



The metabolism of formyl-substituted benzanthracenes to hydroxymethyl metabolites in rat liver in vitro and in vivo

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Abstract

Hydroxylation of benzylic methyl carbon atoms on drugs and carcinogenic polycyclic aromatic hydrocarbons (PAHs) forms benzylic alcohols. Many carcinogenic and mutagenic PAHs bear a primary or secondary benzylic hydroxyl group attached to the meso-region of the molecule. According to the unified theory, PAHs bearing a benzylic hydroxyl group are proximate carcinogenic metabolites. This paper demonstrates that carcinogenic benz[a]anthracenes bearing a formyl group at the meso-region undergo enzymatic reductive metabolism to the corresponding carcinogenic benzylic alcohol in vitro and in vivo. The unified theory would then predict sulfuric acid esterification of such benzylic alcohols as the final common step in their metabolic activation to generate ultimate electrophilic benzylic carbocations. Finally, oxidative metabolism of 7-formylbenz[a]anthracenes gives rise to corresponding carboxylic acids and other oxygenated metabolites that are carcinogenically inert. Thus, oxidative metabolism of meso-region formyl compounds represents an avenue for the elimination of the carcinogen in a detoxified form.

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; 7-FMBA, 7-formyl-12-methylbenz[a]anthracene; 7-FBA, 7-formylbenz[a]anthracene; 7-HBA, 7-hydroxymethylbenz[a]anthracene; 7-HMBA, 7-hydroxymethyl-12-methylbenz[a]anthracene; BA-7-COOH, benz[a]anthracene-7-carboxylic acid; 12-MBA-7-COOH, 12-methylbenz[a]anthracene-7-carboxylic acid; NADPH, nicotinamide adenine dinucleotide phosphate; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; i.p., intraperitoneal; TIC, total ion chromatograph; DMF, *N,N*-dimethylformamide; BA-7,12-dione, benz[a]anthracene-7,12-dione; 7,12-DHMBA, 7,12-dihydroxymethylbenz[a]anthracene.

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1. Introduction

Most, if not all, moderate and strongly carcinogenic polycyclic aromatic hydrocarbons (PAHs) are known to be metabolized to carcinogenic meso-region hydroxymethyl metabolites [1–15]. These types of metabolites have traditionally not been considered important in the carcinogenic activity of the parent unsubstituted and methyl-substituted hydrocarbons because they often did not appear to be more carcinogenic than the parent hydrocarbons [4,16–19]. However, an ever increasing body of evidence indicates that these types of benzylic alcohols are activated by cytosol sulfotransferase to form highly carcinogenic electrophilic metabolites capable of covalently binding cellular nucleophiles [20]. The unified theory of PAH carcinogenesis, originally formulated by Flesher and Sydnor in 1971 [4,16], views these less carcinogenic hydroxymethyl metabolites as important proximate intermediates in the activation of PAH to highly reactive esters and/or halides as ultimate carcinogens (Fig. 1). The lessened biological activity of the more polar hydroxymethyl metabolites is explained, according to this theory, by their decreased ability to penetrate cellular membranes [16]. This explanation is supported by numerous studies, which indicate that various less polar derivatives of the

alcohols, such as acetate derivatives, possess activity rivaling that of the parent hydrocarbons [16,21].

Alternative theories of activation of hydrocarbons such as DMBA include bay-region dihydrodiol-epoxidation and formation of radical cations via metabolic one-electron oxidation [22,23]. The bay-region theory of PAH carcinogenesis predicts that PAH possessing an unsubstituted angular benzo ring will undergo activation to ultimate electrophilic forms through the formation of an M-region dihydrodiol. Activation by biological one-electron oxidation predicts that PAH possessing low ionization potentials will undergo sequential one-electron abstractions and hydrogen losses to form radical cations as the ultimate carcinogenic forms. Specifically, for DMBA these theories predict epoxidation on the 1,2-positions of the M-region *trans*-3,4-dihydrodiol, or a radical cation involving the 12-methyl position, respectively. It is difficult to see how either of these theories could explain the carcinogenicity of PAH substituted with electron-withdrawing groups.

Formyl hydrocarbons constitute an interesting class of oxygenated PAH derivatives. These types of derivatives such as 6-formylbenzo[*a*]pyrene, 7-formyl-12-methylbenz[*a*]anthracene (7-FMBA), 7-formylbenz[*a*]anthracene (7-FBA) and the ketone 1-keto-3-methylcholanthrene are moderate to

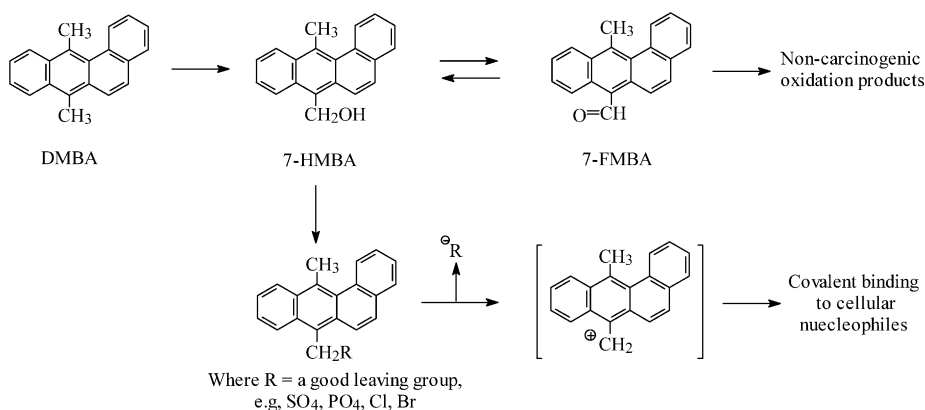


Fig. 1. The unified theory of Flesher and Sydnor [16] states that meso-region, methyl-substituted PAHs are carcinogenic owing to their further metabolism to proximate hydroxymethyl metabolites with subsequent esterification and/or halogenation to form reactive esters and/or halides as ultimate carcinogenic forms. Unsubstituted PAHs undergo meso-region biomethylation as a first step in this chain of substitution reactions [4]. Carcinogenic meso-region formyl derivatives could enter into this pathway of activation if it could be shown that they are readily reduced to the corresponding benzylic alcohol *in vivo*.

strongly active carcinogens, and represent instances in which substitution of an electron-withdrawing group, directly onto a PAH ring, results in pronounced carcinogenic properties [16–18,24]. Although the activity of these aldehydes has not been adequately addressed by any particular theory to date, direct bay-region diol-epoxide activation of strongly carcinogenic formyl hydrocarbons, such as 7-FMBA, seems unlikely owing to the presence of an electron-withdrawing group on the ring system [25]. In addition, one-electron oxidation to radical cations as ultimate carcinogenic forms has been excluded for oxygen-containing PAH derivatives by the model for metabolic activation formulated by Cavalieri et al. [26]. However, these carcinogenic formyl hydrocarbons could be readily incorporated into the unified theory of PAH activation if it could be shown that formyl hydrocarbons can be reduced to hydroxymethyl metabolites *in vitro* and *in vivo*. Thus, aralkylating ester activation, as predicted by the unified theory, might account for the carcinogenicity of formylated compounds, since reduction of carbonyl functional groups has been shown to occur for 1-keto-3-methylcholanthrene in rat liver preparations and for 7-FBA in yeast metabolizing systems ([2,3] and reviewed in [27]). To further develop this proposition, studies were undertaken to test whether carcinogenic formyl hydrocarbons are reduced in biological systems to benzylic hydroxymethyl metabolites. The moderately active 7-FBA and strongly active 7-FMBA aldehydes were chosen for investigations *in vitro*, while 7-FMBA was employed in *in vivo* studies.

