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Novel metabolic pathways for linoleic and arachidonic acid metabolism

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Abstract

Mouse liver microsomes oxidized linoleic acid to form 9,10- or 12,13-epoxyoctadecenoate. These monoepoxides were subsequently hydrolyzed to their corresponding diols in the absence of the microsomal epoxide hydrolase inhibitor, 1,2-epoxy-3,3,3-trichloropropane. Furthermore, both 9,10- and 12,13-epoxyoctadecenoates were oxidized to diepoxyoctadecanoate at apparently identical rates by mouse liver microsomal P-450 epoxidation. Both epoxyoctadecenoates and diepoxyoctadecanoates were converted to tetrahydrofuran-diols by microsomes. Tetrahydroxides of linoleate were produced as minor metabolites. Arachidonic acid was metabolized to epoxyeicosatrienoates, dihydroxyeicosatrienoates, and monohydroxyeicosatetraenoates by the microsomes. Microsomes prepared from clofibrate (but not phenobarbital) -treated mice exhibited much higher production rates for epoxyeicosatrienoates and *vic*-dihydroxyeicosatrienoates. This indicated an induction of P-450 epoxygenase(s) and microsomal epoxide hydrolase in mice by clofibrate and not by phenobarbital. Incubation of synthetic epoxyeicosatrienoates with microsomes led to the production of diepoxyeicosadienoates. Among chemically generated diepoxyeicosadienoate isomers, three of them possessing adjacent diepoxides were hydrolyzed to their diol epoxides which cyclized to the corresponding tetrahydrofuran-diols by microsomes as well as soluble epoxide hydrolase at a much higher rate. Larger cyclic products from non-adjacent diepoxides were not observed. The results of our in vitro experiments suggest that linoleic and arachidonic acid can be metabolized to their tetrahydrofuran-diols by two consecutive microsomal cytochrome *P*-450 epoxidations followed by microsomal or soluble epoxide hydrolase catalyzed hydrolysis of the epoxides. Incubation experiments with the S-9 fractions indicate that the soluble epoxide hydrolase is more important in this conversion. This manuscript is the first report of techniques for the separation and identification of regio and geometrical isomers of an interesting class of oxylipins and their metabolism by liver microsomes and S-9 fractions to THF-diols.

Keywords: Lipid; Oxylipin; Cytochrome *P*-450; Epoxide; Hydrolase

1. Introduction

Long-chain polyunsaturated fatty acids like arachidonic acid (AA, **1**, Fig. 1) form various biologically active metabolites. These fatty acid metabolites include prostaglandins, leukotrienes, lipoxins, and several other hydroxy and epoxy fatty acids [1]. The epoxides of polyunsaturated fatty acids are intermediates in biosynthetic pathways as well as biologically active substances. For example, 5,6-EET (**2**, Fig. 1) is a potent stimulator of prolactin release and an effective vasodilator [2,3]. This compound was also reported to cause insulin release from rat pancreatic islets [4], and somatostatin from the median eminence [5]. Furthermore, 11,12-DHET (**8**, Fig. 1) is an inhibitor of Na^+/K^+-ATPase, while 19- and 20-HETE (ω-1 and ω-

Abbreviations: BSTFA, *N, O*-bis(trimethylsilyl)-trifluoroacetamide; *m*-CPBA, *m*-chloroperbenzoic acid; DEED, diepoxyeicosadienoate; DHET, dihydroxyeicosatrienoate; EDTA, ethylenediaminetetraacetic acid; EET, epoxyeicosatrienoate; ETCP, 1,2-epoxy-3,3,3-trichloropropane (also termed TCPO or trichloropropene oxide); 4-FCO, 4-fluorochalcone oxide; GC/LREI/MS, gas chromatography low resolution electron impact mass spectrometry; HETE, hydroxyeicosatetraenoate; mEH, microsomal epoxide hydrolase; NP HPLC, normal-phase high performance liquid chromatography; P-450, cytochrome *P*-450; PUFA, polyunsaturated fatty acid; THF, tetrahydrofuran; RP HPLC, reverse-phase high performance liquid chromatography; sEH, soluble epoxide hydrolase; TMS, trimethylsilyl; TMCS, trimethylchlorosilane.

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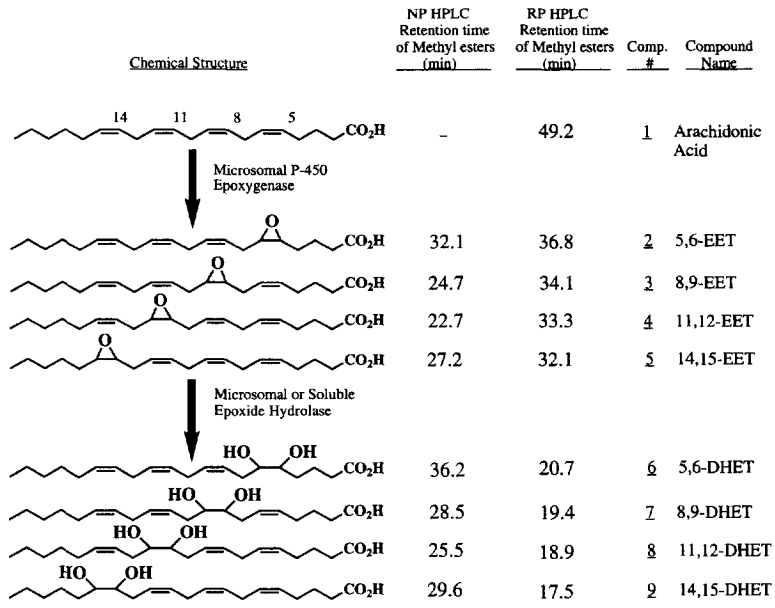


Fig. 1. Structures, abbreviations, and HPLC retention times of epoxides and diols of arachidonic acid formed by incubation of arachidonic acid with mouse microsomes and NADPH or formed synthetically. The position of the epoxide in the EET regioisomers collected as HPLC fractions was determined as the corresponding diol following catalytic reduction and derivatization with analysis by GC-MS.

HETE) are Na^+/K^+-ATPase stimulators [6–9]. Many cytochrome *P*-450 enzymes that oxidize fatty acids have been purified from liver and kidney, and their induction by some hypolipidemic agents such as clofibrate has been reported [10]. Monoepoxides of arachidonic acid which are produced by P-450 epoxygenase(s), have been reported as endogenous constituents of rat liver, rabbit kidney and human urine [11–13].

In in vitro assays, soluble epoxide hydrolase converts synthetic diepoxides (**13M**, Fig. 2) prepared from methyl linoleate (**10M**, Fig. 2) to corresponding tetraols (**14M**, Fig. 2) and tetrahydrofuran-diols (THF-diols, **16M**, Fig. 2) [14,14a]. Under physiological concentrations of sEH, synthetic methyl diepoxyoctadecanoate (**13M**, Fig. 2) was converted to methyl dihydroxyepoxides (**15M**, Fig. 2), which spontaneously cyclized to THF-diols [14].

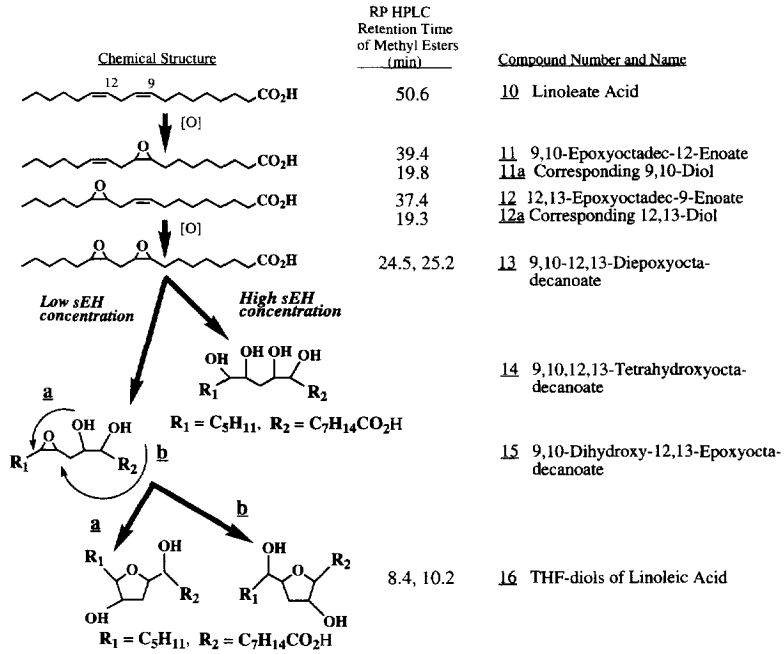


Fig. 2. In vitro metabolism of the synthetic diepoxide of linoleic acid to THF-diols. A compound number followed by 'M' in the text indicates the methyl ester of the compound represented by that number.

