhibitor 1,2-epoxy-3,3,3-trichloropropane was found to eliminate all dihydrodiol formation and markedly inhibit the formation of several dimethylbenzanthracene metabolites. It is proposed that the tentatively identified 3-hydroxy and 4-hydroxy derivatives are formed by an enzymatic mechanism that does not involve epoxides as intermediates. The metabolic pathways of 7,12-dimethylbenz[*a*]anthracene in hepatic microsomal enzymes are proposed.**

7,12-dimethylbenz[*a*]anthracene (DMBA) is a potent carcinogen which induces skin tumors in mice (1) and causes mammary cancer and massive adrenal necrosis in rats (2, 3). It has been found to induce malignant transformation of *in vitro* cell transformation systems (4, 5), and to be mutagenic in bacteria (6) and mammalian cells (7). The carcinogenic and mutagenic activities of polycyclic aromatic hydrocarbons (PAH) require metabolic activation by mixed-function oxidases located in the microsomes of mammalian cells (7–9). The enzyme system is present and inducible *in vivo* and in cells grown in culture (10–13). PAH are metabolized to phenols, dihydrodiols, quinones, and epoxides (14–23). In DMBA metabolism, the hydroxylation of the 7-methyl group is believed to be the key step towards carcinogenesis in the mammary glands and adrenal necrosis in rats (19, 24–26). Administration of certain aromatic compounds to rats prior to the feeding of DMBA prevents the induction of adrenal necrosis by this carcinogen and also inhibits the development of mammary cancer (17, 27).

PAH metabolites have been analyzed mainly by thin-layer chromatography (15, 28, 29), but due to incomplete separation not all the metabolites formed could be examined in detail. Recently high-pressure liquid chromatography (HPLC) has been applied successfully to the analysis of benzo[*a*]pyrene metabolites (30). This paper describes the quantitative analysis of DMBA metabolism with control and 3-methylcholanthrene (MC)-pretreated rat liver microsomes by HPLC.

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; 7-M-12-OHMBA, 7-methyl-12-hydroxymethylbenz[*a*]anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz[*a*]anthracene; 7,12-diOHMBA, 7,12-dihydroxymethylbenz[*a*]anthracene; DMBA-8,9-diol, 8,9-dihydro-8,9-dihydroxy-DMBA; presumed to be *trans* form; 3-OH-DMBA, 3-hydroxy-DMBA; DMBA-phenols, hydroxy derivatives of DMBA; DMBA-5,6-oxide, DMBA-5,6-epoxide; Other derivatives of DMBA, 7-M-12-OHMBA, 7-OHM-12-MBA, and 7,12-diOHMBA, are similarly abbreviated as above; TCPO, 1,2-epoxy-3,3,3-trichloropropane; HPLC, high-pressure liquid chromatography; MC, 3-methylcholanthrene; BP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbons.

MATERIALS AND METHODS

Materials. [^{3}H]DMBA (specific activity 13 Ci/mmol) and [^{14}C]DMBA (specific activity 21.2 Ci/mol) were purified on a silica gel column and HPLC showed them to be 99.8% pure. The specific activities in methanol solution of [^{3}H]- and [^{14}C]DMBA used in the experiments were 115.3 and 24.3 Ci/mol, respectively. Synthetic compounds 7-OHM-12-MBA, 7-M-12-OHMBA, 7,12-di-OHMBA, and DMBA-*cis*-5,6-diol were generously provided by Dr. Harry Gelboin of the National Cancer Institute.

Rat Liver Microsomes and *In Vitro* Incubations. The control and MC-induced microsomes of male Sprague–Dawley rats were prepared according to the procedures of Kinoshita *et al.* (18). The incubation mixture contains 50 mM Tris·HCl, pH 7.5, 3 mM $MgCl_2$, 0.56 mM NADPH, 79.2 μM [^{3}H]DMBA or 88 μM [^{14}C]DMBA, 0.5 mg of protein per ml of control microsomes or 0.4 mg of protein per ml of MC-induced microsomes. Unlabeled 7-M-12-OHMBA, 7-OHM-12-MBA, and 7,12-diOHMBA were used at about 50 μM. For the inhibitor experiments, 1,2-epoxy-3,3,3-trichloropropane (TCPO) was used at 93 mM. Incubation, organic solvent extraction, and sample preparation for HPLC were carried out similarly as described (30).