2. Materials and methods

2.1. Chemicals

7-FMBA, 7-FBA, 7-hydroxymethylbenz[a]anthracene (7-HBA), 7-hydroxymethyl-12-methylbenz[a]anthracene (7-HMBA), benz[a]anthracene-7-carboxylic acid (BA-7-COOH), and 12-methylbenz[a]anthracene-7-carboxylic acid (12-MBA-7-COOH) were synthesized according to methods in the literature [28–33]. All synthesized compounds were analyzed for purity by HPLC and

GC/MS, prior to use as standards or test compounds. All solvents, glucose-6-phosphate and β -NADPH tetrasodium salt were purchased from Sigma-Aldrich Chemicals. *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)+1% trimethylchlorosilane (TMCS) was purchased from Pierce Chemicals.

2.2. S-9 rat liver metabolism

Following the method of Sims [3], the livers of four Sprague–Dawley male rats (age: 50 days, b.w.: 180 g) were excised, washed, minced and homogenized in 200 ml ice-cold 1.15% KCl (w/v) with an Elvehjem-type homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 0 °C for 20 min at $1480 \times g_{av}$ in a Sorvall RC 5B refrigerated centrifuge with a SS-34 rotor. The S-9 supernatant, containing microsomal and cytosolic enzymes, was pooled and diluted with an equal volume of ice-cold 0.1 M phosphate buffer (pH 7.4). Nicotinamide (1.67 g), nicotinamide adenine dinucleotide phosphate (NADPH) (30 mg), and glucose-6-phosphate (250 mg) were added, and the mixture was heated slowly to 37 °C in a shaking water bath. A slow stream of air was continually bubbled through the reaction mixture as 5 mg of formyl hydrocarbon substrate, in a minimum volume of ethanol, was added and incubated for 1 h. The reaction mixture was extracted thrice with equal volumes of ethyl acetate. The ethyl acetate extractions were then pooled, washed with an equal volume of water, dried over sodium sulfate, and evaporated to dryness under reduced pressure. The residue was then analyzed for metabolites by preparative normal-phase thin-layer chromatography (TLC), and GC/MS. Two controls were conducted, one without substrate, to aid in identification of media-derived components, and a second with boiled homogenate incubated with 7-FBA, to identify non-enzymatic products.

2.3. Cytosolic enzyme preparations

Four-week-old female Sprague–Dawley rats (≈ 200 g b.w., Harlan Sprague–Dawley, Indianapolis, IN) were used for the preparation of liver cytosol. Animals were sacrificed by cervical dis-

location, and their livers quickly excised and rinsed with ice-cold 1.15% KCl. The liver preparations were continually kept on ice or at 0 °C until quick frozen with liquid nitrogen. The livers were individually minced and homogenized in 1.15% KCl to form a 10% (w/v) homogenate of the livers. The individual homogenates were fractionated by centrifugation at 11,000 rpm ($\approx 9000 \times g_{av}$) for 45 min on a Sorvall RC 5B refrigerated super-speed centrifuge equipped with an SS-34 rotor. The S-9 supernatant was collected and centrifuged at 32,000 rpm ($\approx 105,000 \times g_{av}$) for 1 h in a Beckman ultracentrifuge in a Ti 50.2 rotor. The cytosol was collected and quickly shell-frozen in 2 ml aliquots in small vials with liquid N₂. All cytosolic preparations were kept frozen at -80 °C until required for experimental incubations or protein determination. Protein determinations were carried out by the method of Lowry et al. [34] for each individual liver cytosol; protein concentrations were found to be in the range 0.155–0.425 mg/ml.

2.4. Reduction assays

Incubations ($n = 3$) were carried out in reaction vials, containing 1 ml cytosolic enzyme preparation (0.155–0.425 mg protein; subsequent concentrations represent value following dilution), 0.869 mM NADPH, 6.67 mM glucose-6-phosphate, 3.48 mM MgCl₂, and 0.926 μ mol 7-FMBA substrate in 0.125 ml ethanol (0.436 mM), diluted to 2.125 ml final volume with phosphate buffer (pH 7.4).

Reaction mixtures were incubated for 42 min in a shaking 37 °C water bath, and were quenched by the addition of an equal, 2.125 ml, volume of ethyl acetate. Reaction incubations were extracted an additional two times with ethyl acetate, and the organic layers combined, washed with an equal volume of water, and dried over sodium sulfate. The organic extract was then dried under reduced pressure and the residue brought up in 0.5 ml CH₂Cl₂ for analysis by GC/MS. Preliminary experiments were conducted which utilized NADH rather than NADPH as a reducing cofactor, and phosphate buffered at pH 6 rather than pH 7.4. Control experiments included 7-HMBA as a substrate ($n = 2$), omission of NADPH cofactor ($n = 2$), “time zero” (mixing of all standard

components, but with zero incubation time) ($n = 2$), “boiled cytosol” (boiling of cytosol for 10 min) ($n = 3$), and exclusion of cytosol ($n = 1$) incubations.

2.5. *In vivo* preparations

Four-week-old female Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) were injected intraperitoneally (i.p.) with 0.13 mmol 7-FMBA/kg b.w. in minimal DMSO. Animals were sacrificed by cervical dislocation 1 h after injection, and their livers quickly excised and rinsed with ice-cold 1.15% KCl. Throughout the following manipulations, the liver preparations were kept on ice. The livers were individually minced and homogenized in 1.15% KCl to form a 10% (w/v) homogenate of the liver. The individual homogenates were then treated immediately with 70% (v/v) acetone in water to precipitate proteins. The precipitated proteins were separated by low-speed centrifugation, and the individual supernatants collected as aqueous/acetone solutions. Acetone was evaporated from these aqueous solutions under reduced pressure, leaving aqueous solutions, which were extracted three times with equal volumes of ethyl acetate. The water-rinsed organic layers were collected, combined, and dried over sodium sulfate. The organic extracts were dried under reduced pressure to yield extraction residues, which were each dissolved into 1 ml CH₂Cl₂ for preparative reverse-phase TLC. Control experiments used rat livers from animals that had not been treated i.p. with 7-FMBA, and either remained untreated (untreated control), were treated with 7-FMBA after homogenation (homogenate time zero control), or were treated with 7-FMBA after acetone protein precipitation (acetone time zero control).

2.6. Preparative TLC

Preparative normal-phase TLC was conducted on silica gel plates with 19:1 benzene:ethanol development and UV (375 nm) visualization. Aliquots of S-9 metabolism reaction residues, dissolved in benzene, were applied as bands to the base line of TLC plates with reference stan-

dards adjacent. After development, UV-visualized bands were scraped from the plates, and individually extracted twice with ether. Ether extracts were then evaporated to dryness to generate S-9 metabolite residues, which were analyzed by GC/MS.