The microsomal and S-9 biosynthesis of diepoxides of polyunsaturated fatty acids and their THF-diols have not been reported, previously. Therefore, we undertook a study of enzymatic oxidation of linoleic acid to mono- and diepoxides as well as other metabolites and the subsequent conversion of the diepoxides into THF-diols by microsomes. Furthermore, because many arachidonate metabolites are important chemical mediators of biological response in mammals, we extended our studies to the metabolism of arachidonic acid diepoxides or DEEDs, and their hydrolysis products in the microsomal fraction of liver homogenates. Although fatty acid epoxides are hydrated much more rapidly by the sEH than mEH as shown by this and other studies, here we emphasize the P450/mEH interaction to minimize hydration of epoxides and diepoxides. In the present study, in vitro production of linoleate and arachidonate mono- and diepoxides and THF-diols were first examined using clofibrate induced and uninduced mouse liver microsomes. The soluble epoxide hydrolase acts on fatty acid epoxides at a much higher rate than microsomal epoxide hydrolase (> 1000 fold in rats and > 13000 fold in mice and humans) [15]. Therefore, other experiments included recombinant and wild-type mouse liver sEH and S-9 fractions. Finally, the purification and identification of individual positional and geometrical isomers of the epoxides, diols, diepoxides and THF-diols reported herein for both fatty acid groups, but especially in the arachidonate series, was challenging.

2. Materials and methods

2.1. Chemicals

Arachidonic acid and linoleic acid were purchased from Nu-Chek-Prep Inc. (Elysian, MN, USA). [1-^{14}C]Arachidonic acid (2.0 GBq/mmol) and [1-^{14}C]linoleic acid (2.0 GBq/mmol) were obtained from DuPont-New England Nuclear (Boston, MA, USA). *m*-CPBA was obtained from Kodak Laboratory and Research Products (Rochester, NY, USA). The soluble epoxide hydrolase inhibitor, 4-FCO, was prepared as reported previously [16]. Microsomal epoxide hydrolase inhibitor, ETCP [17] was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). The silica gel TLC plates were #5715, E. Merck, Darmstadt, Germany. The HPLC system used in this study consisted of the following: pump, series 410 Bio (Perkin-Elmer, Flanders, NJ); injector, model 7125 with 50 μl loop (Rheodyne Inc., Cotati, CA); spectrophotometric detector, Spectroflow 757 (KRATOS Analytical Instruments, Ramsey, NJ) and radiochemical detector, β-RAM (IN/US System Inc, Tampa, FL). All ^{1}H and ^{13}C NMR spectra were obtained on a GE QE300. All GC/LREI/MS spectra were obtained on a VG-Trio2 Quadrupole equipped with a HP 5890 gas chromatograph. A silica gel column, Techsphere (ϕ 4.6 × 250 mm, HPLC Technology Ltd., Cheshire, UK) was used for NP HPLC. In the case of RP HPLC, a non-endcapped WAKOSIL 5C18N (ODS, ϕ 4.6 × 250 mm, Wako Pure Chemical Industries, Osaka, Japan) was used. All other chemicals were of analytical grade.

2.2. Preparation of linoleate derivatives

A non-selective epoxidation of methyl linoleate was performed using *m*-CPBA in CH_2Cl_2 at room temperature [18]. The products were separated on silica gel flash chromatography (10–30% diethyl ether in *n*-hexane). Each fraction was further purified to get distinct positional isomers by silica NP HPLC as described below. In order to prepare the 1,2 or *vic*-diols, the corresponding epoxy fatty acids were hydrolyzed in THF:H_2O:5% aq. $HClO_4$ (3:1:1) at room temperature. Methyl linoleate derivatives, methyl 9,10-epoxy-11(*Z*)-octadecenoate (**11M**, Fig. 2), methyl 9,10-dihydroxy-11(*Z*)-octadecenoate (**11aM**, Fig. 2), methyl 12,13-epoxy-9(*Z*)-octadecenoate (**12M**, Fig. 2), methyl 12,13-dihydroxy-9(*Z*)-octadecenoate (**12aM**, Fig. 2), methyl 9,10-12,13-diepoxyoctadecanoate (**13M**, Fig. 2), and methyl THF-diols of linoleate (**16M**, Fig. 2) were identified as previously described [18]. In short, the purity and identity of these compounds were confirmed using a combination of ^{1}H and ^{13}C NMR, GC, GC/LREI/MS, TLC, and HPLC, as both their free acids and methyl esters. By converting epoxides quantitatively to their diols, unequivocal assignment of the site of oxidation could be made based on the electron impact mass spectral fragmentation pattern of the respective TMS esters. Acetates and n-butylboronates of the diols failed to yield fragmentation patterns as clear as those from TMS esters.

Methyl monohydroxylinoleate standards were prepared by incubation of linoleic acid with mouse liver microsomes using fifty times more substrate than described in the metabolism section below. The oxylipins were then extracted using peroxide free diethyl ether and methylated using TMS-diazomethane. Methylated compounds were purified using preparative TLC (n-hexane:diethyl ether, 4:1) followed with NP HPLC as described below. GC/LREI/MS indicated that they were monohydroxy compounds. Because these compounds were not the main focus of our study, positional isomers were not identified. Next, the resulting compounds were concentrated under N_2, derivatized to their TMS ethers, and analyzed by GC/LREI/MS: Methyl monohydroxylinoleate trimethylsilyl ethers (multiple GC peaks resulted from different positional isomers), *m/z* (obs. fragment, intensity), 382 ($[M^+]$, 0.7%), 367 ($[M\text{-}CH_3]^+$, 3.2%), 309 ($[M\text{-}TMS]^+$, 1.5%), 293 ($[M\text{-}TMSO]^+$, 3.2%), 73 ($[C_2H_5OSi]^+$, 100%).

2.3. Preparation of arachidonate analogs

Methyl EETs and methyl DEEDs were prepared by *m*-CPBA epoxidation of methyl arachidonate. After NP

column separation of these compounds, each group was further purified to get distinct positional isomers by NP HPLC as described, later. Each compound appeared pure based on NP and RP HPLC, NP TLC, and GC. Full spectral data were obtained on each methyl EET and methyl DEED and these data were consistent with assigned structure. The purity and identity of these compounds were confirmed using a combination of ^{1}H and ^{13}C NMR, GC, GC/LREI/MS, TLC, and HPLC. GC/LREI/MS supported the assigned structures but in the case of these epoxides did not provide clear diagnostic fragments for assigning positional isomers. Therefore, each methyl EET and methyl DEED was identified as its DHET and THF-diol, respectively, after acid hydrolysis for the same reasons as were given for the linoleate series. The fragmentation patterns were easily interpreted before and after catalytic reduction.

Each DHET showed a single spot on TLC. These were derivatized to their *bis*-trimethylsilyl ethers and characterized by GC/LREI/MS: Methyl DHETs. Methyl 5,6-dihydroxy-8(Z),11(Z),14(Z)-eicosatrienoate (**6M**, Fig. 1) *bis*-TMS ether, *m/z* (obs. fragment, intensity), 496 ($[M^+]$, 1.5%), 305 ($[M\text{-}C_5H_9O_2]^+$, 15.5%), 293 ($[M\text{-}C_6H_{10}O_3TMS]^+$, 1.5%), 203 ($[M\text{-}C_{15}H_{24}OTMS]^+$, 98.3%), 73 ($[C_2H_5OSi]^+$, 100%). Methyl 8,9-dihydroxy-5(Z),11(Z),14(Z)-eicosatrienoate (**7M**, Fig. 1) *bis*-TMS ether, *m/z* (obs. fragment, intensity), 496 ($[M^+]$, 0.4%), 253 ($[M\text{-}C_9H_{14}O_3TMS]^+$, 5.2%), 243 ($[M\text{-}C_{13}H_{22}OTMS]^+$, 31.9%), 73 ($[C_2H_5OSi]^+$, 100%). Methyl 11,12-dihydroxy-5(Z),8(Z),14(Z)-eicosatrienoate (**8M**, Fig. 1) *bis*-TMS ether, *m/z* (obs. fragment, intensity), 496 (M^+, 0.4%), 283($[M\text{-}C_9H_{16}OTMS]^+$, 5.0%), 213 ($[M\text{-}C_{12}H_{18}O_3TMS]^+$, 18.0%), 73 ($[C_2H_5OSi]^+$, 100%). Methyl 14,15-dihydroxy-5(Z),8(Z),11(Z)-eicosatrienoate (**9M**, Fig. 1) bis-TMS ether, *m/z* (obs. fragment, intensity), 496 (M^+, 0.4%), 323 ($[M\text{-}C_6H_{12}OTMS]^+$, 0.4%), 173 ($[M\text{-}C_{15}H_{22}O_3TMS]^+$, 24.5%), 73 ($[C_2H_5OSi]^+$, 100%).

