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Formation of Cyclic Products from the Diepoxide of Long-Chain Fatty Esters by Cytosolic Epoxide Hydrolase

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Since diepoxides are known metabolites of polyunsaturated fatty acids, the action of the cytosolic epoxide hydrolase purified from liver tissue was examined on these diepoxides. Diepoxymethylstearate was metabolized to the corresponding tetraol by high concentrations of affinity-purified cytosolic epoxide hydrolase. When the enzyme was diluted (1000- to 2000-fold), disappearance of the tetraol metabolite occurred simultaneously with formation of other hydration products with GC retention times and chromatographic mobilities different from those of the tetraol. The hydration products were identified as tetrahydrofuran diols based on comparison of chromatographic properties and mass spectral information with the properties and spectra of chemically generated products. Also, a mixture of diepoxymethylarachidonates was hydrated to tetraols using concentrated enzyme. As the enzyme was diluted (1000- to 2000-fold), a decrease in tetraol formation occurred along with the elevation of other hydration products whose mass spectra were consistent with tetrahydrofuran diol structures. These data are consistent with the epoxide hydrolase at low concentrations acting to open one epoxide followed by nonenzymatic cyclization to the tetrahydrofuran diols. The data also suggest that oxygenated lipids may be endogenous substrates for the cytosolic epoxide hydrolase. Since some oxylipins are known chemical mediators, the *in vivo* presence and role of these novel diols and tetrahydrofuran diols should be examined. © 1992 Academic Press, Inc.

Long-chain polyunsaturated fatty acids form several unique products with various biological functions. These products, which arise both chemically and enzymatically, include prostaglandins, leukotrienes, lipoxins, and several hydroxy and epoxide fatty acids (1). The epoxides of fatty acids, in addition to being key intermediates in the formation of several products, may have potent biological effects. For example, the 5,6-epoxide of arachidonic acid is a potent stimulator of prolactin release and an effective vasodilator (2, 3). Recently, Capdevila *et al.* (4) have shown further conversion of the monoepoxides of arachidonic acid to diepoxides and epoxy alcohols by cytochrome P450 enzymes. Although biological activities of the diepoxides have not been studied, we have shown that diepoxymethylstearate is efficiently metabolized by both crude and affinity-purified liver cytosolic epoxide hydrolase (5).

Epoxide hydrolases are enzymes which act on epoxides and convert them to diols. To date four different epoxide hydrolases have been shown to exist in mammalian systems. Microsomal epoxide hydrolase (mEH)[5] is involved in the metabolism of arene epoxides and epoxides with some *cis* substituents α to aromatic systems (6–8). Cholesterol epoxide hydrolase (also microsomal) selectively hydrolyzes 5,6-epoxy cholesterols and related compounds (9, 10). Cytosolic epoxide hydrolase (cEH) hydrates a variety of aliphatic epoxides including those which are *trans*-substituted and α to aromatic systems (11, 12). Monoepoxide fatty acids are the best known substrates for cEH (5, 13–15). Another form of epoxide hydrolase with high substrate specificity toward leukotriene A_4 has also been described (16). These findings suggest that epoxide fatty acids may be endogenous substrates for cEH.

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[5] Abbreviations used: cEH, cytosolic epoxide hydrolase; mEH, microsomal epoxide hydrolase; TMS, trimethylsilyl; THF, tetrahydrofuran; MSTFA, *N*-methyl-*N*-trimethylsilyl trifluoroacetamide; DFF, diisopropylfluorophosphate; oxylipins, oxygenated fatty acids.

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While studying the catabolism of epoxide fatty acids by cEH using gas chromatography, we found that this enzyme efficiently converts diepoxymethylstearate to the corresponding 9,10,12,13-tetraol (5). However, when the enzyme was diluted 2000-fold, no tetraol was obtained from diepoxymethylstearate. Instead, other metabolites with retention times different than that of the tetraol were formed. The present paper concerns the characterization of these hydration products formed from diepoxides of methylstearate and methylarachidonate by low concentrations of the cEH.

MATERIALS AND METHODS

Materials. All fatty acids and esters (*cis* isomers) were purchased from Nu Chek Prep, Inc. (Elysian, MN). *N*-Methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) was obtained from Pierce Chemical Company (Rockford, IL). *m*-Chloroperoxybenzoic acid (80%) was purchased from Kodak Laboratory and Research Products (Rochester, NY). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and were used without further purification.

Synthesis of 9,10:12,13-diepoxymethylstearate. To an ice-cooled solution of methyl linoleate (10 g) dissolved in 80 ml of dichloromethane, *m*-chloroperoxybenzoic acid (13 g) was slowly added and stirred for 18 h at room temperature. After partitioning with petroleum ether, the organic layer was sequentially washed with 5% aqueous $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed under reduced pressure and the diepoxide was purified by flash chromatography (silica gel 60, 230–400 mesh, E. Merck, Darmstadt, Germany) using hexane:ether (3:1). ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, deformed t), 1.1–1.85 (22H, m), 2.25 (2H, t), 2.75–3.20 (4H, m), 3.60 (3H, s).