High-Pressure Liquid Chromatography. A Dupont model 830 high-pressure liquid chromatograph fitted with a 1 m Permaphase octadecyltrimethoxysilane (ODS) column was used. The column was maintained at 50° and 500 pounds/inch^2 (3.4 MPa). The column was eluted with a linear gradient of methanol in water from 20% to 80%.

Five microliters of prepared metabolite sample in methanol were injected with the solvent flow stopped. The eluent was monitored by ultraviolet absorption at 254 nm. Ten-drop fractions were collected (12–19 sec intervals) and the radioactivity was determined. The counting data was converted to pmol by a simple computer program with calibrated counting efficiency.

RESULTS

Identification of DMBA Metabolites. DMBA metabolites were identified by their chromatographic properties on thin-layer chromatography and HPLC, and their ultraviolet and fluorescence spectra, making use of the properties of known compounds and previous assignments where available (15, 22, 28, 29, 31).

The chromatographic properties of 7,12-diOHMBA, DMBA-5,6-diol, 7-OHM-12-MBA, and 7-M-12-OHMBA as-

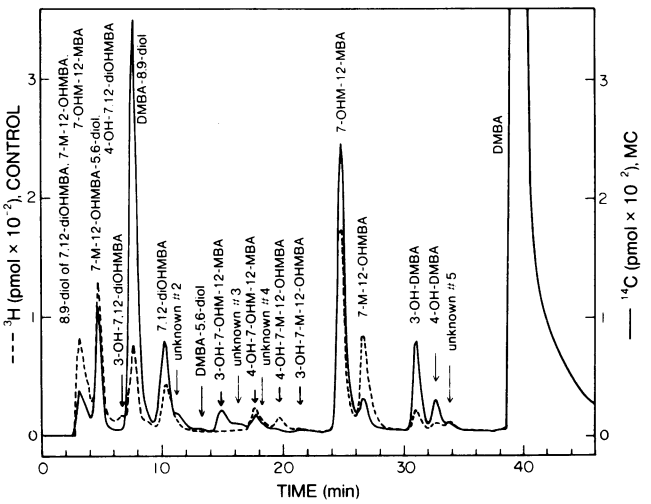

FIG. 1. Separation of DMBA and metabolites by high-pressure liquid chromatography. HPLC was carried out as described in *Materials and Methods*. The quantitative aspect of DMBA metabolism by control and MC-induced (MC) microsomes is shown in Table 2.

signed in Fig. 1 were identical to those of the reference compounds. The assigned 8,9-diols of DMBA and its hydroxymethyl derivatives have absorption spectra similar to those determined by Sims (22, 28) and Gentil *et al.* (31). The 8,9-diols obtained by incubating 7,12-diOHMBA, 7-OHM-12-MBA, and 7-M-12-OHMBA separately with microsomes have identical retention times on HPLC. When 7-OHM-12-MBA or 7-M-12-OHMBA was used as substrate, the relative enzyme activities toward 8,9-diol formation (by mono-oxygenases and epoxide hydratase) and methyl-hydroxylation determine the concentration of 7,12-diOHMBA-8,9-diol relative to that of 7-OHM-12-MBA-8,9-diol (or of 7-M-12-OHMBA-8,9-diol). It will be shown in Table 2 that the rate of DMBA-8,9-diol formation is 2.5 to 13 times higher than that of 7-OHM-12-MBA and 7-M-12-OHMBA, and the rate of methyl-hydroxylation of the second methyl group appears to be lower than that of the first methyl group. Therefore, one would expect that the 8,9-diols obtained from 7-OHM-12-MBA and 7-M-12-OHMBA are mainly monohydroxymethyl derivatives. This is supported by the finding that the 8,9-diol obtained by incubating 7,12-diOHMBA with microsomes has ultraviolet absorption and fluorescence properties distinct from those of the 8,9-diols of 7-OHM-12-MBA and 7-M-12-OHMBA (unpublished results). The absorption and fluorescence properties of the assigned 7-M-12-OHMBA-5,6-diol were similar to those of DMBA-*cis*-5,6-diol and DMBA-*trans*-5,6-diol (31). The phenolic products were extensively studied for their absorption and fluorescence properties in both methanol and 1 N NaOH. The compound assigned as 4-OH-DMBA in Fig. 1 has similar absorption spectrum to the synthetic standard (31). The absorption spectrum of 4-OH-DMBA in methanol has a characteristic absorption maximum at 320 nm. This absorption characteristic prevails in all the 4-hydroxy derivatives and is absent in all the 3-hydroxy derivatives assigned in Fig. 1. As shown in Table 1, the absorption characteristics were reflected in their fluorescence properties. All the assigned 4-hydroxy derivatives have an excitation maximum at around 320 nm which is absent in the assigned 3-hydroxy derivatives. The phenolic characters of the compounds in Table 1