In order to identify methyl DEEDs (**17M** to **22M**, Fig. 3), these eicosanoids were first hydrolyzed with dilute acid to their corresponding *cis* and *trans* THF-diols (**23M** to **28M** and **29M** to **34M**, Fig. 4). This method yielded extremely low quantities of compounds **24M** and **30M**. The GC/LREI/MS fragmentation patterns of the THF-diols were complex and made positional assignments difficult. Therefore, the resulting compounds were hydrogenated with PtO_2/H_2 which was found to simplify the fragmentation pattern dramatically and allow assignment of positional isomers. Following reduction the compounds were then derivatized to their *bis*-trimethylsilyl ethers, and characterized by GC/LREI/MS. This procedure was used because the fragmentation patterns of the resulting THF-diols after hydrogenation yielded unequivocal fragmentation patterns allowing assignment of the position of the THF moiety and by inference the corresponding diepoxide. In each case the THF diols failed to react with n-butylboronic acid to give diesters of higher mobility on TLC. As expected the diepoxides 18, 19, and 21 which lack adjacent epoxides failed to yield any tetrahydrofuran diols. The products of these compounds following acid treatment were of low R_f and reacted with n-butylboronic acid to yield boronate diesters of higher R_f.

Methyl arachidonate THF-diols. cis-Methyl 6,9-dihydroxy-5(8)-oxy-11(Z),14(Z)-eicosadienoate (**23M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 259 ($[M\text{-}C_{12}H_{24}OTMS]^+$, 5.5%), 257 ($[M\text{-}C_{12}H_{18}O_3TMS]^+$, 58.9%), 169 (32.9%), 73 ($[C_2H_5OSi]^+$, 100%). *cis*-Methyl 9,12-dihydroxy-8(11)-oxy-5(Z),14(Z)-eicosadienoate (**25M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 301 ($[M\text{-}C_9H_{18}OTMS]^+$, 4.0%), 215 ($[M\text{-}C_{12}H_{20}O_4TMS]^+$, 91.8%), 73 ($[C_2H_5OSi]^+$, 100%). *cis*-

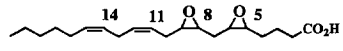
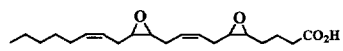
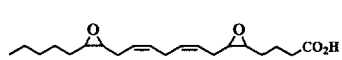
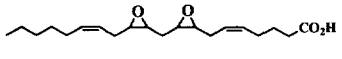
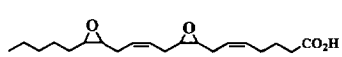
Chemical Structure	RP HPLC Retention Time of Methyl Esters (min)	NP HPLC Retention Time of Methyl Esters (min)	Comp. #	Compound Name
	19.4	37.8	17	5,6-8,9-Diepoxyeicosa-11,14-Dienoate *
	20.8	34.6	18	5,6-11,12-Diepoxyeicosa-8,14-Dienoate
	21.1	29.4	19	5,6-14,15-Diepoxyeicosa-8,11-Dienoate
	21.5	24.6	20	8,9-11,12-Diepoxyeicosa-5,14-Dienoate *
	22.7	27.2	21	8,9-14,15-Diepoxyeicosa-5,11-Dienoate
	24.4	21.0	22	11,12-14,15-Diepoxyeicosa-5,8-Dienoate *

Fig. 3. Structures and HPLC retention times of diepoxides of arachidonic acid (diepoxyeicosadienoic acids or DEEDs). A compound number followed by 'M' in the text indicates the methyl ester of the compound represented by that number. Compounds identified by '*' have methylene interrupted epoxides (1,4-diepoxides) and are converted to THF-diols by both microsomal and soluble epoxide hydrolases.

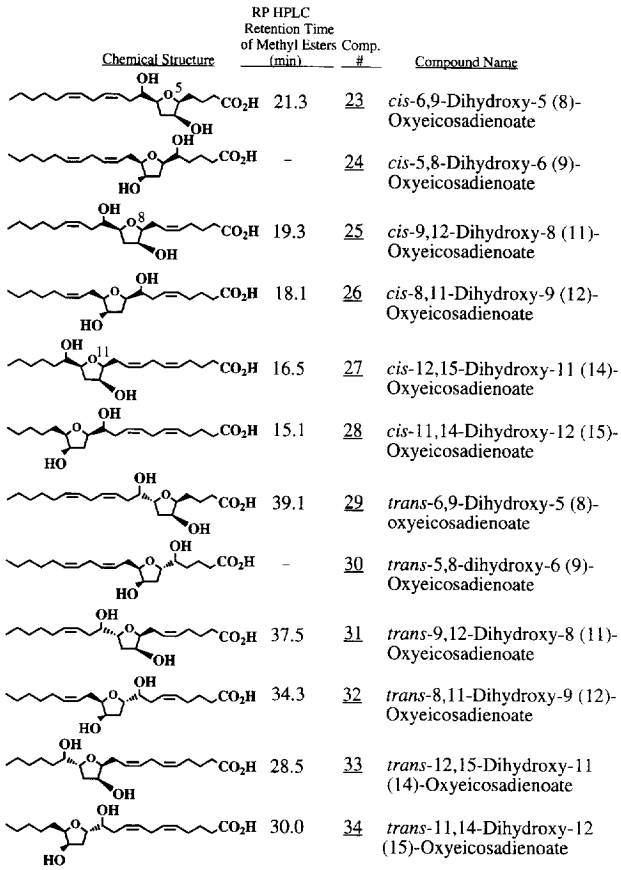


Fig. 4. Structures and HPLC retention times of THF-diols of arachidonic acid (dihydroxy-oxyeicosadienoic acids or DiHOED). A compound number followed by 'M' in the text indicates the methyl ester of the compound represented by that number.

