Journal of Chromatography, 185 (1979) 635–654

CHROM. 12,003

COMBINED REVERSED-PHASE AND NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE PURIFICATION AND IDENTIFICATION OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE METABOLITES

MING W. CHOU and SHEN K. YANG

Department of Pharmacology, School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Md. 20014 (U.S.A.)

SUMMARY

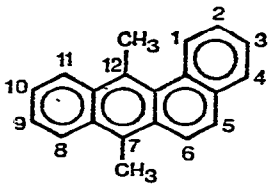
A total of 37 compounds have been identified as rat liver microsomal metabolites of the potent carcinogen 7,12-dimethylbenz[*a*]anthracene and its hydroxymethyl derivatives 7-methyl-12-hydroxymethylbenz[*a*]anthracene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene and 7,12-dihydroxymethylbenz[*a*]anthracene. The metabolites were characterized by: (i) retention times on reversed-phase (with a C_{18} column) and normal-phase (with a silica gel column) high-performance liquid chromatography (HPLC); (ii) ultraviolet absorption and fluorescence spectra; (iii) mass spectral analysis; (iv) optical activity; and (v) comparison of the physicochemical properties of the metabolites with those of some available synthetic standards. The 37 identified metabolites include four *trans*-3,4-dihydrodiols, four *trans*-5,6-dihydrodiols, four *trans*-8,9-dihydrodiols, four *trans*-10,11-dihydrodiols, two methyl carboxylic acids, two methyl aldehydes, two hydroxymethyl aldehydes, four 2-phenols, four 3-phenols, four 4-phenols and three hydroxymethyl derivatives. The *trans* configuration of the dihydrodiols was determined by their inability to form vicinal *cis*-acetonides. Seven dihydrodiol metabolites were found to be optically active. Detailed physicochemical properties, such as ultraviolet absorption spectra, fluorescence spectra measured in methanol and in 0.1 *N* NaOH, major mass ions from mass spectral analysis and the retention times on two HPLC systems, are presented in support of the structural assignments.

INTRODUCTION

7,12-Dimethylbenz[*a*]anthracene (DMBA) is one of the most potent carcinogenic polycyclic aromatic hydrocarbons (PAHs) and is widely used as a prototype carcinogen and mutagen. DMBA, like other PAHs, is inactive by itself and its biological properties such as mutagenicity and carcinogenicity require metabolic activation by the drug-metabolizing enzyme systems[1–3]. The metabolic pathways and products of DMBA have been studied intensively by many investigators in the past 15 years[3–16]. The recent application of high-performance liquid chromatography (HPLC)

in the separation of PAH metabolites has provided some detailed understanding of the activation pathways of several PAHs such as benzo[*a*]pyrene[17–23], 3-methylcholanthrene[24], 5-methylchrysene[25] and DMBA[11–14].

We reported previously the HPLC separation and tentative identification of some DMBA metabolites[11]. Due to the low resolving power of the reversed-phase C_{18} column used previously, many metabolites described in this report were not recognized. With improved HPLC column packings and elution conditions, we have recently reported the identification of four *trans*-3,4-dihydrodiols[12] and four 2-phenols[26] that are derived enzymatically from DMBA,7-methyl-12-hydroxymethylbenz[*a*]anthracene (7-M-12-OHMBA), 7-hydroxymethylbenz[*a*]anthracene (7-OHM-12-MBA) and 7,12-dihydroxymethylbenz[*a*]anthracene (7,12-diOHMBA), respectively. This paper presents the ultraviolet (UV) absorption spectra, fluorescence spectra, mass spectra, optical rotation properties and the structural identification of the metabolites that were purified by two HPLC systems. All properties of the metabolites were obtained from the purified metabolites and were compared with those of the synthetic standards whenever available. We believe that the detailed information presented in this report will be of value to other investigators in the analysis of metabolites by HPLC and in the identification of metabolites in other experimental systems.



Structural formulae of DMBA

MATERIALS AND METHODS

DMBA was purchased from Eastman (Rochester, N.Y., U.S.A.) and was purified by passing it through a silica gel (Bio-Sil A, 100–200 mesh; Bio-Rad Labs., Richmond, Calif., U.S.A.) column with hexane as the eluting solvent. 7-OHM-12-MBA, 7-M-12-OHMBA and 7,12-diOHMBA were synthesized according to a modified procedure[27] of Boyland and Sims[4]. Synthetic DMBA 1-, 2-, 3- and 4-phenols were kindly provided by Professor M. S. Newman of the Ohio State University. The synthetic 8-methoxy-, 9-methoxy-, 10-methoxy- and 11-methoxy-DMBA were gifts of Dr. C. E. Morreal of the Roswell Park Memorial Institute. The methoxy compounds were converted to the corresponding phenols as described[28] and were each purified by the reversed-phase HPLC described below. A mixture of DMBA-*trans*-8,9-diol and DMBA-8,9,10,11-tetrahydro-*trans*-8,9-diol was a gift from Drs. P. P. Fu and R. G. Harvey of the University of Chicago. The DMBA-*trans*-8,9-diol was separated from the tetrahydrodiol by the reversed-phase HPLC system described below. DMBA-7,12-endoperoxide was a gift from Dr. C. Chen of the Northwestern University. DMBA-*cis*-5,6-diol and DMBA-5,6-epoxide were obtained from the Chemical Repository of the National Cancer Institute.

DMBA-*trans*-5,6-diol was obtained by *in vitro* incubation of DMBA-5,6-epox-

ide with rat liver microsomes in the absence of reduced nicotinamide–adenine dinucleotide phosphate (NADPH) and was purified by reversed-phase HPLC. The UV absorption and fluorescence spectra of this 5,6-diol were identical to those of the synthetic DMBA-*cis*-5,6-diol. However, its retention times on two HPLC systems (Table I) were different from those of the synthetic DMBA-*cis*-5,6-diol. Furthermore, this 5,6-diol did not form vicinal *cis*-acetonide whereas the synthetic DMBA-*cis*-5,6-diol formed vicinal *cis*-acetonide quantitatively under identical conditions[12]. We thus concluded that the 5,6-diol derived from DMBA-5,6-epoxide is a *trans* isomer and it was used as a chromatographic standard.

7-Formyl-12-methylbenz[*a*]anthracene (7-CHO-12-MBA), 7-methyl-12-formylbenz[*a*]anthracene (7-M-12-CHO-BA) and 7,12-diformylbenz[*a*]anthracene (7,12-$(CHO)_2$-BA) were prepared from 7-OHM-12-MBA, 7-M-12-OHMBA, and 7,12-diOHMBA, respectively, according to the procedure of Pataki *et al.*[29] and were each purified by HPLC as previously described[27]. 7-Hydroxymethyl-12-formylbenz[*a*]-anthracene (7-OHM-12-CHO-BA) and 7-formyl-12-hydroxymethylbenz[*a*]anthracene (7-CHO-12-OHMBA) were obtained from the reaction of 7,12-diOHMBA with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) as described[29]. A solution of 7,12-diOHMBA (5 mg) in 10 ml dioxane–tetrahydrofuran (THF) (1:1) was stirred while adding 12 mg DDQ. After 24 h at room temperature, the reaction products were partitioned with ethyl acetate (20 ml) and 2% NaOH aqueous solution (20 ml) saturated with NaCl. The ethereal phase was washed twice with the above NaOH solution, three times with water, dehydrated with anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was dissolved in THF–hexane (1:3) and chromatographed on a DuPont Zorbax SIL column (25 cm × 6.2 mm) with THF–hexane (1:3) as the eluting solvent at a flow-rate of 2 ml/min. In addition to the expected product[29] 7-CHO-12-OHMBA (retention time 9.0 min) and the unreacted 7,12-diOHMBA (retention time 18.0 min), we found a third compound (retention time 15.0 min) with a peak height about one-tenth that of 7-CHO-12-OHMBA. The UV absorption spectrum of the third compound was similar to that of 7-M-12-CHO-BA (Fig. 8) and it has a molecular ion at *m/e* 286 (Table I) from mass spectral analysis. We have concluded that this third compound is 7-OHM-12-CHO-BA, which apparently was not recognized previously[29].

