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MICROSOMAL HYDROXYLATION OF 3-METHYLCHOLANTHRENE: ANALYSIS BY COMPUTERIZED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Microsomal metabolism of 3-methylcholanthrene (MC) was examined by computerized gas chromatography–mass spectrometry. Five mono-, four di- and thirteen trihydroxylated metabolites were found after incubation of MC in mouse liver microsomal fraction for 15 min, in the presence of NADPH. Among these metabolites, three mono- and three dihydroxylated metabolites were identified by means of authentic samples. The chemical structures of the other metabolites were deduced from their characteristic mass spectral fragmentations. This is the first description of trihydroxylated metabolites in MC metabolism *in vitro* and *in vivo*.

INTRODUCTION

Arene oxide has been proposed as an intermediate in the microsomal hydroxylation of carcinogenic arene hydrocarbons[1–3]. It is formed by the NADPH-dependent microsomal monoxygenase system and is rearranged to a phenol or dihydrodiol, the latter being mediated by microsomal epoxide hydrase[4]. These metabolites have been considered to be intermediates in the formation of water-soluble conjugates for excretion[5]. Recent interest in chemical carcinogenesis has been focused on the binding of carcinogens to macromolecular tissue components. In the case of 3-methylcholanthrene (MC), 9,10-dihydrodiols of MC or 1-hydroxy-MC have been proposed as potent intermediates in the binding with cellular DNA[6–8].

In the present study, MC metabolites in a reconstructed microsomal hydroxylation system were investigated thoroughly by a computerized gas chromatographic–mass spectrometric (GC–MS) system to detect all hydroxylated metabolites mediated by the reconstructed microsomal system devoid of conjugating agents. In the hydroxylation system, demonstration of the presence of the complete range of MC metabolites is possible.

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EXPERIMENTAL

Chemicals

MC was obtained from Sigma (St. Louis, MO, U.S.A.), and most of its derivatives were prepared in our laboratory[9] by the methods of Sims[10]. Benz[*a*]anthracene (BA), dibenz[*a,h*]anthracene (DBA), β-naphthoflavone (BNF) and purified sterols such as cholesterol, sitosterol and tetrahydroergosterol were obtained from Nakarai Chemicals (Kyoto, Japan).

Microsomal fractions

Ten-week-old male C57BL/6 mice were chosen for the present experiments because they are known to exhibit high responses to arene hydrocarbon hydroxylase induction[11]. The mice were injected with 80 mg/kg of BNF in sesame oil and then fasted. Forty-eight hours later, the liver was removed and microsomal fractions were prepared by a slight modification of the method of Gielen *et al.*[12]. That is, tissues were minced in a buffered solution (0.15 *M* KCl–0.25 *M* potassium phosphate, pH 7.25), freed from contaminating blood corpuscles and homogenized with a PTFE pestled homogenizer. After removal of precipitates by centrifugation at 10,000 *g* for 15 min, microsomal fractions were obtained from the supernatants by centrifugation at 78,000 *g* for 90 min. The precipitates were washed several times in a buffered solution (30% glycerol–0.25 *M* sodium phosphate, pH 7.25) to obtain final pellets of the purified microsomal fraction.

Microsomal oxidation of MC and extraction of MC metabolites

According to the method of Nebert and Gelboin[13], the purified microsomal fraction (240 mg of protein) was suspended in 300 ml of phosphate buffer (50 m*M*, pH 7.2) containing 3 m*M* $MgCl_2$ with or without 0.36 m*M* NADPH. After the addition of 6.432 mg of MC (final concentration, 80 μ*M*) in acetone, the suspension was incubated under an atmosphere of 95% O_2 and 5% CO_2 at 37°C for 15 min, unless otherwise stated. The incubation was terminated by mixing the suspension with 300 ml of cold acetone and then 600 ml of benzene. The residue after evaporation of the solvent was put on a silica gel column and eluted first with benzene (Fl) and then with ethanol (F2).

