

# Cytochrome P-450-Dependent $\omega$ -Hydroxylation of Lauric Acid by Microsomes from Pea Seedlings<sup>1</sup>

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## ABSTRACT

Microsomes from apical buds of pea (*Pisum sativum* L. var. Téléphone à rames) seedlings hydroxylate lauric acid at the  $\omega$ -position. This oxidation is catalyzed by a cytochrome P-450 enzyme which differs from laurate hydroxylases previously described in microorganisms and mammals by its strict substrate specificity and the ability of low NADH concentrations to support unusually high oxidation rates. The apparent  $K_m$  for lauric acid was 20 micromolar. NADPH- and NADH-dependent laurate hydroxylation followed non-Michaelian kinetics with apparent  $K_m$  values ranging from 0.2 to 28 micromolar for NADPH, and 0.2 to 318 micromolar for NADH. When induced by the photomorphogenic photoreceptor phytochrome, the time course for the enhancement of laurate  $\omega$ -hydroxylase was totally different from that of the cinnamic acid 4-hydroxylase, providing evidence for the existence of multiple cytochrome P-450 species in pea microsomes.

Several Cyt P-450-dependent monooxygenases have been described in higher plants. These microsomal enzymes catalyze important oxidative steps in different biosynthetic pathways: hydroxylation of cinnamic acid (1, 13), a precursor of lignins and flavonoids, hydroxylation of fatty acids and cutin constituents (14, 18), oxidation of kaurene and other intermediates in the biosynthesis of gibberellins (7), and hydroxylation of monoterpene precursors of alkaloids (10). Cyt P-450 may also be implicated in the oxidative *N*-demethylation of two foreign compounds, e.g. *p*-chloro-*N*-methylaniline (20) and the herbicide Monuron (3-[4-chlorophenyl]-1,1-dimethylurea) (5).

Cyt P-450 enzymes consist in a complex of membrane-bound amphipathic proteins, the Cyt serving as the terminal oxidase of the system. To achieve catalysis, the Cyt binds the substrate and molecular oxygen which is then activated by reducing equivalents transferred from NADPH to Cyt P-450 via the flavoprotein NADPH-Cyt P-450 reductase. In many cases, NADH may substitute, although with lesser efficiency, for NADPH. A second flavoprotein, NADH-Cyt *b5* reductase and Cyt *b5* may be implicated in this alternative electron transfer.

In recent years, it has become evident that in the mammalian liver, Cyt P-450 exists in multiple molecular forms with broad and overlapping substrate specificities (9). Such evidence is not at hand concerning plant Cyt P-450 enzymes.

We have recently reported that aging Jerusalem artichoke tuber tissues contain an in-chain hydroxylating lauric acid monooxygenase (14) which, unlike the mammalian and microbial fatty acid hydroxylases, showed very strict substrate specificity (15). By

feeding to the tissues metabolic intermediates, the induction of this enzyme could be clearly dissociated from that of the cinnamic acid 4-hydroxylase, thus providing some evidence that the two monooxygenase activities were supported by distinct hemoproteins (15, 16). In this paper, we describe a different lauric acid monooxygenase in pea seedlings. This enzyme has a higher apparent  $K_m$  for laurate, yields  $\omega$ -hydroxylauric acid, and shows no NADPH-NADH synergism although NADH given alone sustains appreciable hydroxylation rates. Comparative induction studies confirm the existence of multiple forms of Cyt P-450 in higher plants.

## MATERIALS AND METHODS

**Plant Material.** Pea seeds (*Pisum sativum* L. var Téléphone à rames) were germinated and grown in moist vermiculite in darkness at 26°C for 7 d. The etiolated seedlings were then irradiated with far-red (730 ± 60 nm) for different times.

**Preparation of Microsomes.** Apical buds were ground with an Ultra-Turrax homogenizer in 0.1 M Na-phosphate buffer (pH 7.4) containing 250 mM sucrose, 1 mM EDTA, 0.4% BSA, and 10 mM 2-mercaptoethanol. The homogenate was centrifuged 30 min at 6000g. The resulting supernatant was centrifuged 60 min at 100,000g. The 100,000g pellets were washed once in 0.1 M Na-phosphate (pH 7.4) containing 1 mM EDTA and 10 mM 2-mercaptoethanol, and resedimented 60 min at 100,000g. Microsomes were suspended in 0.1 M Na-phosphate buffer (pH 7.4) containing 10 mM 2-mercaptoethanol and 30% (v/v) glycerol and stored at -20°C.

