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ω-hydroxylation of *Z*9-octadecenoic, *Z*9,10-epoxystearic and 9,10-dihydroxystearic acids by microsomal cytochrome P450 systems from *Vicia sativa*

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Summary: A microsomal fraction from etiolated *Vicia sativa* seedlings incubated aerobically with [1-^{14}C]oleic acid (*Z*9-octadecenoic acid) or [1-^{14}C]9,10-epoxystearic acid or [1-^{14}C]9,10-dihydroxystearic acid catalyzed the NADPH-dependent formation of hydroxylated metabolites. The chemical structure of compounds formed from oleic, 9,10-epoxystearic or 9,10-dihydroxystearic acids was established by gas chromatography/mass spectra analysis to be 18-hydroxyoleic acid, 18-hydroxy-9,10-epoxystearic acid and 9,10,18-trihydroxystearic acid, respectively. The reactions required O_2 and NADPH and were inhibited by carbon monoxide. As expected for monooxygenase reactions involving cytochrome P450, inhibition could be partially reversed by light and all three reactions were inhibited by antibodies raised against NADPH-cytochrome P450 reductase from Jerusalem artichoke. The ω-hydroxylation of the three substrates was enhanced in microsomes from clofibrate induced seedlings.

Plants are isolated from the outer environment by polymerized layers: cutin and suberin. Major constituents of cutins which are associated with aerial parts of plants, are aliphatic components such as ω-hydroxy and di-hydroxy fatty acids of the C16 family and 18-hydroxy,

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; RP-HPLC, reverse-phase high-pressure liquid chromatography; TLC, thin-layer chromatography.

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18-hydroxy-9,10-epoxy and trihydroxy fatty acids of the C18 family. Suberins, which are associated with the below ground portions of plants, contain aliphatic components but phenolic materials are also found. Aliphatic components of suberin are essentially ω-hydroxy and dicarboxylic fatty acids of the C16 and C18 families (1). The cutins and suberins differ mainly in the relative proportions of monomer classes. It has been shown that the composition of cuticle, in terms of relative proportion of monomers, undergoes constant change during leaf development (2) Most enzymes involved in the biosynthetic pathway of these phytopolymers have been characterized by Kolattukudy's group (3). Based on this work, Kolattukudy proposed a scheme where palmitic acid was first ω-hydroxylated and 16-hydroxypalmitic acid was subsequently in-chain-hydroxylated by a microsomal preparation from germinating *Vicia faba* to give rise to 9 (or 10), 16-dihydroxypalmitic acid (4). Carbon monoxide inhibited these two reactions suggesting the involvement of P450 but only the activity of the in-chain hydroxylase system was reversed by light. Enzymatic conversion of 16-hydroxy- and 16-oxo-palmitic acids to dicarboxylic acids by cell-free preparation from the epidermis of *Vicia faba* leaves was also demonstrated. Moreover, this particulate fraction catalyzed incorporation of 16-hydroxy and 10,16-dihydroxypalmitic acids into polymer fractions. Similarly, it was demonstrated that 18-hydroxyoleic acid was converted to 18-hydroxy-9,10-epoxystearic acid by a particulate fraction (3000*g* sediment) from spinach leaves (5). The reaction required O_2 and NADPH but also ATP and coenzyme-A. A similar particulate fraction from apple skin catalyzed epoxide hydration of 18-hydroxy-9,10-epoxystearic acid to 18-hydroxy-*threo* 9,10-dihydroxystearic acid with high specificity for the substrate (6). Incorporation of oleic acid into ω-hydroxyoleic acid of envelope monomers was also reported but the hydroxylating enzyme system was not characterized (7). Thus it appears that ω–hydroxylation of C16 and C18 fatty acids is a key reaction for the biosynthesis of plant cuticular membranes.

We have recently reported that a microsomal fraction from clofibrate induced *V. sativa* seedlings was able to catalyze ω-oxygenation of medium chain fatty acids (C10-C14) (8) and of a series of monounsaturated (Δ 7-11) lauric acid analogs (9). All these ω-hydroxylation activities were enhanced 30-100 fold when seedlings were treated with clofibrate. In all cases involvement of cytochrome P450 was clearly demonstrated. Moreover, it has recently been shown in our laboratory that a microsomal hydroperoxide-dependent epoxygenase from soybean can catalyze epoxidation of unsaturated stearic acid

analogs (10). A similar reaction was also found in microsomes from *Vicia faba* seeds (11). In the course of our studies of cytochrome P450 monooxygenases catalyzing fatty acids oxydations, we have found that microsomes from *V. sativa* can hydroxylate the methyl end of oleic acid and of oxygenated derivatives.The present paper is concerned with the characterization of the enzyme systems involved in these reactions and with the structure of the metabolites that are formed.