GC–MS analysis

As described previously[14], the extracts after evaporation of the solvents were silylated in a mixture of pyridine–N,O-bis(trimethylsilyl)acetamide–trimethylchlorosilane (2:2:1, v/v/v) at 60°C for 15 min and examined with a comptuterized GC–MS equipment (Japan Electrics Optical Laboratory, JEOL, Tokyo, Japan). The latter consisted of a JGC-20K gas chromatograph, a JMS-D300 mass spectrometer and a JAM-2000 mass analysis system. The chromatograph was furnished with a 6% Dexsil 300 column (1 m × 2 mm) and operated at 290°C with helium as the carrier gas (flow-rate, 20 ml/min). The spectrometer was operated with an emission current of 300 μA and electron energy of 20 eV. The scan range was from *m/e* 200 to 550, unless otherwise specified. The distribution of substances was recorded by total ion monitoring (TIM) and they were analyzed by reconstructive ion chromatography (RIC) and single ion monitoring (SIM) for the characteristic ion currents.

Retention time

The solvent peak was not obtained in the GC–MS system, as it would have a deleterious effect on the electrostatic field in the ionization chamber. Therefore, the retention time of a compound was documented by its retention relative to a standard, DBA (RR = 1.00), added to the test solution immediately before GC–MS analysis.

RESULTS

Tissue sterols

Sterols originating from the microsomal fractions always contaminate the benzene extracts of MC metabolites. Cholesterol, tetrahydroergosterols and sitosterol are the most important contaminants, appearing in TIM chromatograms of F2 extracts as peaks 1, 3, 5 and 8 in Fig. 1. Table I gives mass spectral data of these sterols, which were all identified by GC–MS of authentic substances.

Monohydroxylated metabolites

Three authentic monohydroxylated MC derivatives, 1-, 2- and 11-hydroxy-

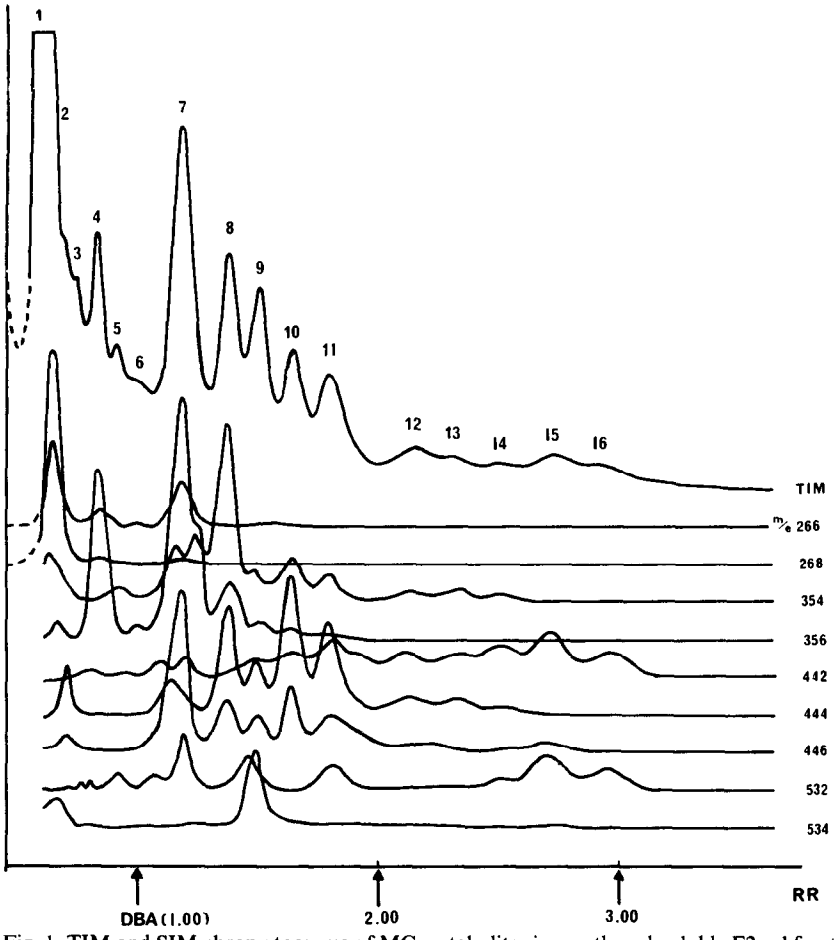


Fig. 1. TIM and SIM chromatograms of MC metabolites in an ethanol-soluble F2 subfraction of benzene extracts of a microsomal incubation mixture.