Table 1. Fluorescence spectra of phenolic derivatives of 7,12-dimethylbenz[*a*]anthracene

Compound[a]	Excitation maximum (nm)[b]	Fluorescence maximum (nm)[b]
4-OH-7,12-diOHMBA	285,322,<u>368</u>,402	460
3-OH-7,12-diOHMBA	305, <u>370</u>,407	470
3-OH-7-OHM-12-MBA	298, <u>365</u>,405	457
4-OH-7-OHM-12-MBA	290,325,<u>383</u>,409	452
4-OH-7-M-12-OHMBA	297,320,<u>373</u>,402	452
3-OH-7-M-12-OHMBA	305, <u>367</u>,405	452
3-OH-DMBA	312, <u>382</u>,410	425,<u>443</u>
4-OH-DMBA	295,325,<u>384</u>,408	425,<u>440</u>

[a] The phenolic products were obtained by incubating each of their corresponding parent compounds with liver microsomes and were subsequently separated by HPLC.

[b] Spectra were measured in methanol solution on an Aminco-Bowman spectrophotofluorimeter. Highest excitation or fluorescence maxima are underlined.

Table 2. DMBA metabolism analyzed by high-pressure liquid chromatography

Metabolite	Retention time (min)	nmol/mg protein[a] MC-induced	nmol/mg protein[a] Control
7,12-diOHMBA-8,9-diol[b]	3.2	4.7	2.4
7-M-12-OHMBA-5,6-diol[c]	4.7	10.3	3.4
Unknown no. 1[d]	6.0	—	—
3-OH-7,12-diOHMBA	6.6	—	0.25
DMBA-8,9-diol	7.4	24.2	2.4
7,12-diOHMBA	10.2	1.5	1.2
Unknown no. 2[e]	11.2	—	—
DMBA-5,6-diol[e]	12.7	—	—
3-OH-7-OHM-12-MBA	14.9	1.1	—
Unknown no. 3[f]	16.3	0.15	—
4-OH-7-OHM-12-MBA	17.5	1.2	0.8
Unknown no. 4[f]	18.2	—	—
4-OH-7-M-12-OHMBA	19.2	—	0.3
3-OH-7-M-12-OHMBA[g]	21.5	—	—
7-OHM-12-MBA	24.5	9.6	4.9
7-M-12-OHMBA	26.6	1.9	3.0
3-OH-DMBA	30.9	2.2	0.5
4-OH-DMBA	32.2	1.0	0.3
Unknown no. 5[f]	33.6	—	0.3
DMBA	39.5	—	—
Total metabolites		57.9	19.8
% Metabolized		29	12

[a] Nanomoles of metabolite detected per ml of incubation mixture per mg of protein microsomes for a 30 min incubation at 37°. Control and MC-induced microsomes were used at 0.5 and 0.4 mg of protein per ml, respectively. Errors of the determined values are estimated to be about 7%.

[b] This chromatographic peak may contain 7-M-12-OHMBA-8,9-diol and 7-OHM-12-MBA-8,9-diol.

[c] A small amount of 4-OH-7,12-diOHMBA is present in this chromatographic peak.

[d] Detected as metabolite when either 7-OHM-12-MBA or 7-M-12-OHMBA was used as substrate in the incubation.

[e] Detected as minor metabolites when DMBA is incubated with MC-induced microsomes in a 30 ml incubation mixture (this is indicated in Fig. 1).

[f] Chromatographic peaks were unidentified due to small quantity present and close association with neighboring metabolites.

