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Characterization of a Cytochrome P-450 Dependent Monoterpene Hydroxylase from the Higher Plant *Vinca rosea*†

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ABSTRACT: A monooxygenase isolated from 5-day old etiolated *Vinca rosea* seedlings was shown to catalyze the hydroxylation of the monoterpene alcohols, geraniol and nerol, to their corresponding 10-hydroxy derivatives. Hydroxylase activity was dependent upon NADPH (neither NADH nor combination of NADH, $NADP^+$, and ATP served as substitutes) and O_2. Geraniol hydroxylation was enhanced by dithiothreitol (monothiols were less effective) and inhibited by phospholipases, thiol reagents, metyrapone, and cytochrome *c*, as well as other inhibitors of cytochrome P-450 systems. Geraniol was hydroxylated at a faster rate than nerol, but the alcohols possessed similar apparent K_m values. The membrane-bound hydroxylase was solubilized by treatment with sodium cholate, Renex-30, or Lubrol-WX. Cholate-treated enzyme was resolved by DEAE-cellulose chromatography and reconstitution of the hydroxylase was effected utilizing different fractions containing cytochrome P-450, a NADPH-cytochrome *c* reductase, and lipid.

The biological conversion of the acyclic monoterpene alcohols, geraniol and its cis isomer, nerol, to cyclopentano derivatives involves allylic oxygenation, dehydrogenation, and cyclization (Scheme I) (Banthorpe et al., 1972). In the higher plant, *Vinca rosea,* this metabolic sequence represents the beginning of indole alkaloid biosynthesis, and one of the initial steps is hydroxylation at the C-10 methyl group to afford the diols, 10-hydroxygeraniol and 10-hydroxynerol (Escher et al., 1970; Battersby et al., 1970). A NADPH-dependent monooxygenase found in crude extracts of *V. rosea* catalyzes this hydroxylation (Meehan and Coscia, 1973), and this enzyme has been identified as one of a growing number of cytochrome P-450 dependent oxygenases which have been discovered in higher plants (Cotte-

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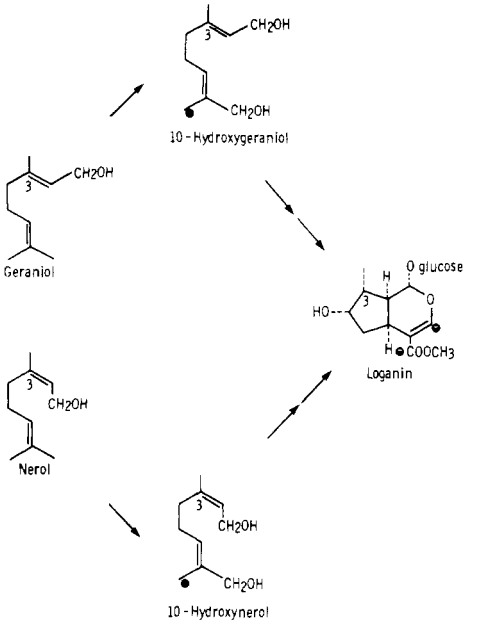
Scheme I



Martinon, et al., 1974; Frear et al., 1969; Markham et al., 1972; Murphy and West, 1969; Potts et al., 1974; Young and Beevers, 1975). While their enzymic role has been defined in most cases, there have been no reports of the solubilization and resolution of this type of plant oxygenase into its individual components. To date most cytochrome P-450 dependent systems belong either to the category in which the heme protein is directly coupled to a flavoprotein as exemplified by the liver microsomal system or to the class in which a three-protein complex is involved. In the latter an iron–sulfur protein mediates electron flow between the reductase and cytochrome P-450 and such complexes have been found in bacteria and adrenal cortex mitochondria (Gunsalus et al., 1975). It was of interest then to designate this plant cytochrome P-450 dependent monooxygenase as either one of the known types or as one having a novel electron transport chain.