The preparation of carboxylic acid derivatives of DMBA was carried out similarly to the preparation of 7-benz[*a*]anthracene carboxylic acid (7-COOH-BA) from 7-formylbenz[*a*]anthracene (7-CHO-BA)[30]. Powdered $KMnO_4$ (3 mg) was added to an ice-cold solution of 7-M-12-CHO-BA (5 mg) in pure acetone (5 ml) and the mixture was stirred for 1 h. The resulting 7-methylbenz[*a*]anthracene-12-carboxylic acid (7-M-12-COOH-BA) was partitioned with diethyl ether (10 ml) and 1 *N* NaOH (10 ml). The aqueous phase was further extracted twice with diethyl ether and the 7-M-12-COOH-BA in the aqueous phase was precipitated by dropwise addition of conc. HCl. The precipitate was collected on a sintered-glass filter and then dissolved in THF. 12-Methylbenz[*a*]anthracene-7-carboxylic acid (7-COOH-12-MBA) and benz[*a*]anthracene-7,12-dicarboxylic acid (7,12-$(COOH)_2$-BA) were prepared similarly from 7-CHO-12-MBA and 7,12-$(CHO)_2$-BA, respectively. Chromatography on a DuPont Zorbax SIL column (25 cm × 6.2 mm) with THF–hexane (2:3) containing 0.4% glacial acetic acid at a flow-rate of 2 ml/min indicated that each of the acids was greater than 99% pure. The UV absorption spectra (not

TABLE I

HPLC RETENTION TIMES, MOLAR EXTINCTION COEFFICIENTS AND MASS SPECTRA OF DMBA METABOLITES

The chromatographic peaks on reversed-phase HPLC are numbered as in Fig. 1. STD = Standard. A DuPont Zorbax ODS column (25 cm × 4.6 mm) was used in the reversed-phase HPLC and the solvent conditions are described in Materials and methods. A DuPont Zorbax SIL column (25 cm × 6.2 mm) was used in the normal-phase HPLC and the elution solvents are numbered from S-1 to S-9. The flow-rate for S-3 was 1 ml/min; the flow-rate for all other elution solvents was 2 ml/min. The volume ratios (tetrahydrofuran–hexane–methanol–glacial acetic acid) for all elution solvents are: S-1 (5:93.2:1:0.8); S-2 (40:59.6:0:0.4); S-3 (60:39:1:0); S-4 (40:55:5:0); S-5 (40:57.5:2.5:0); S-6 (30:67.5:2.5:0); S-7 (30:59:1:0); S-8 (25:75:0:0); S-9 (10:90:0:0). Mass spectral analysis was carried out and the carboxylic acid derivatives of DMBA were isolated as described in Materials and methods.

Peak no.	*Metabolite*	*Retention time on HPLC (min)*		*log ε ($\lambda_{max.}$, nm)**	*Characteristic mass ions, m/e (relative ion intensity, %)*
		Reversed-phase	*Normal-phase*		
	7,12-$(COOH)_2$-BA (STD)	1.8	7.8 (S-2)	ND	316 (M^+, 100%), 299 (13), 297 (9), 272 (27), 255 (12), 226 (70), 215 (31), 213 (31)
	7-M-12-COOH-BA	4.3	19.0 (S-1) 4.5 (S-2)	ND	286 (M^+, 100%), 269 (23), 254 (5), 241 (23), 239 (30), 226 (31), 215 (8)
	7-COOH-12-MBA	5.1	16.6 (S-1) 4.5 (S-2)	5.06 (293.5)	286 (M^+, 100%), 269 (11), 241 (63), 239 (62), 226 (21), 215 (11)
D2	7,12-diOHMBA-*trans*-5,6-diol	3.4	21.3 (S-3)	ND	322 (M^+, 10%), 304 (1), 258 (73), 245 (84), 229 (92), 215 (100), 202 (61)
A2, B3	7-M-12-OHMBA-*trans*-5,6-diol	4.6	7.8 (S-4)	4.80 (268.2)	306 (M^+, 23%), 288 (11), 270 (37), 245 (60), 242 (40), 228 (47), 215 (100), 202 (55)
D3	7,12-diOHMBA-*trans*-10,11-diol	4.7	15.6 (S-4)	ND	322 (M^+, 10%), 304 (4), 288 (50), 286 (47), 257 (75), 241 (71), 239 (59), 229 (100), 226 (84), 215 (81), 202 (65)
A3, B4, C2, D4	7,12-diOHMBA-*trans*-3,4-diol	5.2	16.2 (S-3) 14.3 (S-4)	ND	322 (M^+, 2%), 304 (8), 286 (31), 257 (78), 239 (39), 228 (87), 215 (100), 202 (73)
A4, D5	7,12-diOHMBA-*trans*-8,9-diol	5.6	18.3 (S-4) 25.0 (S-5)	ND	322 (M^+, 16%), 288 (43), 286 (16), 257 (62), 244 (52), 239 (46), 226 (68), 215 (95), 202 (100)
A5, C3	7-OHM-12-MBA-*trans*-10,11-diol	8.7	16.5 (S-5)	4.91 (276.5)	306 (M^+, 48%), 288 (32), 275 (29), 270 (23), 259 (61), 242 (43), 229 (61), 215 (100), 202 (90)
A6, B5	7-M-12-OHMBA-*trans*-8,9-diol	11.7	13.1 (S-5)	ND	306 (M^+, 41%), 288 (47), 272 (23), 259 (79), 244 (72), 227 (60), 215 (100), 202 (92)
A7, C4	7-OHM-12-MBA-*trans*-5,6-diol	12.1	14.4 (S-5)	ND	306 (M^+, 7%), 288 (8), 270 (13), 259 (34), 258 (35), 245 (100), 229 (75), 215 (87), 202 (42)
A8, B6 C5, D6	4-OH-7,12-diOHMBA	13.0	ND	ND	304 (M^+, 30%), 286 (10), 269 (16), 257 (100), 245 (20), 239 (23), 226 (46), 215 (46), 202 (27)

A8, C5	7-OHM-12-MBA-*trans*-8,9-diol	13.3	16.4 (S-5)	4.79 (266.8)	306 (M^+, 24%), 288 (17), 270 (56), 259 (94), 245 (58), 242 (64), 228 (44), 226 (41), 215 (100), 202 (61)
A9, B7	7-M-12-OHMBA-*trans*-10,11-diol	17.8	16.5 (S-6)	ND	306 (M^+, 26%), 288 (40), 270 (31), 259 (100), 245 (40), 227 (45), 215 (58), 202 (47)
D7	2-OH-7,12-diOHMBA	17.8	ND	ND	304 (M^+, 50%), 286 (5), 269 (11), 257 (100), 244 (16), 239 (11), 226 (21), 215 (17), 202 (11)
A10, C6	7-OHM-12-MBA-*trans*-3,4-diol	18.6	19.5 (S-6)	5.20 (270.0)	306 (M^+, 67%), 291 (5), 288 (26), 260 (38), 244 (43), 231 (59), 216 (83), 215 (100), 202 (89)
B8, D8	3-OH-7,12-diOHMBA	19.2	ND	ND	304 (M^+, 46%), 286 (13), 269 (13), 257 (100), 245 (23), 244 (18), 239 (13), 226 (23), 215 (25), 202 (17)
A11	DMBA-*trans*-5,6-diol	19.3	9.2 (S-6)	4.84 (269.3)	290 (M^+, 60%), 272 (20), 257 (38), 243 (58), 239 (10), 229 (100), 215 (38), 202 (29)
A11, B8	7-M-12-OHMBA-*trans*-3,4-diol	19.3	15.5 (S-6)	5.05 (269.8)	306 (M^+, 41%), 291 (0), 288 (19), 275 (9), 270 (14), 259 (60), 244 (65), 242 (26), 239 (17), 229 (31), 226 (30), 215 (100), 202 (70)
A12	DMBA-*trans*-8,9-diol	22.7	12.7 (S-6)	4.80 (266.1)	290 (M^+, 52%), 272 (28), 259 (18), 257 (24), 244 (74), 239 (12), 229 (100), 215 (34), 203 (52), 202 (51)
	DMBA-*trans*-10,11-diol	22.7	11.8 (S-6)	4.83 (275.2)	290 (M^+, 81%), 272 (50), 259 (30), 257 (44), 244 (77), 239 (21), 229 (100), 215 (46), 203 (73), 202 (76)
A13, B9, C7, D9	7,12-diOHMBA	24.6	8.0 (S-6) 9.4 (S-7)	4.91 (293.6)	288 (M^+, 23%), 257 (3), 241 (100), 239 (25), 229 (35), 228 (41), 226 (25), 215 (13), 202 (13)
A14, C8	2-OH-7-OHM-12-MBA	28.1	9.7 (S-7)	4.63 (288.5)	288 (M^+, 92%), 273 (7), 272 (11), 271 (29), 259 (43), 244 (100), 239 (16), 226 (23), 215 (24), 202 (13)
A15, C9	3-OH-7-OHM-12-MBA	30.9	11.3 (S-7)	4.90 (296.4)	288 (M^+, 94%), 273 (7), 272 (12), 271 (26), 259 (43), 255 (15), 244 (100), 239 (20), 226 (26), 215 (32), 202 (20)
A16, B10	4-OH-7-M-12-OHMBA	32.8	7.5 (S-7)	4.66 (280.0)	288 (M^+, 100%), 273 (8), 272 (7), 271 (23), 259 (57), 255 (16), 244 (80), 239 (22), 226 (31), 215 (43), 202 (26)
A17, C10	4-OH-7-OHM-12-MBA	33.7	9.5 (S-7)	ND	288 (M^+, 66%), 273 (6), 272 (13), 271 (27), 259 (58), 244 (100), 239 (28), 226 (36), 215 (58), 202 (23)
A18, B11	3-OH-7-M-12-OHMBA	34.2	8.3 (S-7)	ND	288 (M^+, 100%), 271 (27), 259 (50), 244 (84), 239 (16), 226 (20), 215 (25), 202 (11)
	DMBA-*cis*-5,6-diol.(STD)	34.3	9.9 (S-7)	ND	290 (M^+, 78%), 272 (9), 257 (45), 243 (59), 239 (9), 229 (100), 215 (38), 202 (26)
A19, B12, C10, D10	7-OHM-12-CHO-BA	35.8	6.9 (S-9) 4.6 (S-8)	4.67 (291.8)	286 (M^+, 100%), 285 (67), 269 (32), 268 (33), 257 (34), 255 (38), 241 (26), 239 (98), 229 (86), 228 (95), 226 (93), 224 (28), 215 (12), 202 (25)