[g] Detected as a metabolite when 7-M-12-OHMBA was used as substrate in the incubation.

Table 3. Effect of TCPO on DMBA metabolism

Metabolite	nmol/mg of protein[a] — TCPO	+ TCPO	% Inhibition
7,12-diOHMBA-8,9-diol[b]	4.7	0	100
4-OH-7,12-diOHMBA	—	0.2	—
7-M-12-OHMBA-5,6-diol	10.30	0	100
3-OH-7,12-diOHMBA	—	0.09	—
DMBA-8,9-diol	24.2	0	100
7,12-diOHMBA	1.5	0.14	75
3-OH-7-OHM-12-MBA	1.1	0	100
Unknown no. 3	0.15	0.14	6
4-OH-7-OHM-12-MBA	1.2	0	100
Unknown no. 4	—	0.15	—
4-OH-7-M-12-OHMBA	—	0.2	—
7-OHM-12-MBA	9.6	1.6	83
7-M-12-OHMBA	1.9	0.5	74
3-OH-DMBA	2.2	0.3	86
4-OH-DMBA	1.0	0.2	80
Total Metabolites	57.9	3.8	94
% Metabolized	29	1.7	94

[a] MC-induced microsomes were each used at 0.4 mg of protein per ml.

[b] This chromatographic peak may contain 7-M-12-OHMBA-8,9-diol and 7-OHM-12-MBA-8,9-diol.

Table 4. Metabolism of 7-M-12-OHMBA, 7-OHM-12-MBA, and 7,12-diOHMBA by MC-induced microsomes

Metabolite	Substrate[a] 7-M-12-OHMBA	7-OHM-12-BA	7,12-diOHMBA
7-OHM-12-MBA-8,9-diol		+	
7-M-12-OHMBA-8,9-diol	+		
7,12-diOHMBA-8,9-diol	×	×	+
3-OH-7,12-diOHMBA			+
7-M-12-OHMBA-5,6-diol	+		
Unknown no. 1[b]	+	+	
4-OH-7,12-diOHMBA			+
7,12-diOHMBA	+	+	+
4-OH-7-OHM-12-MBA		+	
3-OH-7-OHM-12-MBA		+	
3-OH-7-M-12-OHMBA	+		
4-OH-7-M-12-OHMBA	+		

[a] The incubations were carried out as described in *Materials and Methods*. Positive sign indicates the presence of that metabolite. The × indicates the presence of 7,12-diOHMBA-8,9-diol, but the amount relative to the monohydroxymethyl derivative is estimated to be small (see *text*). Some minor chromatographic peaks less polar (with longer retention times on HPLC) than the parent compounds were detected. Their identities are unknown.

[b] This metabolite had an absorption maximum at 268 nm and a shoulder at 260 nm, fluorescence maximum at 465 nm with excitation at 375 nm.

were ascertained by their red-shifts of fluorescence maxima in alkaline solution (unpublished results). Although the spectrophotofluorimetric and spectrophotometric properties of the compounds were determined, it should be pointed out that the assignments in Fig. 1 and Table 1 are tentative for those lacking reference standards.

Metabolism of DMBA by Control and MC-Induced Microsomes. Fig. 1 shows the HPLC separation of DMBA and metabolites produced by incubating DMBA with control and MC-induced microsomes. The quantities of each metabolite are listed in Table 2. The detected DMBA metabolites can be divided into three classes: (*1*) methyl-hydroxylated derivatives (e.g., 7-M-12-OHMBA, 7-OHM-12-MBA, and 7,12-diOHMBA), (*2*) phenolic derivatives of DMBA and its methyl-hydroxylated derivatives, and (*3*) dihydrodihydroxy derivatives of DMBA and its methyl-hydroxylated derivatives. Classes *2* and *3* are commonly called ring-hydroxylated derivatives. The activity ratios of Table 2 demonstrate that MC-induced microsomes stimulate the formation of all metabolites with the exceptions of 7-M-12-OHMBA and 4-OH-7-M-12-OHMBA.