Experimental Procedure

Materials. Solvents were of reagent grade quality and were redistilled. Geraniol (99%), citral, and metyrapone were obtained from Aldrich Chemical Co. Nerol (95%) was from Chemical Samples Co., Columbus, Ohio. NAD, $NADP^+$, NADH, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol, iodoacetamide, *p*-chloromercuribenzoate, cytochrome *c*, ATP, sodium deoxycholate, sodium cholate, Triton X-100, Triton N-101, Lubrol WX, phospholipases, and DEAE-cellulose were purchased from Sigma Chemical Co. The DEAE-cellulose (0.95 meq/g) was washed with 0.5 N NaOH, 0.1 N HCl, 0.5 N NaOH, and water in that order. The Renex 30 was a generous gift from Atlas Chemicals Division of ICI, Wilmington, Delaware.

Synthesis of Labeled Compounds. [1-^{3}H]Geraniol and [1-^{3}H]nerol were synthesized by $NaBT_4$ (Amersham Searle) reduction of citral (van Aller and Nes, 1968). Tritiated monoterpenes were purified on thin layers (0.5 mm) of silica gel GF-254 (Merck) impregnated with 3% $AgNO_3$. The chromatoplates were developed with hexane–ethyl acetate (3:2 v/v).

Synthesis of Diols. Synthesis of diols was initially carried out as previously reported (Meehan and Coscia, 1973). In more recent experiments (G. Jamroz, unpublished data), geranyl or neryl acetate was subjected to selenium dioxide oxidation to afford a mixture of 10-aldehydo- and 10-hydroxygeranyl or neryl monoacetates (Meinwald et al., 1971). Ethereal solutions of the latter were reduced to the diols with $LiAlH_4$. 10-Hydroxygeraniol: NMR ($CDCl_3$) δ 1.67 (broad s, H-4, H-9), 2.08 (m, H-5, H-6), 3.98 (s, H-10), 4.13 (d, J = 6.5 Hz, H-10), 5.41 (t, J = 7 Hz, H-2, H-7); high resolution mass spectrum of the diacetate of 10-hydroxygeraniol *m/e* 212.1371 (0.68%; $M^+ - C_2H_2O$, 212.1412), 135.1169 (100%; $M^+ - C_4H_7O_4$, 135.1174), 134.1091 (85%; $M^+ - 2CH_3COOH$, 134.1095). 10-Hydroxygeraniol bis(3,5-dinitrobenzoate), mp 142–145 °C (lit. 143–146 °C, Escher, 1972). Anal. Calcd for $C_{24}H_{22}O_{12}N_4$: C, 51.61; H, 3.97; N, 10.03. Found: C, 51.49; H, 3.91; N, 9.90. 10-Hydroxynerol: NMR ($CDCl_3$) δ 1.65 (s, H-9), 1.75 (m, H-4), 2.16 (m, H-5, H-6), 3.98 (s, H-10), 4.10 (s, H-1), 5.44 (t, J = 6.5 Hz, H-2, H-7); high resolution mass spectrum of the diacetate of 10-hydroxynerol *m/e* 212.1426 (0.14% $M^+ - C_2H_2O$, 212.1412), 135.1172 (25% $M^+ - C_4H_7O_4$, 135.1174), 134.1095 (20%, $M^+ - 2CH_3COOH$), 43.016 (100%). 10-Hydroxynerol bis(3,5-dinitrobenzoate), mp 91–92 °C (lit. 91.5–92.5 °C, Escher, 1972).

Preparation of Cell-Free Extracts. Five-day old etiolated *V. rosea* Linn (*Catharanthus roseus* (L) G. Don) seedlings (100 g, germinated at 30 °C over moist vermiculite) were used as the source of hydroxylase. Seed coats were carefully removed from cotyledons, and seedlings were washed in distilled water. All subsequent operations were carried out at 0–4 °C.

