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Cytochrome P-450LM$_2$ Mediated Hydroxylation of Monoterpene Alcohols†

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ABSTRACT: The enzymatic hydroxylation of geraniol and nerol by rabbit liver microsomal preparations has been demonstrated to occur exclusively at the C-10 (*E*)-methyl group of both acyclic monoterpene alcohols. This activity was greater in microsomes obtained from barbiturate pretreated rabbits than from β-naphthoflavone treated or control microsomal preparations. Kinetic parameters, V_{max} and K_m, for geraniol hydroxylation were consistent with these findings. Additional evidence for cytochrome P-450 mediation included O_2 and NADPH dependence and sensitivity toward typical P-450 heme protein inhibitors such as SKF-525A, metyrapone, CO, deoxycholate, and cytochrome *c*. Upon incubation of a reconstituted system containing highly purified cytochrome P450LM$_2$, Pb-derived P-450 reductase, and dilauroylphosphatidylcholine with geraniol and nerol, V_{max} values of 0.30 nmol of 10-hydroxygeraniol per min per nmol of P-450LM$_2$ and 0.25 nmol of 10-hydroxynerol per min per nmol of P-450LM$_2$, respectively, were obtained. In general, turnover numbers were comparable to those of the microsomes from rabbits administered phenobarbital. Upon reconstitution K_m values for geraniol and nerol (24 and 19 μM, respectively) were 5–10 times lower than the apparent K_m values observed with microsomes, but similar to microsomal dissociation constants derived from binding studies. Purified P-450LM$_4$ was incapable of supporting either monoterpene hydroxylation corroborating data from differential drug treatment. The interactions of representative hemi-, mono-, sesqui-, diterpene, and polyprene alcohols with the cytochrome P-450 fraction of microsomes from phenobarbital-induced animals were tested by optical difference spectroscopy. Many compounds, including the substrate nerol, elicited a type I binding spectrum. The modified type II spectrum was generated by geraniol, 10-hydroxygeraniol, 10-hydroxynerol, and 3-isopentenyl alcohol. In contrast, solanesol, a nonaprenyl alcohol, failed to produce a change in the Soret band of the heme protein.

In mammals cytochrome P-450 dependent monoxygenases are not only capable of hydroxylating an array of structurally different xenobiotics but also a variety of endogenous lipids (Dus, 1976). Included among the latter are many isoprenoid compounds. While cytochrome P-450 systems have not been implicated in the pathway for cholesterol biosynthesis in mammals, they do participate extensively in steroid metabolism. In the adrenal cortex, conversion of cholesterol to corticosteroid hormones entails P-450 heme protein dependent hydroxylations at carbons 11, 17, 18, 20, 21, and 22. Their involvement has also been observed in the synthesis of steroid sex hormones, vitamin D, and bile acids in mammals (Hayaishi, 1974; Gunsalus et al., 1975), of ecdysones in insects (Bollenbacher et al., 1977) as well as in the conversion of diterpenes to giberellins (Murphy & West, 1969) and of monoterpenes to indole alkaloids (Meehan & Coscia, 1973) in higher plants. Finally the studies of Gunsalus & co-workers (1975) on cytochrome P-450 dependent hydroxylation of the monoterpene camphor in bacteria have provided the most detailed knowledge of the mechanism of action of this system.

Investigations on mammalian biotransformation of terpenes extend as far back as a century ago (Williams, 1959). Analysis of urinary metabolites of administered terpenoids have indicated that allylic hydroxylation, oxidation, and glucuronidation are common modes of converting a hydrophobic molecule to a more water-soluble and hence excretable molecule. Examples of such detoxification processes have been observed in the conversion of *d*-limonene to 13 identified metabolites and, in contrast, the transformation of geraniol to predominantly 1,10-dicarboxylic acids (Hildebrandt's acid; Kodama et al., 1976; Kühn et al., 1936). Despite the numerous studies on mammalian terpene metabolism, involvement of cytochrome P-450 has only been based on circumstantial evidence such as the induction of this heme protein after chronic administration of terpenes (Ariyoshi et al., 1975; Bang & Ourisson, 1975; Cinti et al., 1976) and terpene-induced optical difference spectra with microsomal preparations (Leibman & Ortiz, 1973). These difference spectra have been characteristic of substrate–cytochrome P-450 interactions which can be correlated with spin state changes of the latter (Sligar, 1976).

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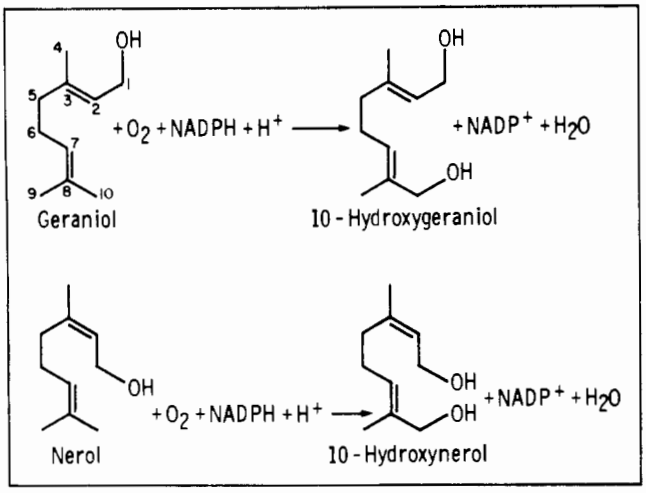
SCHEME I



We wish to report the first unequivocal evidence for the ω-hydroxylation of the acyclic monoterpene alcohols, geraniol and nerol, by a hepatic microsomal enzyme system containing cytochrome P-450LM$_2$. Enzymatic studies with both microsomes and a reconstituted P-450LM$_2$ system exhibited a positional selectivity for the (*E*)-methyl (C-10) group of geraniol and nerol (Scheme I). By generating optical difference spectra of a variety of isoprenoids, we have contributed to the evidence that they are also hydroxylated by P-450 heme protein dependent enzymes. The dissociation constants obtained in such studies suggest certain mono- and sesquiterpenes have high affinity for binding sites on P-450 heme proteins.