MC-induced microsomes yield twice as much 7-OHM-12-MBA as the control microsomes. This stimulatory effect is reproducible. The results presented here agree qualitatively with the data of Sims (28) with respect to increased formation of 7-OHM-12-MBA and decreased formation of 7-M-12-OHMBA with MC-induced rat liver homogenates compared with control homogenates. However, this finding is in disagreement with those of Boyland *et al.* (25), and Jellinck *et al.* (24), who found a decreased level of 7-OHM-12-MBA with MC-induced microsomes.

Metabolism of DMBA by MC-Induced Microsomes in the Presence of TCPO. The epoxide hydratase inhibitor TCPO (32) is known to inhibit effectively the formation of all benzo[*a*]pyrene-diols and other metabolites (30). As shown in Table 3, TCPO has a similar effect on DMBA metabolism. The total metabolism of DMBA is reduced by 94% in the presence of 93 mM TCPO. Formation of phenolic and hydroxymethyl derivatives was drastically inhibited (except phenols of 7-OHM-12-MBA). No dihydrodihydroxy derivatives (presumably formed via epoxides) are produced. Because of the absence of dihydrodihydroxy derivatives, the phenolic derivatives of 7,12-diOHMBA are present as distinctive chromatographic peaks on HPLC.

Metabolism of 7-M-12-OHMBA, 7-OHM-12-MBA, and 7,12-diOHMBA by MC-Induced Microsomes. The metabolites detected and characterized are shown in Table 4. The quantitative metabolism could not be determined because unlabeled parent compounds were used. The metabolites of 7-OHM-12-MBA were produced in approximately equal quantity, estimated by their absorbances (peak area at 254 nm) on the chromatogram. Unknown no. 1 and 7-OHM-12-MBA-8,9-diol were totally absent when the incubation was carried out in the presence of TCPO.

About 80% of the metabolites formed by incubating 7-M-12-OHMBA are 7-M-12-OHMBA-5,6-diol. Formation of this K-region diol (K-region refers to the "electron rich" region) was totally inhibited by TCPO; it is also a major metabolite when DMBA is used as substrate (Table 2). Two phenolic and one dihydrodihydroxy derivatives were detected as metabolites of 7,12-diOHMBA.

DISCUSSION

The efficient separation of DMBA metabolites by HPLC has provided a more quantitative analysis and a better understanding of the metabolic pathways of DMBA in the *in vitro* system. It has been reported that metabolites formed *in vitro* are mainly monohydroxymethyl derivatives of DMBA (14). We have shown that (*1*) the phenolic and dihydrodihydroxy derivatives of DMBA, (*2*) 7-OHM-12-MBA and 7-M-12-OHMBA, and (*3*) the phenolic and dihydrodihydroxy derivatives of 7-OHM-12-MBA and 7-M-12-OHMBA are formed in comparable quantities. A dihydroxymethyl derivative is also formed in a significant amount.