Methyl 8,11-dihydroxy-9(12)-oxy-5(Z),14(Z)-eicosadienoate (**26M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 271 ([M-$C_9H_{16}O_3TMS]^+$, 5.0%), 245 ([M-$C_{12}H_{22}O_2TMS]^-$, 89.0%), 73 ([$C_2H_5OSi]^+$, 100%). *cis*-Methyl 12,15-dihydroxy-11(14)-oxy-5(Z),8(Z)-eicosadienoate (**27M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 343 ([M-$C_6H_{12}OTMS]^+$, 1.8%), 285 ([M-$C_6H_{12}OTMS$-$C_2H_3O_2]^+$, 6.9%), 253 ([M-TMSOH-$C_6H_{12}OTMS]^+$, 35.6%), 173 ([M-$C_{15}H_{26}O_4TMS]^+$, 98.6%), 73 ([$C_2H_5OSi]^+$, 100%). *cis*-Methyl 11,14-dihydroxy-12(15)-oxy-5(Z),8(Z)-eicosadienoate (**28M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 287 ([M-$C_{10}H_{18}O_2TMS]^+$, 54.8%), 271 ([M-$C_{10}H_{18}O_2TMS$-$CH_3]^+$, 6.9%), 229 ([M-$C_{12}H_{22}O_3TMS]^+$, 8.9%), 73 ([$C_2H_5OSi]^+$, 100%). *trans*-Methyl 6,9-dihydroxy-5(8)-oxy-11(Z),14(Z)-eicosadienoate (**29M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 259 ([M-$C_{12}H_{24}OTMS]^+$, 6.3%), 257 ([M-$C_{12}H_{18}O_3TMS]^+$, 68.6%), 169 (35.6%), 73 ([$C_2H_5OSi]^+$, 100%). *trans*-Methyl 9,12-dihydroxy-8(11)-oxy-5(Z),14(Z)-eicosadienoate (**31M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.2%), 301 ([M-$C_9H_{18}OTMS]^+$, 3.6%), 215 ([M-$C_{12}H_{20}O_4TMS]^+$, 93.7%), 73 ([$C_2H_5OSi]^+$, 100%). *trans*-Methyl 8,11-dihydroxy-9(12)-oxy-5(Z),14(Z)-eicosadienoate (**32M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 271 ([M-$C_9H_{16}O_3TMS]^+$, 6.4%), 245 ([M-$C_{12}H_{22}O_2TMS]^+$, 95.9%), 73 ([$C_2H_5OSi]^+$, 100%). *trans*-Methyl 12,15-dihydroxy-11(14)-oxy-5(Z),8(Z)-eicosadienoate (**33M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 343 ([M-$C_6H_{12}OTMS]^+$, 0.2%), 285 ([M-$C_6H_{12}OTMS$-$C_2H_3O_2]^+$, 8.9%), 253 ([M-TMSOH-$C_6H_{12}OTMS]^+$, 32.2%), 173 ([M-$C_{15}H_{26}O_4TMS]^+$, 99.3%), 73 ([$C_2H_5OSi]^+$, 100%). *trans*-Methyl 11,14-dihydroxy-12(15)-oxy-5(Z),8(Z)-eicosadienoate (**34M**, Fig. 4) hydrogenated *bis*-TMS ether, *m/z* (obs. fragment, intensity), 516 (M^+, 0.1%), 287 ([M-$C_{10}H_{18}O_2TMS]^+$, 84.9%), 271 ([M-$C_{10}H_{18}O_2TMS$-$CH_3]^+$, 19.2%), 229 ([M-$C_{12}H_{22}O_3TMS]^+$, 9.6%), 73 ([$C_2H_5OSi]^+$, 100%).

Methyl monohydroxyarachidonate standards were prepared in the same way as described for methyl monohydroxylinoleates. Because these compounds were not the main focus of this study, positional isomers were not separated. The incubation products were concentrated under N_2, derivatized to their TMS ethers and analyzed by GC/LREI/MS: Methyl monohydroxyarachidonate trimethylsilyl ethers, *m/z* (obs. fragment, intensity), 406 ([M^+], 1.7%), 391 ([M-$CH_3]^+$, 2.3%), 375 ([M-$CH_3O]^+$, 7.3%), 333 ([M-TMS]$^+$, 13.3%), 73 ([$C_2H_5OSi]^+$, 100%).

2.4. Preparation of [1-^{14}C]epoxyoctadecenoate, diepoxyoctadecanoate, EETs, and DEEDs

Linoleic acid [1-^{14}C, 185 kBq] was diluted with unlabeled linoleate to yield a 50 mBq/mmol solution which was methylated with excess TMS-diazomethane dissolved in hexane. After evaporation under N_2, the residue was dissolved in 0.5 ml of CH_2Cl_2. To this solution was added 1.6 mg of *m*-CPBA dissolved in 0.5 ml of CH_2Cl_2. At the end of the reaction, 5 ml of CH_2Cl_2 was added and this solution was washed sequentially with 3 ml of each of saturated $NaHCO_3$, brine and distilled water. The resulting organic phase was dried under N_2 and separated into mono- and diepoxides on TLC (*n*-hexane:diethyl ether, 4:1, twice). [1-^{14}C] 9,10- and 12,13-epoxyoctadecenoate methyl esters were separated on silica NP HPLC as described in the following section. Arachidonic acid [1-^{14}C, 370 kBq] was diluted with unlabelled arachidonic acid to yield 50 mBq/mmol and methylated by addition of TMS-diazomethane. After evaporation, the residue was dissolved into 0.5 ml CH_2Cl_2 and epoxidized by addition of 2.26 mg of *m*-CPBA dissolved into 0.5 ml CH_2Cl_2 while stirring for 2 h at ambient temperature. This material was washed with 1 ml of saturated $NaHCO_3$, NaCl and distilled water (twice each), and evaporated under a stream of

N_2. The resulting material was separated on TLC (solvent, *n*-hexane/diethyl ether, 3/2). Each isomer of methyl EET and methyl DEED was obtained on HPLC as described below.

2.5. Separation of 9,10- and 12,13-epoxyoctadecenoate, and EET and DEED isomers, and their hydrolysis products on HPLC

When using a UV detector, all HPLC separations were monitored at 200 nm. Separation of 9,10- and 12,13-epoxyoctadecenoate methyl esters (**11M** and **12M**, Fig. 2) was achieved by silica NP HPLC using 0.3% 2-propanol in *n*-hexane at 2 ml/min. Under these conditions, 9,10- and 12,13-epoxyoctadecenoate methyl esters had retention times of 10.5 and 13.6 min, respectively. Methyl EETs were separated into 4 positional isomers with silica NP HPLC using 0.075% THF in *n*-hexane as a solvent at a flow rate of 2 ml/min. Retention time for each isomer was as follows: Methyl 5,6-EET (**2M**, Fig. 1), 32.1 min; methyl 8,9-EET (**3M**, Fig. 1), 24.7 min; methyl 11,12-EET (**4M**, Fig. 1), 22.7 min; methyl 14,15-EET (**5M**, Fig. 1), 27.2 min. Six positional isomers of DEEDs were obtained by silica NP HPLC using 0.75% THF in *n*-hexane. Retention times for each isomer were as follows: Methyl 5,6-8,9-DEED (**17M**, Fig. 3), 37.8 min; methyl 5,6-11,12-DEED (**18M**, Fig. 3), 34.6 min; methyl 5,6-14,15-DEED (**19M**, Fig. 3), 29.4 min; methyl 8,9-11,12-DEED (**20M**, Fig. 3), 24.6 min; methyl 8,9-14,15-DEED (**21M**, Fig. 3), 27.2 min; methyl 11,12-14,15-DEED (**22M**, Fig. 3), 21.0 min. Attempted separation of the positional isomers of methyl THF-diols of arachidonic acid by NP or endcapped RP HPLC was incomplete or unsuccessful. These compounds were, however, separable on a non-endcapped WAKOSIL 5C18N C-18 RP HPLC column using acetonitrile:water (55:45) as eluant at a flow rate of 1 ml/min. Retention times of methyl AA THF-diols (Fig. 4) were as follows: **23M**, 21.3 min; **25M**, 19.3 min; **26M**, 18.1 min; **27M**, 16.5 min; **28M**, 15.1 min; **29M**, 39.1 min; **31M**, 37.5 min; **32M**, 34.3 min; **33M**, 28.5 min; **34M**, 30.0 min. As mentioned earlier, two methyl AA THF-diol isomers (**24M** and **30M**) were not detected using this method.

2.6. Animal treatment and preparation of microsomal and S-9 fractions

Five-week-old male Swiss-Webster mice were obtained from Bantin Kingman (Fremont, CA) and fed control or 0.5% clofibrate containing rodent chow ad lib for 10 days. During the last three days, 75 mg/kg of saline (0.9% NaCl) or phenobarbital dissolved in saline was injected intraperitoneally every 24 h. After the last injection of phenobarbital or saline, mice were fasted for 24 h before sacrifice. Livers were excised and perfused with ice-cold saline to remove blood. Liver samples were minced and homogenized with 4 volumes of isotonic buffer (1.15% KCl containing 10 mM Tris-HCl and 1 mM EDTA, pH 7.4). To prepare S-9 fractions, the homogenates were centrifuged (9000 × *g* for 10 min). For microsomal preparations, S-9 fractions were further centrifuged (105 000 × *g* for 60 min) [19]. Centrifugally washed microsomes were resuspended into 78 mM K-phosphate buffer (pH 7.4) containing 20% glycerol which were kept at −80°C until use. Protein contents of microsomal fractions were determined according to the method reported previously [20]. Microsomal P-450 content was measured by carbon monoxide differential spectra according to the method of Ohmura et al. [20].