Experimental Section

Materials. Solvents were of reagent grade quality and were redistilled prior to use. Geraniol (99+%), geranyl acetate (99%), citral (redistilled), *d*-carvone (96%), *l*-carvone (98%), citronellol (97%), limonene (97%), linalool (99%), 2-isopentenol (96%), 3-isopentenol (97%), farnesol (95%), *l*-menthol (mp 43–45 °C), and cedrol (mp 86–87 °C) were obtained from Aldrich Chemical Co. Numbers in parentheses represent the manufacturer's assessment of purity which was confirmed by GLC analysis using a Varian Aerograph series 1200 chromatograph equipped with a hydrogen flame detector. A 10 ft. × 1/8 in. column packed with 15% Carbowax supported on acid washed Chromosorb (100–200 mesh) was used with a helium flow rate of 15 mL/min. Technical grade phytol was purchased from Aldrich and further purified by thin-layer chromatography. Nerol (redistilled) was from Chemical Samples Co., Columbus, Ohio. Geranylgeraniol and solanesol were kindly supplied by Dr. R. E. Olson of this department.

Sodium deoxycholate, sodium cholate, di-12GPC,[1] Tween 80, Triton N-101, NaDodSO$_4$, NADP$^+$, NADH, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XV), glucose oxidase (type V), crystalline BSA, horseheart cytochrome *c* (type III), *p*-OH biphenyl (*p*-phenylphenol), DTT, and DEAE-cellulose were purchased from Sigma Chemical Co. The DEAE-cellulose (medium mesh; capacity 0.95 mequiv/g) was washed with 0.5 N NaOH, 0.1 N HCl, 0.5 N NaOH, and water in that order. The pyridine was distilled over NaOH. Aminopyrine (97%), PCMA, BNF (mp 164–166 °C), metyrapone (mp 53–56 °C), acetyl acetone (99%), and ammonium acetate (98%) were purchased from Aldrich Chemical Co., 3,5-dinitrobenzoyl chloride (recrystallized, mp 69 °C), $AgNO_3$, and biphenyl (mp 69–71 °C) were obtained from Eastman, Hepes was from Boehringer Mannheim, PPO from Packard, SKF-525A from Smith, Kline, and French Labs., and 2′-AMP from P-L Biochemicals. Renex 30 (polyoxyethylene (12) tridecyl ether) was a gift of Atlas Chemicals Division of ICI, Wilmington, Del.

Highly purified rabbit liver cytochromes P-450LM$_2$ (specific content = 14.6 nmol/mg) and P-450LM$_4$ (specific content = 12.4 nmol/mg) were generously provided by Dr. M. J. Coon. Both species of heme protein were obtained from Pb-treated animals.

Synthesis of Labeled Compounds. [1-^{3}H]Geraniol and [1-^{3}H]nerol were synthesized by NaB^3H_4 reduction of citral as described elsewhere (van Aller & Nes, 1968; Madyastha et al., 1976). Radiochemical purity was verified by radiochromatographic scan (Packard Model 7201) of a TLC profile.

Synthesis of Diols. 10-Hydroxygeraniol and 10-hydroxynerol were synthesized from geranyl and neryl acetates, respectively, by selenium dioxide oxidation and structural assignments were based on NMR, high resolution mass spectrometry of the diacetates, and melting point determinations of the bis(3,5-dinitrobenzoate) ester derivatives, as described previously (Madyastha et al., 1976).

Treatment of Animals. All compounds were administered intraperitoneally at the following doses: 1% BNF suspension in Mazola corn oil, 80 mg of BNF/kg, Pt in 0.85% NaCl, 100 mg of Pt/kg, and Pb in 0.85% NaCl, 100 mg of Pb/kg. BNF (or corn oil alone) was administered in a single dose 38–40 h before sacrifice; Pt, Pb, or saline were given in two daily half-doses for 3 days. Animals were fasted 24 h preceding sacrifice.

Preparation of Microsomes. Rabbits were sacrificed by injection of Nembutal into the ear vein. The liver was perfused in situ with 0.1 M KCl, 0.1 M sodium citrate, pH 7.7, until the perfusate was clear. All subsequent steps were performed at 0–4 °C.

Liver homogenization and isolation of microsomes were performed according to Haugen & Coon (1976). DTT replaced butylated hydroxytoluene in all buffers. Microsomes were stored at −20 °C.

Protein concentrations were determined by the method of Lowry et al. (1951). Glycerol concentrations were kept below 0.05% in all determinations.

Solubilization and Purification of NADPH-Cyt P-450 Reductase. The method of biospecific affinity chromatography was used in the purification of the detergent-solubilized NADPH-cyt P-450 reductase from BNF-treated and Pb-treated rabbit liver microsomes, as reported by Yasukochi & Masters for rat and pig liver reductase preparations (1976), with the following modifications. Both Pb-microsomes (0.088–1.5 g) and BNF-microsomes (0.49–1.7 g) were mixed for 60 min with either a solution of 5% sodium cholate and 10% Triton N-101 or a solution of 5% sodium cholate and 10% Renex 30 to afford final concentrations of 0.64% of cholate and 1.3% of Triton or Renex. The 100 000*g* × 90 min supernatant, which contained 86–100% of the total microsomal reductase activity, was directly applied onto a DEAE-cellulose column

[1] Abbreviations used: PCMA, *p*-chloro-*N*-methylaniline; BNF, β-naphthoflavone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate; NaDodSO$_4$, sodium dodecyl sulfate; di-12GPC, dilauroylphosphatidyl glycerol; Pb, phenobarbital; Pt, pentobarbital; Pb-microsomes, microsomes from Pb-treated rabbits; Pt-microsomes, microsomes from Pt-treated rabbits; DTT, dithiothreitol; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; BSA, bovine serum albumin; 2′,5′-ADP-Sepharose 4B, Sepharose 4B bound ^{6}*N*-(6-aminohexyl)-adenosine 2′,5′-bisphosphate; P-450 LM, liver microsomal cytochrome P-450; PPO, 2,5-diphenyloxazole.