Preparative reverse-phase TLC of 800 μl aliquots from in vivo extraction samples were banded onto a Whatman KC-18 reverse-phase TLC plate, with reference 7-HMBA and 7-FMBA adjacent, and eluted with 19:1 benzene:ethanol. UV-visible (375 nm) and non-visible bands, which co-chromatographed with 7-HMBA reference sample were individually scraped, and extracted twice with ether. Ether extracts were dried with N_2 , and brought to 0.5 ml with methanol for reverse-phase HPLC and/or GC/MS analysis.

2.7. GC/MS analysis

GC/MS analysis was conducted on a Hewlett-Packard 5890 GC equipped with a HP-5MS column (30 $\mu\text{m} \times 0.25 \text{ mm i.d.} \times 0.25 \mu\text{m}$ film), and interfaced to a 5970 mass selective detector. Injected samples (1 μl) were volatilized at 250 $^\circ\text{C}$, carried at 1 ml/min by He gas to the GC oven which was temperature-programmed from 200 $^\circ\text{C}$ (held for 1 min) to 280 $^\circ\text{C}$ at 20 $^\circ\text{C}/\text{min}$, and kept at 280 $^\circ\text{C}$ for 18 min. Electron impact fragmentation was induced at 70 eV, generating mass spectra in the m/z 50–550 mass range.

PAH standards, reaction residues, and metabolite residues from the S-9 metabolism experiments were derivatized with 50 μl BSTFA+1% TMCS, at 75 $^\circ\text{C}$ for 30 min. Resulting total ion chromatographs (TICs) were then analyzed by single ion monitoring for particular PAH-derived molecular and fragment ions.

Aliquots (100 μl) of the cytosolic experimental and control samples were individually dried, combined with 5 μl *N,N*-dimethylformamide (DMF), and derivatized for 30–45 min with 100 μl BSTFA+1% TMCS at 75 $^\circ\text{C}$. GC/MS analysis was conducted in a manner as outlined for the S-9 metabolism samples except that data were collected by selected ion monitoring for m/z 270, 344, and 358 ions, corresponding to the molecular weights of derivatized 7-FMBA, 7-HMBA, and 12-MBA-7-COOH, respectively. Sample analyte

concentrations were estimated by a process of external standardization [35] according to which increasing concentrations of standards were injected, data collected according to the SIM protocol described above, and the areas of the respective quantitative ions measured on ion chromatograms and plotted as a function of concentration. The success of such external standardization is dependent upon the reproducibility of 1 μl injections by the 7673 Autoinjector, which was judged to be good owing to the linearity of the results. Typical slopes on a linear $y = mx + b$ plot were 7-FMBA ($m = 1.8 \times 10^{-9}$), TMS-derivatized 7-HMBA ($m = 1.6 \times 10^{-9}$) and TMS-derivatized 12-MBA-7-COOH ($m = 5.07 \times 10^{-8}$).

2.8. Reverse-phase HPLC

Reverse-phase HPLC analysis of in vivo extraction samples was conducted on a Waters HPLC system fitted with C-18 (5 μm , 300 \AA) column in series with a Waters 440 absorbance detector and a Waters 474 fluorometer, eluting at 0.8 ml/min with 80% (v/v) methanol in water. Ultraviolet absorbance was monitored at 254 nm, while fluorescence was monitored at 240 nm excitation and 361 nm emission. Chromatographic profiles were analyzed on an IBM computer with Waters Maxima 820 software.

3. Results

Studies were conducted to test the generality of the unified theory of PAH activation for carcinogenic formyl-PAH, by determining if the selected carcinogenic formyl hydrocarbons, 7-FBA and 7-FMBA, were reduced to the corresponding hydroxymethyl metabolites by incubation in male rat liver homogenate (S-9 fraction). TLC and GC/MS analyses of these fractions showed that the formyl-PAH underwent enzymatic reduction to hydroxymethyl metabolites and non-enzymatic oxidation to carboxylic acids as major products. The control without hydrocarbon substrate showed that endogenous media components, such as cholesterol and fatty acids, were present as expected.

TLC of products from 7-FBA incubated with male rat liver S-9 homogenate resulted in six separable UV–visible bands (see Table 1). GC/MS analyses of these bands eluted with ether showed that two bands were media components, with the four remaining bands identified as being PAH derived. In particular, GC/MS analysis of TLC band 2 residue, shown in Fig. 2, resulted in a pronounced peak demonstrative of 7-hydroxymethylBA formation ($R_t = 10.1$ min). Single ion monitoring of the total ion chromatogram of the reaction mixture residue for m/z 330, 434, and 508 molecular ions demonstrated that 7-FBA was converted to 7-hydroxymethylBA (m/z 330) but did not exclude that a dihydrodiol metabolite of 7-FBA (m/z 434) or of 7-HBA (m/z 508) was formed (see Fig. 3). Comparison of these observations with those resulting from 7-FBA incubation in boiled S-9 indicated that reduction to the alcohol was enzymatic and did not occur in the boiled control, but that oxidation to non-carcinogenic phenols, quinones, and carboxylic acids did occur, suggestive of non-enzymatic oxidation (see Fig. 4).

Rat liver S-9 metabolism of 7-FMBA resulted in seven separable UV–visible bands by TLC (Table 2). Analysis of prominent peaks in the total ion chromatograms from scraped TLC band products indicated the pronounced presence of 7-HMBA as the primary metabolic product in band 3 (see Fig. 5), 12-MBA-7-COOH as a non-enzymatic oxidation product in band 1, and various media components. Further inspection of the mass spectra by single ion monitoring for the typical BA-

derived fragmentation ions (m/z 226 and 202) or particular metabolites (m/z 448 and 522) revealed further, less populous species such as benz[a]anthracene-7,12-dione (BA-7,12-dione) and 7,12-dihydroxymethylbenz[a]anthracene (7,12-DHMBA), but no SIM or full scan mass spectral evidence was found for the occurrence of a formyl-dihydrodiol (m/z 448) or hydroxymethyl-dihydrodiol (m/z 522) metabolite (data not shown), but further work with proper standards is required to verify the lack of these potential metabolites, particularly in light of observations of others [36,37].

In attempts to maximize and quantitate the reduction of 7-FMBA to its hydroxymethyl metabolite, further experiments were conducted in female rat liver cytosols. Preliminary experiments to determine the appropriate reducing cofactor and pH demonstrated that the enzymatic reduction of 7-FMBA to 7-HMBA occurred more efficiently in incubations utilizing NADPH as a reducing cofactor rather than NADH (5.2% versus 2.6% conversion of 7-FMBA to 7-HMBA, respectively). Incubations to compare the system at acidic versus physiological pH, and containing an incomplete versus a complete (i.e. glucose-6-phosphate and magnesium chloride added) NADPH regenerating system demonstrated only slight variations in the efficiency of reducing 7-FMBA to 7-HMBA (4.7% versus 5.2% conversion for the pH comparison, and 5.2% versus 5.4% conversion for the NADPH study, respectively).

