

HYDRATION OF CIS- AND TRANS-EPOXYMETHYL STEARATES
BY THE CYTOSOLIC EPOXIDE HYDRASE OF MOUSE LIVER

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SUMMARY: Man is exposed to epoxides of fatty acids from a number of sources, yet their degradative metabolism is not well understood. In mouse liver the 100,000 g supernatant or the cytosolic fraction is the most active fraction in hydrating cis- and trans-epoxymethyl stearates with the oxirane ring opening in a trans manner to give the corresponding threo and erythro diols, respectively. Hydration was also observed in the microsomal, nuclei and cell debris, and mitochondrial fractions in decreasing order of specific activity.

Epoxides of fatty acids occur in fungi and seed oils of numerous plants, they are major constituents of some plant cutins and suberins, and they have also been found in pulp mill effluents (1-7 and included references). In addition, fatty acid epoxides can be formed through hydroperoxide intermediates or apparently by direct oxidation of olefins by plant or mammalian enzymes or by autoxidation (8-10). Since epoxidized fatty acids are undoubtedly present in the environment and in man's diet, in addition to being formed in vivo, an understanding of the enzymatic routes leading to their metabolism is important.

The epoxide hydrases, which hydrate epoxides to 1,2-diols, provide one obvious route for the mammalian metabolism of epoxidized fatty acids. These enzymes are important in the metabolism of many olefinic or aromatic xenobiotics which have been previously oxidized to epoxides.

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Of the epoxide hydrases, the membrane bound microsomal epoxide hydrases have received a great deal of attention due to their ability to hydrate potentially toxic, mutagenic, and carcinogenic xenobiotics (11-13). Previous studies on the metabolism of cis- and trans-9,10-epoxystearic acids in rats and rabbits failed to report extra microsomal hydration (9, 14). The following report concerns the hydration of the methyl esters of cis- and trans-9,10-epoxystearic acid by epoxide hydrases in the cytosolic and other subcellular fractions of mouse liver.

MATERIALS AND METHODS

Chemicals: [1-¹⁴C] Oleic acid (ICN, Irvine, CA, 1.11 GBq/mmol) was esterified with diazomethane and epoxidized with 2 molar excess of m-chloroperoxybenzoic acid to yield cis-9,10-epoxymethyl stearate. The radioactive product was purified by thin-layer chromatography (tlc) in petroleum ether: ether (10:1) to >99% chemical purity and >98% geometrical purity. It showed one radioactive spot on tlc using silicagel G plates in several solvent systems and it co-chromatographed with authentic cis-epoxymethyl stearate, which was synthesized from oleic acid (Mallincrodt, St. Louis, MO) (redistilled as the methyl ester). trans-Epoxymethyl stearate was similarly synthesized from elaidic acid (Sigma Chemical Co., St. Louis, MO). Erythro- and threo-9,10-dihydroxystearic acids were synthesized according to Swern (15, 16) using H₂O₂ and formic acid and were then esterified with diazomethane. All chemical structures were confirmed by their nuclear magnetic resonance spectra, infra-red spectra, and melting points. These data agreed with literature values and their methyl esters demonstrated >98% geometrical purity on gas liquid chromatography (glc).

Enzyme preparation: Livers of male Swiss-Webster mice (30-40 g) were homogenized in sodium phosphate buffer (pH 6.8, I = 0.2M) containing diisopropylfluorophosphate (DFP, 1 x 10⁻⁴M) to give a 10% (w/v) homogenate. Cytosolic epoxide hydrase is not inhibited by DFP at concentrations up to 1 x 10⁻³M. Differential and sequential centrifugation of the homogenate at 800 g for 10 min, the 800 g supernatant at 10,000 g for 10 min, and the 10,000 g supernatant at 100,000 g for 1 hr provided fractions referred to as the cell nuclei and debris, mitochondrial, and microsomal fractions, respectively. The 100,000 g supernatant was used as the crude soluble fraction or it was partially purified by passing it through a Sephacryl S-200 column (86 x 2.5 cm, ID) using sodium phosphate buffer as the elutant (18). Mitochondria and microsomes were resuspended and recentrifuged prior to use. The various subcellular fractions were diluted with cold buffer to give appropriate protein concentrations, which were determined by the method of Lowry et al. (17) using bovine serum albumin as the standard.