Seedlings were ground gently in a cold mortar for about 2 min in 2 volumes of grinding medium (0.1 M Tris-HCl, pH 7.6, containing 0.4 M sucrose, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, 10 mM metabisulfite, and 1 mM DTT[1]). The slurry was mixed with washed polyclar AT (tissue:polyclar AT, 25:1, w/w) and squeezed through two layers of silk and the filtrate centrifuged at 3000*g* for 20 min. The 3000*g* supernatant was then centrifuged at 20 000*g* for 20 min. The pellet was gently washed with small amounts of grinding medium and resedimented. The 20 000*g* pellet (60–70 mg) thus obtained was homogenized in 0.1 M Tris-HCl, pH 7.8, containing 1 mM DTT, 0.2 M sucrose, 1 mM EDTA, and 15% glycerol (w/v). This preparation retained most of its activity for 3 to 4 days when stored at −70 °C. In some initial experiments, a 10 000–100 000*g* pellet (microsomal fraction) was used as the source of enzyme. This was prepared as described earlier (Meehan and Coscia, 1973) and gave lower specific activities.

Solubilization and Resolution of the Hydroxylase System. The resuspended 20 000*g* pellet (3–4 mg of protein/ml) was sonicated for four 20-s intervals in a Branson ultrasonic disintegrator (Model W, 140D) at maximum output. Sodium cholate was added dropwise with stirring to give a final cholate to protein ratio of 0.4:1, and the mixture was stirred for an additional 30 min. After centrifugation at 100 000*g* for 1 h, the clear yellow supernatant (2–2.5 mg of protein/ml) was applied to a DEAE-cellulose column (1.25 × 12 cm) previously equilibrated with 0.1 M Tris-HCl, pH 7.8, containing 1 mM DTT, 0.005% sodium cholate, and 15% glycerol (w/v) (buffer A). The column was then eluted

[1] Abbreviations used: DTT, dithiothreitol; GSH, reduced glutathione; p-CMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide.

Table I: Requirements for Hydroxylase Activity.[a]

Deletion	% Activity
1. None (complete)	100
2. None (boiled enzyme)	5
3. NADPH generating system	10
4. O_2[b]	11
5. DTT from assay	35
6. DTT from total preparation	3
7. DTT (+ 1 mM GSH)	66
8. DTT (+ 10 mM GSH)	75
9. DTT (+ 1 mM mercaptoethanol)	33

[a] 0.25 mg of microsomal protein was used in each assay. For details see Experimental Procedure. One hundred percent activity represents 11.4 nmol of diol/(h mg^{-1}) of protein. [b] Assays were conducted anaerobically in septum-capped tubes by gassing the system with nitrogen and adding 0.5 mM glucose and 200 units of glucose oxidase.

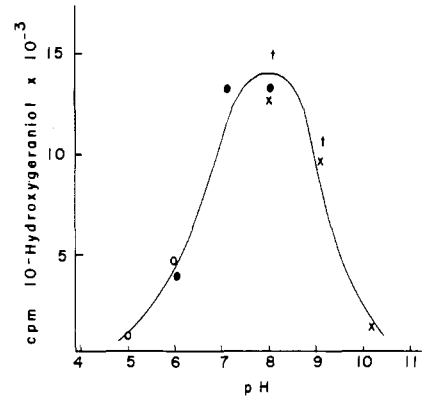


FIGURE 1: Effect of buffer composition and pH on geraniol hydroxylation. Activity is expressed in total cpm of 10-hydroxygeraniol obtained per assay. Microsomal protein (0.3 mg) was used in each assay. Buffers used: (O — O) acetate; (● — ●) phosphate; († — †) Tris; and (X — X) glycine. For details, see Experimental Procedure.

with 100-ml volumes of buffer A alone, then buffer A containing 0.2 M KCl, 0.3 M KCl, and finally 0.5 M KCl. The first eluate (buffer A) contained cytochrome P-450 (in 15 to 20% yield). Significant loss of cytochrome P-450 occurred during DEAE-cellulose chromatography and all attempts to increase the total yield have been unsuccessful. Fractions having cytochrome P-450 were pooled and concentrated by ultrafiltration. This fraction contained low levels of NADPH-cytochrome *c* reductase activity (Figure 3). The 0.2 M KCl eluate exhibited most of the NADPH-cytochrome *c* reductase activity and these fractions were also pooled and concentrated by ultrafiltration. The 0.3 M KCl eluate had very little reductase activity. Cytochrome P-450 eluted from the DEAE-cellulose column was highly labile, whereas the reductase remained stable for several months without appreciable loss of activity when stored at −70 °C.