Synthesis of cyclic compounds from diepoxymethylstearate. Diepoxymethylstearate (4 g) was dissolved in 45 ml tetrahydrofuran (THF):H_2O (7:2) and 5 ml of 5% $VHClO_4$ was slowly added. After 4 h stirring at room temperature, the reaction mixture was partitioned with ether, the organic layer was washed as described above, and the solvent was removed under reduced pressure. On TLC (silica gel 60) using hexane:ethyl acetate (2:1), two distinct spots were observed (A with low R_f and B with higher R_f). These products were separated into two fractions by flash chromatography. Fraction A was eluted using hexane:ethyl acetate (2:1) whereas fraction B was obtained by washing the column with hexane:ethyl acetate (1:1). Final yields for fractions A and B were 1.2 and 1.7 g, respectively. Fraction A ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, deformed t), 1.1–2.0 (21H, m), 2.26 (2H, t, J = 7 Hz), 2.2–2.5 (1H, m), 2.9–3.6 (2H, m, D_2O exchangeable), 3.2–3.7 (2H, m), 3.6 (3H, s), 3.7–4.1 (2H, m). Fraction B ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, deformed t), 1.0–1.7 (20H, m), 1.7–2.0 (2H, m), 2.1–2.3 (1H, broad d, D_2O exchangeable), 2.26 (2H, t, J = Hz), 2.4–2.5 (1H broad d, D_2O exchangeable), 3.2–3.5 (1H, m), 3.6 (3H, s), 3.5–3.8 (1H, m), 3.8–4.05 (1H, m), 4.05–4.3 (1H, m).

Synthesis of a 5,6-epoxide from arachidonic acid. Selective epoxidation of arachidonic acid was performed as described by Corey *et al.* (17) after a slight modification. Briefly, arachidonic acid (1 g) was dissolved in 12 ml THF, and subsequently 6 ml of H_2O containing 1.6 g $KHCO_3$ was added at 0–5°C. To this solution, a mixture of KI (4.3 g) and iodine (6.7 g) dissolved in 30 ml THF:H_2O (1:2) was added and kept in the dark at room temperature for 48 h. After partitioning with petroleum ether, the solvent was removed under reduced pressure and the residue was redissolved in 25 ml of THF:H_2O (3:2). Subsequently, 25 ml of aqueous 0.2 M LiOH was added dropwise at 0–5°C. After 3 h stirring at room temperature, the mixture was cooled and then acidified with oxalic acid to pH 4. The reaction mixture was partitioned twice with ether. The organic layer was washed with $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed under reduced pressure and the monoepoxide was isolated from starting material by flash chromatography using hexane:ether (4:1), yield 36%. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, deformed t), 1.1–3.2 (22H, m), 5.1–5.8 (6H, m), 9.13 (1H, broad s).

Synthesis of diepoxymethylarachidonates from 5,6-epoxide arachidonic acid. To convert 5,6-epoxyarachidonic acid to its methyl ester, excess diazomethane in an ether solution was added to 0.3 g of the 5,6-epoxide derivative and stirred for 15 min. The solvent was removed under reduced pressure and the 5,6-epoxymethylarachidonate was purified by flash chromatography using hexane:ether (10:1). To a cooled (ice bath) solution of 5,6-epoxymethylarachidonate (0.2 g) in dichloromethane, 0.15 g of *m*-chloroperoxybenzoic acid was added slowly and stirred for 15 h at room temperature. The dichloromethane solution was washed with 5% aqueous $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed under reduced pressure and the diepoxides were isolated from the starting material by flash chromatography. The column was washed with hexane:ether (5:1) to remove the starting material. Second elution with hexane:ether (3:1) gave 0.07 g of the diepoxides. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, deformed t), 1.1–3.1 (22H, m), 3.62 (3H, s), 5.2–5.7 (4H, m).

Source and purification of cytosolic epoxide hydrolase. Male and female Swiss Webster mice were obtained from Bantin Kingman (Fremont, CA). Animal treatment, subcellular fractionation of the mouse liver, and affinity purification of cEH on a benzylthiol derivative of Sepharose were performed as described in earlier papers (18, 19). In a typical purification experiment, 30 ml of 20% cytosol in 76 mM Na/K phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM diisopropylfluorophosphate (DFP) was passed through 0.5 ml of affinity gel. After the gel was washed with an equal volume of buffer to remove loosely bound proteins, cEH was specifically eluted with 4 ml of 0.1 mM 4-fluorochalcone oxide using gravity flow. The 4-fluorochalcone oxide was dialyzed against phosphate buffer for 2 h and enzyme purity was checked by discontinuous gel electrophoresis. The enzyme was apparently homogeneous using this technique, giving a single sharp band at approximately 60 kDa with both silver staining and Western blotting. The affinity-purified enzyme gave a single catalytically active peak on analytical isoelectric focusing corresponding to a protein band focusing at a pI of 5.6 pH units. Specific activity was determined using partition assay with *trans*-[^{3}H]stilbene oxide as substrate (11). The affinity-purified cEH was stable for at least 3 months with no significant loss of activity at 5°C.