(Continued on p. 640)

TABLE I *(continued)*

Peak no.	*Metabolite*	*Retention time on HPLC (min)*		*log ε ($\lambda_{max.}$, nm)* *	*Characteristic mass ions, m/e (relative ion intensity, %)*
		Reversed-phase	*Normal-phase*		
A19, C11	7-CHO-12-OHMBA	35.8	4.3 (S-7) 8.6 (S-8) 39.3 (S-9)	4.58 (293.4)	286 (M^+, 56%), 268 (5), 257 (40), 255 (9), 239 (38), 237 (9), 229 (100), 228 (64), 226 (40), 224 (12), 215 (5), 202 (6)
	DMBA-7,12-endoperoxide (STD)	35.8	5.2 (S-7) 5.8 (S-8)	4.76 (232.5)	288 (M^+, 4%), 256 (100), 245 (22), 241 (40), 239 (26), 226 (14), 215 (20), 202 (29)
A20	DMBA-*trans*-3,4-diol	37.8	24.1 (S-8)	5.16 (269.3)	290 (M^+, 100%), 275 (13), 272 (22), 257 (25), 244 (84), 229 (73), 215 (65), 202 (88)
A21, B13	2-OH-7-M-12-OHMBA	38.8	22.6 (S-8)	ND	288 (M^+, 58%), 270 (84), 269 (100), 259 (46), 255 (69), 253 (84), 244 (53), 242 (50), 239 (61), 226 (53)
A22, C12	7-OHM-12-MBA	41.6	9.8 (S-8) 46.0 (S-9)	4.91 (295.0)	272 (M^+, 63%), 257 (7), 255 (20), 243 (46), 239 (40), 228 (100), 226 (21), 215 (11), 202 (10)
A23, B14	7-M-12-OHMBA	42.6	6.0 (S-8) 18.4 (S-9)	4.90 (294.0)	272 (M^+, 60%), 257 (7), 255 (10), 243 (65), 239 (51), 228 (100), 226 (23), 215 (6), 202 (5)
	1-OH-DMBA (STD)	44.3	10.2 (S-9)	ND	272 (M^+, 100%), 257 (54), 255 (31), 242 (40), 239 (44), 228 (26), 226 (39), 215 (13), 202 (18)
A24	2-OH-DMBA	45.7	16.1 (S-9)	4.64 (290.5)	272 (M^+, 100%), 257 (38), 255 (15), 239 (26), 228 (19), 226 (21), 215 (12), 202 (12)
A25	3-OH-DMBA	46.1	17.6 (S-9)	480 (295.2)	272 (M^+, 100%), 257 (43), 255 (14), 239 (18), 228 (15), 226 (15), 215 (7), 202 (7)
A26	4-OH-DMBA	47.0	14.6 (S-9)	4.73 (280.7)	272 (M^+, 100%), 257 (33), 255 (13), 239 (25), 229 (19), 228 (20), 226 (21), 215 (15), 202 (16)
A27, C13	7-CHO-12-MBA	49.2	8.0 (S-9)	4.52 (293.5)	270 (M^+, 97%), 269 (37), 255 (52), 242 (58), 241 (71), 240 (42), 239 (100), 237 (25), 226 (59), 215 (26), 202 (8)
A28, B15	7-M-12-CHO-BA	51.6	7.1 (S-9)	4.67 (292.2)	270 (M^+, 68%), 269 (100), 255 (5), 241 (29), 242 (13), 240 (5), 239 (40), 237 (14), 226 (43), 215 (10), 202 (3)
A29	DMBA	56.2	4.4 (S-9)	4.90 (295.5)	256 (M^+, 92%), 241 (100), 240 (59), 239 (83), 226 (21), 215 (15), 202 (9)

* ND = Not determined.

shown) of the acids showed characteristics of an intact benz[*a*]anthracene (BA) nucleus. Mass spectral analysis (Table I) further confirmed their identities.

[7,12-^{14}C]DMBA (specific activity 112 mCi/mmol) was purchased from Amersham (Arlington Heights, Ill., U.S.A.). Specifically labeled [7-CH_2-^{3}H]-7-OHM-12-MBA, [12-CH_2-^{3}H]-7-M-12-OHMBA and [7,12-CH_2-^{3}H]-7,12-diOHMBA (specific activity 1.67–2.91 Ci/mmol) were prepared as previously described[27] and were diluted to a specific activity of 50–100 mCi/mmol with unlabeled compounds before use.

Preparation of rat liver microsomes

Rat liver microsomes were prepared by a modified procedure of Kinoshita *et al.*[31]. Routinely, 60 male Sprague-Dawley rats weighing 80–100 g were each pretreated with phenobarbital (i.p. injections of 75 mg/kg body weight for each of 3 days). The rats were sacrificed on the fourth day by decapitation, livers were removed and placed in ice-cold 0.25 *M* sucrose–0.05 *M* Tris·HCl buffer, pH 7.5 (sucrose–Tris buffer). Livers were minced with scissors, washed twice with ice-cold sucrose–Tris buffer and were further minced with a blender for 1 min. The minced livers were homogenized in sucrose–Tris buffer, using a Potter-Elvehjem PTFE–glass homogenizer (20–30 strokes). The homogenates were centrifuged at 750 *g* for 20 min, and the supernatant was centrifuged at 10,000 *g* for 20 min. The microsomes were obtained by centrifugation of the supernatant at 105,000 *g* for 60 min. The microsomal pellets were homogenized in sucrose–Tris buffer (*ca.* 10 mg microsomal protein per ml buffer) and frozen at −80° until ready for use. Liver microsomes from corn oil-treated and 3-methylcholanthrene-treated (25 mg/kg 3-MC dissolved in corn oil, i.p. injections on each of 3 days) male Sprague-Dawley rats weighing 80–100 g were prepared as described above. Protein content of the microsomes was determined[32] with bovine serum albumin as the standard.

Large-scale in vitro incubation of the hydrocarbons

Metabolites were obtained by *in vitro* incubation of DMBA, 7-M-12-OHMBA, 7-OHM-12-MBA and 7,12-diOHMBA, respectively (20 μmol in 10 ml methanol) in the dark at 37° for 60 min in a 250-ml reaction mixture (pH 7.5) containing 12.5 mmol of Tris·HCl at pH 7.5, 0,75 mmol of $MgCl_2$, 25 units of glucose-6-phosphate dehydrogenase (Type XII; Sigma, St. Louis, Mo., U.S.A.), 25 mg $NADP^+$, 162.5 mg of glucose-6-phosphate and 250 mg protein equivalent of liver microsomes from phenobarbital-pretreated rats. The above incubation was repeated whenever necessary in order to obtain a sufficient amount of metabolites for characterization. Metabolites were extracted with acetone and ethyl acetate as previously described[12].

Reversed-phase HPLC

A Spectra-Physics Model 3500B liquid chromatograph fitted with a DuPont Zorbax ODS column 25 cm × 4.6 or 6.2 mm I.D.) was used. The 25 cm × 6.2 mm column was used for large-scale isolation of metabolites as previously described[12,13], whereas the 25 cm × 4.6 mm analytical column was used for separation of relatively smaller amounts of metabolites. The analytical column was eluted at ambient temperature with methanol–water (1:1) for 10 min and followed by a 40-min linear gradient of methanol–water (1:1) to methanol at a solvent flow-rate of 0.8 ml/min.

The eluates were monitored at 254 nm. The chromatographic peaks, some of which contained more than one metabolite, were evaporated to dryness and were dissolved in THF–hexane for further purification.

Normal-phase HPLC

Metabolites obtained by reversed-phase HPLC were further separated by normal-phase HPLC on a DuPont Zorbax SIL (silica gel) column (25 cm × 6.2 mm) with various elution solvents containing a different ratio of THF–hexane–methanol–glacial acetic acid at a solvent flow-rate of 1–2 ml/min. The solvent was delivered by an Altex Model 110 pump and the eluates were monitored by a DuPont Model 836 ultraviolet–fluorescence detector as previously described[12]. No special conditioning of the column was necessary for the resolution of metabolites.