**Enzyme Assays.** Lauric acid  $\omega$ -hydroxylation was assayed by a procedure modified from Salaün *et al.* (15). In a final volume of 2 ml, the reaction mixture contained: 1 mM NADPH, 6.7 mM glucose 6-P, 0.4 units glucose 6-P dehydrogenase, [1-<sup>14</sup>C]lauric acid (36 Ci/mol; Commissariat à l'Energie Atomique, Saclay, France) and sodium laurate to a final concentration of 5 to 17  $\mu$ M, 2 to 3 mg microsomal protein, and 0.1 M Na-phosphate buffer (pH 7.4). Incubations were run for 30 min at 26°C and terminated by the addition of 1 ml 4 N HCl. When NADH was the source of reducing equivalents, 80 mM ethanol and 5 units alcohol dehydrogenase were added as a regenerating system. The incubation mixture was extracted with benzene-diethyl ether (9:1, v/v). Products were methylated by diazomethane and spotted on a 0.25 mm layer of silica gel F 254. The developing solvent was an ethyl acetate:cyclohexane (30:70, v/v) mixture. Radioactivity was detected with a Berthold LB 2723 thin-layer scanner. The major polar radioactive peak, which comigrated with authentic  $\omega$ -hydroxylaurate methylester, was scraped off the plate and counted by liquid scintillation using Intertechnique SL 4000 instrument. We have now adopted a different procedure which is less time-consuming: the benzene:diethyl ether extract is directly spotted on chromatoplates which are developed with diethyl ether:light petroleum (b.p., 30-60°C):formic acid (70:30:1, v/v) as solvent.

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Cinnamic acid 4-hydroxylation was measured with the radiochemical method described previously (2).

**Radio GC.** The reaction products were methylated with diazomethane, isolated by TLC, and trimethylsilylated with *N,O*-bis(trimethylsilyl) trifluoroacetamide in pyridine as described elsewhere (14), prior to analysis by radio GC. A glass column (2 m, diameter, 0.32 cm) packed with 10% SE 30 Chromosorb W 60/80 AW was used with a 53 ml/min He carried flow on an Intersmat IGC 120 DFL instrument. The temperature program was 160 to 240°C at 4°C/min. Radioactivity was measured by monitoring 90% of column effluent with a Barber-Coleman radioactivity monitor.

**Kinetic Measurements.** Data were fitted using nonlinear regression programs written in BASIC and run on a PDP-11/03 computer. Apparent  $K_m$  and  $V_{max}$  of enzyme activities following Michaelian kinetics were calculated using the procedure of Wilkinson (19). Biphasic kinetics were fitted with the method outlined by Osmundsen (12).

## RESULTS

**1) Identity of Products.** The polar fraction formed upon aerobic incubation of NADPH and [ $1-^{14}C$ ]lauric acid with a microsomal preparation from pea seedlings was methylated and analyzed by TLC. All the label was associated with one peak comigrating with authentic  $\omega$ -hydroxylaurate methyl ester. This peak was further analyzed, after trimethylsilylation, by radio GC and resolved into three components (Fig. 1). Over 85% of the label was associated with peak A which cochromatographed with the methyl ester-trimethylsilyl derivative of authentic  $\omega$ -hydroxylauric acid. Peak B, although only partially separated from peak A, contained no radioactivity. This peak which was also present in controls stopped at time 0 is likely to be coextracted with the reaction products from the microsomes. A small part of the label (13%) was associated with a very minor compound, peak C, which could not be identified.

**2) Cofactor Requirements for Laurate  $\omega$ -Monooxygenation.** Laurate  $\omega$ -hydroxylation showed an absolute requirement for reducing equivalents and, as usual in Cyt P-450-dependent oxi-

dations, NADPH was more efficient than NADH (Table I). However, the ability of NADH to sustain laurate  $\omega$ -hydroxylation was remarkably high. This is evident from Table I which allows for a comparison between laurate  $\omega$ -hydroxylation and cinnamate 4-hydroxylation, measured under identical conditions on the same microsomal preparation. At a 10  $\mu M$  concentration, NADH supported 30% of the rate obtained with 100  $\mu M$  NADPH (a quasi-saturating concentration) when laurate oxidation was concerned. The corresponding figure for cinnamate 4-hydroxylase was only 5%. The possibility that NADPH could form upon transhydrogenation of NADP<sup>+</sup> which might be present in the microsomes was considered. Although 100,000g supernatant and unwashed microsomes showed low transhydrogenase activity, washed microsomes, even supplemented with ATP, Mg<sup>2+</sup> ions, and NADP<sup>+</sup>, did not catalyze any measurable transhydrogenation. Table I also shows that NADPH, at low concentration, is relatively more efficient in sustaining laurate  $\omega$ -hydroxylation than cinnamate hydroxylation.