Metabolism of diepoxides by cytosolic epoxide hydrolase. For standard incubations the affinity-purified enzyme was diluted 100 to 2000 times in 76 mM phosphate buffer (pH 7.4) containing 0.01% bovine serum albumin and 0.1 mM EDTA. One milliliter of the diluted enzyme was transferred into a test tube and preincubated at 37°C for 1 min. Then, 10 μl of 5 mM diepoxide fatty ester in ethanol was added to give the final substrate concentration of 0.05 mM. After incubation for 10 min, the reaction was stopped by adding NaCl. Unhydrolyzed diepoxide fatty ester together with hydration products was extracted twice with ether (1 ml). The organic layer was pooled and dried over anhydrous Na_2SO_4. All assays were done in duplicate and were repeated independently twice. The substrates were stable to extended incubations in buffer or in heat-inactivated enzyme.

Derivatization, gas chromatography, and gas chromatography–mass spectrometry. Prior to GC analysis, the sample was allowed to dry under a hood and the residue was dissolved in 40 μl of MSTFA:acetonitrile (1:1). The sample was incubated at 37°C for 2 h and the solvent was removed under a stream of nitrogen. The residue was redissolved in 50 μl hexane and 1–2 μl was injected into the GC.

GC analysis was carried out on a Hewlett–Packard 5890A gas chromatograph equipped with a flame ionization detector and a DB-23 or a DB-5 column (30 m × 0.25 mm, film thickness 25 μm, J&W Scientific, Folsom, CA). A temperature program of 150 to 240°C at 5°C/min was used. Mass spectrometric analysis was performed on a VG TRIO-2 with a VG 11-250 data system.

Nuclear magnetic resonance. ^{1}H-NMR spectra were recorded on a Varian EM 390. The spectra were obtained at ambient temperature using $CDCl_3$ as solvent. Chemical shifts are reported in parts per million in reference to tetramethylsilane.

RESULTS

To obtain the metabolic products of 9,10:12,13-diepoxymethylstearate, it was incubated with several dilutions of apparently homogeneous affinity-purified mouse liver cEH. Similar results were obtained with crude cytosol preparations except that some ester hydrolysis due to solubilized esterases occurred. Unhydrolyzed diepoxymethylstearate together with the metabolic products was extracted. Initially, the products were separated as TMS ethers on an OV-101-packed column and a capillary DB-5 column; however, a more polar column (DB-23) was used for later studies. In all cases the metabolites cochromatographed with their corresponding synthetic standards. Figure 1 shows GC traces of the cEH-catalyzed hydration products from diepoxymethylstearate. When the enzyme was diluted 100-fold (protein concentration ≥ 7.5 μg/ml) or less, diepoxymethylstearate gave only one metabolite with a retention time of 16.2 min (peak I in Fig. 1A). In a previous study (5), this product was shown to be 9,10,12,13-tetrahydroxymethylstearate based on mass spectral information; however, when greater enzyme dilution (1 to 2000, protein concentration 0.4 μg/ml) was used, the product with retention time 16.2 min disappeared but other peaks with retention times between 17.1 and 17.3 min were obtained (peak II in Fig. 1B).

For comparison of enzymatically and chemically generated hydration products, acid hydrolysis of diepoxymethylstearate was carried out as described under Materials and Methods. Two products (A with low R_f and B with higher R_f) were detected by TLC. Tetraol was never obtained under these conditions and the standard is a product of independent synthesis. Compounds A and B were separated from each other by flash chromatography using silica gel 60. Both fractions had similar retention times when analyzed as TMS ether derivatives on a capillary column (DB-23) (data not shown). Only products corresponding to peak II in Fig. 1 were obtained from the crude reaction mixture or from fractions A and B following separation by silica gel chromatography.

Direct probe mass spectral analysis of fractions A and B as TMS ether derivatives gave similar fragmentation patterns. In both mass spectra, the highest mass peak at *m/z* 488 was assigned as the parent ion. This parent ion assignment was also supported by data from positive chemical ionization with isobutane (data not shown). The spectra displayed strong signals at *m/z* 398 and 308 corresponding to M-TMS and M-2TMS, respectively. In addition, the mass spectra of fractions A and B contained signals at *m/z* 259/229 and 315/173, referring to cleavage of TMS ether carbons next to a cyclic ether moiety. The mass spectrum of fraction A is shown in Fig. 2. The total parent ion was shifted to 345 *m/z* when fractions A and B were analyzed as free alcohols (data not shown). Again, the presence of two hydroxy groups was supported by signals at 327 and 309 *m/z* referring to M-H_2O and M-2H_2O.