Isolation of carboxylic acids as metabolites

The UV absorption spectra of the early eluted peaks (retention times 1–6 min) collected on reversed-phase HPLC indicated the presence of carboxylic acid derivatives of DMBA. However, because the early eluted peaks contained other materials that were extracted from rat liver microsomes by ethyl acetate, we could not obtain sufficiently pure carboxylic acids for definitive identification. For the purpose of definitive identification, we have developed a procedure which specifically isolates carboxylic acid derivatives from the *in vitro* incubation products of DMBA and its hydroxymethyl metabolites.

DMBA and its metabolites that were extracted with acetone and ethyl acetate from 2 l of incubation products of DMBA with rat liver microsomes were dissolved in benzene (50 ml) and loaded on to a 30 × 1.5 cm silica gel (Bio-Sil A, 100–200 mesh; Bio-Rad Labs.) column previously equilibrated with benzene. The column was eluted with 100 ml benzene and this released most of the DMBA, DMBA phenols, 7-OHM-12-MBA and 7-M-12-OHMBA. The column was further eluted with 100 ml THF and this gave essentially all other DMBA metabolites except the carboxylic acid derivatives. A 100-ml volume of THF containing 1% glacial acetic acid was then applied to the column and the eluates were evaporated to dryness under reduced pressure. When the acetic acid was completely evaporated, the residue was partitioned with 1 *N* NaOH (20 ml) and ethyl acetate (40 ml). The alkaline aqueous solution was extracted twice more with ethyl acetate and the carboxylic acid derivatives obtained were subjected to further purification by reversed-phase and normal-phase HPLC and by mass spectral analysis.

Determination of molar extinction coefficients

Whenever the metabolites were available in sufficient quantity, the molar extinction coefficients (ε, $cm^{-1} \cdot M^{-1}$) were determined from an accurately weighed sample. However, most of the DMBA metabolites described in this report have not yet been synthesized. We have thus determined the molar extinction coefficients of some of the DMBA metabolites that are formed from specifically labeled [7,12-^{14}C] DMBA or [7-CH_2-^{3}H]-7-OHM-12-MBA of known specific activity. The molar extinction coefficients of the [7,12-^{14}C]-7-OHM-12-MBA-*trans*-3,4-diol derived from [7,12-^{14}C]DMBA and the [7-CH_2-^{3}H]-7-OHM-12-MBA-*trans*-3,4-diol derived from [7-CH_2-^{3}H]-7-OHM-12-MBA, for example, were found to be in agreement within

10%. This indicates that the tritium label at the hydroxymethyl group of [7-CH_2-^{3}H]-7-OHM-12-MBA underwent little or no exchange during the incubation procedure. Thus the accuracy of the molar extinction coefficients listed in Table I is within tolerable experimental error (±10%).

Physicochemical properties of the metabolites

Absorption spectra were measured in methanol on a Cary 118C spectrophotometer. Uncorrected fluorescence spectra were measured in methanol or in 0.1 *N* NaOH on a Perkin-Elmer Model 44A spectrophotofluorimeter. Mass spectral analysis of the metabolites was performed on a Finnigan 4000 gas chromatograph–mass spectrometer–data system by electron impact with a solid probe at 70 eV and 250° ionizer temperature. Optical rotations were measured on a Perkin-Elmer Model 241-MC polarimeter. All dihydrodiol metabolites were identified as *trans* isomers by examining their reaction products (2 h at room temperature) with acetone in the presence of anhydrous $CuSO_4$[21]. The reaction products were analyzed by mass spectrometry. The synthetic standard DMBA-*cis*-5,6-diol formed vicinal *cis*-acetonide (m/e of M^+ at 330) quantitatively, whereas none of the other dihydrodiol metabolites reported herein formed any acetonide.

RESULTS

The physicochemical properties of each metabolite described in this report were obtained from samples that had been purified by two HPLC systems. A metabolite is considered pure if it emerges as a single chromatographic peak in both reversed-phase and normal-phase HPLC systems.

Reversed-phase HPLC

The separation of metabolites by reversed-phase HPLC on an analytical Zorbax ODS column (25 cm × 4.6 mm) is shown in Fig. 1. The UV absorption profiles were obtained from *in vitro* incubation products of DMBA (Fig. 1A), 7-M-12-OHMBA (Fig. 1B), 7-OHM-12-MBA (Fig. 1C) and 7,12-diOHMBA (Fig. 1D), respectively, with liver microsomes from phenobarbital-pretreated rats. Chromatographic peaks are numbered in Fig. 1 and their retention times and identities are indicated in Table I. Chromatographic peaks that were not numbered were mostly derived from rat liver microsomes, observed in control experiments. Many extremely minor peaks in Fig. 1 were found to be due to very small amounts of metabolites. The identities of these minor metabolites were determined from pooled fractions that were collected from 10–30 chromatographic runs.

It can be seen from Fig. 1A that a complex mixture of metabolites was produced from DMBA that had been incubated with rat liver microsomes. The complexity is due to the occurrence of further metabolism of the primary and secondary metabolites such as 7-M-12-OHMBA, 7-OHM-12-MBA and 7,12-diOHMBA. The hydroxymethyl derivatives can be enzymatically oxygenated at the ring positions to form dihydrodiol and phenolic products that are structurally similar to those derived from DMBA. For example, peaks A3, A10, A11 and A20 (Fig. 1A and Table I) each contain a *trans*-3,4-diol. The structural differences among these *trans*-3,4-diols are the degree of hydroxylation at the 7- and 12-methyl groups. The UV absorption and fluorescence properties of these *trans*-3,4-diols are closely similar[12].

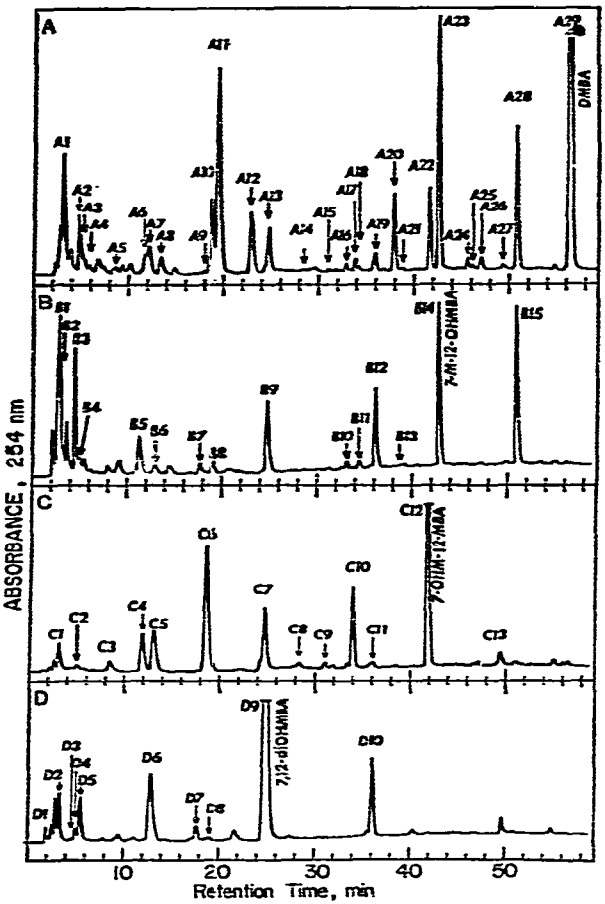


Fig. 1. Reversed-phase HPLC separation of the *in vitro* rat liver microsomal metabolites of DMBA (A), 7-M-12-OHMBA (B), 7-OHM-12-MBA (C) and 7,12-diOHMBA (D). Chromatographic peaks are numbered and their identities are indicated in Table I. HPLC conditions are described in Materials and methods.

HPLC analysis of metabolites that are formed from *in vitro* incubations of 7-M-12-OHMBA (Fig. 1B), 7-OHM-12-MBA (Fig. 1C) and 7,12-diOHMBA (Fig. 1D), respectively, has greatly simplified our task of identifying the metabolites formed from DMBA (Fig.1A). Because all the hydroxymethyl derivatives (peaks A13, A22 and A23 in Fig. 1A) are formed as metabolites of DMBA, further metabolism of these three hydroxymethyl metabolites to more polar products is thus expected. Theoretically, metabolites that have been found in Fig. 1B, 1C and 1D should all be present in Fig. 1A. This has been found to be the case for most metabolites (Table I). Only peaks D2, D3, D7 and D8 in Fig. 1D have not been found directly as metabolites of DMBA. This was not surprising in view of the observation (Fig. 1D) that metabolites D2, D3, D7 and D8 are all minor products of 7,12-diOHMBA. Thus these minor metabolites were probably formed from 7,12-diOHMBA during the metabolism of DMBA, but the amounts were too small to be detected in Fig. 1A.