(4 × 15 cm) previously equilibrated with 25 mM Tris buffer, pH 7.7, containing 0.25% Triton N-101, 0.1% sodium cholate, 0.05 mM EDTA, and 0.05 mM DTT or with the same buffer solution containing 0.8% Renex 30 in place of Triton N-101. After washing with four column volumes of equilibration buffer, reductase was eluted with 1500 mL of a linear gradient of KCl in equilibration buffer (0–0.5 M KCl). Fractions (23 mL each) containing reductase of specific activity >0.3 μmol/(min mg of protein) were pooled and concentrated tenfold in an Amicon Ultrafiltration apparatus equipped with a PM-10 or PM-30 membrane under 20–30 psi of nitrogen, followed by direct application of the concentrate to a 2′,5′-ADP-Sepharose 4B affinity column (bed volume 16 mL). Equilibration, wash, and elution buffers all contained 0.1% Renex 30 or 0.1% Triton N-101. Removal of unbound 2′-AMP from reductase containing eluates was facilitated by passage over Sephadex G-25. Active fractions in the V_0 were applied to a DEAE-cellulose column (4 × 15 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol in order to remove nonionic detergent. In the Triton N-101 preparations, the eluate was monitored until the A_{280} was approximately zero (120 mL). A slightly larger volume (142 mL) was allowed for detergent removal from Renex 30 preparations, as this detergent does not contain a UV-absorbing phenyl moiety. A yellow band comprising the top few millimeters of the cellulose column was resuspended with a Pasteur pipet and transferred to a tube containing 0.5 M potassium phosphate buffer, pH 7.7, in the presence of 20% glycerol and 0.1% sodium deoxycholate. The reductase was dialyzed twice against 2 L of 50 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM EDTA, and 0.1% sodium deoxycholate and stored at −80 °C. The Dulley & Grieve (1975) modification of the Lowry method was employed for determining protein concentrations of all solutions containing nonionic detergents.

Spectral Analyses. Cytochrome P-450 levels in microsomes (3 mg of protein/mL) were determined from CO difference spectrum of dithionite reduced protein using an extinction coefficient of 91 mM^{-1} cm^{-1} for Δ450–490 nm (Omura & Sato, 1964). Spectra were recorded with an Aminco-Chance DWII double-beam recording spectrophotometer equipped with a temperature controlled cell holder equilibrated at room temperature and magnetic stirrers in both cell compartments.

Spectral interactions betweeen isoprenoid compounds and microsomal heme protein were measured at ambient temperature after establishing a baseline. Microsomes were diluted with Tris–acetate buffer to a protein concentration of 3 mg/mL. Compounds were dissolved in acetone and added to the sample cuvette subsequent to addition of an equivalent volume of acetone to the reference cuvette. The volume of acetone added did not exceed 50 μL which represented 2% of the final suspension. A family of curves was obtained by adding increasing amounts of the compound to the microsomes and scanning from 350 to 500 nm until no further increase in absorption occurred. Spectra are designated as type I (peak at 390–397 nm and trough at 426–438 nm) or modified type II (peak at 416–417 nm and trough at 385–392 nm; Yoshida & Kumaoka 1975; Schenkman et al., 1972). The extent of interaction was measured by the difference in absorbance between the wavelengths of maximum and minimum absorption (ΔA_{max}). Binding isotherms generated by plotting reciprocal ΔA_{max} vs. reciprocal concentration permitted an estimation of the dissociation constant.

In those instances where a high affinity for the P-450 heme proteins is exhibited by the ligand, the total ligand concentration can approach the total heme protein concentration. Since K_d should be obtained from a plot of ΔOD vs. the *free* ligand level, this will lead to an overestimated dissociation constant unless compensation is made for the amount of ligand bound. Although we have not corrected for bound ligand, we estimate our maximal errors to be 5–10%. This figure was arrived at by calculations utilizing a $\Delta\epsilon_{385-420}$ of 40–50 mM^{-1} cm^{-1} attributed to typical type I ligands (Jefcoate, 1978). Even if the monoterpenes examined exhibited a $\Delta\epsilon$ as high as that of camphor with P-450_{CAM} (104 mM^{-1} cm^{-1}), our error would only be doubled. However, the calculation of this error was made assuming the total population of P-450 heme proteins will bind with ligand. As suggested by our reconstitution studies, a fraction of the total population of P-450 hemeprotein species present in microsomes, i.e., P-450 LM_4, may not be capable of interacting with these compounds. Furthermore in these studies we have used microsomes from animals treated with phenobarbital which would be expected to be retained at a fraction of the same cytochrome P-450 sites to which monoterpenes bind. This would result in reducing the availability of low-spin heme protein even further (Ebel et al., 1978) which would decrease our error.

All determinations of NADPH-cyt *c* (P-450) reductase activity were measured at room temperature using either an Aminco-Chance DWII recording spectrophotometer or a Heath-Schlumberger Model EU-707-11 double-beam recording spectrophotometer. The reaction was initiated by the addition of NADPH and ΔA_{550nm} was measured (ϵ 21 mM^{-1} cm^{-1}). Initial velocities were dependent upon the amount of reductase added.

Reconstitution of Hydroxylase Activities. The components of geraniol and nerol hydroxylase incubation mixtures resembled those of Haugen et al. (1975) for various other substrates, including biphenyl. Certain assays included 0.2 nmol of cyt P-450LM or up to 150 μg of di-12GPC per mL as indicated. Incubations were carried out for 30 min at 30 °C and contained 2.5–213 nmol of [1-^{3}H]geraniol or 12–272 nmol of [1-^{3}H]nerol and 0.4 μmol of NADPH as the final additive. NADPH was omitted in control experiments.

All reconstitution studies were performed with the Pb-induced reductase which has been shown to support hydroxylation of various substrates in the presence of each of the recognized forms of highly purified cyt P-450LM (Guengerich, 1977a).

Other Enzyme Assays. Biphenyl 4-hydroxylase activity was measured fluorimetrically (Creaven et al., 1965) using a Perkin-Elmer Model 512 double-beam recording fluorescence spectrophotometer with 1-cm^2 quartz cuvettes and 150-W xenon lamp as light source. Aminopyrine and PCMA demethylation activities were assayed as described by Bend et al. (1972). Formaldehyde production was measured spectrophotometrically using the Cochin & Axelrod modification (1959) of the Nash procedure (Nash, 1953).