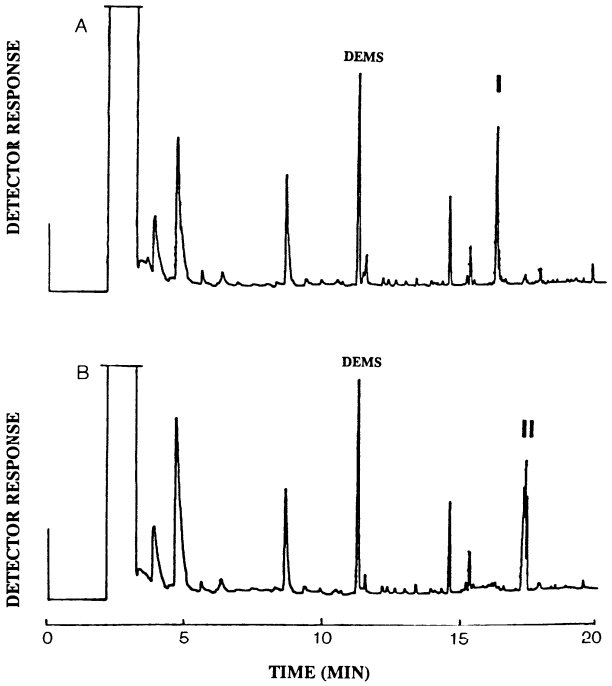

FIG. 1. Gas chromatographic tracing of metabolites of 9,10:12,13-diepoxymethylstearate (DEMS) produced by concentrated (A) and highly diluted (B) affinity-purified cEH from mouse liver. For these tracings, the substrate was incubated with two enzyme concentrations (7.5 and 0.4 μg/ml), and the metabolites were extracted, derivatized to TMS ethers, and separated on a DB-23 column as described under Materials and Methods. Only peaks labeled I (tetraol) and II (cyclic products) were due to substrate metabolism. These products were absent in the absence of catalytically active enzyme. Synthetic standards gave single sharp peaks with similar retention time. In separate experiments, these standards were cochromatographed with the metabolites tentatively identified as tetraol and cyclic products. The other minor peaks in the chromatogram are from organic impurities in the enzyme solution and in solvents used under these conditions. None are metabolically produced or are breakdown products of the substrate.

The mass spectra are consistent with both fractions A and B consisting of a mixture of positional isomers.

^{1}H-NMR spectra of fraction A displayed two hydroxy proton signals downfield (2.9–3.6 ppm) which were exchanged by D_2O. The corresponding chemical shifts for the hydroxy groups in fraction B were in the range 2.1–2.5 ppm. The presence of only two OH groups in each case was also supported by the NMR of acetylated fractions, indicating two characteristic methyl proton signals at about 2.05 ppm and mass spectral analysis of deuterated and acetylated products. Moreover, NMR spectra of fractions A and B exhibited signals at 3.2–3.7 ppm, implying protons bound to oxygen. All epoxides were *cis* in geometry and racemic. Hence, all of the products consist of a complex mixture of optical isomers and diastereomers which are beyond the scope of the current work.

The disappearance of the substrate (diepoxymethylstearate) was linearly dependent on time to at least a