Carboxylic acid derivatives of DMBA (Table I) were not isolated directly by

reversed-phase HPLC shown in Fig. 1. They were isolated by a procedure (see Materials and methods) that specifically isolates the carboxylic acid derivatives from a complex mixture of metabolites. They were then purified by reversed-phase and normal-phase HPLC systems prior to further spectrometric characterization. Two carboxylic acids, 7-M-12-COOH-BA and 7-COOH-12-MBA, were identified as metabolites from the *in vitro* incubation products of DMBA. The amount of 7-M-12-COOH-BA was found to be greater than that of 7-COOH-12-MBA at a ratio of *ca.* 20:1. A product that had identical retention times in two HPLC systems and a UV absorption spectrum identical to that of the synthetic 7,12-$(COOH)_2$-BA was also detected. However, due to unknown reasons, its identity could not be confirmed by mass spectral analysis. Only 7-M-12-COOH-BA was identified as a metabolite from the microsomal incubation products of 7-M-12-OHMBA. Similarly, only 7-COOH-12-MBA was found as a metabolite of 7-OHM-12-MBA. Again, there were indications (retention times on HPLC and UV absorption spectra) for the presence of 7,12-$(COOH)_2$-BA, but its identity could not be confirmed by mass spectral analysis. Because of the difficulties we have encountered, we did not make an attempt to search for 7,12-$(COOH)_2$-BA as a rat liver microsomal metabolite of 7,12-diOHMBA.

Normal-phase HPLC

Many metabolites were either partially overlapped or completely overlapped on the reversed-phase HPLC (Fig. 1 and Table I). The unresolved metabolites can be completely separated from one another on an isocratic normal-phase HPLC system using a DuPont Zorbax SIL (silica gel) column. Mixtures of various ratios of THF–hexane–methanol were used as the elution solvents and these are indicated in the legend to Table I. For the purification of carboxylic acid derivatives, glacial acetic acid (0.1–1%) was added to protonate the acids. The presence of acetic acid in the elution solvent sharpened the elution peak and greatly reduced the retention time of the carboxylic acid derivatives of DMBA. An example of a separation on normal-phase HPLC is shown in Fig. 2. Peaks A11, A12 and A19 in Fig. 1A each containing two metabolites are now completely resolved (Fig. 2). Furthermore, some closely eluted metabolites in Fig. 1 can also be separated completely by the normal-phase HPLC.

The elution orders of metabolites on the reversed-phase HPLC are generally reversed on the normal-phase HPLC when the elution solvents in the latter do not contain methanol (Figs. 1 and 2, Table I). A general rule is not apparent for the elution orders of metabolites in the normal-phase HPLC when methanol is added to the elution solvents (Fig. 2 and Table I).

Mass spectral analysis

Mass spectral analysis was performed on each metabolite that had been purified by the two HPLC systems described above. The major mass ions obtained for each metabolite or synthetic standard are listed in Table I and are in full agreement with the assigned structures.

UV absorption and fluorescence spectra

Saturation of a double bond or hydroxylation at a ring position of the parent hydrocarbon produces a compound which has characteristic UV absorption and fluo-

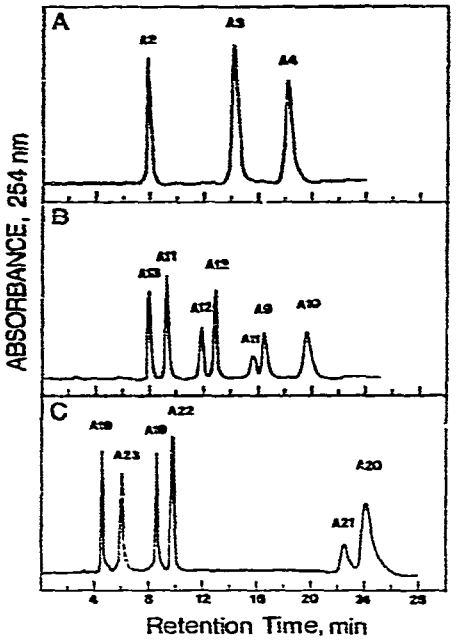


Fig. 2. Normal-phase HPLC separation of some DMBA metabolites. The chromatographic peaks are numbered as in Fig. 1A. The retention times, identities of the metabolites, HPLC conditions and the elution solvents are described in Table I. Elution solvents were S-4 (A), S-6 (B) and S-8 (C).

rescence spectra. We have routinely measured the UV absorption and fluorescence spectra of the collected HPLC peaks as the first clues in the elucidation of the metabolites' structure. The UV absorption spectra of some DMBA metabolites were shown in several previous reports[4–9,12,26]. However, the data were fragmentary and never before was a concentrated effort made to extensively purify and to characterize the number of DMBA metabolites that are reported in this communication. The UV absorption and fluorescence spectra of four *trans*-3,4-diols were reported earlier[12]. We have also reported the nuclear magnetic resonance spectra of DMBA-*trans*-3,4-diol and 7-OHM-12-MBA-*trans*-3,4-diol[13]. None of the four possible *trans*-1,2-diols were detected as metabolites. The UV absorption and fluorescence spectra of four *trans*-5,6-diols, four *trans*-8,9-diols and four *trans*-10,11-diols are shown in Figs. 3–5. Hydroxylation of the 7- and 12-methyl groups apparently did not change the characteristic absorption and fluorescence peaks among the dihydrodiols involving the same ring carbons, but it did shift slightly the absorption and fluorescence maxima. The direction of shifts in absorption maxima seems to depend on the wavelength region (Figs. 3A, 4A and 5A), but hydroxylation of the methyl groups always caused blue shifts in fluorescence maxima (Figs. 3B, 4B and 5B).

The absorption and fluorescence spectra of four 3-phenols and four 4-phenols are shown in Figs. 6 and 7. The UV absorption and fluorescence spectra of four 2-phenols have been reported recently[26]. Although the synthetic 8-, 9-, 10- and 11-phenols of DMBA are available to us for comparisons, we have not found any evidence for the presence of any of these phenols as metabolites of DMBA. Red shifts

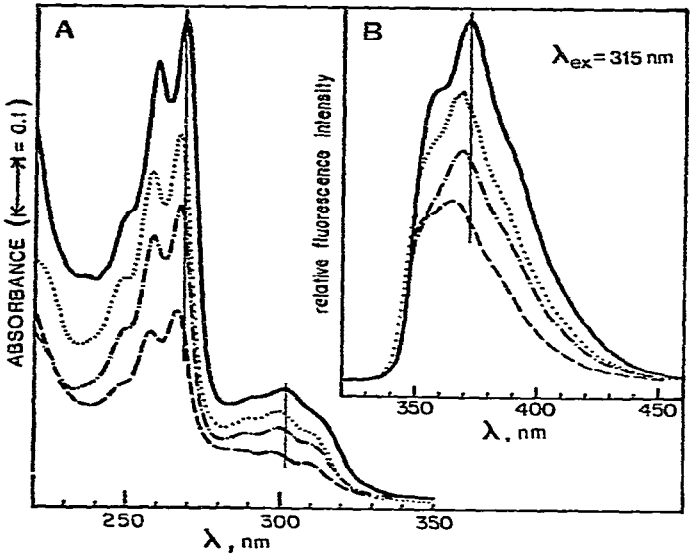


Fig. 3. UV absorption spectra (A, in methanol) and fluorescence spectra (B, in methanol) of DMBA-*trans*-5,6,diol (———), 7-M-12-OHMBA-*trans*-5,6-diol (·····), 7-OHM-12-MBA-*trans*-5,6-diol ((—·—·—) and 7,12-diOHMBA-*trans*-5,6-diol (— — —).

in fluorescence maxima (Figs. 6C and 7C) are indicative of phenolic derivatives of polycyclic aromatic hydrocarbons. However, it should be noted that the fluorescence maxima (Figs. 6C and 7C) vary considerably in alkaline solution for compounds of similar structure. It is also apparent from the data in Figs. 3–7 that the UV absorption

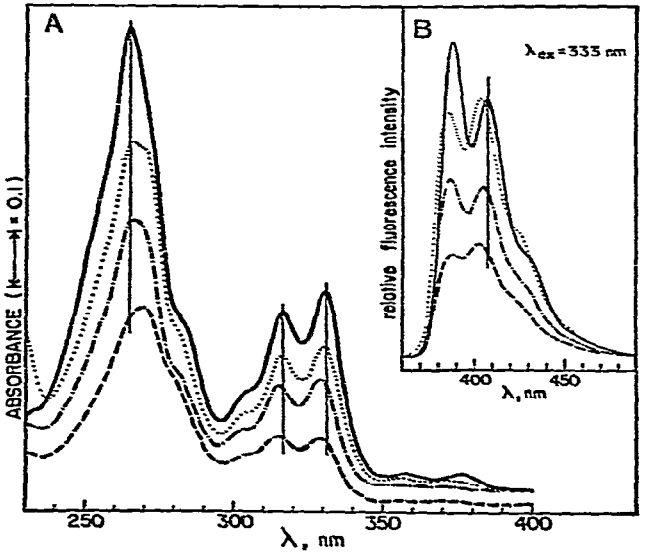


Fig. 4. UV absorption spectra (A, in methanol) and fluorescence spectra (B, in methanol) of DMBA-*trans*-8,9-diol (———), 7-M-12-OHMBA-*trans*-8,9-diol (·····), 7-OHM-12-MBA-*trans*-8,9-diol (—·—·—) and 7,12-diOHMBA-*trans*-8,9-diol (— — —).