The simultaneous presence of NADPH and NADH results in a synergistic effect on the activity of many Cyt P-450 enzymes: the reaction rate is higher than the sum of the rates achieved in the presence of each pyridine nucleotide alone. The cinnamic acid 4-hydroxylase is a representative example of this type of behavior, in contrast to the laurate  $\omega$ -hydroxylase (Table I). For the latter, rates were always lower than expected from the sum of corresponding rates, despite the wide NADPH and NADH concentration range used. However, addition of NADH stimulated laurate  $\omega$ -hydroxylation at the higher NADPH concentrations.

The kinetic properties of lauric acid  $\omega$ -hydroxylase were studied as a function of laurate, NADPH, and NADH concentrations. The reaction followed Michaelis-Menten kinetics when the concentration of lauric acid was varied, with an apparent  $K_m$  of 20  $\mu M$  and a  $V_{max}$  of 17 pmol min<sup>-1</sup> mg<sup>-1</sup> protein. When lauric acid was kept constant, and the velocities were measured as a function of NADPH or NADH, marked deviations from Michaelis kinetics were observed, yielding nonlinear Eadie-Hofstee plots (Fig. 2). Values of  $K_m$  and  $V_{max}$  were estimated by the method of Osmundsen (12). For NADPH, the apparent  $K_m$  ranged from 0.2 to 28  $\mu M$  and  $V_{max}$  from 1.5 to 5.1 pmol min<sup>-1</sup> mg<sup>-1</sup> protein. For NADH, the limits for apparent  $K_m$  were 0.23 and 318  $\mu M$  and those for

Table I. Comparison of the NADPH and NADH Requirements for Cinnamic Acid 4-Hydroxylation (CA4H) and Lauric Acid  $\omega$ -Hydroxylation ( $\omega$ -LAH) by a Microsomal Fraction from Pea Seedlings.

Synergism is calculated as  $(A - B/B \times 100)$ , where  $A$  is the activity observed when both reducers are present simultaneously and  $B$  is the sum of activities achieved in the presence of each nucleotide alone.

Reducing Agent	CA4H		$\omega$ -LAH		
	Concentration $\mu M$	$pmol/min \cdot mg$ protein	$\% activity$	$pmol/min \cdot mg$ protein	$\% activity$
None		0		0	100
NADPH	100	632	100	6.4	89
NADPH	10	381	60	5.7	44
NADH	1,000	85	13	2.8	34
NADH	100	36	6	2.2	30
NADH	10	32	5	1.9	
			<i>% synergism</i>		<i>% synergism</i>
NADPH	10				
+ NADH	10	635	53	6.4	-15
NADPH	10				
+ NADH	100	665	59	5.8	-26
NADPH	10				
+ NADH	1,000	639	37	6.5	-23
NADPH	100				
+NADH	10	818	23	6.8	-18
NADPH	100				
+ NADH	100	831	24	7.7	-10
NADPH	100				
+ NADH	1,000	863	20	7.3	-20

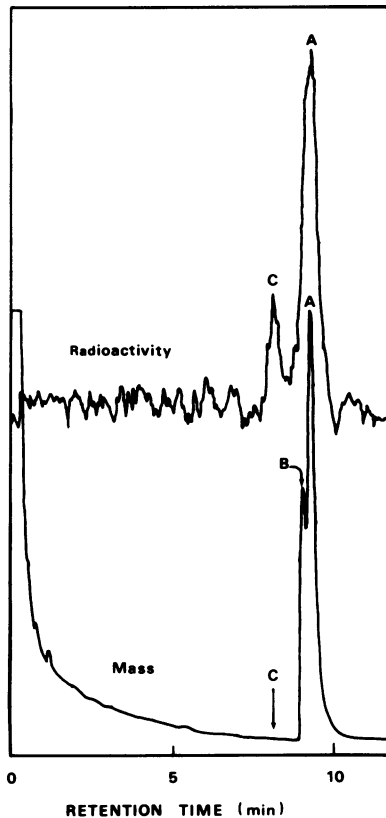


FIG. 1. Radio GC of  $\omega$ -hydroxylaurate obtained from the incubation of  $[1-^{14}\text{C}]$ lauric acid and NADPH with a microsomal preparation from pea seedlings apical buds. Products were methylated, isolated by TLC, and trimethylsilylated as described in "Materials and Methods." The trimethylsilyl derivative of authentic  $\omega$ -hydroxylaurate methylester was coinjected (peak A).