2.7. Linoleic and arachidonic acid and their epoxide metabolism by mouse liver microsomes, and S-9 metabolism of arachidonic acid

Ethanolic solutions of [1-^{14}C] free fatty acids were dispensed into reaction vessels and allowed to evaporate under a stream of N_2 gas. To each reaction vessel, microsomal suspensions were dispensed and kept on ice for 15 min. Reaction mixtures consisted of 100 μl microsomal suspension (0.5–2.0 nmol P-450 equivalent), 1 mM NADPH, 20 μM [1-^{14}C]fatty acid (5 kBq), 78 mM K-phosphate (pH 7.4), 5 mM $MgCl_2$ in a total volume of 0.5 ml. A 20-μM concentration of a fatty acid is below the critical micelle formation concentration. Reactions were initiated by adding NADPH and maintained under O_2 gas at 37°C for multiple times from 5 to 20 min. Unless otherwise indicated, data are reported for 20 min incubations. In some cases, ETCP and/or 4-FCO (final concentration 400 μM, each) were added to reaction mixtures to prevent the metabolism of epoxides by the microsomal and soluble epoxide hydrolases, respectively. The reactions were stopped by acidifying with 50 μl of 5% oxalic acid. Products were extracted three times with 0.5 ml ethyl acetate. The organic layers were combined, methylated with TMS-diazomethane and dried under a stream of N_2 gas. Methylated products were resolved and isolated on the C-18 RP HPLC column using a gradient of acetonitrile:water (60:40 to 100% acetonitrile) at a flow rate of 1 ml/min. In S-9 studies, arachidonic acid was incubated with methyl arachidonate and shaken at 37°C. In S-9 fractions, cytochrome *P*-450 metabolism of methyl arachidonate was initiated by addition of 1 mM NADPH. Incubation mixtures were resupplied with 1 mM NADPH every 20 min. The reaction was stopped by addition of saturated brine and diethyl ether after 60 min. Following the extraction method described above, incubation products were remethylated using TMS-diazomethane. Compounds which exhibited the same elution pattern as standard methyl AA THF-diols were separated from the mixture using Baker-bond spe ethyl (C2) disposable extraction columns and analyzed by GC/LREI/MS. The compounds assigned as THF-diols showed identical retention times and fragmentation patterns to the standard methyl AA THF-diols. The

emphasis of this study was epoxide metabolism to diepoxides and THF-diols and other metabolites such as hydroxylated products received less attention.

2.8. Metabolism of DEED by recombinant and wild-type soluble epoxide hydrolase

In this study, apparently homologous recombinant and mouse liver sEHs were obtained by affinity chromatography according to the method of Wixtrom et al. [21]. The recombinant enzyme was purified from cells of *Spodoptera frugiperda* infected with the recombinant baculovirus as described by Beetham et al. [22]. The clone was isolated from a library prepared from livers of mice treated with clofibrate [23]. Following affinity chromatography, purification was checked by polyacrylamide gel electrophoresis under denaturing conditions. For standard incubations, the enzyme was diluted to 1 μg/ml with 76 mM phosphate buffer (pH 7.4) containing 0.01% bovine serum albumin and 0.1% EDTA. Reaction mixtures consisted of 10 ng affinity purified mouse sEH, 4 nmol [1-^{14}C] DEED (0.2 kBq), 0.2 ml of 76 mM phosphate buffer (pH 7.4) containing 0.01% bovine serum albumin and 0.1% EDTA. Incubations were carried out at 37°C for varying times from 10 to 60 min.

2.9. Identification of the metabolites

The oxylipin metabolites described here were biosynthesized in minute amounts and were present as complex mixtures of isomers. In order to identify these metabolites, standards for mono- and diepoxides as well as the corresponding diols and THF-diols of linoleic and arachidonic acids were prepared and identified unequivocally as described above. Then the identity of each metabolite was determined by comparison to the authentic standards using HPLC and GC retention times and co-injection. Chromatographic evidence does not constitute absolute identification of a metabolite. However, co-chromatography of authentic standards with radiolabeled metabolites in multiple chromatographic systems is strong evidence in favor of the structural assignments; especially when the precursor compounds are known.

3. Results and discussion

3.1. Metabolism of linoleic acid and its oxidized metabolites by mouse liver microsomes

Several replicate analyses of microsomal metabolism of linoleate and epoxylinoleate showed a relative distribution and proportion of metabolites that was the same if monitored by collection of fractions followed by analysis using liquid scintillation counting or by on-line analysis with the β-RAM HPLC Detector. Thus, all subsequent studies were performed with on-line monitoring of radioactivity. Linoleic acid was mainly metabolized in microsomal preparations by epoxydation, hydroxylation, and hydration of epoxides. As shown in Fig. 5a, all of the above metabolites were formed only in the presence of NADPH. Without any sEH and mEH inhibitors, only a small amount of the 12,13-epoxide (**12M**, Fig. 2) was observed and the 9,10-epoxide (**11M**, Fig. 2) could not be detected. However, both 9,10- and 12,13-dihydroxyoctadecenoate (**11aM** and **12aM**, Fig. 2) were present as major metabolites. Although these two positional isomers were not resolved on HPLC (Fig. 5), they were separated on GC and identified separately on GC/LREI/MS by co-chromatography with authentic standards and by fragmentation pattern. ETCP (400 μM), an mEH inhibitor [17], largely suppressed the formation of dihydroxyoctadecenoates and caused accumulation of monoepoxides (Fig. 5d). As expected, 4-FCO, a potent sEH inhibitor [16], did not con-

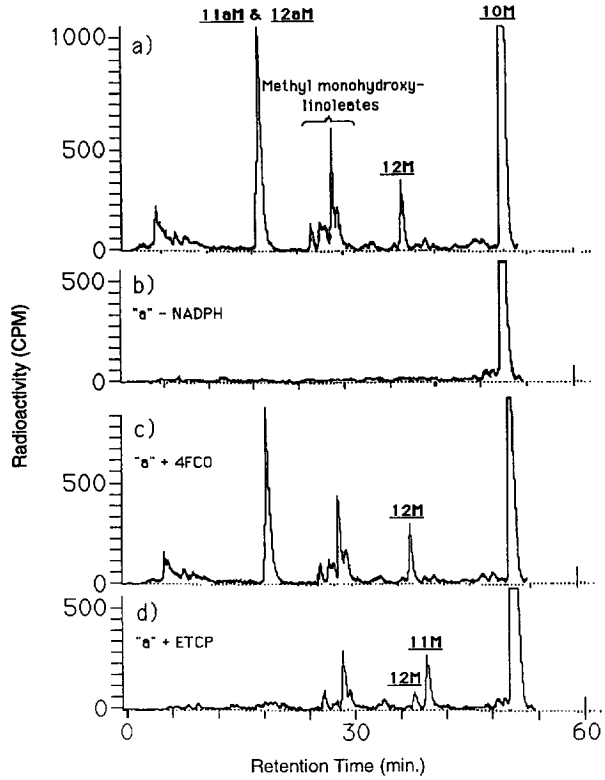


Fig. 5. Chromatograms of [1-^{14}C]linoleic acid metabolized by mouse liver microsomes. Mouse liver microsomes were incubated with [1-^{14}C]linoleic acid for 5–20 min and methylated incubation products were analyzed by RP HPLC as described in Section 2. (a) Metabolites from 10 min incubation with NADPH; (b) same as 'a' except without NADPH; (c) same as 'a' except with 400 μM of sEH inhibitor, 4-FCO; (d) same as 'a' except with 400 μM of mEH inhibitor, ETCP. The hydroxylated linoleic acid metabolites are thought to arise primarily from P-450 ω- and ω-1 hydroxylation. Products identified by co-chromatography are indicated on the figure. Metabolites **11aM** and **12aM** are the 9,10- and 12,13-diols while **11M** and **12M** are the corresponding epoxides. All products were converted to methyl esters before analysis. Diepoxides and hydration products of the mono- and diepoxides are evident at longer incubation times or higher enzyme concentrations.

Table 1
Linoleic acid metabolism by mouse liver microsomes with or without 1 mM ETCP, an mEH inhibitor

Metabolites	Retention time (min)	Rate (pmol/min/nmol P-450) [a]	
		Control	ETCP
Dihydroxyoctadecenoate (**11a** and **12a**)	19.1	98.9 ± 2.4	N.D. [b]
Monohydroxyepoxyoctadecenoate (positional isomers)	20.8	N.D.	19.5 ± 0.8
Monohydroxylinoleate (positional isomers)	29.2	20.4 ± 1.1	9.8 ± 0.3
12,13-Epoxyoctadec-enoate (**12**)	37.4	N.D.	8.5 ± 0.2
9,10-Epoxyoctadec-enoate (**11**)	39.4	3.5 ± 0.3	29.6 ± 0.4
Total linoleic acid metabolism	50.6	274 ± 9	111 ± 2

[1-^{14}C]Linoleic acid was incubated with mouse liver microsomes for 20 min. The methylated incubation products were analyzed by radiochemical RP HPLC as described in the text.
[a] Mean ± S.E.M., $n = 3$.
[b] Not detected; minimum detection = 0.1 pmol/min/nmol P-450.