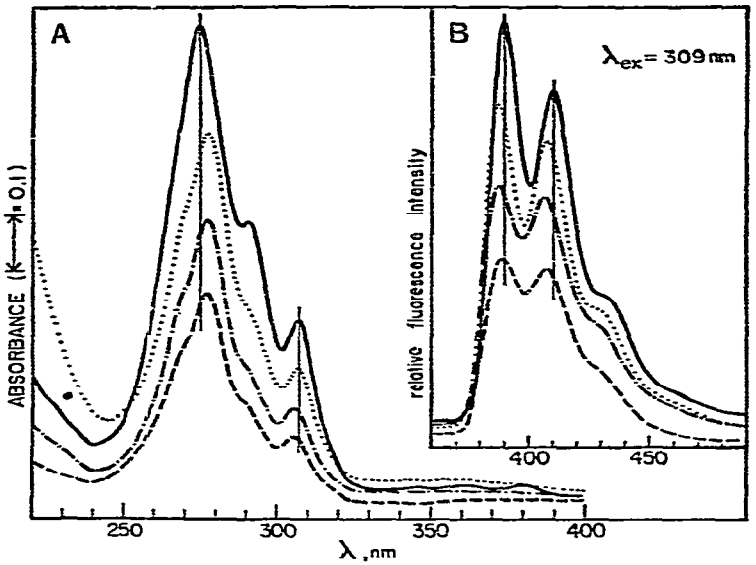


Fig. 5. UV absorption spectra (A, in methanol) and fluorescence spectra (B, in methanol) of DMBA-*trans*-10,11-diol (———), 7-M-12-OHMBA-*trans*-10,11-diol (·····), 7-OHM-12-MBA-*trans*-10,11-diol (—·—·—·) and 7,12-diOHMBA-*trans*-10,11-diol (— — —).

and/or fluorescence spectra of DMBA metabolites cannot be used as the sole source of structural proof of a metabolite in the absence of other data.

Fig. 8 shows the UV absorption and fluorescence spectra of two methyl aldehydes (7-M-12-CHO-BA and 7-CHO-12-MBA) and two hydroxymethyl alde-

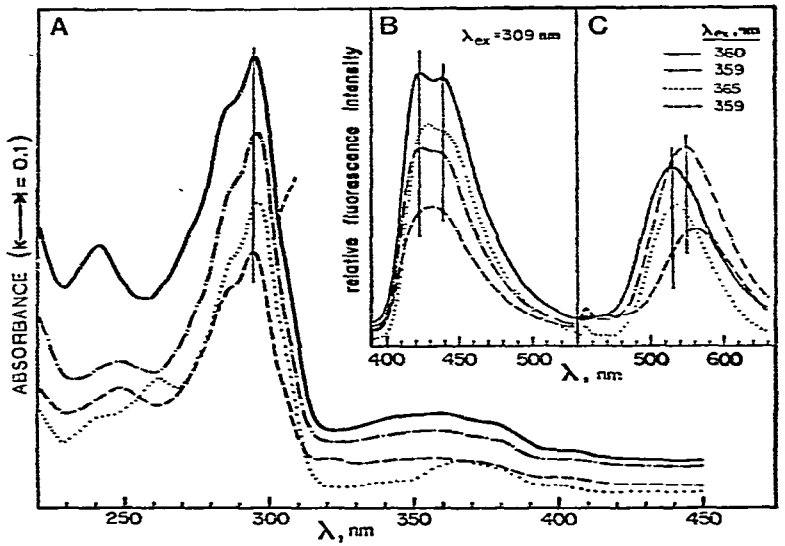


Fig. 6. UV absorption spectra (A, in methanol) and fluorescence spectra (B, in methanol; C, in 0.1 *N* NaOH) of 3-OH-DMBA (———), 3-OH-7-M-12-OHMBA (·····), 3-OH-7-OHM-12-MBA (—·—·—) and 3-OH-7,12-diOHMBA (— — —).

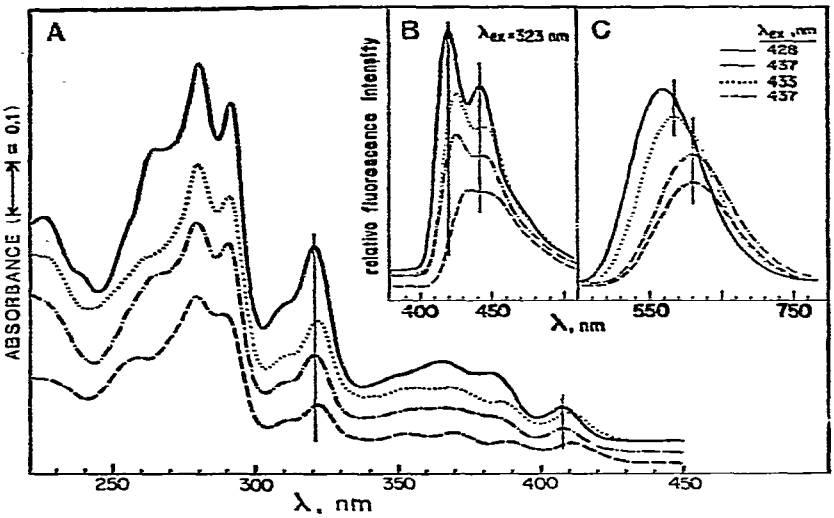


Fig. 7. UV absorption spectra (A, in methanol) and fluorescence spectra (B, in methanol; C, in 0.1 *N* NaOH) of 4-OH-DMBA (———), 4-OH-7-M-12-OHMBA (·····), 4-OH-7-OHM-12-MBA (—·—·—) and 4-OH-7,12-diOHMBA (— — —).

hydes (7-OHM-12-CHO-BA and 7-CHO-12-OHMBA). None of these were previously found as metabolites of DMBA.

Optical activity of dihydrodiol metabolites

Formation of optically active dihydrodiol metabolites indicates the stereospecific or stereoselective properties of the rat liver microsomal mixed-function oxi-

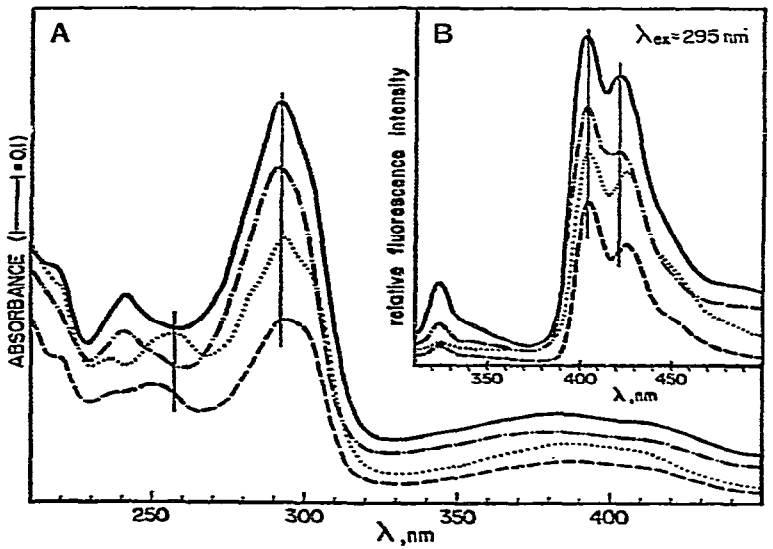


Fig. 8. UV absorption spectra (A, in methanol) and fluorescence spectra (B, in methanol) of 7-M-12-CHO-BA (———), 7-OHM-12-CHO-BA (—·—·—), 7-CHO-12-MBA (·····) and 7-CHO-12-OHMBA (— — —).

dases and epoxide hydra(ta)se[22,23,33]. We obtained several highly purified dihydrodiols in sufficient quantity for optical rotation measurement (Table II). In contrast to the predominantly (—)-dihydrodiol enantiomers formed from benzo[*a*]pyrene[22,23,33], the dihydrodiols formed from DMBA have both (+) and (—) optical rotations. It is also interesting to note that the specific rotation of DMBA-*trans*-5,6-diol is +257° whereas the 7-OHM-12-MBA-*trans*-5,6-diol has a specific rotation of —29°. The results in Table II indicate that the dihydrodiols are formed stereospecifically or stereoselectively from DMBA by rat liver microsomal mixed-function oxidases and epoxide hydra(ta)se. To determine the exact degree of stereospecificity or stereoselectivity would require a major effort in determining the optical purity of the dihydrodiol metabolites.

TABLE II

OPTICAL ACTIVITY OF SOME DMBA METABOLITES

The dihydrodiols of DMBA were obtained from the *in vitro* incubation of DMBA whereas the dihydrodiols of 7-OHM-12-MBA were obtained from *in vitro* incubation of 7-OHM-12-MBA. The enzyme source in each case was liver microsomes from phenobarbital-pretreated male Sprague–Dawley rats.