$V_{max}$  were 0.33 and 4.4  $\text{pmol min}^{-1} \text{mg}^{-1}$  protein. Inasmuch as NADPH and NADH saturation kinetics of the enzyme were measured with a different enzyme preparation from that used to obtain laurate saturation curves, the  $V_{max}$  measured for the latter is not to be compared with those obtained for the former.

3) **Effect of Carbon Monoxide.** Inhibition of the reaction by CO, and its partial reversion by light, is a characteristic of Cyt P-450 enzymes. Laurate  $\omega$ -hydroxylation, when measured under a CO, O<sub>2</sub>, N<sub>2</sub> (2:2:6, v/v/v) atmosphere, was inhibited by 70% in microsomes incubated in the dark and by 55% when the incubation medium was irradiated with white light. The Warburg partition coefficient  $K$  increased from 0.42 in darkness to 0.79 in illuminated microsomes. This can be compared to  $K$  values of 0.43 and 1.58 for cinnamic acid 4-hydroxylase when measured under similar conditions.

4) **Substrate Specificity.** We have investigated the ability of several fatty acids or fatty acid derivatives to compete with lauric acid for the enzyme. Saturated fatty acids with 10, 14, or 16 carbons did not compete to a significant extent with lauric acid (Table II). Likewise, dodecane, 1-dodecanol, laurate methylester, and the C-12 diacid did not inhibit laurate  $\omega$ -hydroxylation.

Kinetic studies were performed to determine if cinnamic acid 4-hydroxylase and lauric acid  $\omega$ -hydroxylase activities could be inhibited by laurate or cinnamate, respectively. No competition or inhibition of one reaction by the substrate of the other could be measured. The apparent  $K_m$  of cinnamic acid 4-hydroxylase for cinnamate was  $6.2 \pm 2.4 \mu\text{M}$  in the absence of laurate and  $6.1 \pm 2.5 \mu\text{M}$  when  $40 \mu\text{M}$  laurate was present. The corresponding  $V_{max}$  were  $871 \pm 170$  and  $977 \pm 210 \text{ pmol min}^{-1} \text{mg}^{-1}$  protein, respectively. Conversely,  $330 \mu\text{M}$  cinnamic acid did not inhibit laurate

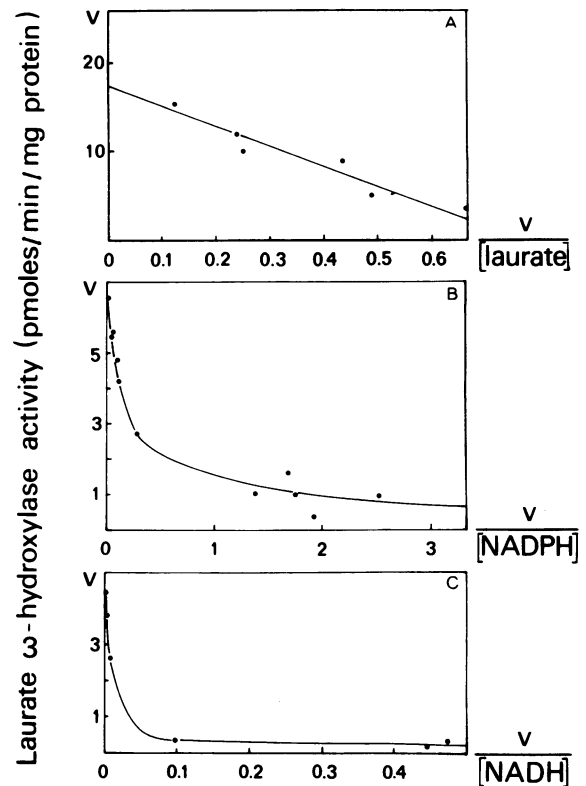


FIG. 2. Eadie-Hofstee plots of lauric acid  $\omega$ -hydroxylase from pea apical buds. Substrate saturation curves were determined for lauric acid (A), NADPH (B), and NADH (C). The microsomal preparation used for A was different from that used for B and C. A, The concentration of lauric acid ranged from 5 to 125  $\mu\text{M}$ . NADPH was maintained constant at 1 mM. Microsomes (2.6 mg protein) were incubated for 20 min at 26°C. B, The concentration of NADPH, ranging from 0.2 to 1000  $\mu\text{M}$ , was kept constant during incubation (45 min at 26°C) by a regenerating system. Laurate concentration was 12.5  $\mu\text{M}$ . C, The concentration of NADH, ranging from 0.13 to 3400  $\mu\text{M}$ , was maintained constant during incubation (45 min at 26°C) by a regenerating system. Laurate concentration was 12.5  $\mu\text{M}$ .