Table 2
Metabolism of monoepoxides of linoleic acid by mouse liver microsomes with or without mEH inhibitor

Metabolites	Retention time (min)	Rate (pmol/min/nmol P-450) [a]			
		9,10-Epoxyoctadecenoate		12,13-Epoxyoctadecenoate	
		Control	ETCP	Control	ETCP
Trihydroxyoctadecenoates (positional isomers)	5.3	337 ± 2	N.D.	395 ± 22	N.D. [b]
THF-diols (**16**)	8.4, 10.2	101 ± 3	N.D.	68 ± 11	N.D.
12,13-Dihydroxyoctadec-enoates (**12a**)	19.3	–	–	788 ± 12	105 ± 6
9,10-Dihydroxyoctadec-enoates (**11a**)	19.8	868 ± 3	68.2 ± 2.1	–	–
Diepoxyoctadec-anoates (**13**)	24.5, 25.2	N.D.	100 ± 6	N.D.	108 ± 8
Total Epoxyoctadecenoate metabolism (**11** and **12**) [c]	37.4, 39.4	1330 ± 9	413 ± 26	1271 ± 28	404 ± 22

[1-^{14}C]Epoxyoctadecenoate was incubated with mouse liver microsomes with NADPH for 20 min and methylated incubation products were analyzed by radiochemical RP HPLC as described in the text.
[a] Mean ± S.E.M., $n = 3$.
[b] Not detected; minimum detectable limit = 0.1 pmol/min/nmol P-450.
[c] Measured as disappearance of radioactive epoxyoctadecenoate.

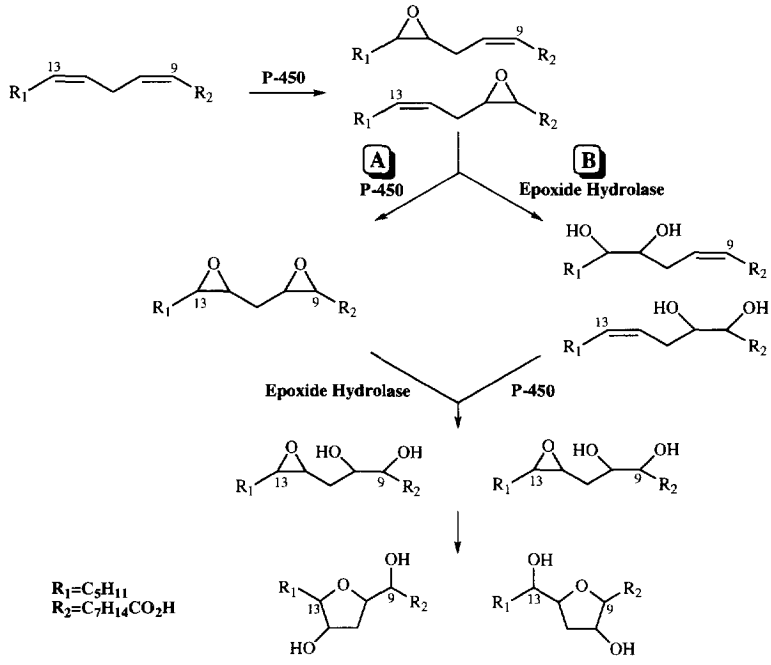


Fig. 6. Proposed in vitro mechanism of THF-diol formation from synthetic monoepoxyoctadecenoic acids.

tribute to the preservation of the monoepoxides in washed microsomes and did not lead to a significant reduction in the total metabolism of the substrate with these microsomal preparations (Fig. 5c). Halarnkar et al. has already reported that epoxides of fatty acids were hydrolyzed with affinity-purified mouse liver sEH [14]. Thus, the ineffectiveness of 4-FCO in preventing epoxide hydrolysis demonstrated the absence of sEH contamination in microsomal preparations. These data support the hypothesis that hydration of monoepoxides is catalyzed by mEH activity in microsomes. Among the two possible epoxides, the 9,10-epoxide was the predominant isomer which was retained as a result of mEH inhibition. The ratio of formation was 3.5:1 measured in the presence of 400 μM ETCP (Table 1). Under these conditions, production of diepoxides or THF-diols from linoleic acid was not observed. The most simple interpretation of our data is that the concentration of ETCP used to inhibit mEH caused significant inhibition of P-450 oxidation, complicating quantitative interpretation of the data. However, at this concentration of ETCP no inhibition of sEH was observed. The observation that the 12,13-epoxide of linoleic acid was metabolized to its triols (products of terminal hydroxylation and epoxide hydrolysis) at a faster rate than the 9,10-epoxyoctadecenoate may indicate that the former epoxide is a better substrate for P-450 hydroxylase (Table 2). ETCP (400 μM) inhibited both the hydrolysis of monoepoxides (Fig. 5) and production of THF-diols from synthetic monoepoxides (Table 2), and it resulted in accumulation of the diepoxide in the reaction system. No inhibition of epoxide hydration was observed with 4-FCO, indicating the absence of sEH in the incubation mixture. Thus, in this experiment THF-diols of linoleic acid seemed to be formed through mEH activity and subsequent cyclization. Assuming equal concentrations of the two monoepoxides, if the pathway leading to the production of THF-diols involved epoxydation of its monoepoxides to a diepoxide and a subsequent epoxide hydrolysis (Fig. 6A), one may expect approximately equal rates of THF-diol formation for both monoepoxides because they have equal rates of diepoxide formation (Table 2). However, the 9,10-monoepoxide of linoleic acid had a higher rate of THF-diol formation and that correlates with its higher rate of conversion to the corresponding diol (Table 1). Based solely on these observations, one may conclude that the formation of THF-diols of linoleate from the monoepoxides involves hydrolysis of

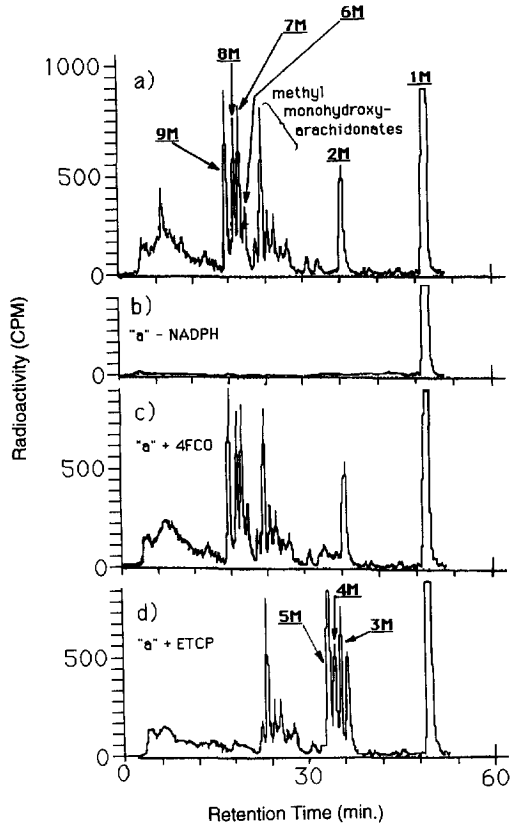


Fig. 7. Chromatograms of [1-^{14}C]arachidonic acid metabolized by mouse liver microsomes. Mouse liver microsomes were incubated with [1-^{14}C] arachidonic acid for 5–20 min and methylated incubation products were analyzed by RP HPLC as described in Section 2. (a) Metabolites from 10 min incubation with NADPH; (b) same as 'a' except without NADPH; (c) same as 'a' except with 400 μM 4-FCO; (d) same as 'a' except with 400 μM ETCP. Products tentatively identified by co-chromatography are indicated on the figure. Metabolites **2M–5M** are methyl esters of monoepoxides of arachidonic acid while **6M–9M** are the corresponding diols. Diepoxides and hydration products of the mono- and diepoxides are evident on longer incubation times.

monoepoxides to diols, epoxydation of the diols to diol-epoxides, and a subsequent cyclization of diol-epoxides to THF-diols (Fig. 6B). However, as indicated in Table 1, the rate of formation of 9,10-epoxyoctadecenoate is more than three times higher than 12,13-epoxyoctadecenoate and these two compounds are not present in equal concentrations. This confuses our analysis, and it is not clear which pathway (Fig. 6A or B) predominates in the biosynthesis of THF-diols at this point. In the absence or presence of

Table 3
Effect of clofibrate and phenobarbital on mouse liver P-450

Treatment	Wet liver weight (g)	relative liver Weight (g/100g)	P-450 content (nmol/g)	Protein [a] (mg/m)
Control	1.63 ± 0.05	4.90 ± 0.08	9.72 ± 0.69	12.7 ± 2.3
Clofibrate	2.38 ± 0.01	7.37 ± 0.44	12.0 ± 1.6	19.8 ± 0.8
Phenobarbital	1.89 ± 0.05	5.67 ± 0.10	12.1 ± 1.8	19.9 ± 1.7

[a] Expressed as mg of microsomal protein per gram wet weight of liver.
Values reported as mean ± S.E.M., $n = 3$.