Metabolite	$[\alpha]_D^{25}$, (°)*
DMBA-*trans*-3,4-diol	—175 (0.8)
DMBA-*trans*-8,9-diol	+ 25 (0.99)
DMBA-*trans*-5,6-diol	+257 (1.52)
7-OHM-12-MBA-*trans*-3,4-diol	—105** (2.0)
7-OHM-12-MBA-*trans*-8,9-diol	+ 21 (2.11)
7-OHM-12-MBA-*trans*-5,6-diol	— 29 (0.68)
7-OHM-12-MBA-*trans*-10,11-diol	— 97 (1.55)

* The concentration (mg/ml in methanol) is given in parentheses.

** A specific rotation of —138° was reported previously[13] from a sample obtained with a different microsomal preparation.

DISCUSSION

The *in vitro* system using rat liver microsomal enzymes is used most commonly in the identification of metabolic products of a chemical carcinogen. The term "chemical carcinogen" is used here to include drugs, environmental pollutants, food additives and other xenobiotics that possess carcinogenic properties. The advantages of using rat liver microsomes as the carcinogen-metabolizing enzyme source are: (i) the relative ease in obtaining a large quantity of microsomal enzymes; (ii) induced liver microsomal enzyme activity from phenobarbital-, 3-methylcholanthrene- or other chemically-treated rats; and (iii) the *in vitro* incubation products of a chemical carcinogen which are qualitatively similar to those obtained with enzyme sources from extrahepatic tissues, some of which are target sites of a chemical carcinogen.

A variety of metabolites are formed from the *in vitro* incubation of a chemical carcinogen with rat liver microsomes. The metabolites formed are usually separated from the microsomal proteins and lipids by various treatments and extraction procedures. In the case of PAHs, unconjugated metabolites are easily separated from the microsomal components and water-soluble products by organic solvent extraction. In earlier studies PAH metabolites were separated for identification by thin-

layer chromatography (TLC) using silica gel-coated plates[3]. Due to incomplete separation on TLC plates not all metabolites could be examined in detail. In the past few years HPLC has been applied successfully to the separation of metabolic products of PAHs such as benzo[*a*]pyrene[17–23], 3-methylcholanthrene[24], 5-methylchrysene[25] and DMBA[11–14]. Reversed-phase HPLC has been used primarily in the analysis of PAH metabolites. Recently it has become increasingly apparent that a single and symmetric chromatographic peak on the reversed-phase HPLC often contains more than one metabolite. The metabolites that are not separable by the reversed-phase HPLC are in most cases resolvable by the normal-phase HPLC. We have applied both HPLC systems in combination to extensively purify such metabolites and have identified more than thirty DMBA metabolites that are formed from the *in vitro* incubation of DMBA and its hydroxymethyl derivatives with rat liver microsomes.

The identified metabolites listed in Table I clearly indicate that all methyl groups and ring carbons numbered 2–6 and 8–11 of DMBA, 7-M-12-OHMBA, 7-OHM-12-MBA and 7,12-diOHMBA are involved in metabolic oxygenations. Four 2-phenols have been identified as metabolites and these may be formed by nonenzymatic rearrangement of the 1,2-epoxide intermediates[26]. However, although unlikely, the 2-phenols may also be formed by a direct insertion mechanism[34]. The involvement of C_1 carbons in metabolism may be demonstrated by the metabolism of the *trans*-3,4-diols to 3,4-diol-1,2-epoxides[12] although this has not been reported.

DMBA-7,12-endoperoxide has been reported as a major metabolite of DMBA[10]. In spite of an intensive search, we have not found this metabolite using our incubation conditions. When a [^{14}C]DMBA was incubated with heat-inactivated (100° for 10 min) rat liver microsomes, a radioactive peak which cochromatographed with the synthetic DMBA-7,12-endoperoxide was substantially higher than the peak that was obtained from the incubation of [^{14}C]DMBA with enzymatically active rat liver microsomes (data not shown). Although two hydroxymethyl aldehydes cochromatographed with DMBA-7,12-endoperoxide (peak A19 in Fig. 1), these hydroxymethyl aldehydes could not have been formed from the DMBA with the heat-inactivated microsomes. We thus concluded that a low level of DMBA-7,12-endoperoxide was formed under our experimental conditions but it was not a microsomal enzyme-dependent product.

Nine metabolites that are derived from the oxidation of the methyl groups of DMBA have been identified. These are the three hydroxymethyl derivatives, two methyl aldehydes, two hydroxymethyl aldehydes and two methyl carboxylic acids (Table I). The pathways for the oxidations of the methyl side chains to carboxylic acids are proposed in Fig. 9. Many other potential oxidation products indicated in Fig. 9 have not been found. Both 7-CHO-12-MBA and 7-M-12-CHO-BA were found to possess moderate to weak skin tumor-initiating activity in mice[35]. Thus oxidation of DMBA to methyl aldehydes may contribute to the overall carcinogenicity of DMBA. The biological effects of the further oxidations of the methyl aldehydes to hydroxymethyl aldehydes and carboxylic acid derivatives are not known.

The metabolic pathways for the formation of dihydrodiols and phenolic metabolites at the ring positions are proposed in Fig. 10. None of the epoxide intermediates has been detected directly. However, it has been well established that epoxides (arene oxides) are the metabolic precursors of PAHs. In addition to the hydra-

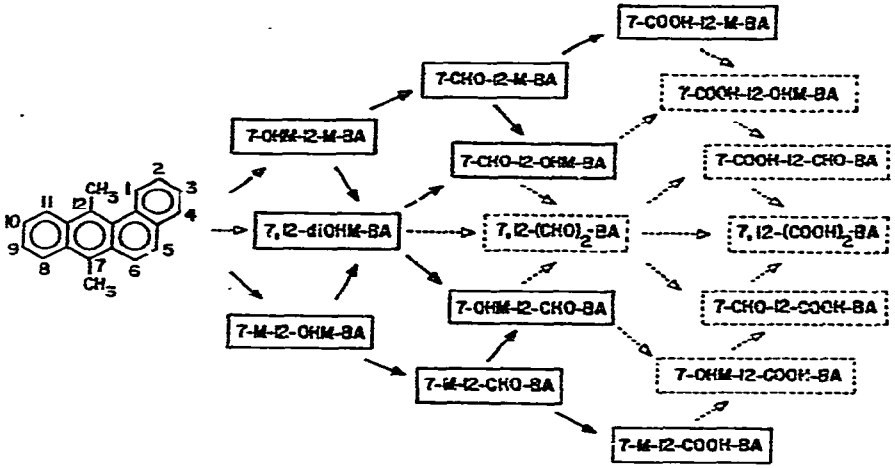


Fig. 9. Proposed pathways of metabolic oxidations of DMBA at the methyl groups. Solid arrows indicate the formation of products which have been identified. Broken arrows indicate the possible formation of products which have not been detected.

tion reaction catalyzed by the microsomal epoxide hydra(ta)se, epoxide intermediates can also rearrange non-enzymatically to phenolic products. Many possible phenolic metabolites indicated in Fig. 10 have not been found. These phenols may be formed in an extremely small amount and thus were not detectable. Although four 2-phenols were found[26], none of the four possible *trans*-1,2-diol metabolites was detected. The absence of *trans*-1,2-diols may be due to the instability of the 1,2-epoxide intermediates. Thus the 1,2-epoxide intermediates may have already been isomerized to 1- and 2-phenols before they can be translocated in the microsomal enzyme complex to the enzymic site of the epoxide hydra(ta)se.

Among the dihydrodiol metabolites, *trans*-3,4-diols are believed to be on the metabolic pathways forming the most reactive, mutagenic and carcinogenic metabo-

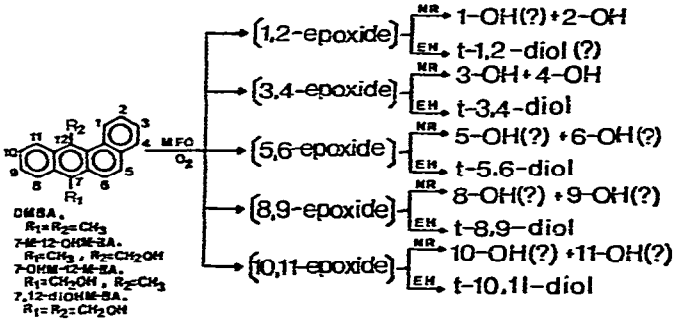


Fig. 10. Proposed pathways of metabolic oxidations of DMBA and its hydroxymethyl derivatives at the ring positions. Epoxide intermediates have not been directly detected.Question marks in parentheses indicate possible formation of a compound not found in this investigation. MFO = Mixed-function oxidases; EH = epoxide hydra(ta)se; NR = non-enzymatic rearrangement; t = *trans*. Further metabolism of the phenolic and dihydrodiol metabolites at other ring positions is not indicated.

lites. According to the "bay region" theory[36], formation of 1,2-epoxides from the *trans*-3,4-diols can lead to the formation of C_1 carbonium ion intermediates which are thought to be the ultimate carcinogenic metabolites of DMBA. Although direct evidence on the metabolism of *trans*-3,4-diols to the 3,4-diol-1,2-epoxides is not available, indirect evidence has been reported suggesting that 3,4-diol-1,2-epoxide(s) is involved in binding to DNA of cells in culture[37,38], to DNA of mouse skin that has been treated with DMBA[39] and to DNA in a rat liver microsomes-mediated *in vitro* system[40]. The mutagenicity of three of the four *trans*-3,4-diols has been tested in a cell-mediated mutagenicity assay with Chinese hamster V79 cells, and only DMBA-*trans*-3,4-diol was found to be more mutagenic than DMBA itself[41]. The mutagenicity of 7-OHM-12-MBA-*trans*-3,4-diol and 7-M-12-OHMBA-*trans*-3,4-diol was found to be less than that of DMBA but more than those of 7-OHM-12-MBA and 7-M-12-OHMBA respectively[41]. In an *in vitro* system, all three of the above mentioned *trans*-3,4-diols produced products that bound more extensively than DMBA upon further metabolism with rat liver microsomes[12]. These results thus indicate that there may be multiple activation pathways in the formation of ultimate carcinogenic metabolites of DMBA.