Isolation of Total Lipid. The 20 000*g* pellet was extracted with a mixture of $CHCl_3$ and MeOH (2:1). The organic layer was evaporated to dryness under nitrogen and then suspended by sonication in 0.1 M Tris-HCl, pH 7.6. This mixture (50 μl, 0.2–0.3 mg of lipid) was used for reconstitution experiments. The total lipid fraction was subjected to TLC on precoated Eastman silica gel chromagram sheets (No. 13181) with chloroform–methanol–concentrated ammonium hydroxide (150:65:8) as developing solvent. The phospholipids were detected with a molybdenum spray (Dittmer and Lester, 1964).

Assay and Incubation Conditions. Protein was incubated in the presence of 5 mM glucose 6-phosphate, 0.5 mM $NADP^+$, 2.5 units of glucose-6-phosphate dehydrogenase, 0.5 μCi of [1-^{3}H]geraniol in 50 μl of acetone (0.066 mCi/μmol), 1 mM DTT, and 0.1 M buffer (potassium phosphate, pH 7.2, for microsomes, Tris-HCl, pH 7.8, for the 20 000*g* pellet) in a final volume of 1.5 ml. Hydroxylation was initiated with protein and the reaction carried out under aerobic conditions for 30 min at 25 °C. The reaction was terminated by the addition of 2.5 ml of methanol and the diols were extracted with 5 ml of $CHCl_3$. The $CHCl_3$ layer was evaporated with a stream of nitrogen at room temperature and an aliquot spotted on an Eastman chromagram sheet (silica gel, 0.1 mm). Authentic 10-hydroxygeraniol was also spotted on the plate which was developed with benzene–acetone–ethyl acetate (2:1:1 v/v/v). Marker diol was visualized with I_2 vapor and the radioactive diol band was cut out. Its tritium content was determined by liquid scintillation spectrometry (Kinard, 1957).

NADPH-dependent cytochrome c reductase was assayed by the procedure of Ernster et al. (1962). The reaction mixture contained in a total volume of 1.0 ml: 500 μmol of Tris-HCl, pH 7.6, 0.05 μmol of cytochrome *c*, 0.15 μmol of NADPH, 0.5 μmol of KCN, and the reductase (50–100 μg of protein). The reaction was initiated by addition of NADPH and ΔA_{550nm} was measured spectrophotometrically (ϵ21 mM^{-1} cm^{-1}).

Cytochrome P-450 and b_5 estimations were made according to the procedures of Omura and Sato (1964) using either a Cary Model 14 or an Aminco-Chance DW-2 split beam spectrophotometer. Protein determinations were made according to the method of Lowry et al. (1951) and, in some cases, a modified Lowry method (Potty, 1969) which corrects for phenolic contaminants. Values of protein content in extracts from 5-day old seedlings obtained by the two methods did not differ appreciably.