Identification of Product Diols. The $CHCl_3$-soluble material from microsomal and reconstitution incubations were independently fractionated on 0.5-mm silica gel preparative plates developed in benzene–ethyl acetate–acetone (2/1/1, v/v/v). The diol band was eluted with acetone, evaporated under N_2, and resuspended in benzene. Upon addition of carrier diol and a benzene solution (0.4–2.9 mL) containing 3,5-dinitrobenzoyl chloride (15–360 mg) and pyridine (<20 μL), the solution was heated at 100 °C for 30 min to provide the corresponding bis(esters). Pyridine and unreacted derivatizing reagent were removed by extraction techniques described by Gasparič and Borecký (Macek, 1963). The radioactive 10-hydroxygeranyl derivative was recrystallized to

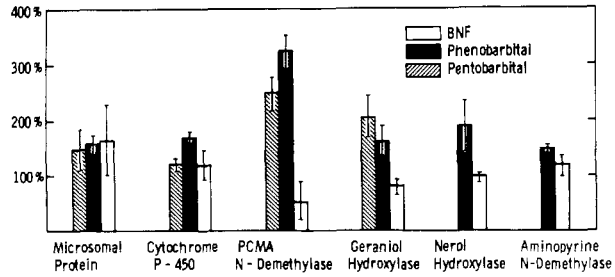


FIGURE 1: Columns represent mean values ±SEM of Pb-treated, Pt-treated, and BNF-treated animals relative to control. Control values were: microsomal protein, 14.4 ± 2.1 mg/g of fresh liver; cytochrome P-450, 1.94 ± 0.21 nmol/mg of protein; PCMA *N*-demethylase, 2.27 ± 0.46 nmol of *p*-chloroaniline/(min mg of protein); geraniol hydroxylase, 0.18 nmol of 10-hydroxygeraniol/(min mg of protein); nerol hydroxylase, 0.52 nmol of 10-hydroxynerol/(min mg of protein); aminopyrine *N*-demethylase, 8.68 ± 2.34 nmol of aminoantipyrine/(min mg of protein). Final concentrations of nerol and geraniol were 100 μM and 32 μM, respectively.

constant specific radioactivity from hexane–benzene solutions. Water–methanolic solutions of the 10-hydroxyneryl derivative crystallized in the cold (−20 °C). Melting point determinations of the crystals were obtained on a Fisher-Johns apparatus and were in agreement with literature values (Escher, 1972; Madyastha et al., 1976).

Results and Discussion

Effects of Inducers on Monoxygenase Activities. Liver microsomes from Pb- or Pt-treated rabbits exhibited higher cytochrome P-450 levels, and elevated *N*-demethylase and monoterpene hydroxylase specific activities (Figure 1). Induction of cytochrome P-450 heme protein by Pt was not as great as that observed following Pb treatment. No increases in *N*-demethylase or monoterpene hydroxylase rates were observed for microsomes from BNF-treated rabbits.

Apparent K_m and V_{max} were derived from kinetic data by Hofstee plots (Lenk, 1976). A comparison of these parameters for control (0.215 mM; 0.92 nmol/(min mg), respectively), Pb (0.123 mM; 1.73 nmol/(min mg), respectively), and BNF (0.181 mM; 1.05 nmol/(min mg), respectively) microsomes reveals an overall increase in substrate affinity and rate of hydroxylation in the Pb-microsomes for geraniol hydroxylase. Similar changes were observed in kinetic parameters of nerol hydroxylase: control (K_m = 0.187 mM, V_{max} = 1.32 nmol/(min mg)), Pb (0.166 mM, 1.70 nmol/(min mg), respectively) and BNF (0.237 mM, 1.39 nmol/(min mg), respectively). This phenomenon has been observed in reference to induction parameters of other monoxygenase activities (Ullrich et al., 1975; Alvares et al., 1968). In the case of nerol hydroxylase BNF treatment appeared to decrease the affinity of the substrate for the microsomes. Differences between the K_m for geraniol in control and BNF microsomes were not statistically significant. V_{max} values for monoterpene hydroxylation by the microsomes are less than some of the best substrates for this system. However, they are six times higher than values for hydroxylation of other isoprenoids such as the cholesterol 7α hydroxylation obtained with microsomes from animals induced with cholestyramine (Boyd et al., 1973).

Reaction rates remained constant in the range of pH 7.0–7.7. A linear relationship between the rate of hydroxylation and microsomal protein concentrations of 150–670 μg/mL was observed. Experiments carried out with 2 or 3 mg of total protein caused 57% and 70% inhibition of specific activity, respectively. Hydroxylation was linear for 30 min and could be detected in incubations employing a substrate concentration of less than 1.7 μM (7500 cpm over a boiled-enzyme blank). An upper limit of approximately 2.4 mM substrate concentration could not be exceeded due to solubility limitations of the incubation mixture. Although DTT was not an absolute requirement for hydroxylation, it was used in all assay buffers to prevent aggregation of microsomes during isolation and incubation.

TABLE I: Effects of Inhibitors on Geraniol Hydroxylation.[a]

incubation/conditions	% act.
1. complete	100
2. minus O_2[b]	4
3. minus NADPH + NADH (0.1 mM)	35
4. + CO[c]	32
5. + SKF525A (1 mM)	48
6. + metyrapone (0.2 mM)	40
7. + sodium deoxycholate (0.05%, w/v)	4
8. + cytochrome *c* (0.1 mM)	5

[a] Microsomal protein (400–800 μg) was incubated in the presence of 5 mM glucose 6-phosphate, 0.5 mM $NADP^+$, 1.25 units of glucose-6-phosphate dehydrogenase, [1-^{3}H]geraniol (15–65 μM, 4.5 × 10^5 to 10^6 cpm) in 10–30 μL of acetone, 1 mM DTT, and 0.1 M Tris-HCl buffer, pH 7.6, in a total volume of 1.5 mL. Hydroxylation was initiated by the introduction of the generating system and the reaction carried out under aerobic conditions for 30 min at 37 °C. The reaction was terminated with methanol and the mixture extracted with chloroform. The chloroform–methanol (2:1, v/v) extract was chromatographed on thin layers of silica gel to separate unreacted substrate from product diol (solvent system: benzene/ethyl acetate/acetone, 2/1/1 (v/v/v)). The specific activity of the complete system was 0.73 nmol of 10-hydroxygeraniol/(min mg of protein). [b] When anaerobic conditions were employed septum-capped test tubes were initially deaerated with N_2. The residual O_2 had to be removed by preincubation of the system with glucose plus glucose oxidase or a considerable amount of enzyme activity was observed (77% of controls). [c] CO (99.9%) was bubbled into the incubation mixture in a screw cap test tube as in experiment 2 but the O_2 scavenging system was omitted.