Materials and Methods. Chemicals. Radiolabeled substrates [1-^{14}C]stearic acid (57 Ci/mol), [^{14}C]triacylglycerol-trioleate (80 Ci/mol) and [1-^{14}C]oleic acid (54 Ci/mol) were from CEA (Gif-sur Yvette, France). Phenylmethylsulfonyl fluoride (PMSF) and thin-layer plates (Silica gel G60 F254 and RP-18 F254 S, 0.25mm) were from Merck (Darmstadt, Germany). NADPH was purchased from Sigma Chimie (La Verpillière, France), tetrahydrofuran (THF) and *bis* (trimethylsilyl)-trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA + 1% TMCS) were from Pierce Europe (Oud-Beijerland, The Netherlands).
Racemic sample of [1-^{14}C]*Z* 9,10-epoxystearic acid was synthetized from [1-^{14}C]oleic acid using m-chloroperoxybenzoic acid. The corresponding diol, [1-^{14}C]*threo* 9,10-dihydroxystearic acid, was prepared by acidic hydrolysis of epoxide.
Microsomal preparations. 4 days old etiolated *Vicia sativa* L. (var. minor) seedlings were aged for 48 h in distillated water or in 1 mM clofibrate solution before isolation of the microsomal fractions as described in (9, 12). In the present studies one half of the final microsomal preparation contained 2.5 mM β-mercaptoethanol.
Cytochrome P450 was measured as in (13) and microsomal proteins were estimated by a microassay procedure from Biorad using bovine serum albumin as a standard.
Enzyme activities and inhibitions. ω-hydroxylase activities were determined by following the rate of hydroxylated products formation. The standard assay contained in a final volume of 0.2 ml, 0.19-0.43 mg of microsomal protein, 20 mM phosphate buffer (pH 7.4), 1 mM NADPH and radiolabeled oleic acid (60 μM), *Z* 9,10-epoxystearic acid (30 μM) or 9,10-dihydroxystearic acid (30 μM). The reaction was initiated at 27°C by adding NADPH and stopped with 0.2 ml acetonitrile/acetic acid (99.8:0.2, v/v) after 30 min incubation period. The reaction products were extracted twice into 1 ml of diethyl ether and initially resolved by TLC and RP-HPLC as described below. Product formation was quantified by liquid scintillation.
For carbon monoxide inhibition studies, the assays were equilibrated under a continuous stream of a CO-O_2-N_2 (2:2:8, v/v/v) gas mixture at 4°C, during 20 min. The vials were transferred to a water bath at 27°C, the reaction was initiated by addition of NADPH either in darkness or under white light from a 15 cm distant 150 W heat filtered quartz lamp.
Rat monoclonal antibodies raised against NADPH-cytochrome P450 reductase from Jerusalem artichoke tubers were preincubated with

microsomes at 20°C during 15 min before enzyme activity measurement as previously described (14).

Chromatographic methods. Reverse-phase high-performance liquid chromatography (RP-HPLC) was performed as described (9, 12). Reaction products were directly injected as 100 µl reaction medium containing 50% acetonitrile (Fig.1; E and F). Alternatively, products were extracted from the incubated medium and dissolved in small volumes of methanol before TLC and RP-HPLC analysis. The metabolites were resolved by RP-HPLC on a 5 µm Ultrasphere C_{18} column (150 x 4.6 mm, Beckman Inst. France) using isocratic solvent at a flow of 2 ml/min. A mixture of acetonitrile/water/acetic acid (46:54:0.2, v/v/v) was used to elute ω-hydroxyoleic acid (*Rt* 25 min) (Fig.1; F, peak 5). A linear gradient (0-100%) of 80% acetonitrile in aqueous acetic acid was applied 45 min after injection at the same flow rate for 10 min to elute residual oleic acid (F, peak 6). A mixture of acetonitrile/water/acetic acid (40:60:0.2, v/v/v) was used to elute (Fig.1, E) 9,10,18-trihydroxystearic acid (*Rt* 3.0 min), 18-hydroxy-9,10-epoxystearic acid (*Rt* 12 min) and 9,10-dihydroxystearic acid (*Rt* 29 min) and a linear gradient, described above, to elute the 9,10-epoxystearic acid (*Rt* 57 min). Radioactivity of RP-HPLC effluents was monitored with a computerized on-line solid scintillation counter (Ramona-D RAYTEST, Germany).