An important subject which has not been explored in detail is the sequence of ring hydroxylation and methyl hydroxylation. For example, 7-OHM-12-MBA-*trans*-3,4-diol is known to be formed from DMBA (Fig. 1A). The 7-OHM-12-MBA-*trans*-3,4-diol may be derived enzymatically either from 7-OHM-12-MBA by epoxidation and hydration reactions or from DMBA-*trans*-3,4-diol by hydroxylation at the 7-methyl group. The former pathway is known to occur (Fig. 1B) but the latter pathway has not been investigated. If the DMBA dihydrodiols can be hydroxylated at the methyl groups to form dihydrodiols of 7-M-12-OHMBA, 7-OHM-12-MBA and 7,12-diOHMBA, then the number of possible pathways is increased severalfold more than what has been proposed in Figs. 9 and 10. Investigation of this subject is now underway in our laboratory*.

The application of both reversed-phase and normal-phase HPLC has made possible the purification of more than thirty DMBA metabolites. We have obtained milligram quantities of many highly purified metabolites for nuclear magnetic resonance spectral analysis[13], optical rotation measurement (Table II), UV absorption and fluorescence spectral analysis (Figs. 3–8) and for mass spectral analysis (Table I). Definitive structural assignments for the metabolites can thus be made on the basis of the above physicochemical properties. Metabolites purified by HPLC procedures described in this report have been used in mutagenicity[41] and carcinogenicity testings. Thus further studies with the purified metabolites can provide information concerning the metabolic pathways, activation and detoxification mechanisms of the potent carcinogen, 7,12-dimethylbenz[*a*]anthracene. The HPLC methods described in this report have been found in our laboratory to be useful in general for the purification and identification of several other PAH metabolic products.

---

* *Note added in proof*: We have found that DMBA-*trans*-3.4-diol can be metabolized by rat liver microsomes to form 7-M-12-OHMBA-*trans*-5,4-diol, 7-OHM-12-MBA-*trans*-3,4-diol, and 7,12-diOHMBA-*trans*-3,4-diol. Both 7-M-12-OHMBA-*trans*-3,4-diol and 7-OHM-12-MBA-*trans*-3,4-diol can be further metabolized to 7,12-di OHMBA-*trans*-3,4-diol.

ACKNOWLEDGEMENT

We thank George Easton and Henri Weems for technical assistance.

REFERENCES

1 E. C. Miller, *Cancer Res.*, 38 (1978) 1479; and references cited therein.
2 C. Heidelberger, *Annu. Rev. Biochem.*, 44 (1975) 79; and references cited therein.
3 P. Sims and P. L. Grover, *Advan. Cancer Res.*, 20 (1974) 165; and references cited therein.
4 E. Boyland and P. Sims, *Biochem. J.*, 95 (1965) 780.
5 P. Sims, *Biochem. Pharmacol.*, 19 (1970) 795.
6 P. Sims, *Biochem. Pharmacol.*, 19 (1970) 2261.
7 P. Sims, *Biochem. J.*, 131 (1973) 405.
8 E. Boyland and P. Sims, *Biochem. J.*, 104 (1967) 394.
9 A. Gentil, C. Lasne and I. Chouroulinkov, *Xenobiotica*, 4 (1974) 537.
10 C. Chen and M.-H. Tu, *Biochem. J.*, 160 (1976) 805.
11 S. K. Yang and W. V. Dower, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 2601.
12 M. W. Chou and S. K. Yang, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 5466.
13 S. K. Yang, M. W. Chou and P. P. Roller, *J. Amer. Chem. Soc.*, 101 (1979) 237.
14 B. Tierney, A. Hewer, A. D. MacNicoll, P. G. Gervasi, H. Rattle, C. Walsh, P. L. Grover and P. Sims, *Chem. Biol. Interac.*, 23 (1978) 243.
15 J. W. Flesher, S. Soedigdo and D. R. Kelley, *J. Med. Chem.*, 10 (1967) 932.
16 C. E. Morreal, V. Alks and A. J. Spiess, *Biochem. Pharmacol.*, 25 (1976) 1927.
17 J. K. Selkirk, R. G. Croy and H. V. Gelboin, *Science*, 184 (1974) 169.
18 G. Holder, H. Yagi, P. Dansette, D. M. Jerina, W. Levin, A. Y. H. Lu and A. H. Conney, *Proc. Nat. Acad. Sci. U.S.*, 72 (1974) 4356.
19 E. Huberman, L. Sachs, S. K. Yang and H. V. Gelboin, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 607.
20 S. K. Yang, D. W. McCourt, P. P. Roller and H. V. Gelboin, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 2594.
21 S. K. Yang, D.W. McCourt, H.V. Gelboin, J. R. Miller and P. P. Roller, *J. Amer. Chem. Soc.*, 99 (1977) 5124.
22 S. K. Yang, P. P. Roller and H. V. Gelboin, *Biochemistry*, 16 (1977) 3680.
23 D. R. Thakker, H. Yagi, H. Akagi, M. Koreeda, A. Y. H. Lu, W. Levin, A. W. Wood, A. H. Conney and D. M. Jerina, *Chem. Biol. Interac.*, 16 (1977) 281.
24 D. R. Thakker, W. Levin, A. W. Wood, A. H. Conney, T. A. Stoming and D. M. Jerina, *J. Amer. Chem. Soc.*, 100 (1978) 645.
25 S. S. Hecht, E. LaVoie, R. Mezzarese, S. Amin, V. Bodenko and D. Hoffmann, *Cancer Res.*, 38 (1978) 2191.
26 M.W. Chou, G. D. Easton and S. K. Yang, *Biochem. Biophys. Res. Commun.*, 88 (1979) 1085.
27 P. P. Fu and S. K. Yang, *J. Labelled Compd. Radiopharm.*, (1979) in press.
28 C. E. Morreal and V. Alks, *J. Chem. Eng. Data*, 22 (1977) 118.
29 J. Pataki, R. Wlos and Y.-J. Cho, *J. Med. Chem.*, 11 (1968) 1083.
30 G. M. Badger and J. W. Cook, *J. Chem. Soc., London*, (1940) 409.
31 N. Kinoshita, B. Shears and H. V. Gelboin, *Cancer Res.*, 33 (1973) 1937.
32 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
33 S. K. Yang, D. W. McCourt, J. C. Leutz and H. V. Gelboin, *Science*, 196 (1977) 1199.
34 D. M. Jerina and J. W. Daly, *Science*, 185 (1974) 573.
35 J. DiGiovanni, T. J. Slaga, A. Viaje, D. L. Berry, R. G. Harvey and M. R. Juchau, *J. Nat. Cancer Inst.*, 61 (1978) 135.
36 D. M. Jerina and R. E. Lehr, in V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney (Editors), *Microsomes and Drug Oxidations*, Pergamon Press, Oxford, 1977, p. 709.
37 R. C. Moschel, W. M. Baird and A. Dipple, *Biochem. Biophys. Res. Commun.*, 76 (1977) 1092.
38 A. Dipple, J. E. Tomaszewski, R. C. Moschel, C. A. H. Bigger, J. A. Nebzydoski and M. Egan, *Cancer Res.*, 39 (1979) 1154.
39 P. Vigny, M. Duquesne, H. Coulomb, B. Tierney, P. L. Grover and P. Sims, *FEBS Lett.*, 82 (1977) 278.
40 V. Ivanovic, N. E. Geacintov, A. M. Jeffrey, P. P. Fu, R. G. Harvey and I. B. Weinstein, *Cancer Lett.*, 4 (1978) 131.
41 E. Huberman, M. W. Chou and S. K. Yang, *Proc. Nat. Acad. Sci., U.S.*, 76 (1979) 862.