Inhibitory Effects on Geraniol and Nerol Hydroxylases. Geraniol was metabolized by the hepatic microsomal fraction in an O_2- and NADPH-dependent process (Table I). The rate of hydroxylation in the presence of 0.1 mM NADH was only 35% as efficient as the NADPH-supported reaction. Significant rates of NADH-mediated metabolism for other substrates (e.g., PCMA, benzo[*a*]pyrene) have been observed (West & Lu, 1977; Correia & Mannering, 1973). Inhibition of oxidation by specific antagonists of cytochrome P-450 systems represents a further criterion implicating involvement of this heme protein in this reaction (Table I). Sodium deoxycholate, which is capable of converting cytochrome P-450 to its inactive form, cytochrome P-420, drastically lowered activity. While CO, metyrapone, and SKF-525A are known to inhibit by interaction with the heme component, cytochrome *c* acts by diverting reducing equivalents from the reductase away from cytochrome P-450. Nerol hydroxylase activities were also sensitive to metyrapone (33% inhibition), SKF-525A (58% inhibition), and cytochrome *c* (88% inhibition), using inhibitor concentrations as in Table I.

Spectral Interactions. Two types of spectral changes were elicited by the addition of various hemiprenes, monoterpenes, sesquiterpenes, and diterpenes to Pb-microsomes as exemplified by the families of binding curves illustrated for geraniol (modified type II) and nerol (type I), Figure 2. The use of Pb-microsomes enhanced production of maximal differences in absorbance between characteristic peaks and troughs (Orrenius et al., 1972; Kumaki et al., 1978). However, some variation in spectral types and binding isotherms have been

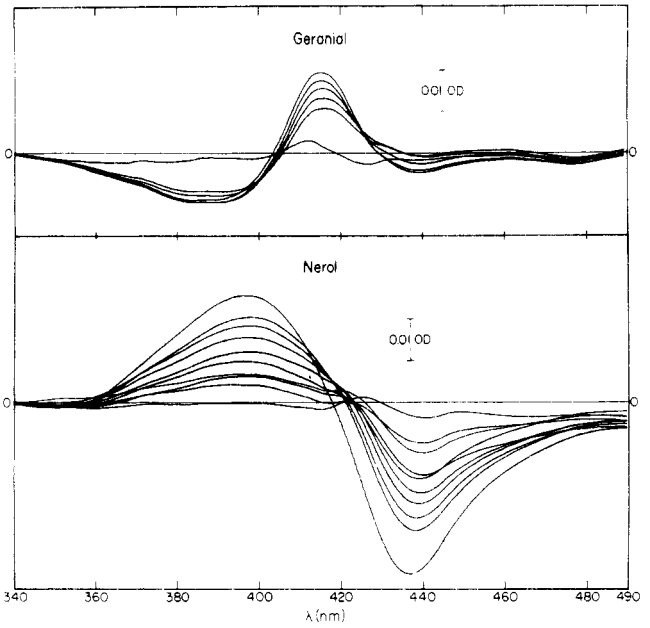


FIGURE 2: Geraniol-induced (modified type II) and nerol-induced (type I) spectral changes in microsomes from phenobarbital-treated rabbits. Each cuvette contained 3 mg of protein per mL maintained at room temperature for the entire experiment. Geraniol concentrations ranged from 15 to 100 μM, whereas nerol levels were varied from 5 to 375 μM.

observed for the same compound when microsomes from differentially treated animals were compared (Orrenius et al., 1972; Powis et al., 1977; Fasco et al., 1977). Reciprocal plots of the peak to trough ΔOD vs. the substrate concentration provides dissociation constants. The results are summarized in Table II. In some instances binding isotherms were biphasic and resulted in the calculation of two dissociation constants (Figure 2 and Table II).

Compounds causing a typical type I spectral change with Pb-microsomes include acyclic (e.g., nerol, farnesol, phytol) as well as cyclic (e.g., limonene, menthol, carvone, cedrol) terpenes. This spectral type is generally associated with hydrophobic molecules that are substrates and in many cases inducers of cytochrome P-450. Most of the terpenes tested are alcohols. Yoshida & Kumaoka (1975) have demonstrated that, at relatively high concentrations, alcohols of 5 carbons or less give modified type II spectra, whereas larger more hydrophobic molecules generate type I spectra with lower affinity constants.[2] The results obtained in this study for the various terpenes are consistent with previous findings with the exception of geraniol (Table II).

The observation of a modified type II spectrum for the trans acyclic alcohol, geraniol, as opposed to the type I spectrum for its cis isomer, nerol, makes an interesting point. Few substrates of cytochrome P-450 elicit a modified type II change in the Soret band, and those that do often show hidden type I interactions at different concentrations (e.g., agroclavine; Wilson & Orrenius, 1972). Higher aliphatic alcohols also exhibit a dual interaction (Yoshida & Kumaoka, 1975) as did 3-isopentenol in this study. Schenkman et al. (1972) have proposed that the modified type II represents a reverse of the type I and therefore could be due to removal of endogenous type I binders from the microsomal cytochrome P-450 in the sample cuvette

[2] 2-Isopentenol consistently produced a type I spectrum. At concentrations below the K_d UV absorption due to this compound interfered with changes in the Soret region of the spectrum. Hence the generation of a modified type II spectrum by concentrations greater than the K_d of the type I spectrum could have gone undetected.

TABLE II: Isoprenoid-Induced Spectral Changes in Pb-Microsomes.

compound	spectral type	absorption peak (nm)	absorption trough (nm)	K_d (μM)
2-isopentenol	I	392	424	4.1×10^5
3-isopentenol	II′	421	390	3.4×10^5
geraniol	II′	416	391	8, 56
nerol	I	397	438	2.2, 45
citronellol	I	393	432	6.7, 100
linalool	I	391	426	25
10-OH geraniol	II′	417	390	100
10-OH nerol	II′	417	385	83
limonene	I	390	428	18.9
l-menthol	I	391	429	5
d-carvone	I	390	427	11, 32
l-carvone	I	390	426	7.8, 48
farnesol	I	391	428	45
cedrol	I	391	427	1.75
geranylgeraniol	I	395	428	115
phytol	I	391	427	286
solanesol	ND[a]	...	...	