As demonstrated in Fig. 6, Table 3, containing an NADPH regenerating system, demonstrates

Table 1
GC/MS results from TLC band products of 7-FBA incubated in rat liver homogenate

Band number	R_f -value	GC/MS R_t (min)	Molecular and fragment ions	Identification
1	0.02	11.721	344, 329, 255, 226, 113, 73	BA-7-COOH
2	0.34	10.109	330, 241, 73	7-HBA
3	0.38	4.73	328, 313, 132, 129, 117, 75, 73	Media component
4	0.59	10.4	256, 228, 226, 113	7-FBA
5	0.78	7.3	228, 113	BA
6	0.81	14.6	459, 443, 368, 329, 129, 73	Media component
Standard		10.04	330, 241, 73	7-HBA
Standard		10.41	256, 228, 113	7-FBA
Standard		7.37	228, 113	BA
Standard		11.6	344, 329, 255, 226, 113, 73	BA-7-COOH

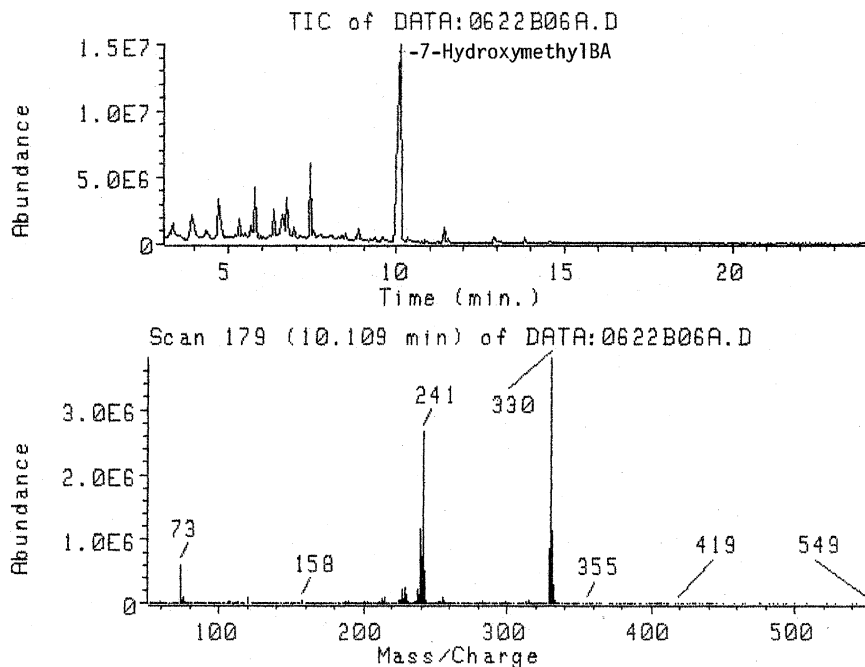


Fig. 2. GC/MS chromatogram of TLC band 2 products from the incubation of 7-FBA in rat liver homogenate. The total ion chromatogram (top) and spectrum (bottom) demonstrate the presence of 7-HBA (compare with reference 7-HBA in Fig. 4).

that at the physiological pH 7-FMBA is reduced to the benzylic alcohol 7-HMBA (average 0.08 ± 0.02 μmol 7-HMBA formed/mg cytosolic protein). This 7-HMBA metabolite co-chromatographed with a

known standard of 7-HMBA, matching the retention time of 14 min, and having the same ion fragmentation pattern. This conversion appears to be catalyzed by aldo-keto reductase, since no 7-

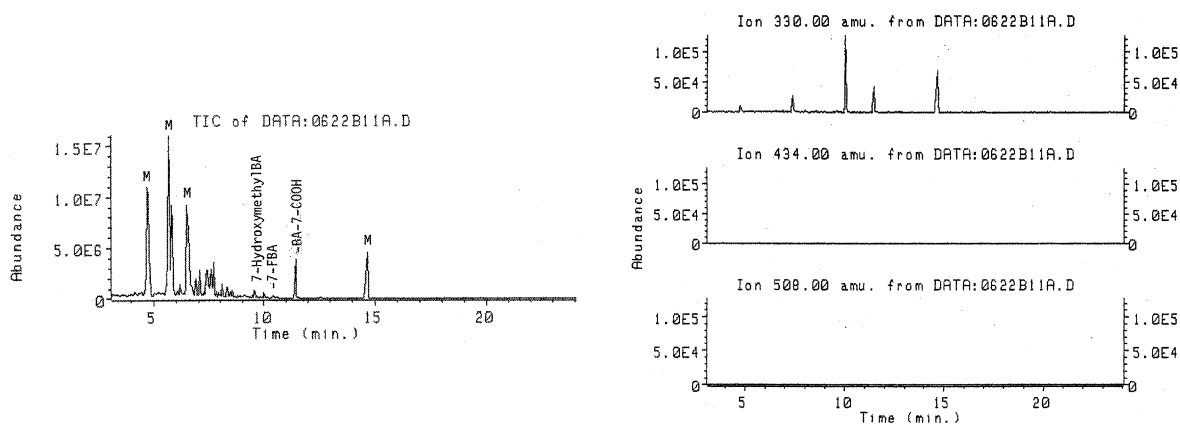


Fig. 3. GC/MS chromatogram of products from the incubation of 7-FBA in rat liver homogenate. GC chromatogram of reaction mixture residue shown on left, single ion monitoring for 330, 434 and 508 ions shown on right. These results indicate that 7-FBA is converted to 7-HBA and BA-7-COOH in liver homogenates (media components are labeled "M") and that no oxidation to 7-FBA-dihydrodiol (434 mw) or 7-HBA-dihydrodiol (508 mw) could be demonstrated.

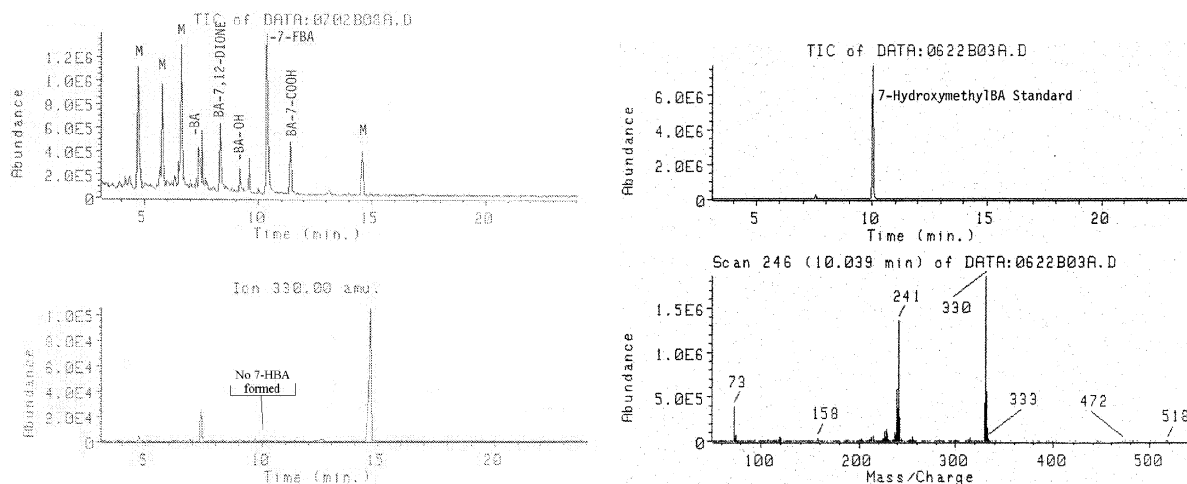


Fig. 4. GC/MS chromatogram of products resulting from the incubation of 7-FBA in boiled rat liver homogenate. The total ion chromatogram for the reaction mixture extract is given in top left. 7-FBA-derived species are noted including BA, BA-7,12-dione and BA-7-COOH, media components, labeled "M", are also seen. Single ion monitoring for the *m/z* 330 molecular ion of 7-hydroxymethylBA ($R_t = 10.04$ min), given in bottom left, demonstrates that no reduction of the formyl hydrocarbon occurred. The total ion chromatogram of reference 7-hydroxymethylBA is given in top right, with the corresponding mass spectrum shown in bottom right.