TABLE I

GC-MS ANALYSIS OF MC METABOLITES AND TISSUE STEROLS IN MICROSOMAL INCUBATION MIXTURES

MC was incubated with mouse liver microsomes in the presence of NADPH for 15 min and benzene extracts of the mixture were examined by computerized GC–MS. Peak numbers correspond to those in Fig. 1. Peaks a, b and c were observed in 5-min incubation mixture. The retention times are relative to that of DBA (RR = 1.00).

Peak No.	*Retention time (min:sec)*	*Relative retention*	*Molecular and fragment ions, m/e (%)*	*Assignment*
BA	1:46	0.19	228(100)	BA
a	3:34	0.38	444(100), 354(34), 266(38)	*trans*-1,2-Dihydroxy-MC
b	4:33	0.49	446(100), 356(69), 266(7)	*cis*-11,12-Dihydro-11,12-dihydroxy-MC
c		0.55	534(100), 444(12), 354(38)	Type 2 trihydroxy-MC
1	5:32	0.59	458(44), 443(12), 368(72), 353(44), 329(100)	Cholesterol
2	5:55	0.64	268(100), 253(15)	MC
		0.689	356(81), 266(100)	1-Hydroxy-MC
		0.75	444(100), 355(18), 266(2)	*cis*-1,2-Dihydroxy-MC
3	7:11	0.77	472(40), 457(18), 382(72), 367(46), 343(100)	Tetrahydroergosteral
4	7:43	0.83	356(100), 341(8), 266(46)	2-Hydroxy-MC
5	8:33	0.92	486(50), 471(8), 396(73), 381(31), 357(100)	Sitostereol
		0.92	532(56), 444(22), 354(100)	Type 1 trihydroxy-MC
6	9:18	1.00	278(100)	DBA
		1.00	356(100), 266(40)	11-Hydroxy-MC
		1.14	532(64), 444(83), 354(100)	Type 1 trihydroxy-MC
7	11:02	1.19	446(22), 356(88), 266(100)	MC *x*-dihydrodiol
		1.21	356(100), 341(12), 266(16)	*x*-Monohydroxy-MC (phenol A)
		1.36	356(100), 341(23), 266(39)	*x*-Monohydroxy-MC (phenol B)
8	12:50	1.38	534(2), 444(60), 429(2), 354(100)	Type 2 trihydroxy-MC
		1.38	472(100), 457(11), 382(27), 367(63)	Tetrahydroergosterol
9	13:43	1.51	534(100), 444(81), 429(23), 354(42)	Type 2 trihydroxy-MC
10	15:29	1.66	534(3), 444(100), 429(22), 354(42)	Type 2 trihydroxy-MC
11	16:50	1.81	534(6), 444(100), 429(24), 354(34)	Type 2 trihydroxy-MC
12	20:00	2.15	534(14), 444(100), 429(8), 354(34)	Type 2 trihydroxy-MC
13	21:39	2.33	534(11), 444(100), 354(57)	Type 2 trihydroxy-MC
14	23:28	2.52	534(11), 444(100), 354(78)	Type 2 trihydroxy-MC
		2.52	532(81), 442(100)	Type 3 trihydroxy-MC
15	25:21	2.73	532(100), 442(57)	Type 3 trihydroxy-MC
16	27:27	2.95	532(100), 442(64)	Type 3 trihydroxy-MC

MC, were examined by GC–MS after silylation. The RR was 0.68 for 1-hydroxy-MC, 0.83 for 2-hydroxy-MC and 1.00 for 11-hydroxy-MC. The mass spectra of these compounds showed the molecular ion at *m/e* 356 and two fragment ions at *m/e* 341 (loss of methyl group) and 266 (loss of trimethylsilanol, M − TMSOH). The fragment at *m/e* 266 indicates the induction of the 1,2-dehydrocompound which seems rather stable in emission spectra, and, therefore, is considered to be a marker of monohydroxylated MC derivatives in GC–MS. These three monohydroxylated derivatives were confirmed[14–16] in tissues or excreta after MC administration. In addition, two other compounds with RR = 1.21 and 1.31, respectively, were deduced to be monohydroxylated MC metabolites from their identical mass spectra to those of 1-, 2- or 11-hydroxy-MC. They were documented as phenol A and phenol B[14].