Thin-layer chromatography plates were developed (Fig.1, A-D) with a mixture of diethyl ether/light petroleum (bp 40-60°)/formic acid.(50:50:1; v/v/v). The area corresponding to 18-hydroxyoleic acid (Rf 0.20; B, peak 3), 9,10-dihydroxystearic acid (Rf 0.1; C, peak 6), 18-hydroxy-9,10-epoxystearic acid (Rf 0.1; D, peak 9) and 9,10,18-trihydroxystearic acid (unmoved; D, peak 8) were scraped into counting vials or eluted (1 ml ether) to separate the unresolved 18-hydroxy-9,10-epoxystearic and 9,10-dihydroxystearic acids (C and D, peaks 6 and 9) as follow. Eluted compounds were spotted on reverse phase-TLC plates (RP-18 F254 S, Merck) and developed with a mixture of acetonitrile/THF/acetic acid (95:5:0.2, v/v/v). The area (not shown) corresponding to 9,10-dihydroxystearic acid (Rf 0.35) and 18-hydroxy-9,10-epoxystearic acid (Rf 0.5) were scraped into counting vials.

Gas chromatography/Mass spectrometry. Oxygenated metabolites were subjected to GC/MS (EI mode, 70 eV) analysis after methylation with diazomethane and silylation with a mixture of BSTFA +1% TMCS and pyridine (1:1; v/v) as described in (9, 12). In order to discriminate between endogenous compounds present in microsomal membranes and the reaction products, incubations were performed with radiolabeled substrates without isotopic dilution. Thus it appeared that fragment ions resulting from the contribution of isotopic ^{14}C were increased by 2 atomic mass units (+2 a.m.u.). Mass spectra analysis of the methylester trimethylsilylether derivatives of products formed from incubation with [1-^{14}C]oleic acid and eluted from silica plate (Fig.1; B, peak 3) showed ions among others at : MS *m/z* (rel. intensity %): *m/z* 386 (21) $[M]^+$; *m/z* 371 (27); *m/z* 339 (48) of peaks containing ^{14}C and others at *m/z* 103 (29); *m/z* 95 (55); *m/z* 81 (66) with a base peak at *m/z* 75. Assuming that the double bond remained in its original position and compared to

published spectra (15), the metabolite was identified as 18-hydroxy-*Z* 9-octadecenoic acid. Products formed (Fig. 1; E, peaks 1-3) from incubation with [1-^{14}C]*Z* 9,10-epoxystearic acid (peak 4) were isolated and analyzed separately. The fragmentation pattern of compound contained in peak 2 was similar to those already described (15) for 18-hydroxy-*Z* 9,10-epoxystearate except for differences of +2 a.m.u. at *m/z* 201 (15); 215 (20); 337 (8); 355 (7); 369 (9) [M-15-18]; 371 (4.5) [M-31] and *m/z* 387 (9) [M-15]. Strong signals were noted for others at *m/z* 81 (76); 95 (51); 103 (39) and base peak at *m/z* 75. Moreover, compound in peak 3 (E) was assigned to 9,10-dihydroxystearate with prominent ions at *m/z* 261 (+2 a.m.u.) (80) and *m/z* 215 (74). Other low intensity fragment ions [M-15]; [M-31]; [M-47] and *m/z* 103 (11) with a base peak at *m/z* 73 were also present. Mass fragmentation of the more polar compound in peak 1 (E), generated in this analysis from isotopically diluted [1-^{14}C] oleic acid (7 Ci/mol), appeared similar to published data (15) with ions at *m/z* 259 (100); 303 (64) and 332 (16) with a base peak at *m/z* 73, corresponding to 9,10,18-trihydroxystearate derivative.