HMBA is formed from 7-FMBA in boiled cytosol controls, and in incubations without NADPH cofactor (see Fig. 7 and Table 4).

12-MBA-7-COOH was identified by comparison with a known standard. Formation of this non-carcinogenic carboxylic acid metabolite appears to be the result of non-enzymatic as well as enzymatic conversion of the formyl parent, since a low level of the carboxylic acid was identified in

control experiment incubations which had either contained boiled cytosol, or did not contain cytosol ($R_t = 17.2$ min). However, the apparent level of this metabolite was significantly increased in the enzymatic incubations in both the presence and absence of NADPH cofactor (Table 4).

Thus, it has been demonstrated in this study that 7-FMBA was enzymatically converted to its benzylic alcohol, 7-HMBA, in rat liver homoge-

Table 2

GC/MS results from TLC band products of 7-FMBA incubated in rat liver homogenate

Band number	R_f -value	GC/MS R_t (min)	Molecular and fragment ions	Identification
1	0.07	13.498	358, 343, 269, 239, 226, 73	12-MBA-7-COOH
2	0.03	6.8	378, 363, 261, 239, 163, 73	Media component
		12.584	432, 417, 343, 329, 253, 239, 73	7,12-DHMBA
3	0.17	11.259	344, 329, 255, 239, 228, 73	7-HMBA
4	0.22	5.8	356, 341, 132, 129, 117, 75, 73	Media component
5	0.33	4.8	328, 313, 132, 129, 117, 75, 73	Media component
6	0.62	12.34	270, 255, 239, 226	7-FMBA
		8.43	258, 230, 202	BA-7,12-dione
7	0.72	14.572	459, 443, 368, 329, 129, 73	Media component
Standard		13.372	358, 343, 269, 239, 226, 73	12-MBA-7-COOH
Standard		11.5	344, 329, 255, 239, 228, 73	7-HMBA
Standard		12.417	270, 255, 239, 226	7-FMBA
Standard		12.610	432, 343, 329, 253, 239, 73	7,12-DHMBA
Standard		8.4	258, 230, 202	BA-7,12-dione

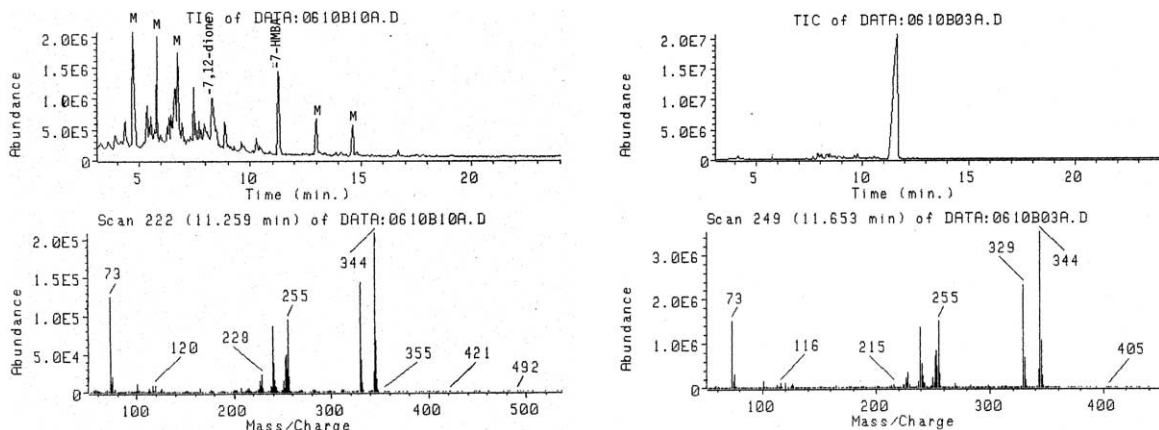


Fig. 5. GC/MS chromatogram of TLC band 3 from the incubation of 7-FMBA with rat liver homogenate. The TIC of the metabolites extracted from the scraped TLC band (3) which co-chromatographed with 7-HMBA reference compound is shown in top left. Prominent and lettered peaks represent 7-HMBA ($R_t = 11.3$ min, mass spectrum shown beneath), BA-7,12-dione ($R_t = 8.4$ min), or media components (labeled M). The TIC for a reference sample of 7-HMBA is given (right) along with corresponding mass spectrum (bottom, right).

nates and cytosols in vitro. The significance of this observation would be enhanced, if it could be shown that 7-HMBA is a metabolite of 7-FMBA

in whole animals. Since 7-HMBA is the metabolic product of particular interest in these studies, and since recovery of starting material and metabolites