In the present microsomal oxidation experiments, five monohydroxylated metabolites were found and confirmed to be identical with those found previously[14]. Fig. 2a shows a mass spectrum of peak 4 in Fig. 1. The intense peaks at *m/e* 356, 341 and 266 indicate a monohydroxy-MC derivative which was identified as 2-hydroxy-MC from its RR = 0.83. The other monohydroxylated metabolites were identified in a similar manner.

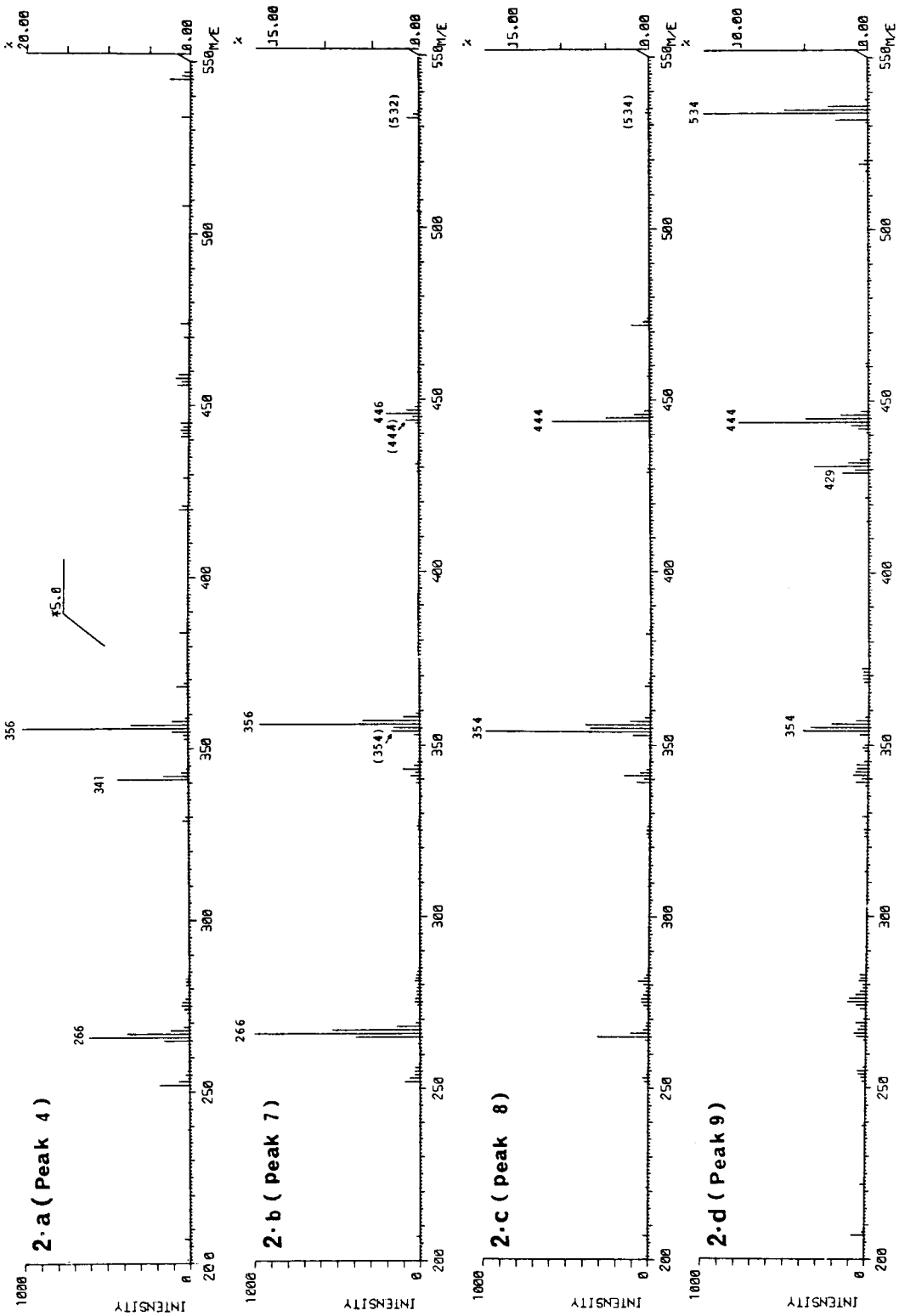
Dihydroxylated metabolites

Three dihydroxylated MC derivatives were used as standard chemicals in GC–MS. The molecular weights, after silylation, of these compounds were calculated to be 444 for *cis*- or *trans*-1,2-dihydroxy-MC and 446 for *cis*-11,12-dihydro-11,12-dihydroxy-MC (*cis*-11,12-dihydrodiol). Fragmentation of 1,2-dihydroxy-MC results in peaks at *m/e* 444 (M^+), 354 or 355 (M − TMSO) and 266 (M − 2 TMSO). In the fragmentation of *cis*-11,12-dihydrodiol, peaks are found at *m/e* 446 (M^+), 356 (M − TMSOH) and 266 (M − 2 TMSOH). The RRs of these dihydroxylated compounds were calculated to be 0.38 for *trans*-1,2-dihydroxy-MC, 0.49 for *cis*-11,12-dihydrodiol and 0.75 for *cis*-1,2-dihydroxy-MC. In a previous experiment the occurrence of *cis*-1,2-dihydroxy-MC was confirmed in extracts of rat and mouse lungs after MC administration. The dihydroxy compound was also found in rat faeces. Two other dihydroxyl metabolites which might be *trans*-1,2-dihydroxy-MC and *cis*-11,12-dihydrodiol were also detected.