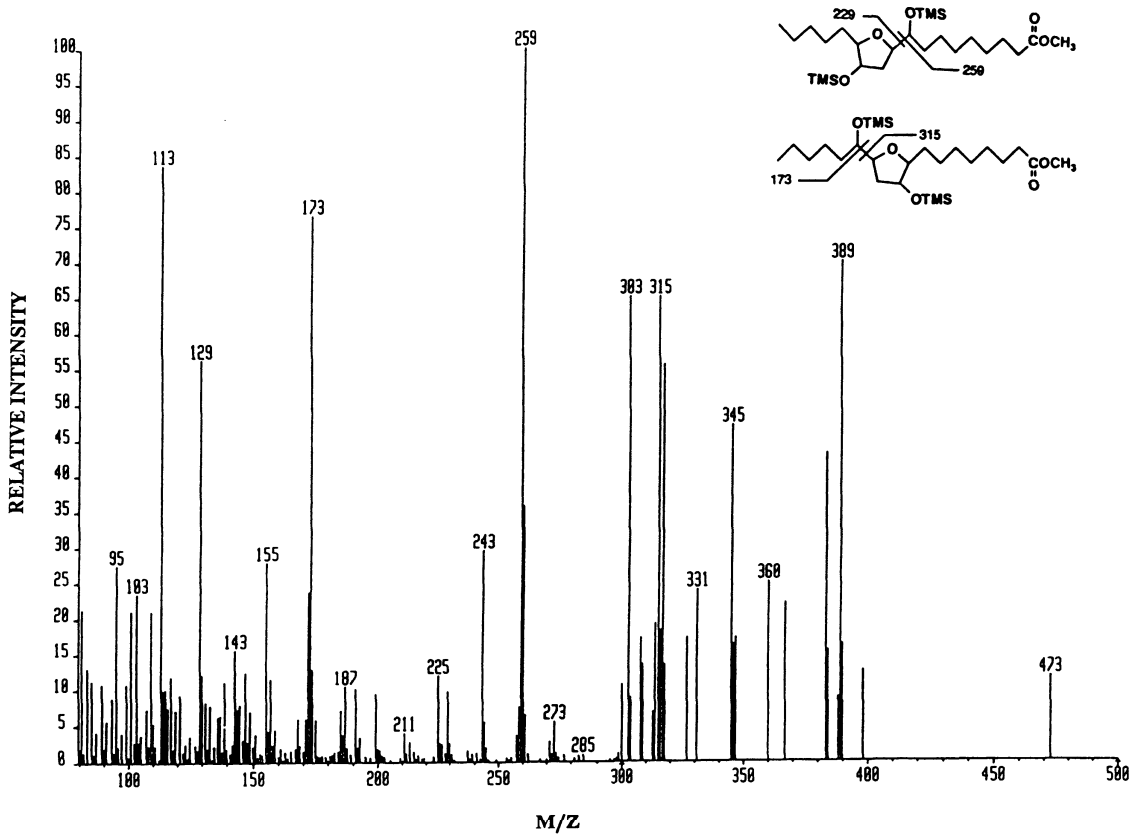

FIG. 2. GC–MS of chemically generated cyclic diols from 9,10:12,13-diepoxymethylstearate. Reaction products were separated into two fractions (A and B) by flash chromatography using silica gel 60 and analyzed as TMS ether derivatives as described under Materials and Methods. Spectra were obtained in the electron impact mode. Spectra of fractions A and B were apparently identical. The spectrum of fraction A is shown. The inset shows the two positional isomers apparently present in both fractions A and B.

level of 40% substrate metabolism at all of the enzyme concentrations used. In addition, substrate disappearance was linearly dependent upon protein concentration over a wide range of cEH concentrations and crude cytosol preparations. The surprising observation was that the mobility of the metabolites produced changed on both TLC and GC when the concentration of the enzyme was reduced.

Because the concentration of the affinity-purified cEH appeared to determine the relative amount of tetraol and putative cyclic products, a concentration study was done (Fig. 3). The diepoxymethylstearate was metabolized to the corresponding tetraol by the concentrated enzyme. As the enzyme was diluted 100-fold ($\geq$7.5 μg/ml) or more, formation of two products with a higher R_f on TLC and a different retention time on GC than the tetraol were observed. Since these metabolites were stable to aqueous acid, the possibility that they were the epoxy diol was ruled out. Because these materials cochromatographed with the cyclic products from synthesis they were tentatively identified as these tetrahydrofuran diols.

As the enzyme concentration was reduced, the amount of tetraol metabolite decreased with an increase in the amount of putative cyclic products. Only cyclic products were obtained with highly diluted enzyme (1000- to 2000-fold, corresponding to a protein concentration of 0.4 μg/ml or below). No metabolites were produced in the absence of catalytically active enzyme, and no chemical decomposition of the starting diepoxides was observed. Prior to or following separation into fractions A and B, both the chemically produced and the enzymatically produced compounds gave similar retention times and apparently identical mass spectra as their TMS ethers.

To characterize the hydration products from diepoxymethylarachidonate, 5,6-epoxymethylarachidonate was synthesized, and then it was converted to a mixture of diepoxides present at the other three possible olefinic sites. This mixture was incubated with concentrated as well as diluted enzyme; unhydrolyzed substrate together with hydration products were partitioned, derivatized, and examined by GC–MS with and without prior TLC separation of metabolites from the parent diepoxides. Exposure

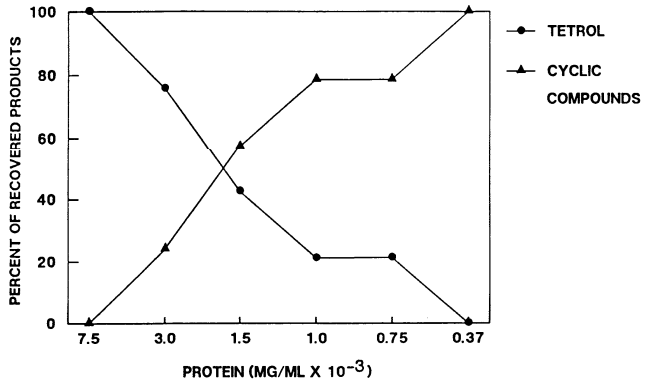

FIG. 3. Effect of enzyme concentration on the formation of tetraol vs cyclic products from 9,10:12,13-diepoxymethylstearate. Affinity-purified cEH from mouse liver was diluted with buffer to several protein concentrations, substrate was added and incubated for 10 min, and the metabolites were extracted, derivatized to TMS ethers, and analyzed on a capillary DB-23 column as described under Materials and Methods. The values are the means of duplicate determinations. At concentrations lower than 0.37 μg/ml cEH only cyclic products were produced. In buffer controls no apparent metabolites were detected.

of the resulting metabolites to aqueous acid did not result in any changes in GC retention times, indicating that none of the products were epoxy diols.

Concentrations of cEH over 7.5 μg/ml yielded primarily products with retention times of 15 and 17 min (Fig. 4). The mass spectra of all peaks in the range 15 to 19 min support the assignment of tetraol structures. As an example, the mass spectrum of the major product with a retention time of 17 min is shown in Fig. 5. The signal at 675 represents the parent ion. The peaks at *m/z* 585, 495, 405, and 315 indicate the presence of four hydroxy groups. The splitting between two hydroxy groups gave major ions at *m/z* 173 and 203. Thus, the peak with a retention time of 17 min in Fig. 4 was identified as consisting largely of 5,6,14,15-tetrahydroxymethylarachidonate, but other positional isomers clearly are present. The peak with a retention time of 15 min consisted at least in part of the 5,6,8,9-tetraol. Additional separation chemistry is needed for positive identification of the other isomers.