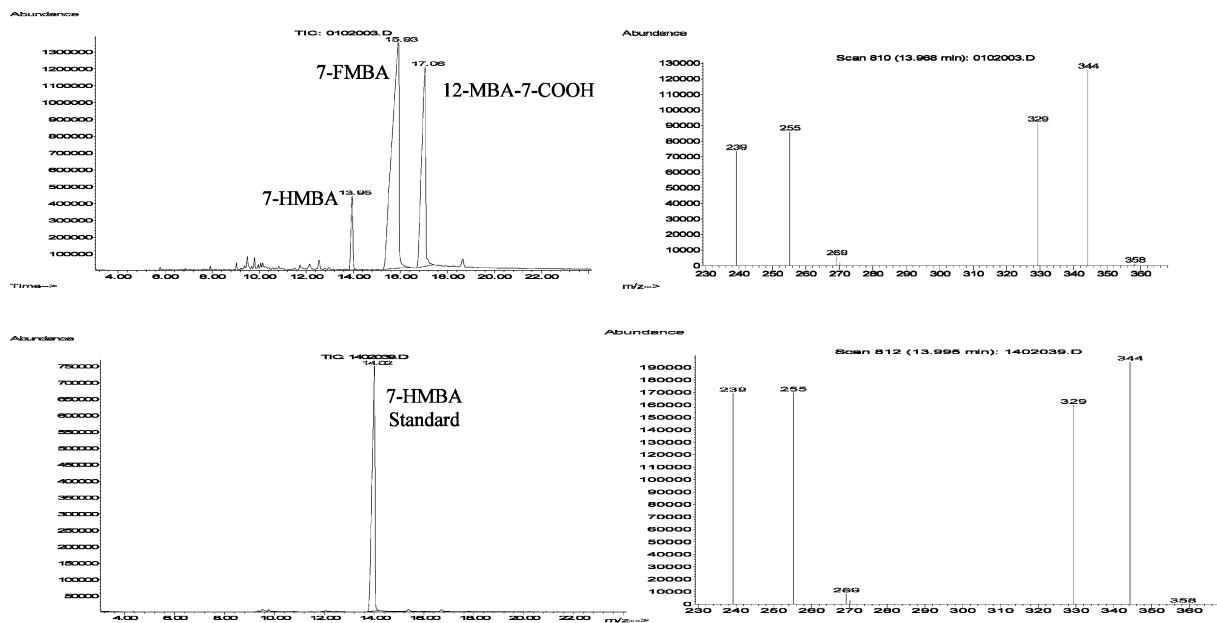


Fig. 6. Representative GC/MS chromatogram and spectra of cytosol incubations with 7-FMBA. A representative GC chromatogram of the reaction mixture ethyl acetate extract of 7-FMBA incubated in rat liver cytosol is given in top left. The three major peaks correspond to 7-HMBA ($R_t = 13.9$ min, mass spectrum shown in top right), 7-FMBA ($R_t = 15.9$ min), and 12-MBA-7-COOH ($R_t = 17.1$ min). 7-HMBA was identified by comparison to a reference standard of 7-HMBA which also had the same retention time and ion fragmentation pattern (bottom).

Table 3
Combined results of 7-FMBA experimental incubations in female rat liver cytosol

	7-FMBA (%)	12-MBA-7-COOH (%)	7-HMBA (%)
7-FMBA incubation-1	62.3	16.0	21.7
7-FMBA incubation-2	58.5	12.1	29.4
7-FMBA incubation-3	73.0	6.7	20.3
7-FMBA incubation-4	62.3	16.0	21.7
Average \pm standard deviation	67.4 \pm 7.3	11.3 \pm 3.3	21.3 \pm 5.6

was anticipated to be relatively low compared with the *in vitro* studies, preparative reverse-phase TLC was conducted. TLC of extracts from the livers of rats treated *i.p.* with 7-FMBA demonstrated that livers taken 24 and 16 h after administration had already cleared all starting material and resulting metabolites (results not shown). On the other hand, ethyl acetate extracts from livers ($n=3$) excised 1 h after 7-FMBA *i.p.* administration possessed a number of UV-visible TLC bands. Primary among these bands was one which fluoresced blue and co-chromatographed with 7-HMBA ($R_f=0.91$), and another which fluoresced yellow and co-chromatographed with adjacent 7-FMBA starting material ($R_f=0.95$). The blue fluorescing material ($R_f=0.81-0.94$) from individual liver extract TLC was scraped, extracted twice into ether, dried and brought to 0.5 ml in methanol. Reverse-phase HPLC analysis of these TLC band extracts demonstrated prominent peaks at $R_t=5.0$ min, matching the retention time of authentic synthesized 7-HMBA (Fig. 8). GC/MS analysis of these same TLC extracts confirmed the

presence of 344 mw species possessing the same R_t and molecular ion fragmentation pattern as authentic 7-HMBA (Fig. 9). Comparison of these results with those obtained from TLC work-up and HPLC analysis of *in vivo* control experiments demonstrated that reduction of 7-FMBA was occurring *in vivo*, and not as a consequence of 7-FMBA reduction during work-up (Fig. 8).

4. Discussion

The studies of rat liver homogenate metabolism demonstrated that both 7-FBA and 7-FMBA are enzymatically reduced to their corresponding hydroxymethyl metabolites, and non-enzymatically oxidized to carboxylic acids and 7,12-benzanthraquinone (BA-7,12-dione) *in vitro*. Neither the formyl compounds nor their hydroxymethyl metabolites appeared to be oxidized to dihydrodiols in this *in vitro* system, although further work is necessary to confirm this latter observation.

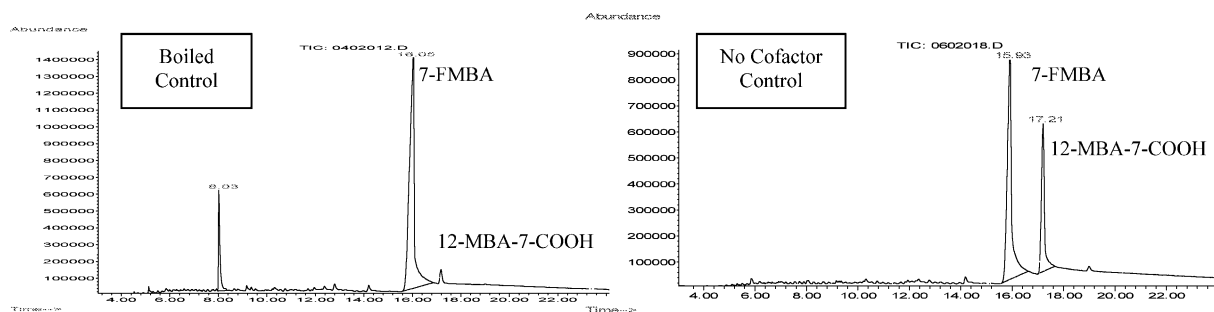


Fig. 7. GC chromatograms of control incubations containing either boiled cytosol (left) or no NADPH (right). Neither demonstrates a 13.9–14.0 min peak representative of 7-HMBA. Single ion monitoring of both controls did not reveal an m/z 334 molecular ion at 14 min, expected of 7-HMBA (chromatograms not shown).

Table 4
Results of control and experimental incubations in rat liver cytosol

	7-FMBA (%)	12-MBA-7-COOH (%)	7-HMBA (%)
7-FMBA incubations (<i>n</i> = 4)	67.4 ± 7.3	11.3 ± 3.3	21.3 ± 5.6
Zero time control (<i>n</i> = 2)	92.9 ± 0.5	5.5 ± 0.1	1.6 ± 0.6
No cofactor control (<i>n</i> = 2)	93.6 ± 2.1	6.4 ± 2.1	0.0 ± 0.0
Boiled control (<i>n</i> = 3)	97.7 ± 0.1	2.3 ± 0.1	0.0 ± 0.0
No cytosol control (<i>n</i> = 1)	99.5	0.5	0

The metabolism of 7-FMBA to 7-HMBA was further investigated in rat liver cytosols since carbonyl reductases, in particular the aldo-keto reductases, are known to be primarily localized within the cellular cytoplasm and possess a wide tissue distribution [38]. It was determined that reduction of 7-FMBA to 7-HMBA did indeed occur in the cytosolic fraction of female rat liver, with reduction occurring more readily in incubations which employed NADPH rather than NADH. Although no attempt was made to identify or characterize the cytosolic enzymes responsible, this particular observation implies that an aldo-keto reductase is probably responsible for the enzymatic reduction, since these enzymes preferentially utilize NADPH as a reducing co-factor [38]. In addition, quantitation of the 12-MBA-7-COOH product formed in cytosolic incubations of 7-FMBA suggested that the bulk of this product was formed enzymatically, rather than non-enzymatically, as determined in boiled and non-cytosolic controls. The identification of this polar derivative was not surprising since the oxidation of methyl groups to carboxylic acids is well documented for methylated aromatics [1,39,40]. Incidentally, this oxidation is at least partially NADPH-independent, since a relatively considerable amount of 7-FMBA was also converted to 12-MBA-7-COOH in incubations not containing NADPH.