Results. Metabolism of [1-^{14}C]oleic acid. The TLC radioactivity profile obtained after incubation of microsomes from clofibrate induced *Vicia sativa* seedlings with [1-^{14}C]oleic acid shows the residual substrate (Fig.1; A and B, peak 5) and metabolites (peaks 1-4) formed in the absence (A) and in the presence (B) of NADPH. No metabolite was formed when boiled microsomes were incubated. Compounds in peaks 1, 2 and 4, which were formed independently of NADPH, have not been identified. The compound in peak 3 (B) or in peak 5 (F) which was formed in the presence of NADPH was identified by GC/MS analysis to be 18-hydroxyoleic acid. Clofibrate treatment of the seedlings stimulated 12.5 fold this activity but had little effect on the cytochrome P450 content. The appearance of ω-hydroxyoleic acid was strongly inhibited by carbon monoxide, and this inhibition could be partially reversed by light (Table 1). Antibodies against the NADPH-cytochrome P450 reductase from *Helianthus tuberosus*, inhibited the hydroxylation of oleic acid (Table 2). Reductase activity and ω-hydroxyoleic acid formation appeared closely correlated. Kinetic studies showed apparent *K*m of 220 μM and *V*max of 1.05 nmol/min/mg protein. Under our conditions of incubation [1-^{14}C]stearic acid (C18:0) and [^{14}C]triacylglycerol-trioleate were not metabolized and neither stearic acid nor elaidic acid (*E*9-octadecenoic acid) were inhibitors of ω–hydroxylation of oleic acid (data not shown).

Metabolism of *Z*9,10-epoxystearic acid. When microsomes were incubated aerobically with racemic [1-^{14}C]*Z*9,10-epoxystearic acid in the absence of NADPH a major metabolite was formed (Fig 1; C, peak 6). This compound has been characterized as *threo* 9,10-dihydroxystearic acid by GC/MS analysis. The reaction did not occur with boiled

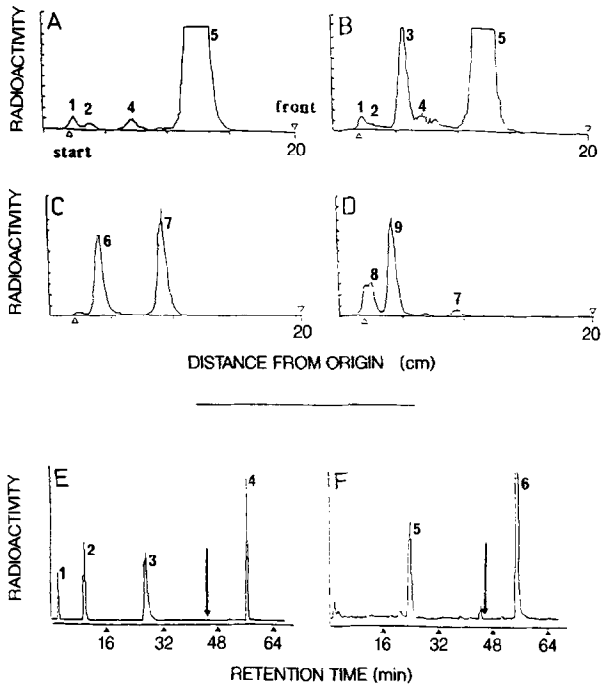


Figure 1. **Chromatographic resolution of metabolites generated by microsomal incubations from *V. sativa* seedlings.**
Radiochromatographic TLC analysis (A-D). Microsomal fractions were incubated with [1-^{14}C]oleic acid (A, B) or [1-^{14}C]9,10-epoxystearic acid (C, D) in the presence of NADPH (B, D) and in the absence (A, C) at 27°C for 30 min. Aliquot parts (100 µL) of incubated medium containing 50% of acidified acetonitrile were spotted on TLC layers. Radiolabeled compounds were resolved into uncharacterized metabolites (peaks: 1,2,4), 18-hydroxyoleic acid (peak 3), residual oleic acid (peak 5), 9,10-dihydroxystearic acid (peaks 6 and 9), residual 9,10-epoxystearic acid (peak 7), 9,10,18-trihydroxystearic acid (peak 8) and a mixture of 9,10-dihydroxystearic acid and 18-hydroxy-9,10-epoxystearic acid (peak 9) as described in Materials and Methods.
RP-HPLC elution profiles of radiolabeled metabolites from incubation of microsomes with [1-^{14}C]9,10-epoxystearic acid (E) and [1-^{14}C]oleic acid (F) using a mixture of $CH_3CN/H_2O/CH_3COOH$ (40:60:0.2, v/v/v) (E) or (46:54:0.2, v/v/v) (F) as mobile phases at a flow rate of 2 ml/min. Metabolites generated from [1-^{14}C]9,10-epoxystearic acid (E) were resolved into 9,10,18-trihydroxy- (peak 1), 18-hydroxy-9,10-epoxy- (2), 9,10-dihydroxy- (3) and residual 9,10-epoxystearic acids (4). Those from [1-^{14}C]oleic acid were resolved into 18-hydroxy-Z9-octadecenoic acid (5) and residual oleic acid (6). The arrows correspond to the run of a linear gradient of 80% CH_3CN as described in Materials and Methods.