Results and Discussion

Requirements for Hydroxylation of Geraniol and Nerol. Under standard assay conditions, enzymatic hydroxylation of geraniol and nerol was dependent upon NADPH and oxygen (Table I). Hydroxylation rates were linear with time for at least 40 min and linear with concentrations up to 0.4 mg of microsomal protein/ml. Examination of the pH dependence of monoterpene hydroxylase activity with geraniol as substrate revealed a rather broad pH optimum (Figure 1). Dithiothreitol (DTT[1]) was routinely included in the extraction buffer and assay medium as a result of investigations on the efficacy of this as well as other reducing agents on hydroxylase activity (Table I). The deletion of metabisulfite from the grinding medium did not lower hydroxylase activity significantly but, if DTT was omitted from the extraction medium and the incubation mixture, hydroxylation was not observed. If DTT was deleted from the assay alone, as little as ⅓ of the control activity was obtained. In experiments 7–9, DTT was present in the extraction media but the indicated reducing agent replaced DTT in the assays. Reduced glutathione (GSH[1]) was less effective than DTT while mercaptoethanol at the above concentrations exhibited no enhancement at all over controls. These results are quite comparable to our findings for other enzymes of this plant (Madyastha et al., 1973) and this suggests that, upon cell disruption, enzymes are released which readily oxidize sulfhydryl groups or yield quinones which can con-

Table II: Effect of Thiol Reagents.[a]

Additive	Concentration (mM)	% Activity
Complete		100
p-CMB	0.1	17
	1.0	7
Iodoacetamide	1.0	100
	10	87
NEM	1.0	58
	10	4

[a] In each assay 0.35 mg of microsomal protein was resuspended in phosphate buffer and after a 10 min preincubation with the inhibitor, DTT, substrate, and NADPH generating system were added. For details see the Experimental Procedure. One hundred percent activity represents 14.0 nmol of diol/(h mg^{-1}) of protein.

Table III: Effect of Inhibitors on Enzymatic Hydroxylation of Geraniol.[a]

Additive	Concentration	% Activity
Metyrapone	0.1 mM	41
	0.2 mM	0
Phospholipase A	2 units	26
Phospholipase C	2 units	47
Cytochrome *c*	3.3 μM	17
	6.6 μM	13

[a] The assay mixture contained 0.15 mmol of Tris-HCl (pH 7.8), 5 mM glucose-6-phosphate, 0.5 mM $NADP^+$, 1.5 units of glucose-6-phosphate dehydrogenase, 0.2 μCi of [1-^{3}H]geraniol in 25 μl of acetone (6 μCi/μmol), 1.5 μmol of DTT, and 0.75 mg of protein (20 000g pellet) in a final volume of 1.5 ml. Protein was preincubated with inhibitor for 10 min before the addition of substrates and cofactors. The assay mixture was incubated at 25 °C for 30 min. Activity determined in the absence of inhibitor (100%) was 36.6 nmol/(h mg^{-1}) of protein.

dense with sulfhydryl groups (Loomis, 1974). The DTT may act to keep sulfhydryl groups in the reduced form and/or it may inhibit enzymes such as the polyphenol oxidases (Stafford, 1975).

Effect of Sulfhydryl Group Reagents. The suggested requirement for reduced sulfhydryl groups for maximal hydroxylation of geraniol was further tested with sulfhydryl reagents (Table II). While *p*-chloromercuribenzoate (p-CMB) and *N*-ethylmaleimide (NEM) were inhibitory, iodoacetamide had little effect on the hydroxylase. If p-CMB were added to an assay mixture containing 1 mM DTT, geraniol hydroxylation occurred to the extent of 47%, i.e., approximately three times that value obtained when the protein was pre-incubated with p-CMB. Subsequent studies of the solubilized NADPH-dependent cytochrome *c* reductase in *V. rosea* extracts reveal it is inhibited by p-CMB to a comparable degree (C. Morrow, unpublished observations). In addition it is known that sulfhydryl reagents convert certain P-450 heme proteins to their P-420 form (Cooper et al., 1965).