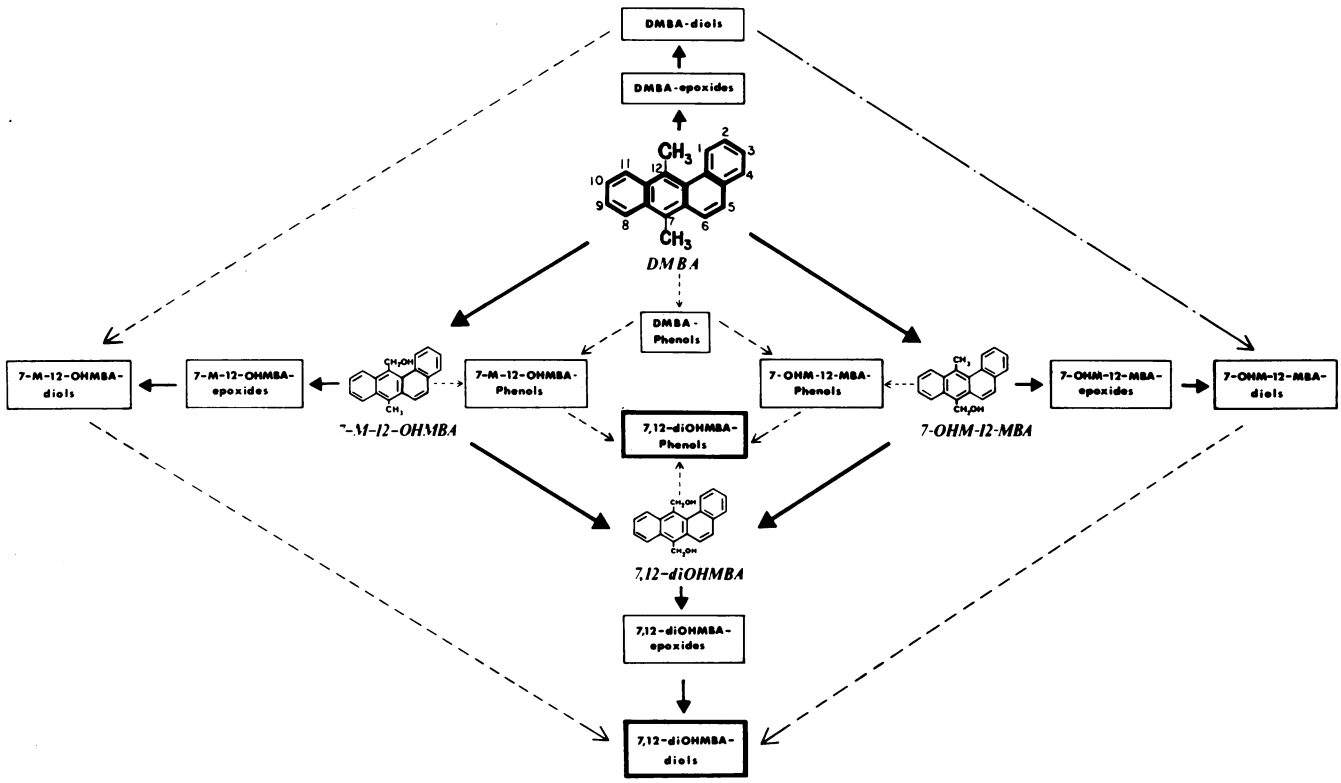

FIG. 2. Metabolic pathways of DMBA in rat liver microsomal enzymes. Diagonal paths indicate methyl-hydroxylation, and vertical and horizontal paths indicate ring-hydroxylation. Experimental evidence, (——▶); partial evidence (ref. 17), (— · — · →); proposed additional pathways, (- - - - - - →).

The detection of a K-region diol, 7-M-12-OHMBA-5,6-diol, is of special interest. Sims (Table 1 of ref. 22) characterized this metabolite as 7-M-12-OHMBA-8,9-diol which was thought to be the major metabolite of 7-M-12-OHMBA. It has been suggested that K-region epoxides are active intermediates responsible for the carcinogenic activities of PAH (5, 33–36). Epoxides are found to be active in inducing malignant cell transformations (5, 34, 36, 37) and are mutagenic (36, 38, 39). The K-region epoxide of 7-M-12-OHMBA has not been tested for malignant and mutagenic activities. However, it was shown that 7-M-12-OHMBA is inactive in inducing adrenal necrosis in rats and mice, and mammary cancer in rats (25).

Some phenolic derivatives of DMBA, 7-OHM-12-MBA, and 7-M-12-OHMBA have been tentatively identified (14, 15, 25). Metabolites that derive from further metabolism of 7,12-diOHMBA have not been reported. The discovery of metabolites derived from 7,12-diOHMBA provides evidence that 7,12-diOHMBA is not the end product of microsomal oxidations. Monohydroxymethyl derivatives can be first hydroxylated on the second methyl group, and the dihydroxymethyl derivative can be further hydroxylated at the ring positions to form dihydrodihydroxy and phenolic derivatives. Boyland *et al.* (14) have found that 7-OHM-12-MBA is more active in inducing adrenal necrosis and mammary cancer in rats. Flesher *et al.* (40) have shown that derivatives capable of being converted to 7-OHM-12-MBA are potent carcinogens. The dihydroxymethyl derivative was found inactive in inducing palpable tumors in rats (40). Some ring-hydroxylated derivatives of DMBA and 7-OHM-12-MBA were found inactive in either inducing tumors (40) or inducing malignant cell transformation (5). Therefore, it appears that metabolism of 7,12-diOHMBA is a route that leads to detoxification.