Table II. *Substrate Specificity of Laurate  $\omega$ -hydroxylase from Pea Seedling Microsomes*

All incubations contained 12.5 nmol of the compound to be tested and 4.25 nmol of  $[1-^{14}\text{C}]$ lauric acid.

Labeled Lauric Acid	Unlabeled Fatty Acids and Derivatives	Radioactive $\omega$ -Hydroxylauric Acid Formed
		<i>pmol/min·mg protein</i>
$[1-^{14}\text{C}]$ Laurate	+ 0	1.65
$[1-^{14}\text{C}]$ Laurate	+ decanoic acid	1.48
$[1-^{14}\text{C}]$ Laurate	+ dodecanoic acid	0.93
$[1-^{14}\text{C}]$ Laurate	+ tetradecanoic acid	1.52
$[1-^{14}\text{C}]$ Laurate	+ hexadecanoic acid	1.66
$[1-^{14}\text{C}]$ Laurate	+ dodecanedioic acid	1.65
$[1-^{14}\text{C}]$ Laurate	+ dodecanoate methylester	1.58
$[1-^{14}\text{C}]$ Laurate	+ dodecane	1.54
$[1-^{14}\text{C}]$ Laurate	+ 1-dodecanol	1.69

$\omega$ -hydroxylation in microsomes incubated with 5  $\mu\text{M}$  lauric acid.

5) **Induction of Lauric Acid  $\omega$ -Hydroxylase Activity by Light.** In a previous report, we studied the effect of light on the activity of the cinnamic acid 4-hydroxylase in pea seedlings. It was shown that the Pr→Pfr transition substantially increased the activities of CA4H and NADPH-Cyt *c* reductase, and the titer of Cyt P-450 and *b5* (3). Here, we compare the evolution of lauric acid  $\omega$ -

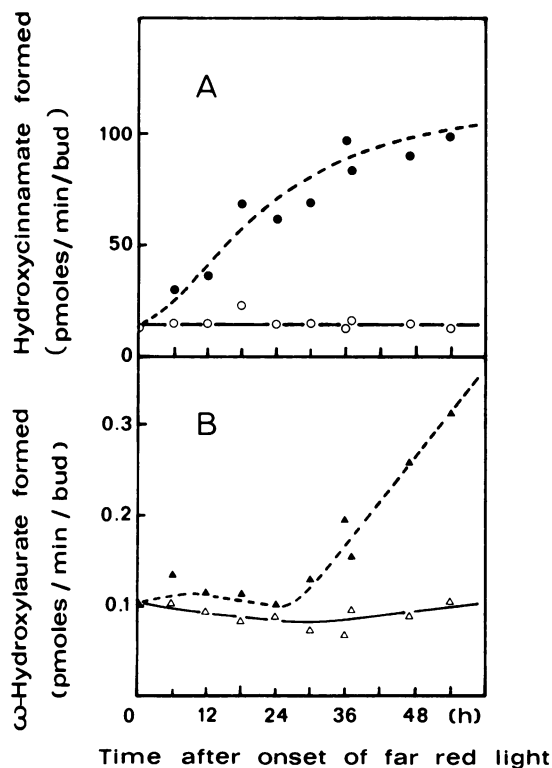


FIG. 3. Time courses of cinnamic acid 4-hydroxylase (A), and laurate  $\omega$ -hydroxylase (B) in 8-d-old etiolated pea seedlings irradiated with continuous far-red light (---) or maintained in the dark (—).

hydroxylase and cinnamic acid 4-hydroxylase during deetiolation under continuous far-red light of 8-d-old etiolated pea seedlings. It is clear from Figure 3 that the two enzymes were induced with totally different time courses. The lag-phase for photoinduction of cinnamic acid 4-hydroxylase was very short, in the order of 45 min (3), whereas laurate  $\omega$ -hydroxylase increased only after 24 h. In fact, changes in the activity of the laurate monooxygenase seem to be entirely related to the phytochrome-mediated growth of the pea bud. When the results are expressed per mg microsomal protein, it appears that only the specific activity of cinnamate hydroxylase, and not that of laurate  $\omega$ -hydroxylase, is increased by light.