[a] ND, not detectable. Addition of terpenes to microsomal suspensions (3 mg of protein/mL) was continued until maximal changes in optical density were established.

(Powis et al., 1977). The modified type II can be elicited by titration of microsomes with BSA or by creating a temperature differential between sample and reference cuvettes (Pierson & Cinti, 1977). However, the maintenance of uniform temperature between sample and reference cuvettes in these experiments was accomplished with the use of a circulating water bath as described in Materials and Methods. In contrast Yoshida & Kumaoka (1975) attribute modified type II spectra to a heme interaction with the alcohol group reminiscent of the nucleophilic amine complexation characteristic of nitrogenous compounds which elicit a type II response. Regardless of its origin the fact that the trans isomer but not the cis acyclic monoterpene alcohol generates this type of change indicates that a critical juxtaposition of hydroxyl (or its equivalent) and hydrophobic groups are prerequisites for its occurrence. Furthermore, the interaction of geraniol is far more specific than that of smaller alcohols on the basis of its much lower affinity constant. Thus to engender a modified type II with 10^{-5} to 10^{-6} M concentrations, an appropriately located hydrophobic group must interact. The absence of this arrangement makes the 10-hydroxygeraniol and 10-hydroxynerol as well as some of the previously mentioned indole derivatives modified type II producers only at higher concentrations (Niwaguchi & Inoue, 1975; Orrenius et al., 1972; Wilson & Orrenius, 1972).

In this study additional evidence for specific geometrical requirements is reflected in the marked difference in affinity between the two sesquiterpene alcohols, farnesol and cedrol. The latter is a tricyclic alcohol and evidence exists to suggest that both it and its dehydration product can induce cytochrome P-450 in mammals (Bang & Ourisson, 1975). The failure of solanesol, an acyclic nonaprenyl alcohol, to interact at 275 μM concentration suggests size limitations exist.

Purification of NADPH-Cytochrome P-450 Reductase. Cytochrome P-450 reductase was purified over 100-fold by affinity chromatography from both Pb-treated and BNF-treated rabbit liver microsomes (Table III). Although purification of the reductase from Pb-microsomes has been reported (Coon et al., 1977), this represents the first attempt at purification of the BNF-reductase (from rabbit). Initial DEAE-

TABLE III: Purification of Pb- and BNF-Reductases by Bioaffinity Chromatography.[a]

preparation	volume (mL)	total protein (mg)	total act. (μmol/min)	sp act. (μmol/(min mg))	yield from microsomes (%)
1. microsomes	98.4 (75)	1704 (1732)	474 (606)	0.278 (0.35)	100
2. solubilized microsomes	182	1384 (1290)	512 (570)	0.370 (0.44)	100 (94)
3. DEAE-cellulose column eluate (0–0.5 M KCl)	935	353	320	0.906	68
4. PM-10 concentrate of DEAE fractions	52	306	75	0.244	16
5. 2′,5′-ADP-Sepharose 4B eluate	10	17	51	30	11
6. Sephadex G-25	20	1.52 (5.8)	46 (90)	30 (15.5)	10 (15)

[a] Purification of BNF-reductase was carried out in the presence of Renex 30, whereas the data for Pb-reductase (numbers in parentheses) were obtained using Triton N-101. Determinations of NADPH-cyt P-450 reductase activity were performed as described in the Experimental Section using an incubation mixture containing 300 μmol of potassium phosphate buffer, pH 7.7, 0.04 μmol of cytochrome *c*, reductase, and 0.1 μmol of NADPH in a final volume of 1 mL. Assays of microsomes and solubilized microsomes contained 0.6 μmol of KCN.

TABLE IV: Reconstitution of the Geraniol and Nerol Hydroxylase System.[a]

components	activity (pmol/(min nmol of P-450)) geraniol hydroxylase	nerol hydroxylase	biphenyl 4-hydroxylase
LM_2	0	0	
reductase	5	0	
LM_2 + lipid	3	0	
reductase + lipid	0	3	
LM_2 + reductase	26	96	
LM_2 + reductase + lipid	93	326	5200[b]
LM_4 + reductase + lipid	0	7	1400[b]

[a] Geraniol, nerol, and biphenyl hydroxylations were determined at 30 °C in a reaction mixture containing, per mL, 0.1 nmol of P-450LM, 288 units (16 μmol/(min mg of protein)) of Pb-reductase, 150 μg of di-12GPC, 50 μg of sodium deoxycholate, 50 μmol of Hepes buffer, pH 7.4, 15 μmol of $MgCl_2$, and 13 nmol of geraniol (420 000 cpm), or 20 nmol of nerol (437 000 cpm), or 1.0 μmol of biphenyl, added in that order. The mixture was preincubated for 3 min at 30 °C, followed by a 30-min incubation for geraniol and nerol hydroxylation and a 15-min incubation for biphenyl hydroxylation initiated by the introduction of 0.4 μmol of NADPH. Maximal rates observed in certain experiments in the presence of 30 μg of di-12GPC were 865 pmol/(min nmol of LM_2) and 722 pmol/(min nmol of LM_2) for geraniol and nerol hydroxylation, respectively. [b] Both of these values were comparable to previously reported data (Haugen et al., 1975).

cellulose chromatography afforded a separation of primarily three colored protein bands. The first yellow band of the 0–0.35 M KCl eluate as well as the following pink component exhibited negligible cytochrome *c* reductase activity. The yellow cytochrome P-450 reductase then emerged sharply followed by some trailing. Increasing the gradient to the 0.5 M KCl final concentration remedied the latter significantly. Whereas ~50% of the total cytochrome P-450 had been converted to cytochrome P-420 during solubilization of the microsomes in the presence of Triton N-101, only ~10% inactivation could be observed in the DEAE eluate if Renex 30 was used. The elution profile of Triton N-101 solubilized reductase and heme protein on DEAE-cellulose resembled the profile obtained by Lu & Levin (1974) for rat liver solubilized microsomal components. Any further trace contamination of reductase by cytochromes P-450 or P-420 was removed during affinity chromatography.