Both the *in vitro* homogenate and cytosolic reduction experiments indicated that 7-HMBA was a major carcinogenic product formed from 7-FMBA in biological systems. The significance of this observation was extended to include whole animals, by qualitative studies, which demonstrated that 7-HMBA was an extractable product

from the liver of female Sprague–Dawley rats treated *i.p.* with 7-FMBA.

The electron-donating methyl groups of the strong carcinogen 7,12-dimethylbenz[*a*]anthracene are known to undergo chemical and biochemical oxidation [21,41]. The benzylic alcohols, which are formed as major products from the metabolism and oxidation of DMBA, are moderate to strongly active carcinogens and thus have been proposed to be intermediates in the activation of DMBA to its ultimate electrophilic and carcinogenic form (Fig. 1) [1,16,42,43]. The further oxidation of methyl and hydroxymethyl groups can also occur to form biologically active 7- and 12-formyl derivatives as has been demonstrated in both biological and chemical systems ([21,41,44] and unpublished work). Therefore, 7-FMBA is a strong chemical carcinogen representative of a class of oxygen-containing PAH derivatives, and possesses a metabolic relationship to the known carcinogens DMBA and its 7-HMBA metabolite.

As a class, only a few formyl-PAH derivatives have been synthesized and tested as complete carcinogens, namely 6-formylbenzo[*a*]pyrene, 7-FBA and 7-FMBA [16–18,24]. These derivatives have been demonstrated to be moderate to potent carcinogens, while other formylated PAH, such as 1-pyrene-carboxaldehyde, have only been classified as potent mutagens in the presence of biological activating systems [45]. Reduction by carbonyl reductases, ubiquitous within the cytosols of many mammalian tissues, has long been recognized as a metabolic route by which endogenous and exogenous carbonyl-containing compounds are transformed into more hydrophilic primary and secondary alcohols [46]. Thus, it seemed likely that a hydroxymethyl metabolite

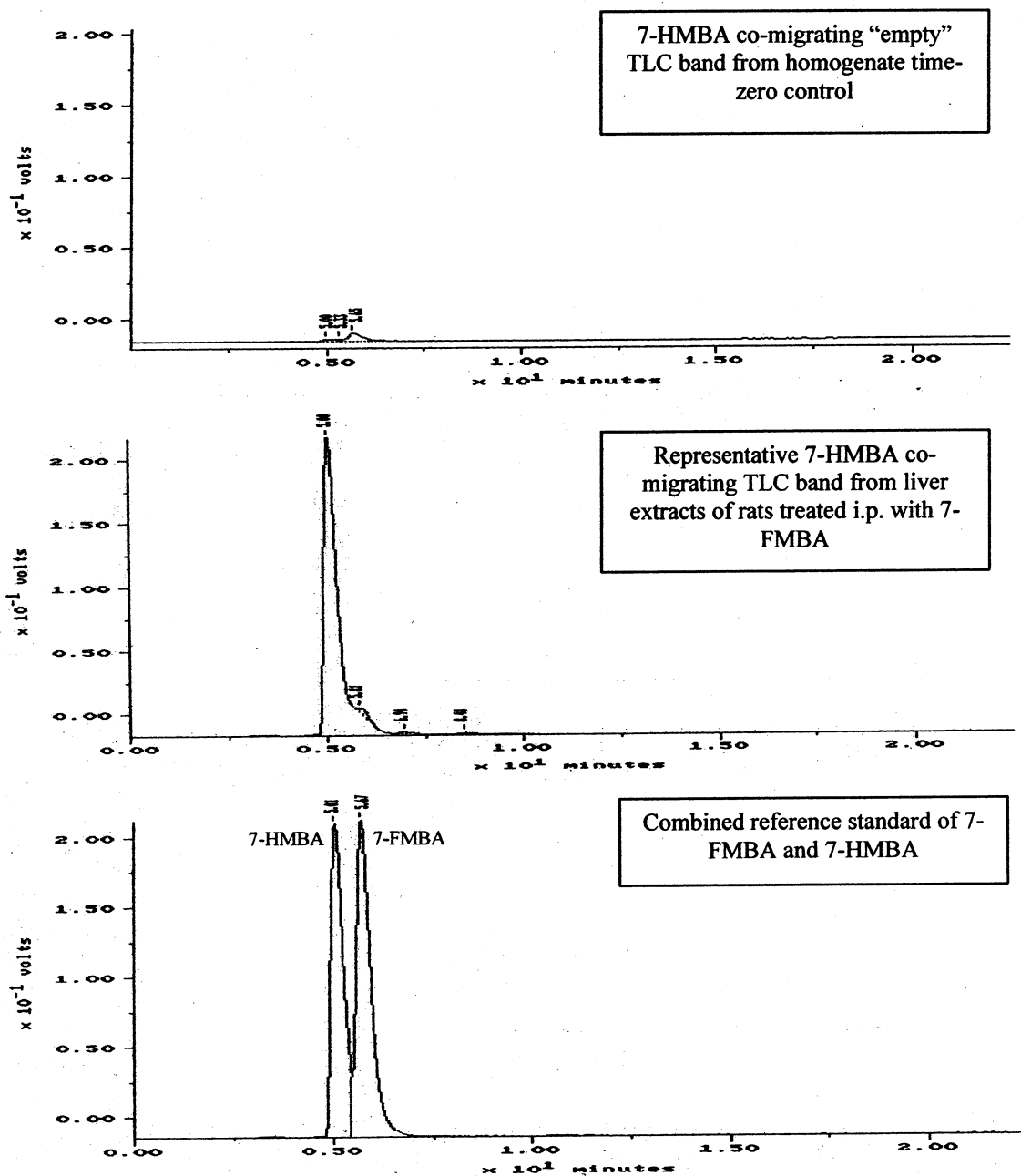


Fig. 8. HPLC chromatograms of 7-HMBA co-chromatographed TLC bands from in vivo experiments. HPLC analysis results are from 7-HMBA co-migrating "empty" or blue fluorescing TLC bands from a control experiment where 7-FMBA was added to liver homogenate before protein precipitation (top) and from liver of a rat treated i.p. with 7-FMBA (middle). A combined reference standard of 7-HMBA ($R_t = 5.0$ min) and 7-FMBA ($R_t = 5.7$ min) is shown at the bottom.