## DISCUSSION

Lauric acid was hydroxylated at the C-12 position by a microsomal enzyme from pea seedlings showing the characteristic behavior of a Cyt P-450-dependent oxygenase.

Hydroxylation of lauric acid in microorganisms (11) and in animals (8) is well documented. Insertion of oxygen onto carbon 11 and 12 have both been reported and were attributed to different Cyt P-450 species (4). We have previously demonstrated the existence of a laurate-monooxygenase which catalyzes the formation of a mixture of 8-OH, 9-OH, and 10-OH lauric acids in microsomes from Jerusalem artichoke tuber tissues (14). The present report is, to our knowledge, the first to describe the  $\omega$ -hydroxylation of lauric acid by a microsomal preparation from a higher plant.

The pea  $\omega$ -hydroxylase is remarkable by its strict substrate specificity, an unusually high ability to utilize NADH as sole source of electrons, and NADPH and NADH substrate saturation curves which do not follow Michaelis kinetics.

The participation of Cyt P-450 in laurate  $\omega$ -hydroxylation is supported by several lines of evidence. The enzyme activity was associated with the microsomal fraction, as are also Cyt P-450 and

NADPH-Cyt *c* reductase in this plant material (3). Carbon monoxide inhibited strongly the reaction, and this effect was partly reversed by light. In this respect, the pea  $\omega$ -hydroxylase resembles the in-chain hydroxylating monooxygenase from tuber microsomes (14), but differs from the palmitate  $\omega$ -hydroxylase from *Vicia faba* which showed no light reversion after CO poisoning (18).

Laurate  $\omega$ -oxidation showed an absolute requirement for an external electron donor. It is noteworthy that, although high NADH concentrations were required to approach  $V_{max}$  (NADH), the enzyme showed a remarkable ability to utilize NADH at low concentrations. This observation prompted kinetic studies which revealed that laurate hydroxylation measured as a function of either NADH or NADPH followed biphasic substrate saturation kinetics. In both cases, the data were best fit assuming the existence of two components, a low  $K_m$  and low  $V_{max}$  component, and a high  $K_m$  and high  $V_{max}$  one. At this stage, it is not possible to assign a physical meaning to these two components. However, it is worthy of note that the low  $V_{max}$  component is saturated at very low NADH or NADPH concentrations compatible with the intracellular concentration of these coenzymes and may be physiologically more relevant than the high  $V_{max}$  component. Laurate saturation curves of enzyme activity were monophasic. This does not imply, however, that the enzyme follows Michaelis kinetics since the range of usable substrate concentration was limited by the sensitivity of the assay and the solubility of lauric acid. Little is known concerning the kinetics of membrane-bound enzymes, and those of Cyt P-450 enzymes are particularly complex because of the many possible interactions between the different microsomal redox components (6). It has been proposed that the kinetics of these monooxygenases are mainly controlled by the redox states of the flavin adenine dinucleotide and flavin mononucleotide of NADPH-Cyt *c* reductase (6).

The totally different time courses of cinnamate 4-hydroxylation and laurate  $\omega$ -hydroxylation during deetiolation of dark-grown seedlings, the former being induced within the 1st h of irradiation and the latter only after 24 h, provides evidence that multiple forms of Cyt P-450 exist in plants.

The high substrate specificity of the  $\omega$ -hydroxylase for lauric acid contrasts with the low specificity of fatty acid hydroxylases from microorganisms (11) and animals (8). On the other hand, it is in line with recent work showing a very strict specificity of the in-chain hydroxylating enzyme from tuber microsomes for lauric acid (15). The relative specificity of plant Cyt P-450 enzymes, also stressed by Sandermann *et al.* (17), may prove a major difference between plant and animal monooxygenases. The intriguing fact that microsomes from *Pisum* and from *Helianthus* (15) oxidize laurate specifically but yield different reaction products prompted us to examine the situation in other plant species. Preliminary results showed that  $\omega$ -hydroxylation and in-chain hydroxylation of laurate were about equally distributed among the 10 species examined and were always exclusive of one another (J. P. Salaün *et al.*, to be published).

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