Concentrating the BNF-reductase by ultrafiltration produced extensive inactivation as seen from the drop in total activity concomitant with total recovery of protein in the retentate. Carrying out this purification on a fourfold lower batch size resulted in considerably less inactivation at this point. The Pb-reductase exhibited less sensitivity to concentration by this procedure. In personal communication with Dr. Y. Yasukochi it was later learned that direct application of the DEAE active fractions onto the Sepharose affinity column alleviates this problem.

Final purification of the BNF-reductase was achieved after elution from the affinity column by increasing the 2′-AMP buffer concentrations to 0.7 mM, 1.0 mM, and 2.0 mM, in a stepwise fashion. The majority of activity was found in the latter two eluates, and, in addition to the reductase present in the 0.7 mM wash, accounted for all of the activity originally loaded.

The purest reductase preparations were treated with mercaptoethanol and $NaDodSO_4$ prior to polyacrylamide electrophoresis. Staining with Coomassie brilliant blue revealed two major bands having molecular weights of approximately 68 000 and 80 000 in the presence of two minor contaminants.

The major bands from both BNF- and Pb-reductase preparations appear to migrate identically. Microsomal cytochrome P-450 reductases from untreated, Pb-, and 3-methylcholanthrene-induced rat livers have been found to be indistinguishable in immunoprecipitation and electrophoretic experiments (Welton and Aust, 1975). However, more recent studies utilizing purified reductase preparations from rabbit or rat liver (Coon et al., 1977; Guengerich, 1977b) and rabbit lung (Guengerich, 1977b) microsomes have demonstrated multiple forms in what were originally considered to be electrophoretically homogeneous samples. Resolution and isolation of two liver reductases have been achieved, and they exhibit different properties such as activity in a reconstituted cytochrome P-450 enzyme system, lability to storage, and molecular weight (Coon et al., 1977). The differences observed in our experiments between reductases purified from BNF- and Pb-treated microsomes (i.e., lability, specific activities) can be attributable to the fact that the Pb-reductase is present in higher concentrations in microsomes. On the other hand, the possibility that they reflect different proportions of multiple species cannot be excluded at this point.

Monoterpene Hydroxylation by a Reconstituted Enzyme System. The catalytic activity of two purified P-450 heme proteins in reconstituted systems were evaluated with respect to monoterpene hydroxylation and found to exhibit the usual requirements of reductase and phospholipid (Table IV). In confirmation of the induction data, cytochrome P-450LM_2 was capable of supporting appreciable monoterpene hydroxylase activity, while P-450LM_4 was not. Biphenyl hydroxylase activities were determined in control assays (Table IV) to ascertain the catalytic activity of each system. The latter was

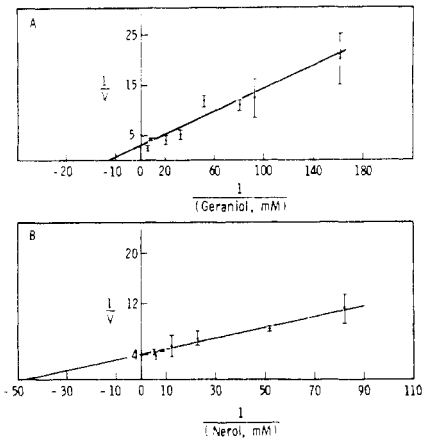


FIGURE 3: Incubations were carried out as described for the LM_2-mediated reconstitution system except that various concentrations of geraniol (A) or nerol (B) were used as indicated. The values represent means ±SD of two to four experiments. v is expressed as nmol of product formed/(min nmol P-450LM_2). Least-squares analyses of data points after replotting these values as a function of v vs. v/s provided K_m values of 24 μM and 19 μM for geraniol and nerol, respectively. Maximal turnover numbers were calculated to be 0.30 nmol of 10-hydroxygeraniol formed/(min nmol of P-450LM_2) and 0.25 nmol of 10-hydroxynerol formed/(min nmol P-450LM_2).

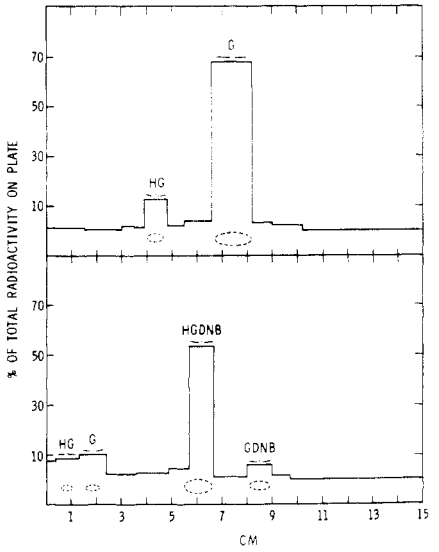


FIGURE 4: (Top) The $CHCl_3$-soluble Pb-microsomal incubation extract was developed on silica gel (0.1 mm) with benzene/ethyl acetate/acetone (2/1/1, v/v/v) and then cut out for liquid scintillation counting. Abbreviations: G, geraniol; HG, 10-hydroxygeraniol; DNB, 3,5-dinitrobenzoate. (Bottom) The $CHCl_3$-soluble Pb-microsomal incubation extract was converted to 3,5-dinitrobenzoate derivatives, separated on silica gel (0.1 mm) sheets developed with benzene/ethyl acetate (60/1, v/v), and cut out for liquid scintillation counting. Standards were cochromatographed for identification purposes. Much of the original geraniol substrate was volatilized in drying the sample down for derivatization.

measured fluorimetrically, whereas our use of the radiometric procedure in the determination of monoterpene hydroxylase activity permitted reproducible estimations of >1 pmol of product per min in reconstituted systems.