microsomes and clofibrate treatement of seedlings decreased by 40-50% the rate of diol formation.
In the presence of NADPH a new polar metabolite appeared (Fig.1; D, peak 8). It was identified by GC/MS as 9,10,18-trihydroxystearic acid. When the lipidic extract of incubated microsomes was analyzed by RP-HPLC,

Table 1. Light-reversible carbon monoxide inhibition of ω-hydroxylation of oleic (C18:1), 9,10-epoxystearic (EPOXI) and 9,10-dihydroxystearic (DIOL) acids

Incubation conditions	% ω-hydroxylation		
	C18:1	EPOXI	DIOL
Air	100[a]	100[b]	100[c]
	+/-8.5	+/-1	+/-1
$CO/O_2/N_2$ (2/2/8)			
Dark	19.1	9.5	38
	+/-1	+/-1	+/-1
Light	75.9	35.3	60
	+/-1	+/-3.5	+/-1.8
Light reversion (%)	70.2	28.5	35.5

Results are expressed as percent of maximal activity which was [a]74.3, [b]254 and [c]154 pmol/min/mg protein. Each value is the mean +/-s.d. of triplicate experiments as described in Materials and Methods.

another metabolite (Fig.1; E, peak 2) was identified by GC/MS as 18-hydroxy-9,10-epoxystearic acid. Thus it appears that the diol and 18-hydroxy-9,10-epoxystearic acid have similar mobilities in silica TLC analysis. Peak 9 (Fig.1; D) contained a mixture of ω-hydroxy-9,10-epoxystearate and diol derivatives which were resolved using a reverse phase-TLC as described or by RP-HPLC analysis (E, peak 2 and 3). As expected for a cytochrome P450-dependent reaction, ω-hydroxylation of 9,10-epoxystearic was inhibited by CO and reversal of inhibition was obtained by light (Table 1). Furthermore, anti-P450 reductase monoclonal antibodies (Table 2) inhibited the reaction. Treatment of seedlings with (1 mM) clofibrate enhanced 8.5 fold this ω-hydroxylase activity. Kinetic studies showed apparent *K*m of 2 μM and *V*max of 0.36 nmol/min/mg protein.

ω-hydroxylation of *threo* 9,10-dihydroxystearic acid. When *V. sativa* microsomes were incubated with NADPH and a racemic mixture of [1-^{14}C]*threo* 9,10-dihydroxystearate, a major polar metabolite was formed. It was identified by GC/MS as 9,10,18-trihydroxystearic acid. This activity was enhanced 7.2 fold when the seedlings were treated with clofibrate. The apparent *K*m value of the reaction was 16 μM and the *V*max 0.25 nmol/min/mg protein. The reaction was inhibited by antireductase antibodies (table 2) and by carbon monoxide. The CO inhibition could be partly reversed by light as shown in Table 1. Without

Table 2. Inhibition of microsomal enzymes activities by rat monoclonal antibodies raised against purified cytochrome P450-reductase from Jerusalem artichoke tubers

Antireductase antibodies	P450-reductase % activity	% ω-hydroxylation		
		C18:1	EPOXI	DIOL
none	100[a]	100[b]	100[c]	100[d]
65µl	67	63	76	61
105µl	37	37	28	35
165µl	20	16	8	15

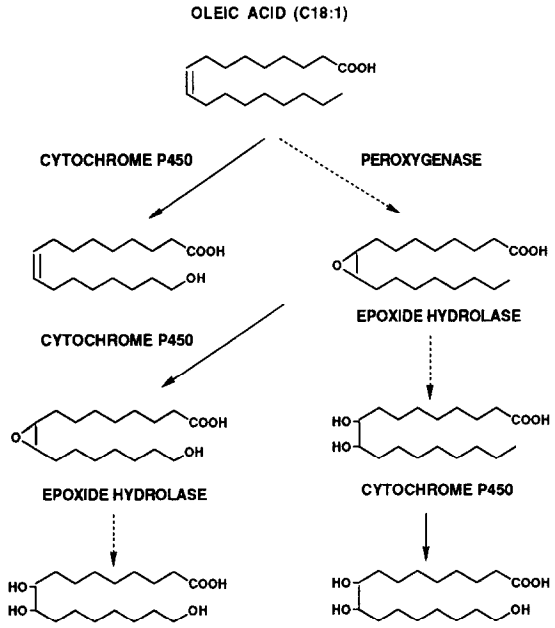
Results are means of duplicated experiments. Enzymes activities are expressed as percent of maximal rate which was: [a]20 nmol/min/mg protein; [b]545, [c]247 and [d]83 pmol/min/mg protein for NADPH-cytochrome P450-reductase and ω-hydroxylation of oleic (C18:1), 9,10-epoxystearic (EPOXI) and 9,10-dihydroxystearic acids (DIOL) respectively.