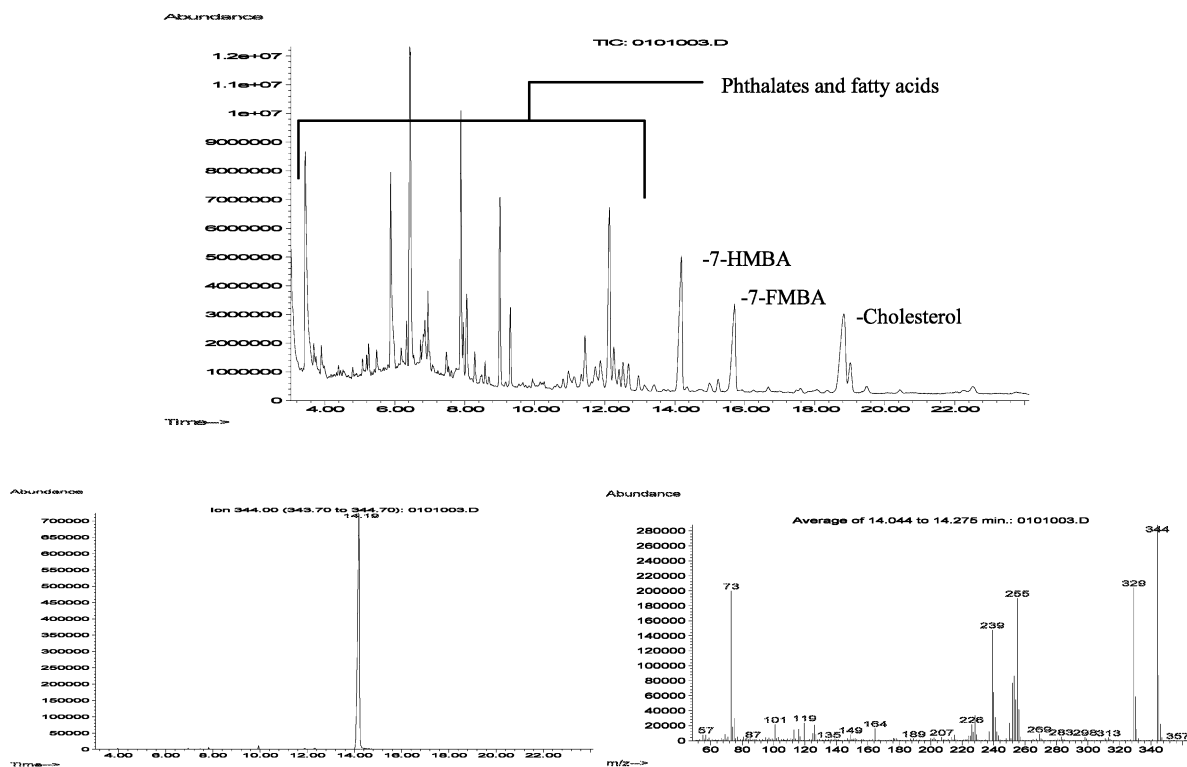


Fig. 9. GC/MS chromatogram of 7-HMBA co-eluting TLC band extract resulting from *in vivo* experiment involving *i.p.* injection of 7-FMBA in a rat. Total ion chromatogram shown at top, with selective ion monitoring for 344 molecular ion shown in bottom left, and mass spectrum shown in bottom right. Other major peaks include 7-FMBA ($R_t = 15.71$ min) and cholesterol ($R_t = 18.83$ min). Remaining peaks are primarily phthalate esters and fatty acid derivatives.

might be an intermediate in the activity of these types of carcinogenic aldehydes. The studies described in this paper confirm this prediction and demonstrate that 7-FMBA, like DMBA, is metabolized *in vitro* and *in vivo* to the carcinogen 7-HMBA. The formation of these hydroxymethyl metabolites from methyl and formyl-substituted PAH most likely represents a common mechanism by which both electron-donating and electron-withdrawing groups can act to sustain and/or enhance the carcinogenic activity of the parent compound.

The reactions catalyzed by aldo-keto reductases, as well as alcohol dehydrogenase and the like, are typically reversible, with their forward and backward reactions markedly pH-dependent [38,47]. Since the reductases generally possess a reducing number: pH optimum of pH 5.5–8, and dehydro-

genases a higher oxidation number: pH optimum range, it seems likely that an equilibrium between 7-FMBA and 7-HMBA exists in biological systems (see Fig. 1), especially given that chemical and biochemical oxidation studies demonstrate that 7-HMBA is oxidized to 7-FMBA in these systems. The finding that 7-FMBA is metabolized *in vivo* and *in vitro* to 7-HMBA, and that a possible equilibrium exists between these two compounds, indicates that 7-FMBA could readily enter into the L-region activation pathway as formulated by Flesher and Sydnor, and that a “reservoir” of the proximate hydroxymethyl metabolite might exist in tissues in the aldehyde form. Since hydroxymethyl metabolites are prime candidates for metabolism to electrophilic, DNA binding, and carcinogenic hydroxymethyl sulfate esters by cytosolic sulfotransferase activity [20,48,49],

further investigation into the role of aldo-keto reductases in the carcinogenicity of formyl hydrocarbons seems worthy of study.

Alternatively, it has been postulated that carcinogenic phenanthrene derivatives are activated to electrophilic ultimate metabolites by the introduction of the dihydrodiol-epoxide on the angular benzo ring [22]. However, metabolic activation of carcinogenic formyl hydrocarbons by the diol-epoxide pathway seems less likely owing to the presence of a meso-region electron-withdrawing group. The presence of an electron-withdrawing group on the BA nucleus would, in theory, lessen the nucleophilic reactivity of the angular benzo ring. Thus, resonance would render both the 1,2- and 3,4-double bonds more electrophilic, hence less likely to undergo addition by electrophilic oxygen across either double bond. In addition, the presence of this electron-withdrawing group at the meso-region would serve to particularly destabilize the benzylic carbocation formed from the epoxide at the 1 position, due to cationic resonance at a position alpha to the carbonyl group and interactions of this electron-withdrawing group at the particular site where the carbocation has the highest absolute value for its lowest unoccupied molecular orbital coefficient [50]. Thus, the metabolic activation of formyl hydrocarbons by reduction to hydroxymethyl metabolites followed by the introduction of a good leaving group for the generation of a benzylic carbocation [51] appears to be as satisfactory a theory for the development of strong carcinogenic properties in this class of compounds as can be formulated on the basis of the facts available.

5. Summary and conclusions

- 1) In vitro and in vivo rat liver metabolism studies indicate that carcinogenic formyl hydrocarbons are reduced enzymatically to carcinogenic hydroxymethyl metabolites, and oxidized both enzymatically and non-enzymatically to non-carcinogenic carboxylic acids.
- 2) Hydroxymethyl hydrocarbons have been proposed to be important proximate carcinogens derived from methyl-substituted PAH.
- 3) Thus, it seems likely that hydroxymethyl metabolites may be important common intermediates in the activation of carcinogenic PAH, substituted in their meso-regions with either electron-donating methyl groups or electron-withdrawing formyl groups.
- 4) The enzymes responsible for reduction of formyl hydrocarbons to hydroxymethyl metabolites are most probably aldo-keto reductases, since reduction proceeded preferentially in the presence of NADPH.
- 5) Since a hydroxymethyl metabolite is, in fact, an important proximate carcinogen in the activation of carcinogenic unsubstituted, methyl-substituted and formyl-substituted hydrocarbons, the interconversion of the aldehyde and alcoholic forms would provide a “reservoir” of the proximate carcinogen, assuming that further oxidation to a carboxylic acid was a minor competing pathway.

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