The molar ratio of reductase to heme protein was maintained at 3 following previously described procedures (Haugen et al., 1975; Kamataki et al., 1976) for reconstituted systems. This ratio is not representative of the value of intact hepatic microsomes which is 1:20–30 in favor of the heme protein (Estabrook et al., 1976). It should be noted that the reductase participates in rat liver microsomal heme oxygenase (Schacter et al., 1972) and squalene epoxidase (Ono et al., 1977) systems which may reduce the availability of reductase for P-450 catalysis. A system containing molar ratios of reductase to P-450 heme protein of 1:4 or less are capable of N-demethylating benzphetamine (Yasukochi & Masters, 1976; Lu et al., 1974). To ensure reproducible turnover numbers at concentrations of geraniol or nerol below their respective K_m required the addition of 150 μg of di-12GPC to the reconstitution assay system (Table IV). However, higher rates of hydroxylation of either geraniol or nerol were observed when 30–50 μg of phospholipid was added (Table IV) which were comparable to microsomal turnover numbers (geraniol = 0.84; nerol = 0.94). Thus V_{max} values obtained by least-squares analyses are somewhat underestimated (Figure 3). The turnover numbers observed are comparable to those of this system for many substrates including isoprenoid compounds (Haugen et al., 1975).

Interestingly K_m values for geraniol and nerol obtained with the reconstituted system (Figure 3) were approximately tenfold less than microsomal apparent K_m values but comparable to the dissociation constants obtained by optical difference spectroscopy (Table II). One would expect that apparent K_m values derived from microsomal turnover numbers would better approximate reconstitution kinetic parameters. Calculation of the heme protein fraction represented by LM_2 alone would further provide more meaningful K_m values. However, little if any LM_2 heme protein may be present in control microsomes making it likely that in uninduced microsomes another species of cytochrome P-450 is involved in hydroxylation.

Identification of Diol Products. The positional specificity of the P-450 heme protein catalyzed hydroxylation of geraniol and nerol was established in the following manner. The $CHCl_3$-methanol incubation extract was taken to dryness under N_2 and resuspended in a known amount of $CHCl_3$. A typical TLC distribution of the resulting radioactivity is shown in Figure 4. Further fractionation on preparative TLC provided a diol band which was identified by using authentic markers on side tracks of the TLC plate. Since the chromatographic system neither resolves geraniol from nerol nor their respective 10-hydroxy derivatives from each other, we can assume it would not resolve a 9-hydroxynerol or -geraniol from a 10-hydroxy derivative. After elution of the diol fraction from the TLC plate, an aliquot was counted to determine total radioactivity in the fraction. Subsequently, the acetone was removed with a stream of N_2 which simultaneously caused considerable reduction in [^{3}H]geraniol counts. Upon addition of authentic 10-hydroxy carrier the products were concentrated, derivatized to their respective bis(3,5-dinitrobenzoates), and recrystallized to constant specific activity. The values determined in the final three crystallizations from incubations with both microsomes and reconstituted enzymes are shown in Table V. Authenticities of the compounds were ascertained by melting point which differ for C-9 and C-10 hydroxyl derivatives and by high pressure liquid chromatography (HPLC). Not only were the bis(dinitrobenzoates) resolved on TLC (Figure 4), but they were also subjected to HPLC, and appropriate fractions were collected and counted. In this manner radioactivity profiles of the product diol derivative from geraniol hydroxylase incubations were shown to coincide with the A_{254} elution profile of authentic derivative (unpublished observations). Using these procedures it was determined that all of the radioactivity in the diol fraction could be accounted for by the corresponding *cis*- or *trans*-10-hydroxy derivative thereby establishing the positional specificity.

It appeared then that the 10-hydroxy derivatives were the sole hydroxylation product of the rabbit liver microsomal P-450

TABLE V: Specificity of Monoterpene Hydroxylation.

preparation	substrate	diol fraction (dpm)	diol deriv. (dpm)	sp act.[b] (dpm/mg)
microsomes Pb-induced	geraniol	6.01×10^5	5.48×10^5	15 594 15 480 15 221
reconstituted system[a]	geraniol	3.77×10^5	3.75×10^5	31 906 31 767 31 745
microsomes Pb-induced	nerol	8.02×10^5	7.77×10^5	51 556 51 312 51 747
reconstituted system[a]	nerol	1.81×10^5	1.78×10^5	8 567 8 505 8 482

[a] Containing P-450 LM_2 heme protein. [b] Values obtained in each of the last three recrystallizations of the bis(3,5-dinitrobenzoate) derivative of 10-hydroxygeraniol or nerol.

dependent system. Furthermore, the aqueous layer derived from hydroxylase incubations did not contain greater levels of radioactivity than that from blank assays.[3] Both mammalian (Gaylor & Delwiche, 1973) and plant microsomes (Murphy & West, 1969) are known to catalyze the oxidation of methyl groups of cyclic isoprenoid compounds to carboxylic acids. As mentioned previously, rabbits force fed geraniol excrete large amounts of 1,10-dicarboxylic acids. Under optimal assay conditions 10-hydroxygeraniol and 10-hydroxynerol were formed in 10–20% yields. Furthermore, sufficient amounts of radioactive substrate were used in these assays to detect a metabolite of the diol formed in 1% overall yield. Since acyclic monoterpene alcohols such as geraniol are good substrates for cytosolic alcohol dehydrogenase (Christophe & Popják, 1961), it seems more consistent that further oxidation of the 10-hydroxy monoterpenes occurs in this manner.

Triol products were also not present in appreciable amounts as they would have been detected at the origin of the TLC plate in Figure 4 or in the aqueous layer. However, conversion of a double bond to a diol is a common feature of in vivo mammalian biotransformation for terpenes as well as other xenobiotics (Williams, 1959; Kodama et al., 1976). Triols can arise from epoxides by the action of microsomal epoxide hydratases or nonenzymatically (Yang et al., 1976) and the ability of epoxides to react chemically under the conditions of our assay is likely. However, both the epoxide and its degradation products other than the triol (including cyclization products) could be hidden under the geraniol band on TLC and go undetected. Thus, if the epoxides are being formed they are not converted to triols. In any case, the absence of detectable amounts of other allylic hydroxylation products (at C-4, 5, 6, and 9) as well as acids suggest the rabbit liver microsomal system exhibits an unusual specificity for the acyclic monoterpene alcohols. Whether this selectivity is of relevance to cholesterol biosynthesis in mammals remains to be investigated.

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[3] Recently we demonstrated the ability of rat liver microsomes to metabolize geraniol and nerol to products which comigrate on TLC with their 10-hydroxy derivatives. Interestingly, the aqueous fraction appears to contain significant amounts of as yet unidentified radioactivity.

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