β-mercaptoethanol, trihydroxystearate was also produced in smaller amount when microsomes were incubated either with [1-^{14}C]oleic acid in the presence of NADPH or with [1-^{14}C]9,10-epoxystearic acid alone.

Discussion. Omega hydroxylation of fatty acids by cytochrome P450-dependent monooxygenases has been described in many organisms but few studies have been devoted to plant systems in spite of the important roles played by oxygenated fatty acids. They have been found in polar lipids such as glycerides and phospholipids (16) and as monomer constituents of cutin and suberin which protect plants against pathogenic fungi and microbes (3). Certain monomers from cutin (ie: dihydroxy fatty acids and 9,10,18-trihydroxystearic acid) were found to be potent inducers of the cutinase of several pathogenic fungi (17). Interestingly, antifungal properties have been assigned to poly-hydroxylated metabolites of the C18 family (18). Moreover, large amounts of ω-hydroxyoleic and ω-hydroxylinoleic acids have been found in plant stigma and may play a role in the mechanism of recognition of the stigma by pollen (19). We report here, for the first time, the characterization of an enzymatic system from plant microsomes which hydroxylates exclusively the methyl end of oleic acid and of two oxidized oleate analogs. Evidence for the involvement of a cytochrome P450 monooxygenase is provided by the microsomal localization of the enzyme activities, the requirement for NADPH and their photoreversible

inhibition by CO. Moreover clofibrate, a well known inducer of lauric acid ω-hydroxylase both in plants and animals, was also an effective inducer of ω-hydroxylation of the three substrates. In contrast, hydrolysis of 9,10-epoxystearate was decreased in microsomes from clofibrate treated seedlings. Inhibitions of hydroxylase activities with antibodies demonstrate the involvement of a NADPH-cytochrome P450 reductase in reactions and confirmed that antibodies against NADPH-cytochrome P450 reductase from *Helianthus tuberosus* cross react with *V. sativa* reductase (20).

When *V. sativa* microsomes were incubated with NADPH and oleic acid under our standard conditions (in the presence of β-mercaptoethanol), 18-hydroxyoleate was the only significant metabolite generated. Incubations with 9,10-epoxystearic acid in the absence of NADPH produced a single metabolite identified as 9,10-dihydroxystearic acid. In the presence of NADPH, two additional metabolites were formed: 18-hydroxy-9,10-epoxystearate and 9,10,18-trihydroxystearate. In sharp contrast, when β-mercaptoethanol was omitted from the incubation medium, all five metabolites, 18-hydroxyoleate, 9,10-epoxystearate, 18-hydroxy-9,10-epoxystearate, 9,10-dihydroxystearate and 9,10,18-trihydroxystearate were formed in a single incubation with oleic acid and NADPH. These results demonstrate that the β-mercaptoethanol



Scheme 1. Proposed scheme for the biosynthesis of 9,10,18-trihydroxystearic acid from oleic acid by microsomes from clofibrate-induced *Vicia sativa* seedlings.

sensitive step is the formation of 9,10-epoxystearate, suggesting the presence in *V. sativa* microsomes of a peroxygenase similar to that described in other plant species (10, 11). Furthermore, our results show clearly that the trihydroxy derivative is generated either through the ω-hydroxylation of the corresponding diol or *via* the hydrolysis of the oxiran ring of the epoxy-hydroxy derivative.

Taken together, our results demonstrate that oleic acid is subjected in *Vicia* microsomes to a cascade of biosynthetic reactions that involves at least three distinct enzymes: a peroxygenase, an epoxide hydrolase and a P450-dependent ω-hydroxylase. The interplay of these enzymes account for the formation of all the C18 fatty acid monomers derived from oleic acid and found in cutin and suberin. Our results differ from those already reported by Kolattukudy's group in two important aspects: i) Neither ATP nor acetyl-CoA are required at any step of this oleate cascade in *Vicia* microsomes, ii) the sequence of events in this material is clearly such that the ω-hydroxylation is always the ultimate reaction in the synthesis of the three ω-hydroxylated oleate derivatives. Our findings are summarized in scheme 1.

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