Enzyme assay: Ten min after the addition of DFP (to 1 x 10⁻³M in 1 μ l ethanol) to the enzyme (100 μ l) held at 37°C, the substrate [1-¹⁴C]epoxymethyl stearate (2 x 10⁻⁶M to 5.2 x 10⁻⁵M, in 2 μ l ethanol) was added. After a second 10 min incubation the reaction was terminated by addition of NaCl followed by immediate extraction with ether (2 x 250 μ l). The ether phase was analyzed by tlc using a hexane:ethyl acetate:acetic acid

(30:20:1) solvent system. The radioactive spots detected by tlc radio-scanning and/or autoradiography were then scraped and analyzed by liquid scintillation counting. Unlabeled standards were detected by spraying with a 0.01% (w/v) aqueous solution of 8-anilino-1-naphthalene sulfonic acid and detecting their fluorescence at 360 nm. All of the reported data come from enzyme reactions run under conditions of substrate saturation. In each case, there was more than 3% but less than 20% hydration of the substrate. The hydration of cis-epoxymethyl stearate is linearly dependent upon the substrate and enzyme concentrations as well as the incubation times utilized until 50-60% of the substrate is consumed. Epoxide hydration of unlabelled cis- and trans-epoxymethyl stearate was similarly analyzed by incubating the substrate in enzyme (1 ml) for appropriate times followed by addition of NaCl and extraction with ether (2 x 1 ml). The ether phase was dried with Na₂SO₄ and evaporated under nitrogen. The residue was reacted with n-butylboronic acid (20 µg) in isooctane (50 µl), and subsequently analyzed by gas-liquid chromatography (glc) (Varian 1400 interfaced with a Hewlett-Packard 3380A recording integrator; 2% OV-101 on Gas-Chrom Q in a 2 mm x 2 m glass column; column temperature 250°C; nitrogen flow 20 ml/min); to determine the ratio of epoxide:diol n-butylboronic ester. Identification of the erythro and threo diols of methyl stearate formed by enzymatic hydration of epoxymethyl stearate was confirmed by glc of the n-butylboronic esters of authentic standards.

RESULTS AND DISCUSSION

All of the subcellular fractions of male Swiss-Webster mouse liver are capable of hydrating cis-epoxymethyl stearate with the highest epoxide hydrase activity observed in the 100,000 g supernatant or cytosolic fraction (Table 1). Significant activity is also found in the three other subcellular fractions: the microsomal, nuclear and cell debris, and mitochondrial in decreasing order of activity. However, when activity is calculated on a tissue weight equivalent, the lowest activity is found in the microsomal fraction. The rates of hydration reported here for the microsomal fraction compare favorably with published data (9, 14). Activity in the microsomal and mitochondrial fractions is probably membrane bound or trapped in organelles, since it is not possible to remove all the inherent activity by resuspending and recentrifuging either the microsomal or mitochondrial fractions. A significant amount of activity in unwashed microsomes is due to contamination from the cytosolic fraction. Contamination of the mitochondrial pellet by some microsomes is possible. However, preparation of mitochondria by established methods in isotonic sucrose (19) still resulted

TABLE I
 HYDRATION OF CIS-9,10-EPOXYMETHYL STEARATE BY THE
 SUBCELLULAR FRACTIONS OF MALE SWISS-WEBSTER MOUSE LIVER^a

Subcellular Fraction	Specific Activity ^b	
	nmoles min ⁻¹ mg protein ⁻¹	pmoles min ⁻¹ mg tissue equiv. ⁻¹
Cell nuclei and debris	3.7 ± 0.7	460 ± 82
Mitochondria	3.5 ± 0.6 ^c	380 ± 61
Microsomes	5.2 ± 0.6	95 ± 10
Soluble fraction	21.1 ± 0.6 ^d	1300 ± 40

^aMice used were approximately 10 weeks old. ^bDetermined at a minimum of two protein concentrations done in triplicate from pooled mice livers of three mice. Values reported are means ± S. D. The entire experiment was repeated twice. The presence of DFP does not change the subcellular distribution of enzyme activity. Substrate level used $5.2 \times 10^{-5}M$, 10 min incubation. ^cThe specific activity of the mitochondrial fraction can be increased by repeated washing. ^dHydration of the trans-isomer was ~10% faster than the cis-isomer.

in significant activity being present in the mitochondria with minimal microsomal contamination. The presence of epoxide hydrase activity in the nuclear fraction and debris has recently been demonstrated to occur in the nuclear membranes (20, 21), and hydration of styrene oxide and 4,5-benzopyrene oxide is thought to be due to the same enzyme present in the microsomal fraction (22).