Carbon Monoxide Sensitivity of the Hydroxylase. Although a number of inhibitors elicit characteristic cytochrome P-450 responses from the monoterpene hydroxylase (Meehan and Coscia, 1973; Table III), the photoreversible CO sensitivity of the monoterpene hydroxylase is a major criterium in defining such systems (Omura and Sato, 1964). In experiments where precise amounts of CO were added, inhibition increased with increasing CO concentrations and was almost completely reversed with the aid of a

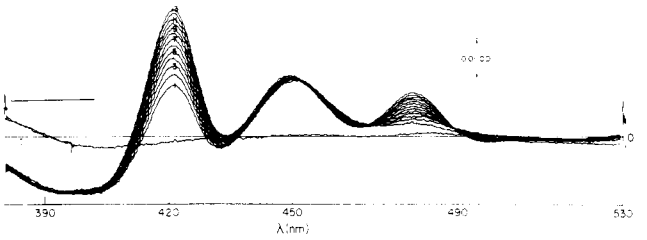


FIGURE 2: CO difference spectra of dithionite-reduced membrane bound cytochrome P-450 from *V. rosea*. Both sample and reference cells contained dithionite reduced microsomal suspensions (3.1 mg of protein/ml in 0.1 M potassium phosphate buffer, pH 7.2, containing 1.15% KCl). CO was bubbled into the sample cell for 30 s, and the spectra were taken. Repetitive (1→13) scans were made every 1.5 min.

light source having a spectral intensity which was optimal between 420 and 450 nm (Meehan and Coscia, 1973). White light from an incandescent light source had no effect on CO inhibition.

Dithionite reduced CO difference spectra (Figure 2) indicated 0.1 nmol of P-450/mg of protein in the 10–100 000g pellet. This concentration is higher than that reported for a number of plants (Markham et al., 1972) but comparable to levels observed in sorghum (Potts et al., 1974) and potato tubers (Cotte-Martinon et al., 1974). In subsequent purification of the hydroxylase, including sucrose density gradient centrifugation, difference spectra were obtained with all fractions containing hydroxylase activity (K. M. Madyastha, unpublished observations). Cytochrome b_5 was also present in such fractions as determined by oxidized vs. reduced visible spectra.

The band observed in the 420-nm region cannot be attributed to denatured P-450 alone. In time course studies (Figure 2) it was discovered that, while the 420-nm band increased with time after introduction of CO, the 450-nm absorption remains the same. A smaller peak at 485 nm also appeared with time and the time-dependent changes at this wavelength and at 420 nm have been attributed to the CO inhibition of enzymic degradation of a carotenoid in the sample cuvette (Cotte-Martinon et al., 1974). As the dithionite-dependent depletion of the carotenoid occurs in the reference cuvette, its presence in the sample cuvette becomes apparent. Metyrapone can replace CO in producing this time dependent change at 420 nm but the carotenoid degradative system does not seem to contain cytochrome P-450. Solubilized and purified fractions of this putative carotenoid degradative system have been isolated which exhibit no measurable CO-dependent 450-nm band. Carotenoids with visible spectra similar to that of lutein have been obtained from fractions containing hydroxylase by extraction with chloroform–methanol (2:1).

Pyridine Nucleotide Specificity. The *V. rosea* monoterpene hydroxylase did not significantly differ in activity with either an NADPH generating system or the free reduced nucleotide (Table IV). Replacing the NADPH generating system with NADH resulted in a loss of hydroxylase activity. To test for transhydrogenase activity the NADPH generating system was replaced with NADH and $NADP^+$. This combination also failed to support hydroxylation. In the course of studying hydroxylation, Murphy and West (1969) have reported that *Echinocystis macrocarpa* microsomes contain transhydrogenase activity which is ATP dependent. This type of activity was not detected in the *V. rosea* system when incubations were carried out in the pres-

Table IV: Pyridine Nucleotide Specificity of Geraniol Hydroxylation.[a]

Generating System Replaced with:	% Activity
None (complete)	100
0.1 mM NADPH	95
0.1 mM NADH	3
5 mM NADH	10
0.1 mM NADPH + 0.1 mM NADH	99
5 mM NADH + 5 mM $NADP^+$	20
5 mM NADH + 5 mM $NADP^+$ + 0.1 mM ATP	11