When the enzyme was progressively diluted, the overall rate of substrate disappearance decreased. In addition, as the enzyme was diluted to 1000- to 2000-fold, the progressive appearance of peaks with new retention times occurred. The major peaks had retention times of 12 and 13 min (Fig. 4). The mass spectra of these materials gave fragments consistent with the presence of tetrahydrofuran diols (Fig. 4). As with higher enzyme concentrations, the GC mobility of the products was not influenced by treatment with aqueous acid, indicating the absence of epoxy diols. The progressive disappearance of the tetraol peak with a retention time of 15 min, with the concomitant appearance of the putative tetrahydrofuran- diols at retention times of 12 and 13 min, is consistent with the mass spectral evidence indicating that the peak at 15 min consists largely of the 5,6,8,9-tetraol and the peaks at 12 and 13 min are largely a mixture of 5,8 and 6,9-tetrahydrofuran diols. One would expect that putative epoxy diols not involving the corresponding 5,6 and 8,9 positions would be less likely to cyclize and could yield a variety of products if not metabolized to the corresponding tetraols. Structural assignment of the other minor peaks shown in Fig. 4 based only on their mass spectra was felt conjectural at this time. The chromatographs in Fig. 4 come from long incubation times to eliminate the possibility that thermal rearrangement of the substrate leads to the observed products. Shorter incubation times give similar chromatograms except for the presence of the substrate peak. Intermediate protein concentrations yielded chro-

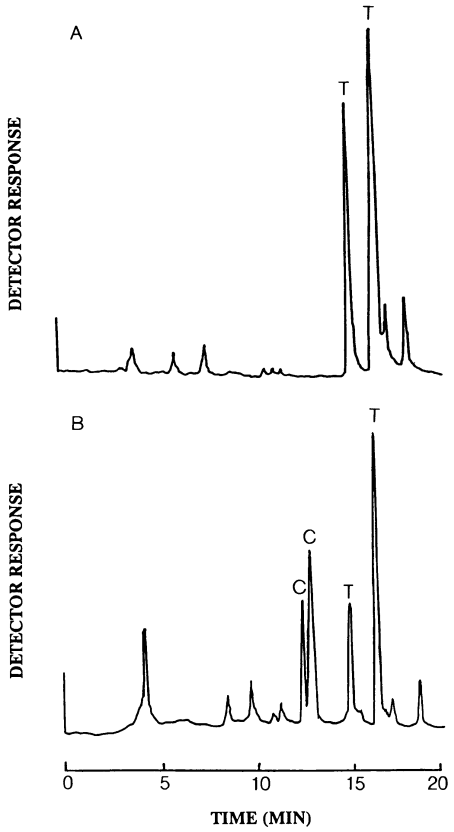

FIG. 4. Gas chromatographic tracing of TMS derivatives of metabolites produced from incubation of diepoxymethylarachidonate with high concentrations (A) or low concentrations (B) of affinity-purified cEH (7.5 and 0.4 μg/ml, respectively). The mass spectra of peaks marked "T" (primary retention times of 15 and 17 min) gave key fragments (*m/z* 585, 495, 405, 315, 203, and 173) indicative of tetraol structures, whereas the spectra of peaks marked "C" (primary retention times of 12 and 13 min) gave key fragments (*m/z* 423, 333, 259, and 203) indicative of cyclic tetrahydrofuran-containing moieties based on analogy with the cyclic products from the diepoxymethylsterate series. The metabolites were extracted, derivatized to TMS ethers, and separated on a DB-5 capillary column as described under Materials and Methods.