In the present microsomal hydroxylation system, *cis*-1,2-dihydroxy-MC was a constant member of MC metabolites and *trans*-1,2-dihydroxy-MC and *cis*-11,12-dihydrodiol were found in mixtures incubated for 5 min but not in those incubated for 15 min. The RRs of these dihydroxylated metabolites were 0.75, 0.38 and 0.49, respectively. Peak 7 (RR = 1.19) is the most intense peak in the TIM chromatograms of mixtures incubated for 15 min (Fig. 1). The mass spectrum of this peak shows three distinct ion peaks at *m/e* 446 (M^+), 356 (M − TMSOH) and 266 (M − 2 TMSOH). This indicates the presence of a dihydrodiol other than those described above. The peak was contaminated with a preceding trihydroxyl-MC and succeeding phenols. The marker fragment also appears at *m/e* 266 in the fragmentation of dihydroxylated MC derivatives.

Trihydroxylated metabolites

On the basis of the GC–MS analyses of mono- and dihydroxylated MC derivatives, trihydroxylated derivatives were classified into three types. Type 1 includes


2·a (Peak 4)
2·b (Peak 7)
2·c (peak 8)
2·d (Peak 9)
INTENSITY
M/E
356
341
266
(354)
(444)
446
(532)
(534)
444
429
534
354