[a] Assays were conducted with 0.3 mg of microsomal protein as described in the Experimental Procedure. One hundred percent activity represents 9.2 nmol of diol/(h mg^{-1}) of protein.

ence of NADH, $NADP^+$, and ATP. Geraniol hydroxylation is no greater in the presence of both NADPH and NADH than with NADPH alone at 0.1 mM concentrations. In subsequent experiments, however, a synergistic effect of 30% was observed at 10 and 20 μM concentrations (not shown). Since saturation plots reveal that 0.1 mM NADPH is saturating, the synergism was not apparent initially. The fact that cytochrome b_5 has been detected by oxidized vs. reduced difference spectra in all fractions containing cytochrome P-450 may be of significance to the cytochrome P-450 electron transport chain. A direct interaction of an NADH-cytochrome b_5 system with the cytochrome P-450 hydroxylase may explain the above results, but experiments with crude membrane fractions cannot provide unequivocal answers. Studies (van der Hoeven et al., 1974) on the liver microsomal P-450 system, which has now been obtained in a highly purified state, rule out the requirement of cytochrome b_5 for certain cytochrome P-450 dependent hydroxylations but not synergistic effects on electron transfer within the intact membrane (Sasame et al., 1974; West et al., 1974).

Kinetics of Hydroxylation. The kinetics of geraniol and nerol hydroxylation were determined under standard assay procedures. Unlabeled substrate was used for saturating the hydroxylase while the amount of tritiated geraniol and nerol was kept constant. Lineweaver–Burk plots were linear. The apparent K_m for geraniol (5.5 μM) and nerol (11 μM) determined by the method of least-squares were similar and indicated a relatively high affinity of the substrates for the hydroxylase. The fact that the maximum velocity with geraniol as substrate (0.43 nmol/(min mg^{-1}) of protein) was greater than that for nerol (0.33 nmol/(min mg^{-1}) of protein) may be of significance to the in vivo metabolism of the acyclic alcohols. Evidence from in vivo tracer experiments suggests, however, that both cis and trans isomers are converted to indole alkaloids equally well in *V. rosea* (Scheme I) (Banthorpe et al., 1972). Enzyme specificity for the C-10 methyl group of both geraniol and nerol has been demonstrated and no cis–trans isomerase activity was detected in this partially purified system under the conditions of the assay (Meehan and Coscia, 1973).

Solubilization and Reconstitution. Before detergent solubilization studies were initiated, a series of assays was conducted in the presence of ionic and non-ionic detergents to determine their effect on hydroxylase activity. As seen in Table V, cholate and Renex-30 were least inhibitory at concentrations comparable to those used in the solubilization of the hydroxylase (see Experimental Procedure). However, prolonged storage of membrane bound or solubilized en-

Table V: Effect of Various Detergents on Geraniol Hydroxylation.[a]

Detergent	Concentration (μg/assay)	% Activity
Sodium cholate	200	94
	400	83
Sodium deoxycholate	200	85
	400	50
Lubrol-WX	200	71
	400	29
Renex-30	200	95
	400	64
Triton X-100	200	85
	400	39
Triton N-101	200	50
	400	19

[a] In each assay 1.0 mg of protein from the 20 000g pellet was preincubated with detergent for 10 min. See footnote *a*, Table III, for details. Activity in the absence of detergent (100%) was 26.8 nmol/(h mg^{-1}) of protein.

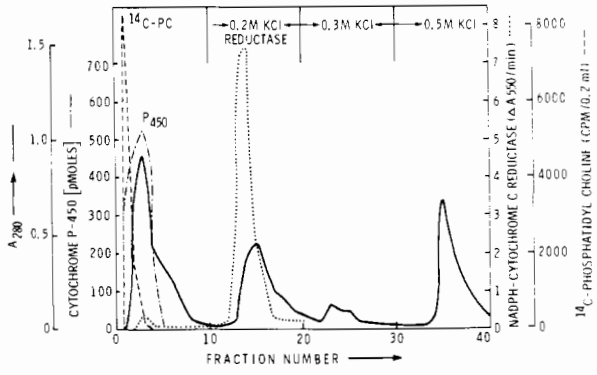


FIGURE 3: Elution profile of cholate solubilized hydroxylase from a DEAE-cellulose column. Fraction size, 10 ml, flow rate, 30 ml/h. See Experimental Procedure for details.