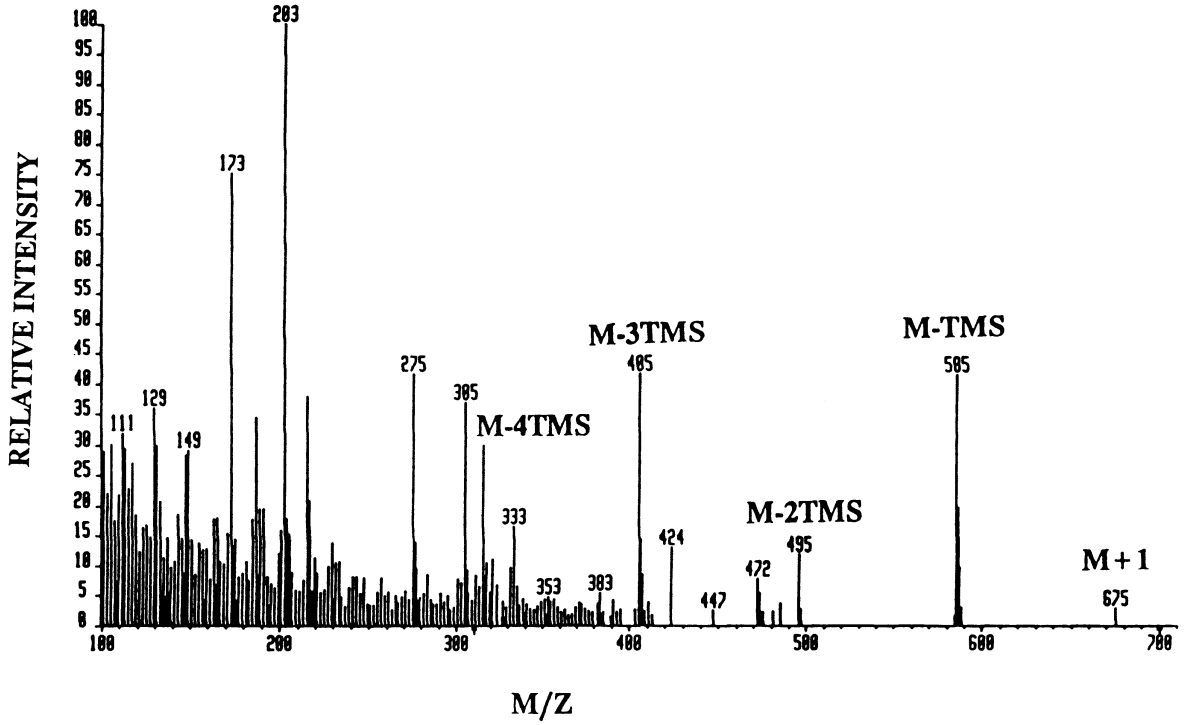

FIG. 5. Mass spectrum of a tetraol of methylarachidonate. A mixture of diepoxymethylarachidonates was incubated with affinity-purified cEH (7.5 μg/ml), and the products were isolated, derivatized with TMS, and analyzed by GC-MS. The spectrum was obtained in the electron mode impact. The parent ion was verified using isobutane to obtain positive chemical ionization in a separate spectrum.

matograms with higher concentrations of the tetraol peak with a retention time of 15 min and lower concentrations of the peaks marked C at 12 and 13 min corresponding to putative tetrahydrofuran diols.

DISCUSSION

Formation of cyclic diols from olefinic 1,5-diepoxides occurs when one of the epoxides is opened, and then one of the hydroxy groups attacks the remaining epoxide to form a 5-, 6-, or 7-membered ring. Analogous materials can form from hydroxy epoxides. Hammock *et al.* (20) have shown that when diol analogs of juvenile hormone are oxidized under neutral conditions, an epoxy diol intermediate is formed. The epoxy diol was rapidly cyclized to form *cis*- and *trans*-tetrahydrofuran diols as the predominant products. Similar reactions have been demonstrated both chemically and enzymatically for terpenoid oxides and related compounds (20–25). Structure of the *cis*- and *trans*-tetrahydrofuran diols for the terpenes has been established unequivocally by synthesis (20) and two-dimensional NMR (21).

In this study, diepoxymethylstearate was used as the model compound for analogous 1,4-diepoxides because it is easily synthesized and because this oxidized fatty ester is an excellent substrate for cEH. Acid hydrolysis of diepoxymethylstearate gave exclusively two products (A with low R_f and B with higher R_f on TLC). These were isolated and purified by column chromatography on silica gel 60. Based on low-field ^{1}H-NMR structure determination, both fractions A and B contained two hydroxy groups and a cyclic ether moiety. Initially, we hypothesized that these fractions were tetrahydrofuran diols and tetrahydropyran diols based on chromatographic behavior on silica gel 60 and slight differences in ^{1}H-NMR signals; however, when fractions A and B were analyzed as TMS ether derivatives by mass spectrometry, apparently identical fragmentation patterns were obtained (Fig. 2). The highest signal at *m/z* 488 is assigned as the parent ion. The signals at *m/z* 398 and 308 correspond to M-TMS and M-2TMS, respectively. These findings suggest that tetrahydropyran diols from chemical hydrolysis of diepoxymethylstearate can in theory form; however, they were very minor products if present at all. Interestingly, both spectra of fractions A and B contained strong signals at *m/z* 259/229 and 315/173, indicating fragmentation between the TMS ether carbon attached to a cyclic ether moiety.

Enzymatic hydrolysis of diepoxymethylstearate led to tetraols and/or cyclic diols depending on the concentration of affinity-purified cEH. At an enzyme concentration