The epoxide hydrase activity in the cytosolic fraction is not due to contamination with glutathione S-epoxide transferases since similar activity is observed in fractions passed through Sephacryl S-200 and no dependence on GSH is observed (18, 23-25). There was no hydration of styrene oxide or cis-stilbene oxide in the cytoplasmic fraction indicating that contamination with microsomal epoxide hydrase(s) was insignificant. The cytosolic epoxide hydrase also has a higher molecular weight (130,000) and a different substrate specificity (26, 27) when compared to the microsomal epoxide hydrase.

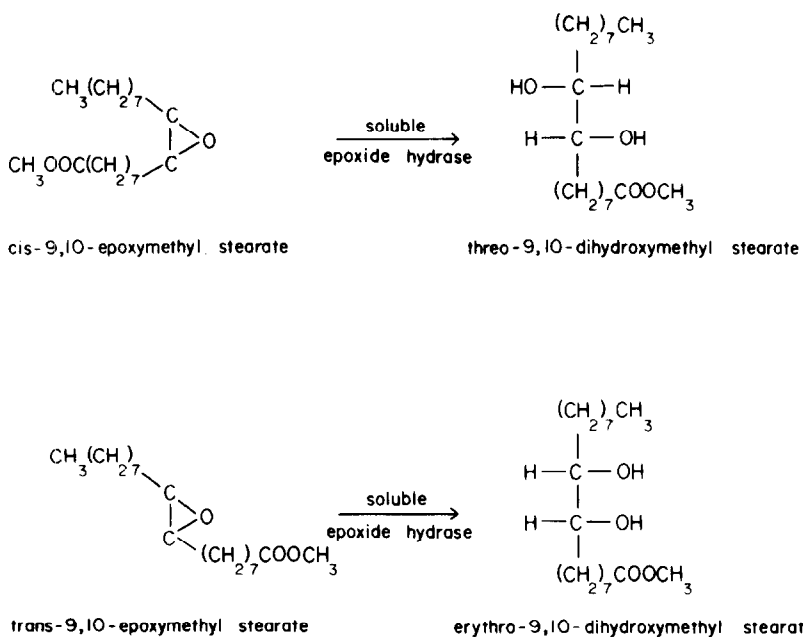


FIG.1. Stereospecific hydration of cis- and trans-9,10-epoxymethyl stearates to their respective threo and erythro diols.

The cytosolic epoxide hydrase is capable of hydrating both cis- and trans-epoxymethyl stearates with the hydration of the trans-isomer slightly more rapid than the cis-isomer at 4 different incubation times ($p = 0.05$). cis-Epoxy stearic acid is also rapidly hydrated. Hydration of the isomers is stereospecific since only the threo diol is obtained from the cis-epoxymethyl stearate while the trans-epoxymethyl stearate gives only the erythro diol. These data indicate that the oxirane ring opening of epoxymethyl stearates occurs in a trans manner (Fig. 1) as previously observed with microsomal epoxide hydrase mediated hydration of cis- and trans-epoxystearic acids (9, 14) and the cytosolic epoxide hydrase acting on other substrates (26, 27).

This study provides further evidence against a widely held misconception that all mammalian epoxide hydrase activity is microsomal or at least membrane bound based on a few studies of subcellular distribution of enzyme activity using a limited number of substrates (11, 28).

It is therefore imperative that investigations involving epoxide hydrase activity in mammals and other vertebrates begin with a thorough study of subcellular distribution of epoxide hydrase utilizing the epoxide under study as substrate. Such studies with mouse liver have demonstrated that cis- and trans-epoxymethyl stearates are most rapidly hydrated in the cytosolic fraction although they are rapidly hydrated in other subcellular fractions.

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