NADPH, diepoxyoctadecanoate was converted to THF-diols at the rate of 938 ± 29 pmol/min/nmol P-450. The rate of tetraol production was 130 ± 6 pmol/min/nmol P-450. These results suggest that linoleic acid can be metabolized by mEH to both its THF-diols and tetraols through diepoxyoctadecanoate intermediates (Fig. 6). Because multiple enzymes are undoubtedly involved in the metabolism of both linoleic and arachidonic acid derivatives and kinetics of these enzyme substrate interactins have not been studied, we do not know how the concentration used in this study relates to K_m. It is unlikely that all the reported rates approached V_{max} conditions. However, disappearance of the substrate was linear with time and protein concentration under the conditions of these assays.

3.2. Metabolism of arachidonic acid by mouse liver microsomes and S-9 fractions obtained from induced and uninduced mice

Both clofibrate and phenobarbital induced mouse liver microsomal P-450 (Table 3). The doses employed in this study were near optimal for maximal induction levels. Microsomes catalyzed the formation of a number of metabolites from arachidonic acid (**1**) only in the presence of NADPH (Fig. 7). As depicted in Fig. 7, the major metabolites were hydroxyeicosanoids, EETs, DHETs (**2-5, 6-9**). Loss of arachidonic acid due to microsomal metabolism was linear for 20 min (data not presented). Secondary metabolites such as DHET and more polar products began to form after 5 min, and their formation was linear with time for 20 min. Multiple incubation times ranging from 10–20 min were employed in this study. As shown in Fig. 7a, 5,6-EET (**2**) was the only EET which accumulated at the end of a 10-min incubation. However, the DHETs corresponding to the positional isomers of the other EETs predominated as a result of EET hydrolysis by the microsomes. In the presence of 400 μM ETCP (Fig. 7d), peaks of DHETs diminished and EETs accumulated. Production of these oxylipins were not affected by 4-FCO. Thus as was the case for epoxyoctadecenoates, the hydrolysis of EETs was due to the mEH activity in microsomes. 5,6-EET appears to be a poor substrate for both mEH and sEH [24].

The epoxydation of arachidonic acid by purified rat, rabbit and human P-450 has been reported [12,13,25]. It has been reported that rat liver microsomes oxidized arachidonic acid to 8,9-, 11,12- and 14,15-EETs. Capdevila et al. [25] were not able to observe production of 5,6-EET by rat liver microsomes. It was suggested that 5,6-EET was unstable and underwent a spontaneous rearrangement to a δ-lactone. In this study we present evidence for the production of 5,6-EET (Fig. 7) in the murine system. In order to increase the longevity of our oxylipins, these metabolites were derivatized to their methyl esters immediately after extraction which may explain their detection.

There were no significant qualitative differences in the distribution of metabolites produced by the microsomes from control and clofibrate-treated animals. Rates of metabolite formation by microsomes from control, clofibrate-induced and phenobarbital-induced animals are summarized in Table 4. The hydroxylation of arachidonic acid was not significantly induced by clofibrate or phenobarbital treatment. On the other hand, clofibrate treatment induced the rate of epoxydation deduced by summing the formation rates of EET and DHET (Table 4). According to these data, arachidonate metabolites with oxidations at 8,9- and 14,15-positions predominated (60.8 and 71.5 pmol/min/nmol P-450, respectively). In addition, clofibrate treatment seems to induce mEH activity as indicated

Table 4
Arachidonic acid metabolism by mouse liver microsomes

Metabolites	Retention times (min)	Rate of formation (pmol/min/nmol P-450) [a]		
		Control	Clofibrate [b]	Phenobarbital [b]
14,15-DHET (**9**)	17.5	53.7 ± 4.6	71.5 ± 7.9 (113)	42.2 ± 3.8 (79)
11,12-DHET (**8**)	18.9	34.5 ± 2.9	43.7 ± 4.6 (127)	36.5 ± 3.9 (106)
8,9-DHET (**7**)	19.4	34.5 ± 3.8	60.8 ± 6.1 (176)	22.5 ± 2.0 (65)
5,6-DHET (**6**)	20.7	10.3 ± 1.0	16.2 ± 1.1 (157)	9.8 ± 1.0 (95)
monohydroxides of arachidonic acid (positional isomers)	23.4	58.0 ± 4.4	52.6 ± 4.8 (91)	55.4 ± 7.1 (96)
14,15-EET (**5**)	32.1	2.9 ± 0.4	4.0 ± 0.4 (137)	4.4 ± 0.7 (152)
11,12-EET (**4**)	33.3	trace [c]	trace	trace
8,9-EET (**3**)	34.1	trace	trace	trace
5,6-EET (**2**)	36.8	16.4 ± 1.2	38.3 ± 5.0 (238)	15.5 ± 1.3 (95)
Total AA (**1**) metabolism [d]	49.2	248 ± 21	358 ± 39 (144)	560 ± 28 (226)

[1-^{14}C]Arachidonic acid was incubated with mouse liver microsomes and NADPH for 20 min and methylated incubation products were analyzed by radiochemical RP HPLC as described in Section 2.

[a] Mean ± S.E.M., $n = 3$.

[b] Values in parentheses represent the percentage versus control.

[c] Minimum detection = 0.3 pmol/min/nmol P-450.

[d] Measured as disappearance of radioactive AA. Much of the metabolism was to unidentified metabolites and espicially at long incubation times to very polar materials either not extracted or eluting at the solvent front from reversed phase HPLC.

by an elevation in the rates of production of DHETs. Furthermore, our data suggest that 5,6-EET hydrolysis by mEH does not take place as readily as it does for the other EETs. Finally, our data indicate that phenobarbital does not induce the formation of epoxides of arachidonate in mice to a significant degree. This is in contrast to data reported in other species. For example, it has been reported that phenobarbital significantly induces EET as well as HETE production activity in livers from chicken embryos [26]. In rat liver, phenobarbital and ciprofibrate (one of the fibrate hypolipidemic agents) were reported to induce arachidonic acid epoxygenase and ω-hydroxylase, respectively [27]. Furthermore, rabbit kidney P-450 was reported to generate only 11,12- and 14,15-EET [28].

Our GC/LREI/MS analysis of semi-purified mixtures of products from incubation of arachidonic acid with S-9 fractions of control and clofibrate fed mice provides evidence for in vitro S-9 production of the THF-diols from arachidonic acid (Fig. 8). As mentioned before, fatty acid epoxides are much better substrates for soluble epoxide hydrolase than microsomal epoxide hydrolase and soluble epoxide hydrolase is more active in murine and human liver than it is in rat liver [15]. Therefore, production of AA THF-diols by mouse liver S-9 fractions is most likely dominated by the catalytic activity of the soluble epoxide hydrolase.