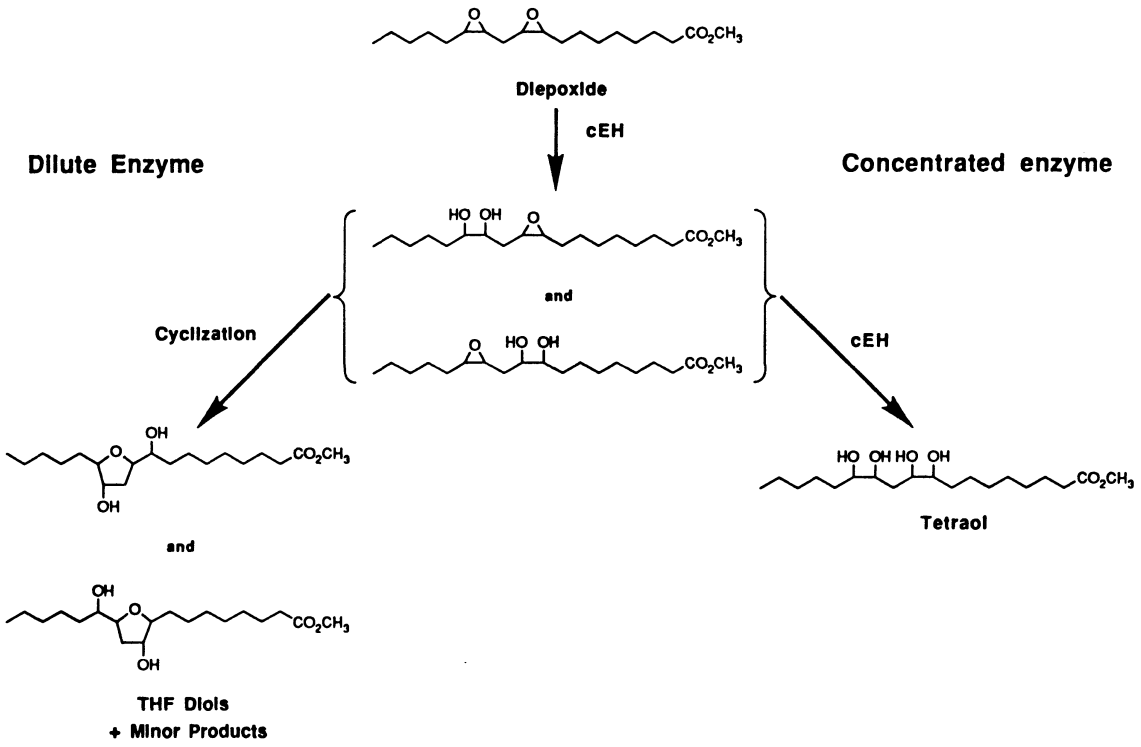

FIG. 6. Proposed pathway for the metabolism of 9,10:12,13-diepoxymethylstearate by mouse liver cEH. The metabolites obtained at both high (7.5 μg/ml) and low (0.4 μg/ml) concentrations of the affinity-purified enzyme are consistent with the presence of an epoxy diol intermediate.

of ≥7.5 μg/ml, the diepoxymethylstearate was metabolized exclusively to tetraols. As enzyme concentration decreased to levels more typical of the *in vivo* situation, disappearance of tetraol occurred simultaneously with the elevation of cyclic products (Fig. 3). Enzymatically and chemically generated cyclic products had similar retention times, co-chromatographed on TLC and GC, and gave virtually identical mass spectra. In the case of methylarachidonate, the apparent difficulty in synthesizing one positional diepoxide led us to prepare a mixture of diepoxides. Since the diepoxymethylarachidonate mixture was synthesized from the 5,6-epoxide derivative, the 5,6:8,9-diepoxymethylarachidonate is the only compound which corresponds to a 1,4-diepoxide and thus could form tetrahydrofuran diols in addition to the corresponding tetraol. Other diepoxides can form epoxy diols and/or tetraols but cannot cyclize in a manner analogous to the 1,4-systems. The diepoxymethylarachidonate mixture was efficiently metabolized to the corresponding tetraols with concentrated enzyme even though the 5,6-monoepoxide is not a preferred substrate among the monoepoxyarachidonate esters (13). With diluted enzyme, several hydration products were formed out of which at least two peaks had mass spectra indicating structures likely to be tetrahydrofuran diols based on the fragmentation pattern of cyclic products from diepoxymethylstearate.

Figure 6 shows the proposed pathway for *in vitro* catabolism of diepoxymethylstearate by affinity-purified mouse liver cEH. The fact that the tetraol is the sole product from diepoxide fatty esters with concentrated affinity-purified cEH suggests that epoxy diols are intermediates. In this study, the samples were kept under a hood for at least 24 h before derivatization and GC analysis. Therefore, epoxy diol intermediates could not be seen in any of our samples. Thus, conversion of diepoxides to cyclic products by cEH presents an entirely new set of lipids which may have certain unique functions in biological systems. Although many of the secondary metabolites of arachidonic acid have not been isolated from tissues, there is a definite possibility of their occurrence *in vivo* from either 1,4-diepoxides or from monohydroxy derivatives which can cyclize to yield 5- or 6-membered ring structures.

In conclusion, the results of this study show conclusively that at high concentrations of cEH, diepoxides in the lineolate and arachidonate series are metabolized exclusively to a presumably diastereomeric mixture of tetraols. As the concentration of enzyme is reduced there is a progressive increase in the proportion of less polar metabolites. At low enzyme concentrations diepoxymethylstearate yields exclusively these metabolites whose structures were shown to be complex mixtures of tet-

rahydrofuran diols based on a combination of mass spectral evidence and chromatographic comparison with products of acid-catalyzed hydration. Production of both the tetraols and tetrahydrofuran diols from the corresponding 1,4-diepoxide systems clearly is enzymatic. Mass spectral evidence and chromatographic behavior strongly suggest that similar products are formed in the arachidonate series. The production of tetraols at high concentrations of cEH and tetrahydrofuran diols at low concentrations of cEH is consistent with the enzyme-dependent formation of a putative epoxy diol intermediate whose cyclization could be chemical or enzymatic. The isolation of this epoxy diol intermediate awaits further study using less harsh analytical conditions than those for GC.

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