Pretreatment of rats with some aromatic compounds protects the animals from adrenal necrosis (2, 3, 11, 15, 26, 27). It has been suggested that induced hepatic microsomal enzymes decrease the effective concentration of 7-OHM-12-MBA, which is believed to be the adrenocorticolytic agent that causes adrenal damage in rats (25). Our results further confirm the earlier reports (15, 24, 29) that MC-induced microsomal enzymes metabolize DMBA more effectively at the ring positions and the rate of further metabolism of monohydroxymethyl derivatives is accelerated. However, our results show that the net concentration of 7-OHM-12-MBA produced with MC-induced microsomes is two times higher than that produced with control microsomes under identical conditions. Therefore, the increased ring-hydroxylation is not at the expense of hydroxylation of the methyl groups as has been suggested by earlier reports (15, 28). Wheatley and Sims (26) have proposed that 7-OHM-12-MBA requires further metabolism or conjugation in the liver to exert its necrotic effect in adrenal cortex. Recently, 7-OHM-12-MBA-5,6-oxide was shown to be inactive in inducing adrenal necrosis in Sprague–Dawley rats (17). Further investigation is clearly needed before 7-OHM-12-MBA can be implicated as the ultimate metabolite that causes adrenal necrosis and mammary cancer in experimental animals.

A number of K-region epoxides have been shown to be intermediates in the *in vitro* metabolism of carcinogenic PAH (16, 17, 20, 41, 42). Epoxides are shown to: (*1*) bind to cellular macromolecules (33), (*2*) undergo nonenzymatic rearrangement to phenolic derivatives (42–44), (*3*) form a glutathione conjugate in a reaction catalyzed by the cytoplasmic

S-epoxide transferase (32), and (4) form dihydrodiols in reactions catalyzed by the microsomal epoxide hydratase (32). Although NIH shift mechanism is shown to be involved in the nonenzymatic rearrangement of epoxides to phenolic derivatives (42–44), there have been examples of direct enzymatic formation of a phenol (42). In the *in vitro* metabolism of benzo[*a*]pyrene (BP), there has been direct evidence for the presence of BP-4,5-oxide (17) and indirect evidence for the presence of BP-7,8-oxide and BP-9,10-oxide (18, 30). However, there has been no indication of the formation of BP-2,3-oxide (30). 3,4-oxides of DMBA, 7-OHM-12-MBA, 7-M-12-OHMBA, and 7,12-diOHMBA have not been detected, either directly by the detection of the epoxide itself or indirectly by the detection of its dihydrodiol. Yet 3-hydroxy-BP (30), 3-hydroxy and 4-hydroxy derivatives of DMBA, 7-M-12-OHMBA, 7-OHM-12-MBA, and 7,12-diOHMBA are formed even in the presence of TCPO, which inhibited the formation of all dihydrodiols. It is probable that 3,4-oxides formed are very unstable and quickly rearranged to 3- and 4-hydroxy derivatives. However, an alternative explanation is that the phenol formation is via an unknown enzymatic mechanism which does not involve epoxides as intermediates. If this were the case, there exists in the microsomal complex a "ring-hydroxylase" which catalyzes the formation of phenolic derivatives from DMBA and its hydroxymethyl derivatives. One can also speculate, for reasons of enzyme–substrate specificity, that the enzymes responsible for the methyl-hydroxylation and phenol formation are different entities in a microsomal multi-enzyme complex. If this is indeed the case, there exists in the microsomal complex a "methyl-hydroxylase" that catalyzes the formation of hydroxymethyl derivatives from DMBA and its ring-hydroxylated derivatives (including phenols and dihydrodiols). This hypothesis is indicated in Fig. 2, the proposed metabolic pathways by microsomal enzymes. The well-established nonenzymatic rearrangement of epoxides to phenols (42–44) is not shown in Fig. 2. Keysell *et al.* have reported that DMBA-*cis*-5,6-diol is metabolized to 7-OHM-12-MBA-*cis*-5,6-diol by rat liver microsomes. This is the only known case where a ring-hydroxylated derivative can be further methyl-hydroxylated. The action of "ring-hydroxylase" is exemplified by the formation of phenolic derivatives from DMBA, 7-OHM-12-MBA, 7-M,12-OHMBA, and 7,12-diOHMBA. However, this concept requires further investigation to substantiate the existence of a "methyl-hydroxylase" and a "ring-hydroxylase" as components of microsomal mixed-function oxidases.

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