3.3. Metabolism of EETs by mouse liver microsome fraction

Mouse liver microsomes converted EETs to their corresponding DHET without NADPH. The rate at which 5,6-EET was hydrolyzed to 5,6-DHET was 0.77 nmol/min/mg protein. The rate of hydrolysis of 14,15-EET to its corresponding DHET was 7.4 nmol/min/mg protein; about 9 times faster than that of 5,6-EET. This finding is consistent with a previous report [28]. As shown in Table 5, in the presence of NADPH and ETCP, all of

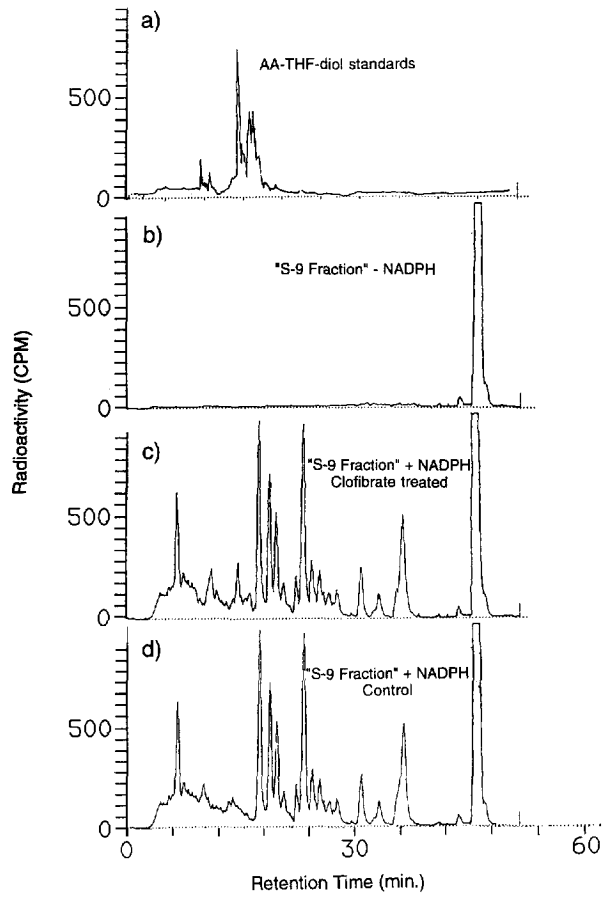


Fig. 8. Chromatograms of [1-14C]arachidonic acid metabolized by mouse liver S-9 fractions. Mouse liver S-9 fractions were incubated with [1-14C]arachidonic acid for 60 min and methylated incubation products were analyzed by RP HPLC as described in Section 2. The structures of compounds with the same retention time as methyl arachidonate THF diols (23–34) were confirmed by GC/LREI/MS. (a) Synthetic mixture of methyl AA THF-diol standards; (b) Liver S-9 fractions from both clofibrate fed and control mice incubated with AA and without NADPH; (c) Liver S-9 fractions from clofibrate-fed mice ($n = 3$) incubated for an hour with AA and NADPH; (d) Liver S-9 fractions from control mice ($n = 3$) incubated for an hour with AA and NADPH.

Table 5
Metabolism of EETs by mouse liver microsomes in the presence and absence of 0.4 mM ETCP, a mEH inhibitor

Substrates	Incubation products				
	Rate (pmol/min/nmol P-450) [a]				
	Mono-hydroxy-EETs	DEED	Un-identified	Total product formation	*vic*-Diols [b]
5,6-EET	6.0 ± 0.8	9.7 ± 1.0	11.7 ± 2.0	27.5 ± 4.8	0.77 ± 0.01
8,9-EET	8.1 ± 0.7	8.2 ± 1.1	16.8 ± 2.0	33.1 ± 7.8	4.45 ± 0.36
11,12-EET	7.6 ± 0.6	13.0 ± 0.6	10.0 ± 0.8	30.6 ± 5.7	7.14 ± 0.48
14,15-EET	9.8 ± 0.9	12.1 ± 0.8	16.1 ± 1.1	38.0 ± 5.7	7.14 ± 0.43

[1-^{14}C]EETs were incubated with mouse liver microsomes and NADPH for 20 min and methylated incubation products were analyzed by radiochemical RP HPLC as described in the text.

[a] Mean ± S.E.M., $n = 3$. Minimum detection = 0.01 pmol/min/nmol P-450.

[b] *vic*-Diol formation studies performed in separate incubations and in the absence of ETCP. These data are not included in the column labeled total product formation. Data are reported as nmol/min/nmol P-450.

[c] Since the acid diol spontaneously cyclizes in part to the δ-lactone, the data represent the combined acid diol and cyclic lactone peaks.

Table 6
Metabolism of diepoxides of arachidonic acid (DEEDs) by affinity purified soluble epoxide hydrolase from mouse and by microsomes

Enzyme	Substrate	Rate (nmol/min/μg protein) [a]			
		Tetraol	Epoxy-diol	THF-diol	DEED metabolism [b]
sEH	5,6-8,9-DEED	1.12 ± 0.02	N.D.	4.11 ± 0.09	5.23 ± 0.13
	8,9-11,12-DEED	0.53 ± 0.02	N.D.	8.33 ± 0.17	8.86 ± 0.17
	11,12-14,15-DEED	0.61 ± 0.19	N.D.	3.56 ± 0.60	4.17 ± 0.79
	5,6-11,12-DEED	5.31 ± 0.19	12.6 ± 0.19	N.D.	17.9 ± 0.33
	5,6-14,15-DEED	9.97 ± 0.27	12.7 ± 0.62	N.D.	22.7 ± 0.6
	8,9-14,15-DEED	7.09 ± 0.27	17.0 ± 0.8	N.D.	24.2 ± 0.6
	Rate (nmol/min/mg) Protein				
Microsomes	5,6-8,9-DEED	0.73 ± 0.02	N.D.	1.66 ± 0.02	2.39 ± 0.04
	8,9-11,12-DEED	0.52 ± 0.03	N.D.	2.15 ± 0.33	2.67 ± 0.35
	11,12-14,15-DEED	0.35 ± 0.05	N.D.	1.84 ± 0.17	2.18 ± 0.21
	5,6-11,12-DEED	1.33 ± 0.17	1.53 ± 0.22	N.D.	2.86 ± 0.38
	5,6-14,15-DEED	1.31 ± 0.09	1.23 ± 0.06	N.D.	2.54 ± 0.14
	8,9-14,15-DEED	1.61 ± 0.01	1.77 ± 0.11	N.D.	3.39 ± 0.12

[1-^{14}C]DEEDs were incubated with the above enzymes for 20 min and methylated incubation products were analyzed by radiochemical RP HPLC as described in the text.
[a] Mean ± S.E.M., $n = 3$.
[b] Measured as disappearance of radioactive DEEDs.

the EETs were converted to monohydroxyepoxy-eicosatrienoates and/or diepoxyeicosadienoates. The four EET positional isomers exhibited similar rates of hydroxylation. Among the four isomeric EETs, 11,12-EET had the highest rate of epoxydation to its corresponding DEED. As shown in Fig. 7c, hydrolysis of EETs to the corresponding DHETs was not inhibited by 400 μM 4-FCO. However, as Fig. 7d shows, DHET formation was completely blocked by addition of 400 μM of ETCP. This observation suggests that mEH, in addition to sEH, is responsible for the hydrolysis of epoxy fatty acids in vivo but probably to a limited extent. The relative extent of mEH activity on EETs is probably determined by the relative abundance and K_m's of the EH's as well as the subcellular distribution of the EETs or other epoxy fatty acids. Considering the proximity of microsomal P-450 and mEH, it is possible that mEH plays a significant role in epoxy fatty acid hydrolysis in vivo in spite of the higher catalytic rate and protein concentration of the sEH.

3.4. Metabolism of DEEDs by mouse liver microsome fraction and affinity-purified recombinant mouse liver soluble epoxide hydrolase

Mouse liver microsomes as well as affinity-purified recombinant sEH hydrolyzed DEEDs to epoxydiols, tetraols and THF-Diols (Table 6). Only methylene-interrupted diepoxides were converted to THF-diols. The larger heterocyclic rings which could possibly form from other regioisomers of DEEDs were not detected while the corresponding tetraols were. Formation of THF-diols from 1,4-diepoxides occurred via the intermediacy of epoxydiols as shown in the analogous linoleate series [14a]. Diol-epoxides and tetraols were most likely formed from oxylipins with more distant diepoxides which could not cyclize as easily (**18**, **19**, **21**). Collectively, cloned mouse sEH hydrolyzed the non-adjacent diepoxides more rapidly than adjacent ones (Table 6). On the other hand, microsomal preparations hydrolyzed adjacent and non-adjacent diepoxides at apparently identical rates; suggesting more specificity in the metabolism of DEEDs by sEH than mEH. Further work to isolate these compounds in vivo is under way.

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