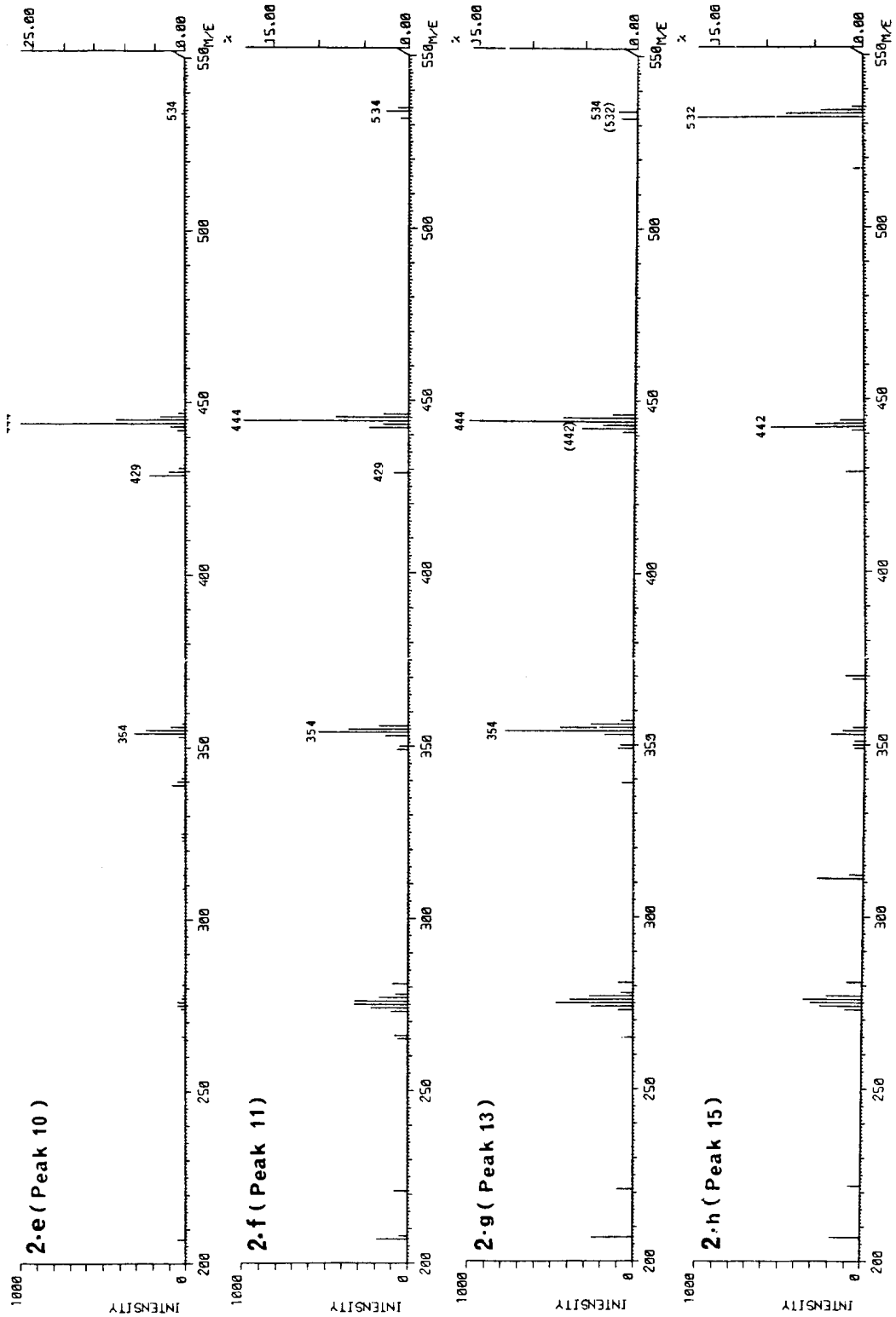


Fig. 2. Mass spectra of several peaks in Fig. 1. Each chromatogram was reduced to one fifth of the actual size.

MC derivatives hydroxylated at 1, 2 and another position of the cholanthrene skeleton. They can be described as 3-methyl-1,2,*x*-trihydroxycholanthrene (1,2,*x*-trihydroxy-MC). A molecular ion at *m/e* 532 (M^+) and two fragment ions at *m/e* 443 (or 442) (M − TMSO) and 354 (M − 2 TMSO) are expected in the mass spectra of these compounds. The ion at *m/e* 354 should represent 1,2-dehydro-*x*-hydroxy-MC which is stable in the emission spectra. Type 2 includes 4,5-, 7,8-, 9,10- or 11,12-dihydrodiol derivatives of *x*-hydroxy-MC. The molecular weight of these compounds is calculated to be 534, and ion peaks in the mass spectrum appear at *m/e* 534 (M^+), 444 (M − TMSOH) and 354 (M − 2 TMSOH). The peak at *m/e* 354 indicates the occurrence of a dehydro derivative of monohydroxylated MC. Type 3 includes trihydroxylated derivatives of MC other than those in types 1 and 2. They are hydroxylated at the 3 positions of the cholanthrene skeleton, avoiding adjacent parallel positions. The molecular weight after silylation is 532, and the mass spectrum show only two peaks at *m/e* 532 (M^+) and 442 (M − TMSOH). The fragment at *m/e* 442 is due to a dehydro compound. Two other hydroxy radicals remain on the dehydrocholanthrene skeleton, but further removal of TMSOH cannot occur due to the lack of transferable hydrogen ions. In the mass spectra of trihydroxylated MC derivatives, marker fragments occur at *m/e* 354 for type 1 and type 2 trihydroxy compounds and at *m/e* 442 for type 3 compounds.