zyme with detergents resulted in considerable loss of hydroxylase activity. By sonicating the membrane fraction and then treating with detergents (cholate, Renex-30, or Lubrol-WX), 50–60% of the original hydroxylase activity was solubilized and remained in a 100 000g supernatant fraction. The solubilized membrane fraction gave characteristic CO difference spectra, but the specific activity of the hydroxylase was considerably lower than that of the original 20 000g pellet. This could be due to the slow inactivation of the cytochrome P-450 component. Supernates were then subjected to DEAE-cellulose chromatography which effected a good separation of the fractions rich in P-450 heme protein from the NADPH-cytochrome *c* reductase, especially when cholate was used as detergent (Figure 3). While added radioactive phosphatidylcholine eluted in the void volume and some phospholipid was found in each protein fraction, the yellow-colored lipid, associated with the hydroxylase in all previous purification steps, remained on the column. In contrast when Renex-30 or Lubrol-WX was utilized, the yellow pigment pervaded all fractions. Furthermore, the bulk of the cholate could be removed by ultrafiltration, whereas Renex-30 and Lubrol-WX cannot be separated in this manner. Since the cytochrome P-450 fraction obtained after the DEAE-cellulose chromatography was highly unstable, attempts to remove the non-ionic detergents from it by Sephadex LH-20 chromatography (Gaylor and Delwiche, 1969) led to a significant loss of hy-

Table VI: Reconstitution of the Geraniol Hydroxylase System.[a]

Fraction	NADPH-Cyt *c* Reductase Activity [nmol/(min mg^{-1}) of protein]	Hydroxylase Activity (pmol/h)
P_{450}	3.8	50
Reductase	190	280
P_{450} + reductase		340
P_{450} + lipid		50
Reductase + lipid		400
P_{450} + reductase + lipid		940

[a] In reconstitution assays cytochrome P-450 (~50 pmol, 0.22 mg of protein), reductase (0.04 mg of protein) and/or lipid (0.25–0.3 mg) were added in that order. For conditions of incubation, see footnote *a* of Table III.

droxylase activity. Thus cholate was used routinely for subsequent solubilization work.

Assaying the various column fractions revealed that buffer A eluent contained most of the cytochrome P-450 and exhibited only 5% of the optimal hydroxylase activities realized in reconstitution studies (Table VI). The reductase fraction, while demonstrating less than 6 pmol/mg of protein of P-450 heme protein (the limit of detection by CO difference spectra), possessed more hydroxylase activity than the cytochrome P-450 eluent, but optimal activities were observed with a combination of reductase, heme protein, and lipid extract (Table VI). However, the reconstituted system yielded only 10–15% of hydroxylase activity present in solubilized membrane fractions which agair is probably due to the highly labile nature of the cytochrome P-450. Protein which was eluted after the reductase neither exhibited hydroxylase activity nor enhanced activity of earlier fractions.

Phosphatidylcholine, phosphatidylethanolamine, lesser amounts of phosphatidylserine and lysolecithin, and an unidentified phospholipid occur in the lipid fraction as determined by TLC using a phospholipid-specific reagent for detection (Dittmer and Lester, 1974). Phospholipase A and C mediated inhibition of hydroxylase (Table III) suggests that the active constituent of the lipid fraction is a phospholipid, and further studies are underway to ascertain this.

Thus on the basis of chromatographic mobility on DEAE-cellulose in the presence of cholate or Renex (Lu and Levin, 1974) and the apparent lipid dependency, this partially purified plant cytochrome P-450 system resembles the liver microsomal more than the bacterial or adrenal cortex mitochondrial complexes.

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