In a previous paper[14] three trihydroxylated metabolites of MC were suggested. Their RRs on Dexsil 300 were 0.92, 1.19 and 1.40. The first compound was found in the lung, the second in the lung, bile and faeces and the last in faeces. In the present microsomal oxidation experiments, the first two of these compounds were confirmed to be type 1 trihydroxylated metabolites with RR = 0.92 and 1.14, and the third to be a type 2 compound with RR = 1.38. In addition, seven new type 2 trihydroxylated metabolites and three type 3 compounds were found in GC–MS analyses of the F2 fraction of the incubation mixture of MC with microsomal fraction supplemented with NADPH. Their chromatographic migrations are shown in Fig. 1 and some of their mass spectra in Fig. 2. Demethylation on the way to degradation of the compounds was observed for type 2 trihydroxy-MC derivatives, their RRs being between 1.38 (peak 8) and 2.15 (peak 12). The other type 2 compounds with RR = 2.33 (peak 13) and 2.52 (peak 14) exhibited no fragment suggestive of demethylation. Thus, fragmentations of some type 2 compounds seemed to follow the fragmentation of monohydroxylated MC derivatives with irregular demethylation under the conditions used. Type 3 compounds were easily identified by their simple and distinct markers.

Tetra- or further hydroxylated metabolites

Molecular weights, after silylation, of tetrahydroxy-MCs are 624, 622 and 620 for compounds with two dihydrodiols, with one dihydrodiol and free of dihydrodial, respectively. According to the fragmentation of mono- and dihydroxy-MC, the mass spectra of tetrahydroxy-MC compounds should reveal peaks at *m/e* 624 (M^+), 534 (M − TMSOH), 444 (M − 2 TMSOH) and 354 (M − 3 TMSOH), at *m/e* 622 (M^+), 532 (M − TMSOH) and 442 (M − 2 TMSOH), at *m/e* 620 (M^+), 531 (M − TMSO) and 442 (M − 2 TMSO), or at *m/e* 620 (M^+) and 530 (M − TMSOH). A peak at *m/e* 354, 442 or 530 indicates the formation of a 1,2-dehydro compound in the fragmentation, and the stable compounds become marker fragments for the tetrahydroxylated MC derivatives.

Calculated molecular weights of pentahydroxylated metabolites after silylation are 712, 710 or 708 and their marker fragments in fragmentation must occur at *m/e* 442, 530 or 618.

In GC–MS analyses of the microsomal incubation mixtures, no evidence for the presence of tetra- or further hydroxylated metabolites was found, even when the mass scanning range was extended to *m/e* 800.

Metabolism in NADPH-free microsomal fraction

When the microsomal oxidation system was not supplemented with NADPH, only small amounts of 1- and 2-hydroxy-MC were detected by SIM analysis, in addition to the tissue sterols found in the control experiments using microsomal incubation mixtures without the addition of MC. Some ketones were found in the mixture, but they were considered to be due to chemical oxidation during the process of identification.

DISCUSSION

The metabolism of arene hydrocarbons has been studied in our laboratory by fluorometric[15], macroautoradiagraphic[16] and radiometric[17] methods. Chromatography is one of the most powerful tools in analytical chemistry, and recent progress in HPLC has allowed demonstration of the presence of mono- and dihydroxy metabolites of arene hydrocarbons in microsomal incubation mixtures[18]. In our laboratory, GC–MS was introduced to study MC metabolism, and preliminary experiments on the identification of MC derivatives were carried out[9,19]. Since then, silylation of test substances and a computer system have been added to the method, and we have tried to identify MC metabolites in tissues or excreta of animals after MC administration[14]. Five mono- and three dihydroxy metabolites were detected, and three trihydroxy compounds were probably also present.

In the present *in vitro* microsomal hydroxylation experiments, five mono-, four di- and thirteen trihydroxylated MC metabolites were identified. Among these, five mono-, three di- and three trihydroxy compounds had already been found or suggested in *in vivo* metabolism experiments. Fragmentation profiles showed that the trihydroxy metabolites could be classified into three types: two type 1 compounds (1,2,*x*-trihydroxy-MC), eight type 2 compounds (*x*-hydroxy-MC dihydrodiol) and three type 3 compounds (trihydroxy-MC without dihydrodiol). We have no authentic samples of trihydroxylated MC metabolites, but their chemical structure can be presumed when the substrate and its metabolites are characteristic and their mass spectra are readily interpretable. The demonstration of such a large number of trihydroxy metabolites may be due to the accumulation of intermediates in the formation of conjugated metabolites for carcinogenesis and excretion. Unlike the cellular oxidation system, the reconstructed system is known to be devoid of conjugated agents.

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